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DEVELOPMENT OF A CHARGE-SHIFTING POLYION-MEDIATED INTRAMAMMARY GENE TRANSFER SYSTEM FOR PRODUCTION OF RECOMBINANT PROTEINS IN MILK

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

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*****

The Ohio State University
1997

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ABSTRACT

A novel charge-shifting polyion-based gene transfer method was developed which permitted non-germline, non-viral recombinant gene transfer directly into secretory mammary epithelia in vivo after direct intramammary infusion. Mixed polycation transfection complexes [DEAE-dextran (DEAE):poly-L-ornithine (Orn_n):DNA] gave increased human growth hormone (hGH) reporter gene expression over that from individual polycations alone. Addition of a charge-shifting polyion, histamine derivatized poly-L-glutamic acid (Glu_n-HisN), to mixed polycation transfection complexes further increased hGH expression five fold in primary bovine mammary cells transfected in vitro. Controlling the order of polyion addition to DNA to place weaker polycations (DEAE) adjacent to DNA also increased hGH expression. Optimal hGH expression was attained when primary bovine mammary cells were transfected with ordered charge-shifting polyion transfection complexes: [DNA:DEAE:Glu_n-HisN:Orn_n]. Transfection medium (Hanks’ Balanced Salt Solution or 0.3 M sucrose, 10 mM HEPES, pH 7.4) also dramatically influenced hGH expression in primary bovine mammary cells transfected in vitro.
Fluorescently labeled polyions alone (not complexed with DNA) were rapidly internalized into virtually all exposed cells and preferentially localized within the nucleus. Fluorescently labeled polyion:DNA transfection complexes, although rapidly internalized, were asymmetrically clustered in the perinuclear region. Transfection complexes were bound and internalized by virtually all cells. DNA escape from endosomes and entry of DNA into the nucleus appear to be the major limitations to polyion-mediated mammary cell transfection in vitro.

Although in vivo transfection of the bovine and ovine mammary glands with charge-shifting polyion transfection complexes failed to give recombinant gene expression, transfection did not irritate or elicit inflammation within the gland. Prepartum guinea pig mammary glands transfected with optimized charge-shifting polyions or DEAE-dextran gave hGH expression in milk (75 and 300-500 ng hGH/ml, respectively) which persisted for the duration of lactation. Cationic lipids were ineffective for transfection of mammary cells both in vitro and in vivo. The ability to express recombinant proteins in milk without germline insertion or viral infection should facilitate gene expression studies in mammary tissue and provide guidance for transfection efforts in larger ruminants.
DEDICATION

To my wife, Kay, and my parents
William and Nancy Amstutz
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PUBLICATIONS

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FIELDS OF STUDY

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Abbreviations

ABC............. avidin-biotin complex
α-lac.............. alpha-lactalbumin
amp.............. ampicillin
BSA........... bovine serum albumin
cDNA.......... complementary deoxyribonucleic acid
CIAP........... calf alkaline intestinal phosphatase
Comma-D.... mouse mammary cell line
Cos-1........... SV40-transformed African green monkey kidney cells
DAPI.......... 4,6-Diamidino-2-phenylindole
DEAE........... diethylaminoethyl dextran (500,000 kDa)
DEPC......... diethyl pyrocarbonate
DIEA........... N,N-diisopropyl ethylamine
DMEM........ Dulbecco’s modified Eagle’s medium
DMF............. dimethyl formamide
dNTP........... deoxyribonucleotide triphosphate
DMSO......... dimethyl sulfoxide
DNA............. deoxyribonucleic acid
dNTP......... deoxyribonucleotide triphosphate
DTT........... dithiothreitol
EDAC......... 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride
EDTA........ ethylenediaminetetraacetic acid
EGF............ epidermal growth factor
ELISA........ enzyme linked immunosobant assay
EMEM........ minimum essential medium (Eagle)
Eth. Br........ ethidium bromide
EtOH......... ethanol
FCS........... fetal calf serum
FITC.......... fluorescein isothiocyanate isomer I
GITC........... guanidine isothiocyanate
Glu........... poly-L-glutamic acid sodium salt (15,000-50,000 kDa)
Glu/HisN...... histamine derivitized poly-L-glutamic acid (15,000-50,000 kDa)
GP............. guinea pig
HBSS.......... Hanks’ balanced salt solution
HBTU.......... [2-(1-H-benzo-triazol-1-yl)-1,1,3,3-tetramethylmonium hexafluorophosphate]
HEPEs......... N-(2-Hydroxyethyl)piperazine-N’-2-ethanesulfonic acid
Abbreviations cont.

HepG2.......... human hepatocellular carcinoma cells
hGH.............. human growth hormone
HPLC............. high pressure liquid chromatography
hTF.............. human transferrin
HTRS............. heat treated rabbit serum
I................ insulin (from bovine pancreas)
IgG.............. immunoglobulin G
IPTG............. isopropyl β-D-thiogalactopyranoside
Lf................. lactoferrin
LAL.............. limulus amebocyte lysate assay
LPS.............. lipopolysaccharide (endotoxin)
Lys............. poly-L-lysine hydrobormide (30,000-70,000 or 150,000-300,000 kDa)
MDBK............ Madin-Darby bovine kidney cells
MDCK............ Madin-Darby canine kidney cells
MeOH............ methanol
MFGM........... milk fat globule membrane
NMP............. N-methylpyrrolidone
Orn........... poly-L-ornithine hydrobromide (5,000-200,000 kDa)
PBS.............. phosphate buffered saline (0.015M Na₂PO₄, 0.145M NaCl, pH 7.2)
PBMC........... primary bovine mammary cells
PCR............. polymerase chain reaction
PEG............. polyethylene glycol (8,000MW)
Prl............. prolactin
QFITC........... quinolizino-substituted fluorescein isothiocyanate
RIA............. radioimmunoassay
RNA............. ribonucleic acid
RNase A........ ribonuclease A
RT-PCR......... reverse transcription polymerase chain reaction
SDS............. sodium lauryl sulfate
TBS............. tris buffered saline (20 mM Tris-HCl, 0.145 M NaCl, pH 7.4)
TEMED........ N,N,N',N'-tetramethylethylenediamine
tet.............. tetracycline
Texas Red........ sulforhodamine 101 acid chloride
TFA............. trifluoroacetic acid
WAP............. whey acidic protein
X-gal........... 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
293............. adenovirus type 5 transformed primary human embryonic kidney cells
CHAPTER 1

INTRODUCTION

Although many recombinant gene expression systems have been developed, most have inherent limitations which either make them infeasible or severely limit their utility for large-scale recombinant protein production. For example, bacterial and yeast fermentation systems either lack the ability to make essential posttranslational modifications (Bebbington and Hentschel, 1985) or do so improperly (Gellerfors et al., 1989), and although mammalian cell cultures systems are proven effective for recombinant protein production (Datar et al., 1993), they are difficult to scale, subject to microbial contamination, and technically complex to maintain (Colman, 1996). Therefore, many interests press for ways to produce large quantities of biologically active eukaryotic proteins.

The ruminant mammary gland’s capacity for large scale protein synthesis and secretion make it an excellent vehicle for recombinant protein production (Wilmut et al., 1990; Yom and Bremel, 1993). Recombinant protein production in milk offers several advantages over recombinant protein production in other gene expression systems. First, harvest of recombinant proteins secreted in milk would be easy since milk collection is
simple, non-invasive, and could utilize milk handling and transport systems already in place. Secondly, most recombinant proteins and glycoproteins produced in the mammary gland should be properly posttranslationally modified and folded into proper conformations (Bawden et al., 1994, and references therein; Meade et al., 1990), unlike those produced in bacterial and yeast fermentation systems. Finally, given the extensive knowledge of milk biochemistry, recombinant protein purification from milk should be easier (Gordon et al., 1990) and safer than purification from human blood fractionation (Colman, 1996).

Following the development of pronuclear microinjection (Gordon et al., 1980) many transgenic animals have been created which secrete biologically active eukaryotic proteins in milk, with secretion of recombinant proteins rivaling or exceeding endogenous milk protein concentrations (Bawden et al., 1994; Houdebine, 1994; Wilmut and Whitelaw, 1994). However, despite the development of transgenic animals almost two decades ago (Gordon et al., 1980), there are still no published reports of recombinant gene expression in bovine milk. This is likely due to the high cost, inefficiency of large animal transgenesis, and extended generation times (from DNA injection to birth of G1 offspring and milk production) of ~15 months (swine), ~18 months (goats/sheep), and ~32 months (cattle) required to produce transgenic livestock (Colman, 1996).

As an alternative to transgenesis, Patton et al. (1984) proposed that direct intramammary infusion of recombinant transgenes might be used to transfer genes directly into mammary epithelial cells responsible for milk protein synthesis and secretion. Direct
intramammary infusion is routinely used in dairy cattle to administer antibiotics for the treatment of bacterial infections without causing undue pain, discomfort or irritation in the mammary gland.

However, somatic tissues (cells) do not ordinarily internalize large and/or charged macromolecules such as DNA. Therefore, a variety of different gene transfer techniques have been developed to facilitate DNA transfer into various somatic cells, including direct microinjection, electroporation, and use of calcium phosphate coprecipitation, cationic liposomes, or polyamine complexes (reviewed by Keown et al., 1990). Direct transfection of the mammary epithelium by direct intramammary infusion of recombinant transgenes will likely require facilitation by these or similar methods.

The unique anatomy of the mammary gland may allow intramammary infusion to be utilized for recombinant gene transfer. The mammary gland consists of a ductular tree which exits through the teat and ascends into the mammary gland with ever finer bifurcation, terminating in spherical secretory alveoli. A single layer of epithelial cells lines each alveolus and vectorially secretes milk proteins into the lumen of the alveolus which drains into the ductular tree for exit through the teat. The apical surfaces of secretory epithelia are therefore directly accessible by intramammary infusion. Thus, unlike most other tissues, the functional unit of the mammary gland (the secretory mammary epithelial cell) is directly accessible for gene transfer by direct intramammary infusion of recombinant transgenes.
Direct intramammary infusion of recombinant transgenes would eliminate the need for mammary specific promoters and regulatory elements, since only mammary epithelial cells responsible for protein synthesis would be exposed to transfection components. Secondly, intramammary gene insertion could be performed on any animal, and would therefore not be limited by the fixed genetic background inherent to the founder lines of transgenic animals. Furthermore, intramammary gene insertion may allow simultaneous expression of multiple recombinant proteins and can be implemented in the current lactation, which allows a much quicker response to rapidly shifting markets than do transgenic animals.

Therefore, the primary objective of this study was to develop a safe, efficient, non-germline, non-viral recombinant gene transfer method compatible with direct intramammary infusion for the purpose of expressing recombinant genes in bovine mammary epithelia in order to modify mammary function and/or the production, composition, and qualities of milk.

To be practical, any form of direct intramammary gene insertion in dairy animals must meet certain criteria. First and foremost it must be safe, both for personnel administering the treatment, and for animals being treated, the food chain, consumers, and the environment. Secondly, it should be cost effective and efficiently produce the desired mammary modification or recombinant protein. Finally, it should be deliverable and implementable in the field using a minimum of technology and technical skills, as is routinely done for artificial insemination.
The anatomy, polarity of the mammary epithelium, and motion of the bovine mammary gland preclude the use of gravity-dependent transfection systems (such as calcium phosphate:DNA coprecipitation) in vivo (Keown et al., 1990). Transfection procedures which require short-duration cell shock (e.g., glycerol or DMSO exposure, etc.) by rapid short-term application and exchange of shock medium are also not feasible in the bovine mammary gland due to its size and the consequent difficulty of rapid introduction and complete and rapid removal of fluid by teat drainage or aspiration (Keown et al., 1990; Malienou-Ngassa et al., 1990). The high cost of selective agents, average size of the mammary gland (~ 50 lbs.) (Schmidt, 1971, and references therein), and limited postparturient mammary cell replication (Schmidt, 1971) will likely preclude survival selection of transfected cells in the bovine mammary gland in vivo (Kauffman, 1990; Keown et al., 1990). Therefore, priority must be placed on the development of gene transfer systems capable of transfection and expression of recombinant transgenes in most or all cells of the mammary gland without the need for selection or cell replication.

The only description of recombinant gene expression in milk attributable to direct transfection of mammary epithelium following direct intramammary infusion comes from Archer et al. (1994) who used replication-deficient retroviral vectors. Unfortunately, recombinant gene expression levels were low, peaking at ≤ 118 ng hGH/ml on day one of lactation and rapidly declined to 12 ng/ml by day three of lactation.

Additionally, use of recombinant retroviral vectors such as those described by Archer et al. (1994) in food animals may not be acceptable to regulatory agencies or the
public given the potential for hazardous viral recombination events (Smith et al., 1991). Retroviral-mediated gene transfer is also limited by the size of the recombinant gene which can be inserted into the viral genome, high titer requirements, and a requirement for cell division for efficient infection.

Of the many gene transfer systems available, polyamines offer several advantages over other transfection systems for intramammary gene transfer. Polyamines are non-infectious, non-toxic, compatible with intramammary infusion, easy to prepare, and simple to use. Furthermore, polycations should effectively deliver recombinant transgenes to all mammary epithelial cells via ionic charge interaction with both the negatively charged DNA and the negatively charged cell surface (Farber et al., 1975; Pagano, 1970).

Polycations such as poly-L-ornithine (Ornₙ), poly-L-lysine (Lysₙ), and DEAE-dextran (DEAE) can mediate efficient gene transfer in several mammalian cell lines (Bond and Wold, 1987; Davis et al., 1986; Farber et al., 1975), including mouse (Danielson et al., 1989) and bovine (Gorewit et al., 1987) mammary cell lines in vitro. However, only DEAE, which is toxic to some cells (Rosenthal, 1987), has been used to transfect primary mammary cells in vitro (Malienou-Ngassa et al., 1990).

Poly-L-ornithine enhanced the uptake and expression of exogenous DNA more reproducibly and to a greater extent than did DEAE or poly-L-lysine in Chinese hamster ovary (Dong et al., 1993) and lung (Farber et al., 1975) cells. Furthermore, polyornithine can mediate efficient transfection of non-replicating quiescent cells (Dong et al., 1993) as might be encountered in the lactating mammary gland in vivo.
The ability to deliver and express recombinant transgenes in most or all mammary cells is critical because recombinant protein expression level and value will likely define potential applications of gene transfer into mammary tissue and recombinant protein expression in milk. In addition to the production of highly valuable pharmaceutical proteins, high to moderate level recombinant gene expression in the mammary gland resulting from direct intramammary gene transfer may be used to alter bulk milk quality and composition for the purpose of increasing the nutritional value, manufacturing, and/or processing characteristics of milk. Furthermore, lower level recombinant gene expression in the mammary gland could be used to alter mammary function or regulation, mammary development, maintenance of lactation, and involution.

The dissertation describes a series of studies aimed at developing a non-viral recombinant gene transfer system which would be compatible with direct intramammary infusion of recombinant transgenes, for the purpose of expressing recombinant genes in the mammary gland to modify mammary function and/or milk composition as described above. The dissertation has been divided into chapters of related material and is not necessarily presented in chronological order. Therefore, some projects undertaken early in the study were terminated once they proved infeasible, while others were initiated later in the study only after pertinent information became available from previous experiments.

Potential transfection methods and expression vectors capable of facilitating recombinant gene expression in ruminant mammary cells were to be screened in vitro using
a primary bovine mammary cell culture system, capable of strong milk protein synthesis and secretion (Talhouk et al., 1990; Talhouk et al., 1992) prior to their use in vivo.

Section one (Chapter 4) describes the initial studies aimed at identification of a polycation-based transfection system capable of efficient targeting of transfected genes to primary bovine mammary cells in vitro, and of inducing strong and sustained expression of the transfected gene under conditions compatible with those anticipated as required for transfection of the bovine mammary gland in vivo. Polyamines were tested for cell targeting, subcellular distribution, and ability to induce expression of the transfected recombinant gene. These studies show that mixtures of polycations may offer transfection and expression advantages over individual polycations alone. To facilitate polycation:DNA dissociation and further increase recombinant gene expression, a novel charge-shifting polion was synthesized and incorporated into mixed polycation transfection complexes used to transfect primary bovine mammary cells in vitro. Recombinant gene expression was increased further when the order of polycation addition to DNA was controlled to allow polycations with lower charge density to complex first with DNA before addition of higher charge density polycations. All developments in this chapter were achieved within the constraint of the requirements perceived for in vivo bovine mammary transfection.

Section two (Chapter 5) focused on investigation of the transfection and expression efficiency and definition of intracellular trafficking and fate of transfected DNA of polion-mediated transfection in mammary cells transfected in vitro. Mammary cells were transfected with fluorescently-labeled polyions or radiolabeled DNA and fluorescent
reporter transgenes whose expressed proteins could be visualized. From the intracellular fate and subcellular distribution of various polyion transfection complexes in mammary cells in vitro, escape of DNA from endosomes and nuclear entry of DNA were identified as potential limitations to efficient polyion-mediated gene transfer. To investigate if nuclear exclusion of transfection complexes was size related and to study transfection complex formation, the morphology and size of polycation:DNA transfection complexes were examined by transmission electron microscopy. In an effort to facilitate DNA escape from endosomes, a pH-dependent endosomolytic peptide was synthesized and incorporated into mixed polyion transfection complexes used to transfect primary bovine mammary cells in vitro. The serendipitous observation that fluorescently labeled polycationic transfection complexes were bound to and immobilized on plastic led to the development of an in vitro polycation:DNA dissociation assay with which to rapidly test dissociation of transfection complexes in response to pH and ionic strength.

Section three (Chapter 6) describes efforts to construct a secretable version of the green fluorescent protein (GFP) as an alternative to the human growth hormone (hGH) reporter gene, the investigation of prokaryotic expression elements, and eukaryotic expression vectors capable of episomal replication to develop an alternative fluorescently-detectable secreted reporter gene which would facilitate expression assays and to further increase and prolong the duration of recombinant gene expression in primary bovine mammary cells transfected in vitro. Two eukaryotic expression vectors were constructed in an effort to develop a secretable form of the green fluorescent protein; one encoding a
human growth hormone secretion signal-green fluorescent protein (GFP) fusion protein, and a second encoding a full length human growth hormone-green fluorescent protein chimera. A bacteriophage T7 RNA polymerase-dependent recombinant gene expression system was examined for its ability to facilitate recombinant gene expression in primary bovine mammary cells transfected in vitro. Eukaryotic expression vectors capable of episomal replication in human and canine cells were examined for their ability to prolong the duration of recombinant gene expression in primary bovine mammary cells transfected in vitro.

Section four (Chapter 7) describes the in vivo transfection of ovine, bovine, and guinea pig mammary glands in an effort to demonstrate the in vivo feasibility of mammary transfection and production of recombinant proteins in milk by direct intramammary infusion of polycation:DNA transfection complexes, and to correlate in vitro recombinant transgene expression results with in vivo transgene expression. As promising polyion-mediated recombinant gene transfer methods were developed in vitro, they were tested in parallel in vivo for recombinant protein production in milk following direct intramammary infusion. Polyion-mediated transfection of the lactating bovine mammary gland in vivo gave no detectable recombinant gene expression, while in vivo guinea pig mammary gland transfections gave high and sustained levels of recombinant gene expression. Guinea pig mammary transfections were used to identify optimal transfection time (2 days prepartum) and transfection component levels (5x the in vitro levels). Ovine mammary transfections performed utilizing optimal guinea pig mammary gland transfection conditions gave no
detectable recombinant gene expression. Validation of the in vitro cell culture model system in the guinea pig mammary gland in vivo indicated that polycation:DNA transfection complexes are likely handled much differently in vivo than in vitro and resulted in very high level recombinant gene expression > 500 ng hGH/ml in milk.

The various phases of these studies involved several additional people and other laboratories. Preliminary polycation transfections, development of the eukaryotic pcDNAI/hGH expression vector and the development of mixed polycation transfection systems by Ms. Peggy Hiltner provided the foundation for beginning this recombinant gene transfer study. Following her departure, I undertook the completion of this project. Dr. Crispin Bennett assisted with the development of transfection complexes with ordered polyion addition and formation of transfection complexes in osmotically balanced sucrose/HEPES transfection medium. Synthesis of the human rhinovirus lytic peptide (HRV2 LP) was conducted with the assistance of Dr. Charles Brooks. Guinea pig transfections were performed by I. Mather and J. Hens at the University of Maryland as a collaborative study, using transfection polymers and DNA prepared and provided by M. Amstutz.
CHAPTER 2

LITERATURE REVIEW

Gene transfer, initially described by Avery et al. (1944) involves the introduction of exogenous genes into cells either in vitro or in vivo for experimental or therapeutic purposes. Recombinant gene transfer technology has since progressed to allow individual protein encoding genes from one species to be expressed with proper hormonal and developmental regulation in a tissue-specific manner in a second species. Such heterologous recombinant gene expression can be used to complement existing genes, replace missing or non-functional genes, introduce novel genes, and study gene function and/or the regulation of gene expression. Although several types of recombinant gene expression systems have been developed, the gene expression system which best fits a particular application will depend on the type of recombinant protein produced, posttranslational modifications required, quantity and quality of recombinant protein needed, its intended use, and overall development and production costs (Colman, 1996).

Central to the development of recombinant protein production systems is the goal of producing recombinant human proteins which could be used for human gene therapy. However, many of the systems currently available for recombinant protein
production have inherent limitations which either severely limit their utility or make them infeasible for producing many human pharmaceutical recombinant proteins. For example, although bacterial fermentation systems have been useful for the production of small unmodified recombinant proteins such as insulin and human interleukin 2 (hIL-2) (Reisman, 1993), many human proteins require posttranslational modifications such as phosphorylation, glycosylation, amidation, and proteolytic cleavage in order to acquire or maintain biological activity (Gordon et al., 1990). Generally, bacteria do not possess these capabilities and are therefore unable to synthesize complex proteins requiring such modification (Bebbington and Hentschel, 1985). Additionally, the high reducing environment within bacteria such as *Escherichia coli* can inhibit proper disulfide bond formation thus yielding improperly folded proteins which may affect protein solubility and lead to protein deposition in inclusion bodies (Marston, 1986). Considerable cost and effort is then required to restore protein solubility and biological activity (Bebbington and Hentschel, 1985; Datar et al., 1993).

Although yeasts possess the ability to glycosylate heterologous proteins, they do so with low efficiency (Cabezon et al., 1984; Wood et al., 1985) and sometimes improperly (Gellerfors et al., 1989). Aberrantly glycosylated recombinant proteins have a drastically reduced serum half-life (Casolaro et al., 1987) and may be hyperallergenic, both of which severely limit their usefulness for human therapy (Tekamp-Olson and Valenzuela, 1990). Finally, large-scale mammalian cell culture systems such as those currently used to produce recombinant tissue plasminogen
activator (t-PA) (Datar et al., 1993) are effective, but difficult to scale, subject to microbial contamination, costly, and technically complex to maintain (Colman, 1996; Werner et al., 1992).

The difficulty of expressing and purifying large quantities of biologically active recombinant proteins in bacterial, yeast, and mammalian cell culture systems, coupled with the development of pronuclear injection to produce transgenic animals (Gordon et al., 1980) focused much attention on the production of recombinant proteins in animal body fluids. Recombinant proteins produced in animals should be properly posttranslationally modified, biologically active, and easy to collect. Although foreign proteins have been secreted into the blood of transgenic animals, the volume of blood available for harvest is limited (Clark et al., 1987) and biologically active foreign proteins circulating in the bloodstream could have adverse effects on animal health (Bawden et al., 1994).

Unlike recombinant protein production in blood, recombinant proteins produced in milk would be anatomically isolated in the mammary gland where they could exert little direct systemic effect. Moreover, the ruminant mammary gland’s capacity for large scale protein synthesis and secretion, producing 0.1, 0.2, and 1 kg of protein per day for ovine, caprine, and bovine mammary glands respectively, make it an excellent vehicle for large-scale recombinant protein production (Archer et al., 1994; Wilmut et al., 1991). Indeed, transgenic animals have been created which secrete biologically active, eukaryotic proteins in milk at levels which rival or exceed
endogenous milk protein concentrations, (reviewed by Houdebine (1994); Bawden et al., (1994); and Wilmut and Whitelaw (1994). Furthermore, these proteins contained proper posttranslational modifications, including β-hydroxylation (Clark et al., 1989), glycosylation (Ebert et al., 1991; Wright et al., 1991), and vitamin K-dependent γ-carboxylation (Velander et al., 1992). The transgenic mammary gland is also able to synthesize heterodimeric protein complexes (DiTullio et al., 1992) and cysteine-rich foreign proteins which fold into functional conformations (Meade et al., 1990).

Recombinant protein production in milk offers several other advantages. First, harvest of recombinant proteins in milk would be easy since milk collection is simple, non-invasive, and could utilize milk handling and transport systems already in place. Secondly, given the extensive knowledge of milk biochemistry, purification of recombinant therapeutic proteins from milk should be easier (Gordon et al., 1990) and safer than purification of therapeutic proteins from human blood fractionation (Colman, 1996).

However, despite the development of transgenic animals almost two decades ago (Gordon et al., 1980), there are still no published reports of recombinant gene expression in bovine milk. This is likely due to the inefficiency of large animal transgenesis, and the cost and extended generation times (from DNA injection to birth of G1 offspring and milk production) of ~15 months (swine), ~18 months (goats/sheep), and ~32 months (cattle) required to produce transgenic livestock (Colman, 1996).
As an alternative to transgenesis, Patton et al. (1984) proposed that intramammary infusion of recombinant transgenes could be used to transfer genes directly into the mammary epithelial cells responsible for milk protein synthesis and secretion. Intramammary infusion of the bovine mammary gland, routinely used in the dairy industry to administer antibiotics for the treatment of bacterial infections, causes no pain, discomfort or irritation in the mammary gland.

The unique anatomy of the mammary gland may allow intramammary infusion to be utilized for recombinant gene transfer. The mammary gland consists of a ductular tree which exits through the teat and ascends into the mammary gland with ever finer bifurcation, terminating in spherical secretory alveoli. A single layer of epithelial cells lines each alveolus and vectorially secretes milk proteins into the lumen of the alveolus which drains into the ductular tree for exit through the teat. The apical surfaces of the mammary secretory epithelial cells are therefore directly accessible by intramammary infusion. Thus, unlike most other tissues, the functional unit of the mammary gland (the secretory mammary epithelial cell) is directly accessible for gene transfer by direct intramammary infusion of recombinant transgenes.

Direct intramammary infusion of recombinant transgenes would eliminate the need for mammary specific promoters and regulatory elements, since only mammary epithelial cells responsible for protein synthesis would be exposed to transfection components. Secondly, intramammary gene insertion could be performed on any animal, and therefore would not be limited by the fixed genetic background inherent to
the founder lines of transgenic animals. Furthermore, intramammary gene insertion would allow simultaneous expression of multiple recombinant proteins and can be implemented in the current lactation, thus allowing a much quicker response to rapidly shifting markets than do transgenic animals.

The level at which a recombinant protein can be expressed in milk, and its value, will likely define potential applications of gene transfer into mammary tissue and recombinant protein expression in milk. Hence, the ability to deliver and express recombinant transgenes in most or all mammary cells is critical. In addition to the production of pharmaceutical proteins, high to moderate level recombinant gene expression in the mammary gland may be used to alter bulk milk quality and composition for the purpose of increasing the nutritional value or manufacturing and/or processing characteristics of milk. Furthermore, lower level recombinant gene expression in the mammary gland could be used to alter mammary function or regulation, mammary development, maintenance of lactation, and involution.

Applications for High to Moderate Level Recombinant Gene Expression in Mammary Tissue

Pharmaceutical Protein Production in Milk

High to moderate level recombinant protein expression in milk (i.e. approaching endogenous milk protein concentrations) would be useful for the production of pharmaceutical proteins, which may have market potential of up to $2
billion annually (Rudolph, 1995). Major biotechnology companies actively pursue the
development of genetically engineered sheep, goats and cattle capable of producing
high concentrations of α-1-antitrypsin, tissue plasminogen activator, Factor IX and
other highly valuable pharmaceutical proteins in milk (Rudolph, 1995). Direct
transfection of the ruminant mammary gland in vivo by direct intramammary infusion
of recombinant transgenes could lead to production of pharmaceutical proteins in milk
without the cost and extended time frames required to generate transgenic animals, or
the safety concerns associated with viral-mediated gene transfer.

Modification of Caseins

The most predominate milk proteins in dairy species are the caseins (αs1, αs2, β,
and κ) which account for approximately 80% of total milk protein (Bawden et al.,
1994). Caseins are a group of proline-rich phosphoproteins whose main function is to
provide calcium, phosphorus, and protein for rapid growth and development of the
suckling neonate. Although milk composition differs greatly between species for both
the types of proteins expressed and their relative concentrations, one or more of the
caseins has been found in the milk of all mammals examined thus far (Bawden et al.,
1994). In the presence of physiological levels of calcium, αs1, αs2, and β-casein
aggregate to form micelles. Micelle size is controlled by the abundance of κ-casein,
the substrate for rennin which proteolytically disrupts casein micelles and precipitates
caseins for cheese making (Wilmut et al., 1991).
Casein micelle stability during heat processing depends on the relative amounts of $\kappa$-casein and $\beta$-lactoglobulin. Thus, it has been proposed that decreasing $\kappa$-casein concentrations in milk may facilitate thermal processing and cheese making (Choi et al., 1996).

Likewise, expression of additional $\beta$-casein in cows’ milk should increase the speed of curd formation and cheese curd firmness (Clark, 1996; Richardson et al., 1982). Expression of modified caseins in milk may provide additional manufacturing and/or processing advantages. For instance, incorporation of “designer” proteolytic cleavage sites into transfected transgenes may facilitate cheese ripening by enhancing the proteolytic breakdown of milk proteins to yield a more desirable texture (Kang et al., 1986), while addition of a glycosylation site at amino acids 7 or 27 of $\beta$-casein is postulated to increase the emulsifying, foaming, and gelling characteristics of milk (Richardson et al., 1982) which would enhance its utility in other food manufacturing processes.

Alteration of milk composition may not be as straightforward as anticipated. Bleck et al. (1995) altered mouse milk composition by overexpressing a recombinant bovine $\beta$-casein transgene in transgenic mice. These $\beta$-casein transgenic mice had increased milk viscosity and exhibited premature mammary involution, the latter of which correlated with the $\beta$-casein concentration in milk, suggesting that there are limits to the content of some recombinant proteins in milk, and possible physiological consequences as well.
Modification of Whey Proteins

α-lactalbumin (α-lac) and β-lactoglobulin (β-lac) are the principle whey proteins in dairy species, while whey acidic protein (WAP) is the major whey protein in rodents (Romagnolo and DiAugustine, 1994). α-lactalbumin interacts with golgi bound β1,4-galactosyl transferase to form the lactose synthetase complex (Keenan et al., 1970). Lactose, the secretion product of this enzyme-modifier protein complex is the major osmoregulatory element in milk (Linzell and Peaker, 1971) and thereby is the major control for milk volume. Thus, a reduction in α-lac concentration in the mammary gland or milk would not only reduce lactose concentrations in milk, but milk volume as well. Unfortunately, a large portion (70-90%) of the world's population is deficient in intestinal lactase which digests lactose in milk (Dahlqvist, 1983; NDC, 1985). Adults with severe lactose intolerance may suffer from abdominal cramps, flatulence, and diarrhea, while children may experience malnutrition or even death in extreme instances (Kretchmer, 1972; Saavedra and Perman, 1989).

Reducing the lactose concentration in milk is more desirable than its complete elimination for several reasons. First, lactose-free milk has been shown to reduce the intestinal absorption of calcium in infants (Tamm, 1994). Secondly, α-lactalbumin-deficient transgenic mice had such viscous milk that it could not be secreted (Stinnakre et al., 1994). L’Huilier et al. (1992) reported that cytoplasmic delivery of anti-α-lactalbumin ribozymes reduced α-lac mRNA levels in C1271 mouse cells in
vitrō. Reducing lactose concentrations in milk would allow a large portion of the world’s population to resume milk consumption without undue discomfort. Lactose concentrations in milk also could be reduced by expressing recombinant lactase or β-galactosidase transgenes in mammary tissue with subsequent enzyme secretion into milk.

“Humanizing” Cow’s Milk

For various reasons, some women either are unable or choose not to breastfeed their infants. Many of these infants are therefore reared on infant formula composed largely of bovine milk. Infant formula is routinely supplemented with iron and vitamin-D as both human and bovine milk contain insufficient levels to meet the nutritional needs of growing human infants (Packard, 1982).

Additionally, human milk has a higher whey:casein ratio, lacks β-lactoglobulin, and contains higher levels of lactoferrin (~1 g/l) than bovine (0.1 g/l) milk (Sanchez et al., 1992, and references therein). Given the numerous functions attributed to lactoferrin, including its iron-binding and bacteriostatic activity, it has been suggested that human lactoferrin supplementation of neonate formula may, among other things, facilitate iron transport and establishment of normal gastrointestinal microbial flora (Bellamy et al., 1992; Sanchez et al., 1992).

A transgenic bull containing a human lactoferrin transgene driven by a mammary specific casein promoter was produced in 1991, and reports of lactoferrin
secretion in the milk of his progeny are anticipated soon (Krimpenfort et al., 1991).
Similar expression of other human milk proteins in bovine milk may provide better
nutritional support, facilitate digestion, iron transport, and the establishment of normal
gastrointestinal microbial flora in the growing infant fed bovine milk-based infant
formula (Bellamy et al., 1992; Sanchez et al., 1992).

Other opportunities for the “humanization” of bovine milk include expression
of other recombinant bioactive peptides (casomorphins, lactorphins, etc.) and
protective proteins (lysozyme, immunoglobulins) in bovine milk which might improve
satiety, gut motility, and/or immunity (Fiat et al., 1993; Maga and Murray, 1995).
Maga et al. (1994) recently described expression of the human lysozyme gene in
transgenic mouse mammary glands to study the effects of lysozyme on bacterial
growth in the mammary gland and milk and to determine if lysozyme expression in
milk altered the physical properties of milk.

Applications for Low Level Recombinant Gene Expression in Mammary Tissue

Low level expression (i.e. levels similar to endogenous growth or transcription
factors) of recombinant genes for growth or transcription factors, hormone receptors,
or regulatory enzymes in mammary tissue could be used to alter gland development,
function, and milk composition.
Growth Factors/Regulatory Enzymes

With each lactation the mammary gland undergoes successive cycles of proliferation, differentiation, and involution. Mammary development, maintenance of lactation, and involution are all regulated by coordinated expression of growth factors, hormones, extracellular matrix components, and other regulatory enzymes and their receptors. Therefore, proper temporal and spatial expression (or inhibition) of appropriate regulatory components could facilitate mammary development or inhibit involution.

Hepatocyte growth factor and neuregulin, which are active at picomolar concentrations, stimulate mammary ductal branching and lobulo-alveolar development, respectively (Soriano et al., 1995; Yang et al., 1995). Hence, it is reasonable that developmentally staged mammary expression of recombinant hepatocyte growth factor and/or neuregulin at even low levels could dramatically increase lobulo-alveolar and ductal development in the mammary gland and therefore milk production. Similar strategies could be utilized with low level mammary expression of other growth and development factors.

Extracellular matrix protease inhibitors

The onset of mammary involution is regulated in part by the ratio of extracellular matrix (ECM) proteases and their inhibitors (tissue inhibitor of metalloproteinsases (TIMP), interleukin-1 converting enzyme (ICE), or stromelysin)
(Talhouk et al., 1992). Theoretically, temporal intramammary expression of ECM protease inhibitors could extend lactation by delaying apoptosis and involution (Lund et al., 1996; Quarrie et al., 1995; Talhouk et al., 1992).

**Alteration of milk composition**

Inhibition or elimination of endogenous mammary enzyme expression by either antisense oligonucleotide (Liebhaber et al., 1992) or ribozyme inhibition of mRNA translation (L'Hullier et al., 1992), or homologous recombination knockout in embryonic stem cells (Capecchi, 1989) may provide additional opportunities to modify milk composition. Reduction or elimination of mammary proteins which are critical regulators of fat synthesis and saturation (acetyl CoA carboxylase, and fatty acid desaturase) could profoundly affect both the amount and saturation of lipids found in milk, thereby altering bulk milk composition and nutrition (Bremel et al., 1989).

Thus, recombinant gene expression in the mammary gland following transfection of mammary epithelium by direct intramammary infusion of recombinant transgenes affords many opportunities for modification of mammary development or milk composition which could prove economically, physiologically, medically or nutritiously advantageous.
Gene Transfer Approaches

In spite of the potential advantages and anatomical feasibility for direct transfection of secretory mammary epithelial cells in the mammary gland in vivo, somatic cells in tissues such as the mammary gland do not normally internalize large and/or charged macromolecules such as DNA. Therefore, a variety of different gene transfer techniques have been developed to facilitate DNA transfer into somatic cells both in vitro and in vivo.

Recombinant gene transfer techniques fall into two basic categories; transgenic and non-transgenic or somatic gene transfer. Transgenic animals are defined as any animal with foreign DNA integrated into its own genome (Smith et al., 1987). Transgenic animals can be created three ways; pronuclear microinjection, homologous recombination in embryonic stem (ES) cells, and retroviral vector insertion into the early embryos (reviewed by Thompson et al., 1993). Although viral gene transfer systems can be used with embryos to produce transgenic animals, they are most often used for insertion of recombinant genes into somatic cells and will be discussed in the somatic gene transfer section. A brief description of each transgenic gene transfer method follows, with special focus on work done in or applicability to recombinant gene expression in the mammary gland.
**Pronuclear Microinjection**

Gordon et al. (1980) were the first to produce transgenic animals (mice) using pronuclear microinjection. As pronuclei are easily visualized using differential interference contrast microscopy, transgenesis by the direct microinjection of recombinant transgenes into single-celled embryos was quickly accomplished in other species, including rabbits, sheep, goats (Bawden et al., 1994, and references therein) and even fish (Zhang et al., 1990). Bovine and porcine embryos however, are much more opaque and require centrifugation for pronuclear visualization to allow successful male pronuclear microinjection (Wall et al., 1985).

The efficiency of transgenesis is low (−1-5%) for large mammals such as sheep and cattle (Clark et al., 1992), but has been improved with the development of in vitro fertilization (Lu et al., 1987) and embryo maturation (Krimpenfort et al., 1991). Transgenic offspring are easily detected using either Southern hybridization or polymerase chain reaction (PCR). The latter has also been used to screen pre-implantation bovine embryos for recombinant transgene integration (Hyttinen et al., 1994). Additionally, microinjection does not appear limited by transgene size, as Schedl et al. (1993) successfully produced transgenic mice containing a 230 Kb pair insert.

Numerous transgenic animals have been created that secrete biologically active eukaryotic proteins in milk at levels which rival or exceed endogenous milk protein concentrations (up to 35 g/L) (Archer et al., 1994; Bawden et al., 1994, and references...
therein; Wilmut et al., 1991). However, early recombinant transgenes lacked important tissue-specific regulatory elements, and thereby allowed the constitutive expression of recombinant transgenes in many tissues. Such widespread unregulated expression of recombinant growth hormone transgenes led to many joint and reproductive problems in transgenic swine (Hammer et al., 1984).

Mammary-specific expression has now been achieved from expression constructs having target recombinant genes downstream of mammary-specific promoter sequences derived from several milk protein genes such as whey acidic protein (WAP), ovine $\beta$-lactoglobulin, bovine $\alpha$-lactalbumin, bovine $\alpha_{\text{S}}$-casein, and caprine $\beta$-casein (Houdebine, 1994). These promoter sequences were capable of driving persistent high level recombinant gene expression in the milk of transgenic mice (23 mg/ml), goats (6 mg/ml), sheep (35 mg/ml), and probably soon in cattle (Bawden et al., 1994; Jones and Cordle, 1995, and references therein).

Pronuclear microinjection of gene constructs results in the integration of uncontrolled amounts of foreign DNA at random sites into the host genome, giving each transgenic animal a unique genotype. Therefore, the level of recombinant gene expression in different animals containing identical transgenes is difficult to predict (Carver et al., 1993). Levels of recombinant gene expression in milk were largely controlled by randomness of integration site and transgene copy number until Dale et al. (1992) showed that proximal cis-acting whey acidic protein (WAP) sequences can direct high level WAP recombinant gene expression independent of integration site in
transgenic mice. Similarly, McKnight et al. (1992) used matrix attachment regions (MARs) of chicken lysozyme to buffer mouse WAP transgene expression from position effects in 4 of 5 transgenic mouse mammary glands.

Cost, inefficiency of transgenesis, and extended generation times are the major limitations to transgenic livestock. Other disadvantages to the production of transgenic livestock include the fixed genetic background of founder animals which limits population penetrance, and the potential for undesirable endogenous gene activation or inactivation following recombinant transgene integration into the host genome. Despite these drawbacks, transgenic animals are capable of high level, mammary specific, and developmentally regulatable expression of recombinant genes which can persist for the duration of lactation.

**Embryonic Stem Cells (ES)**

A second method of germline DNA insertion is to transfect embryo derived pluripotent stem cells and reimplant them in the developing blastocyst (Bradley et al., 1984; Evans and Kaufman, 1981). Coupled with Capecchi’s (1989) development of homologous recombination, deletion or replacement of endogenous genes with modified genes is now possible. However, homologous recombination is a rare event, occurring with a frequency of $10^{-4}$ vs. $10^{-2}$ for random integration (Porter, 1989). Successful homologous recombination also requires extensive uninterrupted sequence homology (at least 250 base pairs) at both the 5’ and 3’ ends; even two mismatched
base pairs per 250 bases can dramatically reduce recombination frequency (Waldman and Liskay, 1988). Recently, Danoff, et al. (1997) developed an improved method to screen ES cells for homologous recombination using RT-PCR.

Many embryonic stem cell experiments have focused on the disruption or "knockout" of target genes to assess gene function, while more recent advances permit precise gene (Stacey et al., 1994) and single base pair replacement (Hasty et al., 1991). Unfortunately, embryonic stem cells have only been reported in mice and sheep (Campbell et al., 1996), but efforts to find bovine equivalents are ongoing (Chemy and Merei, 1994; Strelchenko and Stice, 1994).

A disadvantage of ES cell gene transfer is that the resulting transgenic animals are mosaic in that not all cells or tissues contain or express the recombinant transgene. Consequently, some animals fail to properly transmit intact transgenes to progeny in classical dominant Mendelian fashion (Colman, 1996; Velander et al., 1992). Precise germ-line engineering, as offered by ES cell gene transfer, will be required for modifications that call for removal, modification, or replacement of endogenous mammary genes (Clark, 1996).

Non-transgenic (Somatic) Gene Transfer Methods

Non-transgenic gene transfer can be conducted in vitro, in vivo, or ex vivo. Many methods have been developed for the transfer of genetic material into somatic cells. For the most part, these methods are largely non-toxic, non-antigenic and safe,
and are potentially able to transfect all exposed cells. Additionally, most methods are also non-integrating, scalable, not limited by gene size, and with the exception of ligand-targeted transfection, are not specific for cell type or species. Unfortunately, most of these methods are also inefficient (Keown et al., 1990; Treco and Selden, 1995) resulting in unpredictable recombinant gene expression levels.

Recombinant gene transfer into somatic tissues can lead to either transient or stable gene expression. Transient gene expression is typically defined as expression for up to 72 hours post transfection, and is believed to arise from conversion of transfected plasmid DNA to non-replicating “minichromosomes” which are gradually degraded (Rosenthal, 1987). The main advantage of transient transfection systems is the speed with which results can be obtained.

Conversely, stable gene expression requires random integration into the host genome, which in somatic cells occurs with low frequency (10⁻⁸) but often results in sustained and high level gene expression. A brief description of each non-transgenic gene transfer method follows, with special focus on work done or applicability to mammary tissue.

**Viral-mediated Gene Transfer**

Retroviral vectors, modified so as to be incapable of replication, have been used to introduce recombinant transgenes into the germlines of mice (Jahner et al., 1985; van der Putten et al., 1985) and swine (Petters et al., 1987) by infection of early
embryos. They have also been used for gene transfer into somatic tissues, including mammary cells, in vitro (Strange et al., 1989), in vivo (Archer et al., 1994; Wang et al., 1991), and ex vivo (Edwards et al., 1988; Smith et al., 1991). However, many of these studies were conducted not to assess gene transfer potential, but to determine oncogene function and/or role in mammary neoplasia.

Gunzburg and Salmons (1986) were the first to describe a hormonally regulated, mammary specific retroviral gene delivery system utilizing the mouse mammary tumor virus (MMTV). They also employed the defective retrovirus packaging system developed by Mann et al. (1983), to prevent uncontrolled viral proliferation. More recently, Archer et al. (1994) used direct intramammary infusion of replication-defective retroviral vectors (GalV pseudotype) to express human growth hormone (hGH) during lactation in hormonally induced goat mammary glands and milk. Multiple intramammary infusions were performed in conjunction with hormonal induction of mammary gland development to facilitate transgene integration. Growth hormone concentrations in milk were highest on day one of lactation (~118 ng/ml), then rapidly declined to 12 ng/ml for days 9-15. Unfortunately, the mammary infusate used in this experiment contained both the retroviral vectors and the viral packaging helper cells which also secreted recombinant hGH at significant levels (224 ng hGH/ml) (Archer et al., 1994). Thus, it is uncertain whether the recombinant hGH detected in milk was produced solely by transfected mammary epithelium and not by viral-packaging helper cells.
A second lactation was induced in two of the virally transfected goats, one of which produced no hGH while the second secreted 0.4-2.3 ng hGH/ml on days 5-15 of the second lactation after intramammary retroviral transfection. Recombinant hGH expression in a subsequent lactation without further viral infusion suggests integration of recombinant transgenes into mammary stem cells with expression in mammary epithelium during the subsequent lactation, or survival of transfected epithelial cells into the subsequent lactation.

Viral-mediated gene transfer can be very efficient and is applicable in the mammary gland in vivo (Archer et al., 1994; Levinson, 1990). However, its application is presently restricted by limitations to the size of the inserted gene, high titer requirements for intramammary infusions, a requirement for cell division, and concerns by the public and regulatory agencies for the potential for hazardous viral recombination events (Smith et al., 1991).

**Direct Injection of “Naked” DNA**

Direct injection of “naked” DNA can be conducted either on single cells or on whole tissues. Somatic cells, like their germ-line counterparts, can be directly microinjected either in the nucleus or in the cytosol with DNA, RNA, or oligonucleotides encoding sequences of interest (Ansorge, 1982). Nuclear injection is considered superior, due to concern that DNA injected into the cytosol might be degraded during translocation from the cytosol to the nucleus (Wake et al., 1984).
However, since somatic cells are not totipotent, each cell of interest must be injected separately, making this technique very time consuming. Microinjection of somatic cells is essentially the same as microinjection of embryos for the generation of transgenic animals.

Direct injection of unmodified "naked" DNA or RNA into some organs or tissues can result in successful gene transfer (Doh et al., 1995; Wolff et al., 1990). Plasmid DNA injected intramuscularly resulted in recombinant β-galactosidase gene expression which persisted for 19 months post-injection in vivo (Wolff et al., 1992). Additionally, injected plasmid DNA retained its bacterial methylation pattern, indicating that the foreign DNA did not replicate in muscle cells, and that it was maintained extrachromosomally (Wolff et al., 1992).

Although most direct gene transfer experiments have utilized muscle as the tissue of choice, it is conceivable that this technique would be applicable to mammary epithelium as well. Furth et al. (1992) originally reported that direct injection of recombinant DNA into the mammary parenchyma, as opposed to intraductal intramammary infusion, with a needle and syringe failed to give detectable chloramphenicol acetyl transferase (CAT) expression in mammary tissue from late lactation ewes. However, the same group was subsequently able to detect hGH mRNA by RT-PCR following direct injection of naked recombinant DNA into the mammary parenchyma with a needle and syringe (Kerr et al., 1996). While direct
injection is one of the simplest gene transfer techniques, its inefficiency limits its practicality.

**Particle Bombardment**

Particle bombardment gene transfer is closely related to direct injection in that both make use of “naked” DNA or RNA, can be used in vitro or in vivo, and are applicable to a variety of tissues (Qiu et al., 1996). Briefly, DNA or RNA is precipitated onto inert gold or tungsten beads which are then propelled at high velocity into target organs, tissues, or cells (Klein et al., 1988).

Yang et al. (1990) reported transient gene expression in vivo from particle bombardment-mediated gene transfer into rat and mouse liver, skin, and muscle. The same group has also reported transient gene expression with particle bombardment of freshly isolated rat and human mammary ductal segments and primary cultures derived from these explants. Using similar particle bombardment, Thompson et al. (1993) transfected primary cultures of rat mammary epithelial cells which expressed the transfected transgene for five days post injection. Thompson et al. (1993) also reported particle bombardment transfection to be fivefold more efficient than lipofectin, calcium-phosphate co-precipitation, or electroporation. However, particle bombardment, like direct injection, can reach only a limited number of cells in vivo, can cause damage in surrounding tissue, and typically results in low and transient recombinant gene expression in animal organs.
Gene Gun Transfection

Gene gun-mediated transfection (also referred to as hydraulic jet injection) differs from particle bombardment in that the DNA or RNA is not coated onto gold or tungsten particles, but instead a solution containing the genetic material is injected by an air pressure propulsion system, as first described by Brandsma et al. (1991). Jet injection does not use any metal particles and is reported to penetrate deeper (3-5 cm) than particle bombardment (Kerr et al., 1996). Like particle bombardment, jet injection has been shown to give transient gene expression in numerous tissues (muscle, skin, fat) and in the mammary epithelium of mice, sheep, and swine (Furth et al., 1995; Furth et al., 1992; Kerr et al., 1996). However, Kerr et al. (1996) is the only group to report detection of recombinant gene expression in milk (1 ng hGH/ml) from jet injected animals (sheep).

Direct gene injection, as described above, will probably not be useful for altering mammary function or bulk milk composition in dairy animals since recombinant gene expression is strictly limited to the injection site.

However, because low level recombinant gene expression like that from direct injection often leads to the production of serum antibodies to the expressed recombinant protein in inoculated animals (Kerr et al., 1996 and references therein), direct injection techniques may be most useful for “genetic immunization” (Nabel and Felgner, 1993). Additionally, direct injection methods may be used to test expression constructs in vivo prior to the generation of transgenic animals.
Calcium Phosphate Coprecipitation

Calcium phosphate coprecipitation was originally used to facilitate infectivity of adenoviral DNA (Graham and van der Eb, 1973). Calcium phosphate-mediated transfection relies on the mixing of purified DNA with solutions containing phosphate and calcium ions to form fine DNA:calcium phosphate coprecipitates which sediment by gravity atop cells in culture. Although calcium phosphate:DNA coprecipiation can yield a high number of stably transfected cells (10-15%) (Chen and Okayama, 1987), its dependence on gravity is predicted to limit its use in vivo. Precipitate size, and therefore transfection efficiency, can be controlled by altering pH and DNA concentration in vitro (Keown et al., 1990). Graham and van der Eb (1973) described cellular internalization of calcium phosphate:DNA coprecipitates by phagocytic vesicles, presumed by most to be endocytosis. Non-secretory epithelial cells lining the teat and lactiferous sinuses of the bovine mammary gland (Brooker, 1983) and mouse mammary epithelium (Chandler et al., 1980) have been shown to phagocytose milk constituents and bacteria, respectively. Transfection complex internalization is facilitated by dimethyl sulfoxide (DMSO), polyethylene glycol (PEG) or glycerol shock in many cell lines (Rosenthal, 1987). Although virtually all cells internalized calcium phosphate:DNA complexes, very few contained detectable complexes in the nucleus (Loyter et al., 1982). Loyter et al. (1982) and Maes et al. (1967) also demonstrated early on that DNA contained in calcium phosphate:DNA coprecipitates was nuclease resistant.
Although a gravity-dependent transfection method like calcium phosphate coprecipitation is not likely practical for in vivo mammary transfection in large animals, it can be used effectively in vitro, particularly for generating populations of stably transfected cells (Chen and Okayama, 1987). Calcium phosphate coprecipitation has been used successfully on two mouse mammary cells lines (Comma-1D) (Danielson et al., 1989), and HC11 cells (Doppler et al., 1989), as well as primary mouse (Yoshimura and Oka, 1990), rat (Thompson et al., 1993), rabbit (Malienou-Ngassa et al., 1990), and bovine (Ahn et al., 1995) mammary epithelial cells. However, expression levels resulting from calcium phosphate-mediated transfection of mammalian cells are difficult to interpret since these experiments utilized the CAT reporter gene and were not quantitated.

Electroporation

Mediation of DNA entry into cells by an electric field, commonly referred to as electroporation, is easy, reproducible, and applicable to many cell types in vitro. However, there is some debate concerning how DNA actually enters the cell during electroporation. Keown et al. (1990) suggest that cell membranes subjected to transient electric fields with sufficient voltage undergo reversible partial membrane breakdown which creates transient pores in the membrane large enough for diffusion of macromolecules, including DNA into the cell. In contrast, Xie and Tsong (1993) claim that DNA in solution does not diffuse through pores in the membrane, but
describe electroporation as simply “the electrophoresis of surface bound DNA across the plasma membrane”. Regardless, electroporation results in the internalization of biologically active DNA which remains free in the cytosol and nucleoplasm (Bertling et al., 1987), making it an effective gene transfer method in vitro.

Electroporation has been used to introduce CAT and luciferase transgenes into primary mouse (Yoshimura and Oka, 1990), rat (Thompson et al., 1993), and bovine (Ahn et al., 1995) mammary epithelial cells in vitro. Thompson et al. (1993) report a maximum expression of 6 ng luciferase/mg total extractable protein in primary rat mammary epithelial cells, but CAT expression in primary mouse and bovine mammary epithelial cells was not quantitated (Ahn et al., 1995; Yoshimura and Oka, 1990).

Scrape, Syringe and Sonication Loading

Scrape, syringe, and sonication loading are additional in vitro gene transfer techniques that rely on the disruption of the cell membrane by physical force. Scrape loading involves scraping cells from a culture dish with a rubber policeman in the presence of DNA, while sonication utilizes high-frequency sound waves (Fechheimer et al., 1987). Additionally, a new technique, syringe loading of DNA, has been described in which a suspension of cells and DNA is repeatedly passed through a narrow bore needle (Waldman et al., 1996). These techniques have not been used in mammary cells.
**Laser-mediated Transfection**

Lasers have also been utilized to introduce exogenous DNA into mammalian cells in vitro. Kurata et al. (1986) first used a finely focused laser beam to create small holes in the cell membrane which if done in the presence of DNA in solution allows diffusion of DNA through the laser-created holes and entry of DNA into the perforated cells. These holes are self-sealing, presumably after DNA entry. Laser gene transfer allows the treatment of a large number of cells in a short time but must still be done to individual cells. Laser gene transfer has not been reported in mammary epithelial cells.

**Liposome and Cationic Liposome-mediated Gene Transfer**

Many reports describe lipid-mediated gene transfer into mammalian cells. Initial liposome transfection procedures utilized unilamellar phospholipid vesicles (liposomes) which contained DNA solution in the internal aqueous phase (Fraley et al., 1980). Since then, numerous permutations have been developed to test the effects of fatty acid length and saturation, phospholipid head group, and combinations of phospholipids on transfection efficiency and expression level.

Felgner et al. (1987) showed that cationic-lipid-mediated transfection using N-[1-(2,3-dioleyloxy)-propyl]-N,N,N-trimethylammonium chloride (DOTMA) is more efficient than transfection with uncharged lipids. Most liposome transfection mixtures also contain equimolar amounts of non-bilayer-preferring phospholipids like
dioleoloyl phosphatidylethanolamine (DOPE) which is reported to promote escape of DNA from endosomes (Treco and Selden, 1995). Some studies have included sterols, like cholesterol, to improve mechanical properties of the liposome lipid bilayers while others have created pH sensitive and fusogenic liposomes (Lasic and Templeton, 1996, and references therein). Fusogenic liposomes contain viral proteins that facilitate destabilization and fusion of lipid membranes, and thereby facilitate DNA release from endosomes or liposomes into the cytosol where it can be translocated into the nucleus for transcription (Brigham and Schreier, 1993).

The mechanism by which liposomes transfer DNA into cells is still contested. Evidence has been provided for a) direct liposome-plasma membrane fusion and delivery of liposome DNA into the cytosol (Felgner et al., 1987); b) transfer of lipid:DNA complexes across cell membranes into the cytosol (Bertling et al., 1991); and the most accepted explanation, c) fusion with or disruption of endosomes following endocytosis of lipid:DNA complexes (Friend et al., 1996, and references therein; Hui and Zhao, 1995; Wrobel and Collins, 1995; Zelphati and Szoka Jr., 1996). Cationic liposome:DNA complexes prevent nuclease degradation of DNA (Bertling et al., 1991) and have recently been combined with polycations (polylysine) to increase transfection efficiency (Gao and Huang, 1996; Mack et al., 1996).

Liposomes offer additional approaches in that they can also be used for ligand-targeted gene transfer. Select glycolipid ligands such as lactoslyceramide can be
included in the lipid bilayer to allow cell or tissue specific targeting and internalization of transfected DNA in vivo (Soriano et al., 1983).

Lipid-mediated transfection has been used in a mouse mammary epithelial cell line (HC11) to create a stable population of cells expressing a recombinant GFP fusion protein (Thonabulsombat et al., 1996) and to transiently transfect primary mouse and rat mammary epithelial cells in vitro (Thompson et al., 1993). Recombinant transgene expression efficiency and complexity of cationic liposome preparations continue to be major limitations to lipid-mediated gene transfer, and their cost is prohibitive for use in intramammary transfection of livestock.

Ligand-mediated Gene Transfer

Ligand-mediated gene transfer utilizes specific receptor-ligand interactions to attain cell and tissue specific gene delivery. This is accomplished by covalently crosslinking receptor-specific ligands either to cationic liposomes or polycations such as polylysine which then form electrostatic complexes with the DNA or polynucleotides being delivered. The best characterized ligand-targeted gene delivery system is the ligand-polylysine transfection system which targets transferrin (Wagner et al., 1990) or asialoglycoprotein receptors (Wu et al., 1989). An asialoglycoprotein-targeted CAT transgene was successfully delivered to rat hepatocytes following intravenous injection, demonstrating feasibility in vivo (Wu et al., 1989).
Recombinant gene expression from ligand-mediated gene transfer can be dramatically increased (~500 fold) (Zauner et al., 1995) by incorporating endosomolytic peptides into ligand-mediated gene transfer complexes (Wagner et al., 1992a). Endosomolytic peptide incorporation into ligand-mediated gene transfer complexes is presumed to facilitate recombinant gene expression by pore-formation in endosome membranes upon endosome acidification, allowing DNA to escape from the endosome into the cytosol (Wagner et al., 1992a; Zauner et al., 1995). Prchla et al. (1995) showed that the HRV2 lytic peptide from human rhinovirus could release 27% and 2% of 10 and 70 kDa dextrans, respectively, from isolated HeLa cell endosomes acidified to pH 5.5 in vitro, suggesting that release is via formation of size-limited pores as opposed to large-scale disruption of endosomal membranes.

Ligand-mediated gene transfer allows cell and tissue specific gene transfer, but also requires availability of suitable and specific cell surface receptors, which in some tissues may be developmentally or temporally regulated. Additionally, intravenous administration of ligand-targeted liposomes results in their rapid clearance from circulation unless coated with masking polyethylene glycol (L. Huang, personal communication, 1996). Ligand-mediated gene transfer has not been used to transfect mammary cells in vitro or in vivo.
Polyamine-mediated Gene Transfer

Many polyamines such as spermine, spermidine, polylysine, polyornithine, and DEAE have been used to facilitate polynucleotide transfer into mammalian cells in vitro. Like calcium phosphate:DNA coprecipitation, polyamines were originally used to enhance viral infection of cells and were subsequently found to be useful for transfection of DNA into mammalian cells (Keown et al., 1990).

Leng and Felsenfeld (1966) were the first to demonstrate that polylysine can reversibly bind DNA. Subsequently, Shapiro et al. (1969) determined that at subsaturating polylysine concentrations, polylysine binding to DNA was cooperative: binding of one polylysine chain with a DNA molecule makes it easier for successive polylysine chains to bind with the same DNA molecule. At saturating polylysine concentrations DNA was bound by polylysine with 1:1 stoichiometry (one lysine residue/nucleotide) and resulted in condensation of DNA:polylysine complexes into uniformly sized (40-100 nm diameter) highly soluble “torroids” whose size was independent of polynucleotide size (Arscott et al., 1990; Bloomfield, 1996; Shapiro et al., 1969). Approximately 90% of the DNA charge must be neutralized for condensation to occur (Arscott et al., 1990).

Eickbush and Moudrianakis (1978) subsequently reported the presence of rod-like DNA:polycation condensates (30 nm diameter and 200-300 nm length) in conjunction with the torroids described previously. Similarly, Maes et al. (1967) had
shown earlier that DEAE-dextran caused precipitation or colloid formation when mixed with nucleic acids.

Torroid and rod size is independent of DNA molecular weight (length) because a decrease in DNA molecular weight was compensated by an increase in the number of DNA molecules/torroid or rod (Arscott et al., 1990). Multimolecular DNA:polyamine torroids or rods contain an average of 13 ± 4 DNA molecules for condensates of 2700 base pair fragments and 26 ± 11 for those of 1350 base pair fragments (Arscott et al., 1990).

Pagano (1970) and Farber et al. (1975) propose that polyamines mediate gene transfer by both neutralizing negatively charged DNA, and by simultaneously interacting with the negatively charged cell surface. The charge interaction of cationic polyion:DNA complexes with the anionic cell surface probably explains how polyamines enhance DNA binding on the cell surface (Kabanov and Kabanov, 1995). Polyamine binding to the plasma membrane may serve as a “signal” to induce endocytosis by virtue of the effects on plasma membrane properties, including structure and ion permeability (Ohki and Duax, 1986; Takahashi et al., 1991; Walter et al., 1986). Furthermore, polylysine has been reported to induce pH-dependent fusion of acidic phospholipid vesicles (Walter et al., 1986). Polycation-induced endocytosis has been used to facilitate protein (albumin and horseradish peroxidase) (Shen and Ryser, 1978), drug (methotrexate) (Ryser and Shen, 1978), and oligonucleotide (Leonetti et al., 1990) internalization in cultured cells.
Although Borenfreund et al. (1973) proposed that DEAE facilitated DNA entry into cells without the DEAE itself entering the cell, there has been no subsequent evidence in the literature to support the proposed exclusion of DEAE entry. In contrast, Holter (1961) demonstrated that amoebae can be stimulated by simple addition of proteins to the extracellular environment to selectively internalize proteins by pinocytosis. Pinocytotic vesicles like those described by Holter (1961) can internalize macromolecules of up to 150 nm in diameter in cultured mammalian cells (Steinman et al., 1983). Therefore, pinocytotic vesicles should easily internalize the 40-100 nm DNA:polylysine torroids described by Shapiro et al. (1969) and Lasic and Templeton (1996). Given the large number (~2500 coated vesicles/fibroblast) and short lifespan (~1 min.) of pinocytotic coated vesicles (Goldstein et al., 1979), pinosome-mediated internalization of extracellular components and delivery to endosomes should be rapid. Additional support for pinocytotic internalization comes from Pagano (1970) and Loyter et al. (1982) who used polyamine and calcium phosphate DNA-coprecipitation, respectively, to enhance macromolecule uptake by erythrocytes and monkey kidney cells.

Polycations such as poly-L-ornithine (Orn$_n$), poly-L-lysine (Lys$_n$), and DEAE-dextran (DEAE) can also mediate efficient gene transfer into several mammalian cell lines (Bond and Wold, 1987; Davis et al., 1986; Farber et al., 1975), including mouse (Danielson et al., 1989) and bovine (Gorewit et al., 1987) mammary cell lines in vitro. Like calcium phosphate DNA-coprecipitation, polyornithine
(Farber et al., 1975) and DEAE (Loyter et al., 1982; Pagano, 1970) condensation of DNA also protects DNA from nuclease degradation.

Poly-L-ornithine enhanced the uptake and expression of exogenous DNA more reproducibly and to a greater extent than did DEAE or Lysₙ in Chinese hamster ovary (Dong et al., 1993) and lung (Farber et al., 1975) cells. Furthermore, polyornithine can mediate efficient transfection of non-replicating quiescent cells (Dong et al., 1993) as might be encountered in the mammary gland in vivo. Bond and Wold (1987) described a DNA:polyornithine mass ratio of 0.3:1 as optimal for poly-L-ornithine-mediated transfection of NIH 3T3 and L cells in vitro, and indicated that DNA:polyornithine complexes were less toxic to cells than polyornithine alone.

Polycations other than DEAE-dextran, which is toxic to some cells (Rosenthal, 1987), have not been used for gene transfer in mammary epithelial cells. DEAE-mediated transfection of primary mouse (Yoshimura and Oka, 1990) and rabbit (Malienou-Ngassa et al., 1990) mammary epithelial cells has been reported in vitro, with the latter producing hGH (−1 ng/ml) for 5 days post transfection. Gorewit et al. (1987) reported much more success with DEAE-mediated transfection of bovine mammary myoepithelial cell lines yielding up to 120 ng recombinant bovine growth hormone/ml medium.

Of the many gene transfer systems available, polyamines offer several advantages for intramammary gene transfer over other transfection systems. Polyamines are inexpensive, non-infectious, relatively non-toxic, non-gravity
dependent, easy to prepare, and simple to use. Polyamines should be capable of
delivery of recombinant transgenes to all cells indiscriminately via cell surface ionic
charge interactions (Farber et al., 1975; Pagano, 1970).

Limitations to efficient gene transfer into mammalian cells

Direct transfection of somatic cells in situ as proposed here for epithelial cells
within the mammary gland requires that DNA: a) be efficiently targeted for delivery
and uptake by the desired cell type; b) be protected from nuclease attack while in
transit to the target cell; c) be internalized; d) escape endosome or lysosomal
entrapment and degradation following internalization; and e) be translocated to the
nucleus in an expression competent form.

However, little is known of the mechanisms or details of recombinant gene
transfer and expression for many of the somatic gene transfer systems described
above, particularly for DNA internalization, the intracellular trafficking and
localization, and transgene fate and expression within the nucleus. Twenty two years
ago, Farber et al. (1975) pointed out the need for a comprehensive, definitive study
utilizing the same cell system which correlates a) transfection efficiency b)
intracellular fate of exogenous polynucleotides, and c) specific expression resulting
from the transferred transgene. Yet to date, such a study has not been reported and
only limited advances have been made in the understanding of gene transfer
mechanisms in the intervening 22 years.
DNA delivery to target cells

Presently, recombinant DNA transgenes have only been targeted to specific cell types using viruses, direct microinjection, and specific receptor-ligand interactions. Direct microinjection will likely not be feasible for large-scale recombinant protein production in the mammary gland in vivo as recombinant gene expression is strictly limited to the immediate injection site (Wolff et al., 1990; Wolff et al., 1991). The best characterized ligand-mediated transgene delivery systems utilized transferrin (Wagner et al., 1990) and asialoglycoprotein (Wu et al., 1989) to target hepatocytes both in vitro (Wagner et al., 1990) and in vivo (Soriano et al., 1983; Wu and Wu, 1988).

Direct intramammary infusion of recombinant transgenes into the mammary gland in vivo would present recombinant transgenes directly to the apical surface of mammary epithelial cells which are responsible for milk protein synthesis and secretion. Therefore, if polyamine-mediated transfection is dependent on ionic charge interaction with cell surfaces as proposed by Pagano (1970) and Farber et al. (1975), then polyamine:DNA complexes should ionically interact with virtually all mammary apical cell surfaces indiscriminately. Since the mammary epithelium and ductular epithelium are the only cells in the mammary gland which would be directly exposed to polyamine:DNA complexes by intramammary infusion, specific cell targeting should not be necessary with direct intramammary transfections unless
polyamine:DNA complexes bind anionic milk proteins or milk fat globule membranes, or cellular internalization of transfected DNA is inefficient.

Patton et al. (1984) and Keenan et al. (1974) have shown that concanavalin A (con-A), a plant lectin, can bind apical surfaces of lactating rat mammary cells by specific interaction with cell surface glycoproteins containing α-D-manno- or α-D-glucopyranoside residues (Lis and Sharon, 1973; Nicolson, 1974). Hence, concanavalin A or similar plant lectins might be used to target recombinant transgenes to mammary epithelium by direct intramammary infusion (Patton et al., 1984). Unfortunately, lectins, including con-A, appear to be mitogenic when bound to some cell surfaces (Akiyama et al., 1983; Barnett et al., 1974) and may also alter post-endosomal fate (Rabinowitz et al., 1992) of internalized lectin-targeted transfection complexes. Furthermore, other lectins such as wheat germ agglutinin occlude nuclear pores (Akey and Goldfarb, 1989) and would presumably block entry of DNA into the nucleus, thereby preventing transgene expression.

DNA protection from degradation

Although relatively little is known about DNA damage during transfection, Kao et al. (1973) have shown that bacteriophage T7 DNA undergoes extensive degradation following internalization by human skin fibroblasts. For gene transfer techniques that deliver DNA directly to the nucleus (microinjection) DNA degradation is not a major concern. In contrast, gene transfer techniques utilizing exposed DNA
(particle bombardment, gene gun, laser, and electroporation) may be more susceptible to DNA degradation by extracellular or intracellular extranuclear nucleases.

In general, gene transfer techniques that form DNA:carrier complexes provide protection from nuclease degradation. For example, calcium phosphate coprecipitation (Loyter et al., 1982), DEAE (Loyter et al., 1982; Maes et al., 1967; Pagano, 1970), Orn
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(Farber et al., 1975), and cationic liposomes (Bertling et al., 1991) have all been reported to protect DNA from nuclease degradation in vitro. However, neither Orn
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or DEAE completely protect DNA from nuclease degradation. Farber et al. (1975) reported that only 25-30% of Orn
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-complexed DNA became nuclease resistant when Orn
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-DNA complexes were used to transfect cells in vitro. Similarly, Wake et al. (1984) showed that DEAE-transfected DNA suffers one break per 5-15 kilobase pairs of transfected DNA. Hence, DNA degradation either en route to the cell or intracellularly may be a major limitation to mammalian cell transfections.

DNA internalization

Direct microinjection, particle bombardment, gene gun, and viral-mediated gene transfer rely on physical penetration of DNA through the cell plasma membrane and into the cytosol to achieve cell entry of DNA, while other methods such as electroporation or laser-mediated gene transfer rely on diffusion through pores created in the plasma membrane (Kurata et al., 1986). The predominate mechanism for entry of DNA into cells for ligand-targeted (Wagner et al., 1994), cationic liposome
(Zabner et al., 1995), or polyamine-mediated (Kabanov and Kabanov, 1995, and references therein; Marciano-Cabral et al., 1981) gene transfer appears to be endocytosis. In contrast, Loyter et al. (1982) suggest phagocytosis is responsible for calcium phosphate:DNA complex entry into murine fibroblast L cells (Ltk'Apff) in vitro.

**DNA escape from endosomes and/or lysosomes**

Following in vitro (Mislick et al., 1995; Rosenkranz et al., 1992) and in vivo (Chowdhury et al., 1993) ligand-targeted DNA internalization, DNA may become entrapped in endosomes or lysosomes. Similar endosomal entrapment has been reported for cationic liposome-mediated gene transfer both in vitro (Wrobel and Collins, 1995; Zabner et al., 1995) and in vivo (Cudd and Nicolau, 1985). Zabner et al. (1995) found that endocytosed cationic liposome:DNA complexes showed perinuclear localization with subsequent fusion of complexes into large aggregates in transfected COS cells. Similar aggregation has been observed for internalized Orn_\text{n}:DNA transfection complexes (Marciano-Cabral et al., 1981). Therefore, DNA escape from endosomes and/or lysosomes appears to be a major limitation for mammalian transfection from DNA internalized by endocytosis.

However, polylysine has been reported to induce aggregation and pH-dependent fusion of acidic phospholipid vesicles (Walter et al., 1986). Polylysine-induced vesicle fusion is peptide (Lys_n) size-independent, and increases as the pH is
lowered toward pH 3.0 with an apparent pKa near pH 4.0 suggesting the pH-dependence resides in the lipids rather than in polylysine (Walter et al., 1986). Apparently, fusion of acidic phospholipid vesicles occurs following polylysine-induced vesicle aggregation and neutralization of membrane charge through polylysine binding or phospholipid protonation (Walter et al., 1986).

Incorporation of viral lytic peptides (capable of perturbation or disruption of lipid membranes in vitro (Prchla et al., 1995)) into polycation-ligand transfection complexes dramatically increased (~500 fold) recombinant gene expression in ligand-targeted (Wagner et al., 1992a; Wagner et al., 1992b; Zauner et al., 1995) gene transfer in vitro.

The lysomotropic agent, chloroquine, has also been used to facilitate DNA escape from endosomes and/or lysosomes, presumably by buffering the contents of membrane bound endosome vesicles so as to induce their osmotic swelling and eventual lysis to release intraendosomal transfected DNA into the cytoplasm (Kukowska-Latallo et al., 1996; Mislick et al., 1995).

DNA translocation to the nucleus

Although DNA entry into cells and escape from endosomes/lysosomes are critical to efficient recombinant gene expression, the greatest barrier to efficient recombinant gene expression may be DNA entry into the nucleus. The importance of DNA entry into nuclei is readily apparent from the early demonstration by Capecchi
(1980) that injection of plasmid DNA into the nuclei of cells from a mouse cell line led to recombinant gene expression in 50-100% of cells, whereas injection of plasmid DNA into the cytoplasm led to expression in < 0.01% of cells. Zabner et al. (Zabner et al., 1995) reported similar results when a secreted alkaline phosphatase transgene was injected into the nucleus and cytoplasm of *Xenopus oocytes*. Unfortunately, neither study excluded the possibility that DNA injected into the cytosol may have been degraded before it could translocate into the nucleus (Capecchi, 1980).

DNA entry into cell nuclei is also a major limitation to liposome (Cudd and Nicolau, 1985) and cationic liposome-mediated transfection (Zabner et al., 1995). Cudd et al. (1985) showed that only 14% of liposome-mediated transfected DNA was localized within the nucleus. Strain et al. (1984) observed similar inefficiency of nuclear localization of DNA (4%) following transfection of CV-1 cells with calcium phosphate:DNA coprecipitation in vitro.

In contrast, Farber et al. (1975) showed that 95% of internalized Orn-transfected DNA was rapidly (15 min.) localized to the nuclear fraction. Such rapid and complete plasmid nuclear localization suggests that polycations may mimic nuclear localization signals, often identified as repeated short tracts of basic amino acids in proteins destined for nuclear localization (Davis, 1992). For example, the SV40 large-T antigen nuclear localization signal consists of eight amino acids (Pro Lys Lys Lys Arg Lys Val Glu) (Colledge et al., 1986; Kalderon et al., 1984). Consensus sequences for nuclear localization signals are not highly conserved, and can
consist of repeated but non-contiguous regions of basic amino acids (Davis, 1992, and references therein). However, these observations are inconsistent with Marciano-Cabral et al. (1981) who observed large cytoplasmic accumulations of $^3$H-radiolabeled DNA following Orn$_n$-mediated transfection. Inclusion of the SV40 large-T antigen nuclear localization signal in polycation-based transfection complexes facilitates rapid nuclear accumulation of transfected DNA which can be seen passing through the nuclear pore (Malecki and Skowron, 1995).

**Regulation of transgene expression**

Upon nuclear internalization, heterologous recombinant transgenes become subject to the same transcriptional and translational constraints as endogenous gene expression, including but not limited to initiation of transcription and translation, mRNA 5'-capping (Shatkin, 1987) and polyadenylation (Friedman et al., 1987), mRNA localization (St. Johnston, 1995; Wilhelm and Vale, 1993), and mRNA degradation (Beelman and Parker, 1995; Sachs, 1993). However, expression of heterologous transgenes may also be subject to other regulatory constraints. For instance, in vitro heterologous transgene expression can be inhibited by methylation of promoters such as CMV (Prosch et al., 1996) and SV40 (Muiznieks and Doerfler, 1994), or methylation within the transcribed region of the reporter gene (Komura et al., 1995). DNA can become methylated in either bacterial amplification systems, or
eukaryotic cells cultured in vitro (Alberti and Herzenberg, 1988), or cells in vivo (Gibbs et al., 1994).

Association of recombinant transgene DNA with nuclear DNA binding proteins or chromatin structure may also be important for recombinant transgene expression. For instance, chromatin is less condensed in areas that are transcriptionally active (Yaniv and Cereghini, 1986). Chromatin condensation presumably prevents interaction with transcription factors, thereby inhibiting transcription of genes within condensed DNA. While polyamines also condense DNA (Leng and Felsenfeld, 1966), Wagner et al. (1991) showed that condensation of DNA in ligand-polycation:DNA complexes was essential for efficient endocytosis and expression. Nevertheless, polycations will likely also have to dissociate from DNA intracellularly prior to initiation of transcription. Zabner et al. (1995) showed that dissociation of DNA:cationic liposome complexes was important for efficient transgene expression in COS cells in vitro. Hence, the foregoing suggests that efficient transgene expression will require polycation-mediated condensation of DNA for its cellular uptake and nuclear delivery, and subsequent dissociation of DNA from its complex with polycations to allow access to transcriptional factors in the nucleus.

Additionally, there is a growing body of evidence suggesting that cDNAs are not expressed as efficiently as complete gene sequences containing introns. The presence of an intron, even a heterologous intron, located near the 5' end of the transgene appears to be advantageous for its expression (Brinster et al., 1988). These
intervening intron sequences are reported to increase the efficiency of RNA 3' splicing (Huang and Gorman, 1990) leading to an increase in the levels of both nuclear and cytoplasmic poly(A)+ RNA (Buchman and Berg, 1988).

Recombinant gene transfer and expression in somatic cells in vitro is a complex process compounded by transfection method and cell-specific differences. Recombinant gene transfer and expression in somatic cells in vivo is even more difficult, as many tissues are either inaccessible, do not readily internalize DNA, or will not express high levels of the recombinant transgene.

Polyamine-mediated transfection of primary bovine mammary cells in vitro should identify potential transfection methods and expression vectors to be tested in the mammary gland in vivo. Similarly, transfection of mammary cells in vitro should also allow study and optimization of transfection complex composition, internalization, subcellular distribution, and the regulation of recombinant gene expression to identify potential rate-limiting steps to polyamine-mediated transfection in vitro or in vivo.
CHAPTER 3

MATERIALS AND METHODS

Materials

Transformation Competent Bacterial Host Cells

Transformation competent *Escherichia Coli* strains were purchased as follows. TOP 10 F’ (for pSE380 superlinker vector and derivatives), MC1061/P3 (for pcDNA1 eukaryotic expression vector and derivatives), NM522 (for pCEP4 eukaryotic expression vector and derivatives), and TOP 10 (for pCMV/EBNA eukaryotic expression vector) were purchased from Invitrogen (San Diego, CA). JM109 (for pGEM cloning vector and derivatives) was from Promega Corp. (Madison, WI). XL1-Blue (for pBS cloning vectors and derivatives) was obtained from Stratagene (La Jolla, CA). NovaBlue (for pCITE4 expression vectors and derivatives) was from Novagen (Madison, WI). DH5α (for pCMV/β eukaryotic expression vector) was from Clontech Laboratories, Inc. (Palo Alto, CA).
Eukaryotic plasmid expression vectors

Eukaryotic expression vectors pcDNA1, pCEP4, pCMV/EBNA, and cloning vector pSE380 were purchased from Invitrogen (San Diego, CA). pCMV/β was from Clontech (Palo Alto, CA). Cloning vectors pGEM7ZF(-) and pBSKS+ were from Promega (Madison, WI) and Stratagene (La Jolla, CA) respectively. pCITE-4B was from Novagen, Inc. (Madison, WI). pGP1-2 was a gift from Dr. Charles Brooks (Ohio State University).

Cell Lines

Adenovirus type 5 transformed primary human embryonic kidney cells passage 30, (293 cells); SV40-transformed African green monkey kidney cells passage unknown, (COS-1 cells); Human hepatocellular carcinoma cells passage 75, (Hep G2); Madin-Darby bovine kidney cells passage 112, (MDBK); and Madin-Darby canine kidney cells passage 52, (MDCK) were purchased from American Type Culture Collection (Rockville, MD). Comma-D mouse mammary cell line (Danielson et al., 1989), passage 22, were kindly provided by Dr. Craig Baumrucker (Pennsylvania State University). Primary bovine mammary cells were prepared and cultured as described by Talhouk et al., (1990; 1992).
**Amino Acids and Peptides**

Poly-L-glutamic acid sodium salt (15-50 kDa) (Gluₙ), poly-L-lysine hydrobromide (30-70 kDa) (Lysₙ), poly-L-ornithine hydrobromide (43-200 kDa) (Ornₙ), L-methionine was obtained from Sigma Chemical Co. (St. Louis, MO). Fmoc-Cys(Trt)-NovaSyn® TGA resin was purchased from Novabiochem (San Diego, CA). Synergy™ amino acid columns were purchased from Perkin Elmer Corp. (Foster City, CA).

**Nucleic Acids, Oligonucleotides, and Nucleotides**

Deoxynucleoside triphosphates, and dideoxynucleoside triphosphates were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). (Alpha-³²P) deoxyadenosine 5'-triphosphate tetratriethylammonium salt (α-³²P-dATP) was obtained from DuPont (Boston, MA). (Alpha-³²P) deoxycytidine-5'-triphosphate tetratriethylammonium salt (α-³²P-dCTP) was purchased from ICN Biomedicals, Inc. (Irvine, CA). Oligonucleotides were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX).

**Enzymes**

Calf intestinal alkaline phosphatase (CIAP) was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). *E. coli* DNA polymerase I (Klenow enzyme, large fragment), T4 DNA Ligase, and Tth or Tfl thermostable DNA polymerases were obtained from Promega Corp. (Madison, WI). Restriction endonucleases were purchased from
either Boehringer Mannheim Corp. (Indianapolis, IN), Promega Corp. (Madison, WI), or New England Biolabs (Beverly, MA). Sequenase Version 2.0 Sequencing kit was purchased from United States Biochemical (Cleveland, OH).

**Equipment**

Model TJ-6 and J2-21M centrifuges and model 35 spectrophotometer were from Beckman Instruments, Inc. (Arlington Heights, IL). Cell culture incubators were from Queue Systems, Inc. model 2210 (North Branch, NJ) or Forma Scientific model 3185 (Marietta, OH). A Bacharach Fyrite Gas Analyzer was obtained from Bacharach United Technologies (Pittsburgh, PA). The inverted IMT microscope, model C-35A2-2, 35mm camera, and PM-10ADS automatic photomicrographic system were obtained from Olympus Corp. of America (New Hyde Park, NY). The enzyme immunoassay 96 well plate autoreader model EL 310 was purchased from Bio-Tek Instruments, Inc. (Winooski, VT). The Tracor Analytic model 6892 liquid scintillation system was obtained from TM Analytic, Inc. (Elk Grove Village, IL). Applied Biosystems 432A peptide synthesizer was from Perkin Elmer (Foster City, CA). Gel Dryer model 583 and Sequi-gen nucleic acid sequencing unit were from Bio-Rad Laboratories (Richmond, CA). GNA-100 horizontal gel apparatus was obtained from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). TempCycle model 50 thermocycler was from Coy Laboratory Products Inc. (Grass Lake, MI). The Super Sub-HE100 electrophoresis unit was from Hoefer
Scientific Instruments (San Francisco, CA). The Cobra model 5005 auto-gamma counting system was purchased from Packard Instrument Co. (Meriden, CT).

Reagents

Acetylated Bovine Serum Albumin (BSA), Prime-A-Gene Labeling kit, and Wizard miniprep DNA purification kits were obtained from Promega Corp. (Madison, WI). Acrylamide, ammonium acetate, ammonium chloride, ammonium persulfate, ampicillin (amp), beta-mercaptoethanol, bromophenol blue, boric acid, DEAE-dextran (500 kDa) (DEAE), D-glucose, D-sorbitol, dimethyl sulfoxide (DMSO), ethidium bromide, ethylenediaminetetraacetic acid (EDTA), fluorescein isothiocyanate Isomer I (FITC), formamide, glutaraldehyde, glycerol, N-(2-Hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), hexamminecobalt (III) chloride, histamine, horse serum, insulin (from bovine pancreas), luteotropic hormone (prolactin) from sheep pituitary glands (Prl), magnesium acetate, magnesium chloride, magnesium sulfate, manganese chloride, (2-[N-morpholino] ethanesulfonic acid) monohydrate (MES), mineral oil, N,N’-methylene-bis-acrylamide, nonidet P-40, paraformaldehyde, polyethylene glycol (8 kDa), polyoxyethylene sorbitan monolaurate (Tween 20), potassium ferrocyanide, potassium chloride, QFITC (XRITC) a quinolizino-substituted fluorescein isothiocyanate, rubidium chloride, sodium acetate (NaOAc), sodium lauryl sulfate (SDS), sodium phosphate (Dibasic, Anhydrous and Monobasic), α-D-glucopyranosyl β-D-fructofuranoside (sucrose), N,N,N’,N’-tetramethylethylene-diamine (TEMED), tetracycline (tet),
holo-transferrin, trizma base, X-OMAT XR5 X-Ray films, polymixin B immobilized on agarose beads and xylene cyanole FF were purchased from Sigma Chemical Co. (St. Louis, MO). Agarose (Ultra Pure: electrophoresis grade was from either Gibco-BRL Life Technologies (Gaithersburg, MD) or Amresco (Solon, OH). Bacto-Agar, Bacto-Peptone, Bacto Yeast Extract, glycerol, isopropyl β-D-thiogalactopyranoside (IPTG), sterile polypropylene tubes (15 and 50 ml), 5-bromo-4-chloro-3-indoly-l-β-D-galactopyranoside (X-gal), were obtained from Fischer Scientific (Pittsburgh, PA). Chloroform, formaldehyde solution, isopropyl alcohol, potassium acetate, potassium ferricyanide, potassium phosphate dibasic anhydrous, potassium phosphate monobasic crystals, were obtained from Mallinckrodt (Paris, KY). Compressed carbon dioxide (CO₂) bone dry grade was from Perry Corp. (Wooster Welding) (Akron, OH). Sulforhodamine 101 acid chloride (Texas Red) was purchased from Molecular Probes Inc. (Eugene, OR). 4-pregnen-11β,17α,21-triol-3,20-dione (cortisol) was purchased from Steraloids, Inc. (Wilton, NH). Dulbecco’s Modified Eagles’s Medium (DMEM) with 4.5g/L glucose and L-glutamine, Minimum essential medium (Eagle) with Eagle’s balanced salt solution (EMEM) with non-essential amino acids and sodium pyruvate, fetal bovine serum (FCS), fungizone, gentamycin sulfate, Ham’s F-12 with L-glutamine, Hanks’ balanced salt solution (HBSS) with calcium, magnesium and phenol red (1x and 10x), Medium 199 (M199) with Earle’s Balanced Salt Solution, sodium bicarbonate and L-glutatamine, trypsin-versene mixture were purchased from BioWhittaker, Inc. (Walkersville, MD). 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDAC) was purchased
from Pierce (Rockford, IL). Epidermal growth factor (receptor grade) and mouse laminin were from Collaborative Biomedical Products (Bedford, MA). Absolute Ethyl alcohol was purchased from Quantum Chemical Corp. (Tuscola, IL). Four well tissue culture plates were from Nunc, Inc. (Naperville, IL). Twentyfour well tissue culture clusters were purchased from either Belloco (Vineland, NJ) or Costar (Cambridge, MA). Falcon 25 and 75 cm² tissue culture flasks were from Becton Dickinson Labware (Lincoln Park, NJ). GeneScreen plus nylon membrane was from DuPont (Boston, MA). Guanidine isothiocyanate (GITC) was purchased from Fluka Chemika-BioChemika (Ronkonkoma, NY). Kodak NTB-3 nuclear emulsion, Kodak Dektol developer and fixer were from Kodak Scientific Imaging Systems (New Haven, CT). Limulus Amebocyte Lysate assays (LAL) (Pyrotell) were purchased from Associates of Cape Cod, Inc. (Woods Hole, MA). Phenol (Ultra Pure) was purchased from either Gibco-BRL Life Technologies (Gaithersburg, MD), Amresco (Solon, OH), United States Biochemical (Cleveland, OH) or Fisher Scientific (Pittsburgh, PA). N,N-diisopropylethylamine (DIEA)/dimethyl sulfoxide (DMSO)/NMP mixture, [2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HBTU), 1-hydroxybenzotriazole (HOBt)/dimethyl sulfoxide (DMSO)/NMP mixture, and pipridine were from Perkin Elmer Corp. (Foster City, CA). Nunctrap Push columns were from Stratagene (La Jolla, CA). Nusieve GTG and SeaPlaque GTG agarose were from FMC Corporation Bioproducts (Rockland, ME). TMB microwell peroxidase substrate system was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Vecastain ABC kits were from Vector Laboratories.
Inc. (Burlingame, CA). Lipofectin was from Gibco-BRL Life Technologies (Gaithersburg, MD).

All reagents were molecular biology or cell culture grade unless otherwise indicated. All water was ultrapure and endotoxin free after ion exchange and reverse osmosis filtration (Nanopure, Barnstead Co., Boston, MA) and autoclave sterilized unless specified otherwise.
Cell Culture Methods

Casting Collagen Gels

Type I collagen gels like those used for mouse mammary cell culture by Emerman and Pitelka, (1977), and for bovine mammary acinar cell culture (Baumrucker et al., 1988) were cast as described by Talhouk et al. (1990). Briefly, tendons were stripped from 4-5 adult rat tails, minced into 1 mm pieces and digested in 17.5 mM glacial acetic acid (Bornstien, 1958), 1/1000 v/v, pH 3.25 (0.0175 M/g of original rat tail weight) for 48 hrs at 4°C with continuous gentle stirring. The solution was centrifuged (4000 x g for 30 min. at 4°C), the upper 1/3, watery portion of the solution was discarded and the remainder of the supernatant was centrifuged (5000 x g for 30 min. 4°C) and clear supernatant was collected and supplemented with Gentamycin (50 μg/ml) and Fungizone (2.5 μg/ml) and protein concentration was determined by BCA protein assay. Protein concentrations ranging from 2-3 mg/ml gave gels of acceptable consistency. Gels were cast by mixing 13.4 ml rat tail collagen solution in 0.17 M HAc, 0.72 ml 10x HBSS and 1.2 ml 0.3 N NaOH on ice and applying 0.5 ml/2 cm². Gels were placed at 37°C for 30 min. then covered with 1 ml 1x HBSS/well until use.

Cell Culture

Comma-D mouse mammary cells, kindly provided by Dr. Craig Baumrucker (Pennsylvania State University), were cultured according to Danielson et al. (1989) in 24 well plates in a 1:1 mixture of DMEM:Ham's F-12 with 50mM HEPES pH 7.2,
supplemented with transferrin (10 μg/ml), insulin (10 μg/ml), selenium (0.01 μg/ml), EGF (0.02 μg/ml), and gentamycin (25 μg/ml). Cells were maintained at 37°C in 5% CO₂, 95% air with changes of media every 48 hours.

Lactating primary bovine mammary cells were cultured as described (Talhouk et al., 1992) in 5% CO₂, 95% air at 37°C or 39°C on plastic or Type I rat tail collagen in a 1:1 mixture of M199:Ham’s F-12 supplemented with 15% FCS and laminin (8 μg/ml) for plating and growth. Two days post plating, primary bovine mammary cells were switched to serum free medium supplemented with lactogenic hormones; insulin (5 μg/ml), cortisol (5 μg/ml), prolactin (1 μg/ml), L-methionine (5 μg/ml), and gentamycin (50 μg/ml), with change of medium every 48 hours. Collagen substratum was detached immediately after transfection on day 6 postplating by rimming with a glass pipette to induce cell differentiation (Talhouk et al., 1992).

Hep G2, human hepatocyte carcinoma cells were cultured in 90% EMEM with Earle’s BSS, non-essential amino acids, and sodium pyruvate, 10% FCS, and gentamycin (25 μg/ml). 293 human embryonal kidney cells were cultured in 90% EMEM, 10% horse serum, supplemented with L-glutamine (292 g/l), and gentamycin (25 μg/ml). Cos-1 African green monkey kidney cells were cultured in 90% DMEM, 10% FCS and gentamycin (15 μg/ml). Madin-Darby canine kidney cells were cultured in 90% EMEM with Earle’s BSS, 10% FCS, and gentamycin (25 μg/ml). Madin-Darby bovine kidney were cultured in 90% EMEM with Earle’s BSS, 10% horse serum, and gentamycin (25 μg/ml). All cells were cultured in a 5% CO₂, 95% air, water saturated atmosphere
maintained at 37°C or 39°C as indicated. Spent culture medium was aspirated from wells at 48 hr feeding intervals, and stored at -80°C until assayed for proteins of interest.

Transfection

All cell lines were transfected two days after plating on plastic as cells approached confluence, while primary bovine mammary cells were transfected on day 6 postplating on Type I collagen substratum, with release of collagen substratum to induce differentiation (Talhouk et al., 1992) immediately following removal of transfection medium. Transfections were done by addition of 0.2 ml transfection medium containing polyion:DNA complexes to cells in 2.0 cm² wells from which spent medium had been removed by aspiration immediately before transfection. Transfection medium was left in contact with cells for 4 hrs at 37°C unless specified otherwise. Transfection complexes were prepared by mixing plasmid DNA with combinations of the following polyions: DEAE-Dextran (DEAE) (460 kDa), poly-L-Ornithine (Orn⁵) (141 kDa), poly-L-Lysine (Lys₅) (50 kDa), or poly(glutamylhistamineglutamate) (Glu₅-HisN) (45 kDa) formed in either 0.3 M sucrose, 10 mM HEPES, pH 7.4, or HBSS. Unless otherwise indicated, the concentrations of polyions used were: Orn⁵, 16.6 or 37.5 μg/ml; DEAE 12.5 μg/ml; Lys₅, 8.3 μg/ml; and Glu₅-HisN at 75 μg/ml. When used as the sole polycation, DEAE-Dextran was used at 250 μg/ml (Davis et al., 1986). Plasmid DNA was used at 5 μg/ml unless indicated otherwise. Lipofectin was used at 25 μg/ml per the manufacturer's instructions (Gibco Brl) (Murray, 1991). Transfection solutions were mixed following addition of
each component (at ~ 2 min. intervals), and applied to cells within 10 minutes after addition of the last component. At the end of the 4 hour transfection period, transfection medium was removed by aspiration and cells were fed with 0.5 ml complete medium, and fed each 2 days thereafter. Spent culture medium was analyzed for recombinant hGH gene expression by RIA (Nichols Institute) or ELISA.

**Visualization of Nuclei**

Nuclei were visualized after staining fluorescent blue with 0.2 ml DAPI stain solution (10 μg DAPI/ml, 98% PBS, 1% methanol, 1% DMSO) applied to cells at 37°C for 2 hrs. The DAPI staining solution was then removed and cells were fed 0.5 ml of the appropriate culture media. Labeled nuclei were visualized by fluorescent microscopy.

**Cellular Uptake and Subcellular Localization of Transfected Plasmid**

Cellular uptake and subcellular distribution of transfected radiolabeled plasmid DNA was determined by recovery of ³H-Thymidine or ³²P-labeled plasmid DNA from transfected cells, as modified from Ausubel et al. (1994). Cells were transfected as previously described with radiolabeled plasmid DNA. Following the four hour transfection period, spent transfection medium was aspirated (post transfection medium) and cells were washed with 200 μl HBSS (Ca and Mg free, pH 7.4) before trypsinization for 15 min. at 37°C to release cells from the culture plates. After centrifugation of the trypsinized cell suspension (12,000 x g, 30 sec), the cell free supernatant was collected.
by aspiration (trypsinization supernatant) and the pelleted cells were washed twice by resuspension in 200 μl HBSS (Ca and Mg free, pH 7.4) and centrifugation as before (washes 1 and 2). Washed cell pellets were then resuspended in cell lysis buffer (0.5% Nonidet P-40, 0.5 mM dithiothreitol, 0.14 M NaCl, 1.5 mM MgCl₂, 10 mM Tris, pH 8.6), from which an aliquot was removed for analysis of the whole cell fraction (cell), and the remaining cell suspension was vortexed for 15 sec. and placed on ice for 5 min. to allow cell lysis and release of intact nuclei. Cell lysates were centrifuged at 12,000 × g for 10 sec. to yield nuclear (pellet) and cytosolic (supernatant) subcellular fractions. For determination of radiolabeled plasmid distribution by liquid scintillation counting, all fractions (pre- and post-transfection medium, trypsinization supernatant, washes 1 and 2, cell, cytosolic, and nuclear fractions) were dissolved in 200 μls 0.5 M NaOH. Cytosolic lactate dehydrogenase (LDH) analysis of nuclear fractions confirmed their purity from cytosolic contamination.

**β-galactosidase Detection**

β-galactosidase expression was detected using the histochemical assay described by MacGregor et al. (1991), with minor modifications. Cultured mammary cells were transfected as described in methods and examined for β-galactosidase expression at selected times post transfection as follows. Cell culture media was aspirated and cells were rinsed twice with 1 ml PBS. Transfected cells were fixed with 2% paraformaldehyde, 0.2% gluteraldehyde in 0.5 M Na₂PO₄, pH 7.3, 200 μl per well for 3
min. at 4°C, and washed twice with PBS as described above. Color development was initiated by addition of 200 μl X-Gal stain (100 mM Na₂PO₄, pH 7.3, 1.3 mM MgCl₂, 3 mM K₃Fe(CN)₆, 3 mM K₄Fe(CN)₆, 0.5 μl NP-40, 2.4 μm X-Gal in N,N-dimethyl formamide) per well and incubated at 37°C until development of suitable color.

Radioimmunoassay for human growth hormone

Radioimmunoassays for hGH reporter gene expression in milk were performed according to manufacturers instructions (Nichols Institute Diagnostics). Briefly, 100 μl skim milk sample was mixed with 100 μl ¹²⁵I-hGH monoclonal antibody, biotin-hGH monoclonal antibody solution in a 12 x 75 mm round bottom plastic tube and incubated with an avidin coated polystyrene bead for 4 hrs. at room temperature. Beads were washed twice with 2 ml of the wash solution provided in the kit before quantification of ¹²⁵I by gamma radiation counters.

Enzyme-Linked Immunosorbant Assay (ELISA)

Concentrations of specific endogenous and transfected recombinant secreted proteins were determined by sandwich enzyme-linked immunoadsorbant assay (ELISA) with biotin-avidin-horseradish peroxidase coupling and color detection as described by Talhouk et al. (1990). Antibodies were polyclonal IgG fractions purified from monospecific rabbit (LF, αs₁-casein, hGH, WAP, MFGM, α-lac), sheep (αs₁-casein) or...
goat (hTF) as described by Harboe and Ingold, (1973), and biotinylated with N-hydroxysuccinimidobiotin in dimethyl formamide according to Guesden et al. (1979).

Ninety-six well microtiter plates were coated with 50 µl/well of 10 µg/ml polyclonal IgG specific for the protein of interest in coating solution (0.05 M NaHCO₃, pH 9.5) for 1 hour. After aspiration of the primary antibody coating solution, non-specific binding was blocked by addition of 200 µl 1% heat-treated nonimmune rabbit serum (HTRS) in TBS (20 mM Tris-HCl, 0.145 M NaCl, pH 7.4) to each well for 10 min and washed 3 x with TBS. Aliquots (75 µl) of culture medium, milk samples, or protein specific standards were added to each well after dilution with (5X premix) 0.1 M Tris-HCl, 0.85% NaCl, 0.02% NaN₃, and 1% HTRS, pH 7.4 for all protein assays except αₛ₁-casein, which contained additional 1.6 M urea, and 1% Dextran T-70. For analysis of media from cultures with FCS, the above ELISA diluent was supplemented with 10% FCS to adjust both samples and standards to uniform FCS concentrations. ELISA protein standards were prepared (10x diluent) in 20 mM Tris, 0.85% NaCl, 1% HTRS, 0.02% NaN₃, pH 7.4. Duplicate samples and standards were incubated for 1 hr. or overnight as indicated, then wells were washed 4 x with TBS and incubated with 75 µl of 10-12 µg/ml biotinylated polyclonal IgG specific for the protein of interest for 30 min. After washing (3 x TBS), 60 µl of freshly prepared ABC reagent (prepared per manufacturer’s instructions, Vector Labs) was added to each well and incubated 15 min. Unbound ABC complex was removed by washing (5 x TBS) and 100 µl TMB peroxidase substrate solution containing 3,3',5,5'-tetramethylbenzidine was added to each well for color
development. Absorbance was read at 690 nm on a Bio-Tek EL310 automated microplate reader. All coating, washing, and incubation steps were done at room temperature unless noted otherwise.

Molecular Biology Methods

Competent Cell Preparation

Competent *E. Coli* were prepared as described by Hanahan, (1983). Briefly, pure *E. coli* cultures were streaked on SOB agar plates and incubated overnight at 37°C. An isolated colony was transferred to a 2 liter fluted flask containing 500 mls SOB medium and grown with shaking (200 rpm) until O.D. is 0.500 - 0.550. Cells were then transferred to 250 ml centrifuge bottles, cooled on ice 30 min., and centrifuged at 2500 rpm for 15 min. Cell pellets were resuspended in 20 ml ice-cold FSB (100 mM KCl, 45 mM MnCl₂, 3 mM HACOCl₃, 10 mM CaCl₂, 10% (w/v) redistilled glycerol, pH 6.4) iced for 15 min. and centrifuged as above. Cells pellets were resuspended in 3.5 mls ice-cold FSB and 0.35 ml DMSO and iced for 5 min. An additional 0.35 ml DMSO was added and cells iced for an additional 10 min. Cells were aliquoted in 1.5 ml sterile microcentrifuge tubes and stored at -80°C until use.

Bacterial Transformation

Transformation of all competent cells was performed according to the Stratagene Instruction Manual protocol for transformation of Epicurian Coli, catalogue # 200233,
with minor modifications. Briefly, 20 µl of competent cells per transformation were treated with freshly diluted beta-mercaptoethanol (final concentration 25 mM) for 10 min. on ice, with mixing every 2 min. in sterile polypropylene tubes. DNA (0.1-50 ng/transformation) was then added, followed by gentle mixing and incubation on ice for 30 min. The transformation mixture was heat shocked by immersion in a 42°C water bath for exactly 50 seconds and placed on ice for 2 min. 200 µl of sterile SOC medium (20 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g NaCl in one liter of water, and to which glucose was added to 40% after autoclaving; (Maniatis et al., 1989)), was added to transformation mixtures prior to incubation for 1 hour at 37°C in an orbital shaker (250 rpm). Transformation mixtures were plated on Luria-Bertani (LB) medium (10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl in one liter of water with pH adjusted to 7.0) (Maniatis et al., 1989), with 1.5% agar plates containing appropriate antibiotics for selection of transformed plasmids encoding antibiotic resistance. Plates were incubated inverted, overnight at 37°C.

**Purification of Plasmid DNA (Minipreparation)**

Isolated colonies were picked from overnight cultures of transformed bacteria and inoculated in 3 ml of Terrific Broth (TB) (12 g bacto-tryptone, 24 g bacto-yeast extract, 4 ml glycerol in 900 ml water, autoclave, then add 100 ml 0.17 M KH₂PO₄, 0.72 M K₂HPO₄) (Maniatis et al., 1989), with appropriate antibiotics and grown overnight on an orbital shaker (250 rpm) at 37°C. Glycerol stocks of transformed cultures were prepared
by mixing 1 ml of the overnight culture with 1 ml Glycerol Storage Buffer (65% glycerol, 0.1 M MgSO₄, and 0.025 M Tris-HCl, pH 8.0) (Ausubel et al., 1993).

Plasmid DNA minipreparations were prepared either as described by Maniatis et al. (1989), or using the Promega Wizard miniprep DNA purification resin system. Both methods utilized standard alkaline lysis procedures. Briefly, bacterial cell pellets were obtained by centrifugation (13,000 x g for 10 min.) of 1.5 ml of the overnight culture. Cell pellets were resuspended in 200 μl of cell resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 μg/ml RNAse A), lysed by addition of 200 μl of lysis solution (0.2 M NaOH, 0.1% SDS), and neutralized by addition of 200 μl of 1.32 M potassium acetate, pH 4.8. Cell lysates were centrifuged (13,000 x g for 10 min.) and the supernatant aspirated. Maniatis based minipreps were then treated with RNAase (40 μg/ml) and incubated 30 min. at 37°C followed by phenol:chloroform extraction and EtOH precipitation.

For Promega Whizard minipreps, the supernatant from the alkaline step was mixed with 1 ml of Wizard miniprep resin and collected in the column provided. The resin was washed once with 2 ml of wash solution (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, in 95% ethanol) before elution of purified plasmid DNA by application of 50 μl of warmed (65-70°C) water and centrifugation (13,000 x g for 30 seconds).
Purification of Plasmid DNA (Maxipreparation)

Large-scale DNA preparation was essentially as described by Ausubel et al. (1993). A two liter fluted flask containing 500 ml of terrific broth was inoculated with 100 µl of a glycerol stock, supplemented with appropriate antibiotics and grown in an orbital shaker (250 rpm) overnight at 37°C. Cell pellets were collected by centrifugation (6000 x g for 10 min.), resuspended in 30 ml Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0), lysed with 30 ml lysis solution (0.2 M NaOH, 0.1% SDS), and neutralized by addition of 30 ml of 1.32 M potassium acetate, pH 4.8. Cell lysates were centrifuged (13,000 x g for 10 min.) and the supernatant was decanted through several layers of cheesecloth. Nucleic acids were precipitated by addition of 0.6 vol. isopropanol for 10 min. at room temperature and recovered by centrifugation (15,000 x g for 10 min.). Nucleic acid pellets were resuspended in 8 ml Solution I and RNAsase was added at 20 µg/ml and incubated at 37°C for 30 minutes. Solutions were then repeatedly extracted (4-5 times) with phenol:chloroform, and precipitated with 1/4 vol. 10 M ammonium acetate and 2 vol. EtOH, recovered by centrifugation (10,000 x g for 30 min.), resuspended in 2 ml TE (10 mM Tris-HCl, pH 8.0, EDTA 1 mM, pH 8.0). DNA was then precipitated with 0.8 ml PEG solution (30% PEG, 1.6 M NaCl) overnight at 2°C, recovered by centrifugation (10,000 x g for 30 min.) and resuspended in TE.
**Phenol:Chloroform Extraction**

Phenol:chloroform extractions were as described by Maniatis et al. (1989). An equal volume of phenol:chloroform was added to impure DNA solutions, vigorously vortexed, and centrifuged (13,000 x g for 5 min) until interface remained free of white debris. Supernatant was aspirated and re-extracted with an equal volume of chloroform before EtOH precipitation.

**DNA Precipitation and Concentration**

Ethanol precipitation of DNA was according to Maniatis et al. (1989). Briefly, DNA was precipitated from solution by adding 0.1 volume of 3 M NaOAc, pH 7.5, and two volumes of absolute ethanol (EtOH). Solutions were mixed and stored at -80°C for 15 minutes or -20°C overnight unless specified otherwise. Solutions were then centrifuged (13,000 x g 10-30 min.), washed with 70% EtOH, and vacuum dried 10 min. DNA pellets were then resuspended in H₂O and stored at 4°C until use.

**Nucleic Acid Quantitation**

Nucleic acids were quantitated by spectrophotometric absorbance as described by Maniatis et al. (1989). Briefly, nucleic acids were diluted in water or TE and assayed for absorbance at 260 and 280 nm. Concentrations of DNA, RNA or oligonucleotides were then calculated using extinction coefficients and appropriate dilution corrections. Additionally, the 260:280 ratio provides an estimate of nucleic acid purity with pure 76
preparations having ratios of 1.8-2.0. It has been noted that accurate quantitation is not always possible unless the nucleic acid has been PEG purified. Contamination is presumably due to residual phenolate ions.

**Restriction Endonuclease Digestions**

Restriction endonuclease digestion reactions typically contained 1-50 μg of DNA, 0.1 vol. of a 10x concentration of the appropriate restriction endonuclease buffer, and an appropriate amount (units) of endonuclease in 10-100 μl of dH₂O. Digests were incubated for 30-240 minutes at 37°C to assure full cleavage unless otherwise indicated. Digested DNA was stored at 4°C until use.

**Agarose Gel Electrophoresis**

Miniprep, maxiprep and restriction endonuclease digested DNA was analyzed by agarose gel electrophoresis. Briefly, a 400-1000 ng aliquot of DNA in 10 μl of dH₂O was mixed with 0.1 vol. of 10x gel-loading buffer (20% Ficoll 400, 0.1 M EDTA, pH 8.0, 1.0% SDS, 0.25% bromophenol blue) before resolution on 1% agarose gels at 50 volts for 2-2.5 hours.

**Isolation of DNA Fragments From Agarose Gels**

DNA fragments were resolved in low melting temperature agarose gels at agarose concentrations suitable for recovery of the appropriate size fragment. SeaPlaque GTG
agarose gels of 0.8 to 1.4% were used to isolate fragments $\geq 1$kb, whereas 3% Nusieve GTG agarose gels were used to isolate DNA fragments $\leq 1$ kb. Gels were prepared in either 25 or 50 ml of 1x TAE solution (40 mM Tris-acetate, pH 8.5, 1 mM EDTA) and cast in the 50x75 or 75x100 mm gel trays of the GNA-100 horizontal gel apparatus. DNA fragments (400-1000 ng/lane) were resolved by electrophoresis at 40-50 volts for 2-2.5 hours. To prevent possible mutagenesis arising from ethidium bromide intercalation, sample lanes containing size markers and a DNA aliquot were excised from the gel, stained in 1x TAE containing 0.5 $\mu$g/ml ethidium bromide for 1 min., and destained in 1x TAE for 10-15 min. Following gel reconstruction, the DNA band of interest was identified by UV illumination and the corresponding portion of the unstained gel was excised with a scalpel.

DNA fragments were purified from low melting temperature agarose gels by GELase enzyme digestion or phenol:chloroform extraction as described by Maniatis et al. 1989. GELase enzyme digestion was conducted by adding 2$\mu$l of 50x GELase buffer (40 mM bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane, pH 6.0, 1 mM EDTA, 40 mM NaCl) per 100 mg of agarose gel in a 1.5 ml microfuge tube and incubating at 65-75°C until the gel slice is completely molten. Molten gel was equilibrated in a 40°C water bath and GELase was added at a rate of 1 unit GELase per 300 mg of molten 1% LMP-agarose and incubated for 1-3 hr. Digests were then extracted with phenol (pH 8.0) and phenol (pH 8.0):chloroform (1:1). DNA was then EtOH precipitated as described above.
Phenol:chloroform extraction of DNA from LMP-agarose gels was initiated by adding 5 volumes of 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0 to the gel slice in a 1.5 ml microfuge tube and heating to 65°C until the gel is molten. The gel was then equilibrated to room temperature, extracted once with phenol (pH 8.0) once with phenol (pH 8.0):chloroform (1:1), and once with chloroform. Purified DNA was EtOH precipitated as described above.

**Dephosphorylation of Plasmid DNA**

Agarose gel purified linear plasmid DNA was dephosphorylated in a 100 μl reaction containing 1-20 pmol of 5'-termini, supplemented with 10 μl of 10x dephosphorylation buffer (500 mM Tris-HCl, pH 8.5, 1.0 mM EDTA, pH 8.5), 1 mM ZnCl₂, 1 mM MgCl₂, and 2 units of calf intestine alkaline phosphatase (CIAP) as described by Maniatis et al. (1989). Reactions were incubated at 37°C for 30 min. and terminated by addition of 5 mM EDTA, pH 8.0 and heating at 75°C for 10 min. DNA containing recessed or blunt end termini received a second aliquot of CIAP after 15 min. at 37°C and were then placed at 55°C for another 45 min. DNA was then phenol:chloroform (1:1) extracted and EtOH precipitated as described.
Klenow Fill-in reaction and 3'-5' exonuclease activity

When required, restriction endonuclease digested DNA having 5' protruding termini were blunt-ended with DNA polymerase I large fragment (Klenow) as described by the manufacturer (Promega protocol TB525).

Briefly, 5' protruding termini were filled by incubating 40 μg of restriction endonuclease digested DNA with 0.1 vol. 10x reaction buffer (50 mM Tris-HCl, pH 7.2, 10 mM MgSO_4, 0.1 mM DTT), 40 μM dNTP's, 20 μg/ml acetylated BSA and one unit of Klenow per μg of DNA in 100 μl dH_2O for 10 minutes at room temperature. Fill-in reactions were terminated by heating the mixture at 75°C for 10 minutes. Protein contamination was removed by phenol:chloroform extraction. DNA was then ethanol precipitated, resuspended in water and stored at 4°C until further use.

The Klenow enzyme was also used for removing unwanted 3' nucleotide overhangs on restriction endonuclease digested DNA. Briefly, DNA containing 3' nucleotide overhangs was incubated with Klenow under conditions optimized for 3'-5' exonuclease activity. Typical reactions contained 10 μg DNA in 33 mM Tris acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM DTT, BSA (20 μg/ml) and Klenow (1 unit/μg). Reactions were incubated at room temperature for 20 min., heat inactivated 75°C for 10 min. and phenol:chloroform extracted and precipitated as above.
DNA Ligation

Ligation reactions were essentially as described by Maniatis et al. (1989). Ligation of DNA with cohesive termini was performed in a total volume of 10 µl by addition of dephosphorylated vector DNA to insert DNA at molar ratios of 1:1 for inserts (≥ 1500 bp) or 1:10 for inserts (≤ 1500 bp), and mixtures were placed at 45°C for 5 min. DNA mixtures were then cooled at room temperature for 5 min. before addition of 0.1 vol. 10x ligase buffer (300 mM Tris-HCl, pH 7.8, 100 mM MgCl₂, 100 mM DTT, and 5 mM ATP) and 1-3 Weiss units of T4 DNA ligase. Ligations were completed by incubation in a 14-18°C water bath for 6 hours to overnight.

Blunt-end ligations differed in that they contained 15% PEG and were incubated at 20°C overnight. Both cohesive and blunt end DNA ligation reactions contained 1-2 µg total DNA (vector and insert) per reaction.

DNA Sequencing

All ligations were confirmed by dideoxyn sequencing using the Sequenase Version 2.0 DNA sequencing reaction kit per manufacturers instructions. Briefly, 3-5 µg double stranded plasmid DNA was denatured at 37°C for 30 min. with 0.1 vol. 2 M NaOH, 2 mM EDTA, pH 8.0, and neutralized with 0.1 vol. 3 M NaOAc, pH 5.0 before EtOH precipitation. Denatured DNA pellets were resuspended in 7 µl of water, mixed with 2 µl of Sequenase buffer (200 mM Tris-HCl pH 7.5, 100 mM MgCl₂, 250 mM NaCl), 0.5-1 pm of an appropriate primer, and allowed to anneal by heating in a 68°C water bath.
followed by gradual cooling to \( \leq 30^\circ C \). Annealed primer-template reactions were briefly centrifuged and placed on ice until use. Sequencing reactions were labeled by addition of 1 \( \mu l \) of 0.1 M dithiothreitol (DTT), 1.5 \( \mu M \) each dGTP, dCTP, dTTP, 5 \( \mu Ci \) alpha-\(^{33}\)P-dATP, 3.25 units of the Sequenase version 2.0 T7 DNA polymerase, before incubation at room temperature for 3-5 min. Aliquots (3.5 \( \mu l \)) of the labeling reaction were added to prewarmed (37°C) termination reaction tubes; a) 2.5 \( \mu l \) of ddGTP mixture (8 \( \mu M \) ddGTP, 80 \( \mu M \) each dATP, dGTP, dCTP, dTTP, 50 mM NaCl), b) 2.5 \( \mu l \) of ddCTP mixture (8 \( \mu M \) ddCTP, 80 \( \mu M \) each of dATP, dGTP, dCTP, dTTP, 50 mM NaCl), c) 2.5 \( \mu l \) of ddATP mixture (8 \( \mu M \) ddATP, 80 \( \mu M \) each dATP, dGTP, dCTP, dTTP, 50 mM NaCl), d) 2.5 \( \mu l \) of ddTTP mixture (8 \( \mu M \) ddTTP, 80 \( \mu M \) each dATP, dGTP, dCTP, dTTP, 50 mM NaCl), and incubated at 37°C for 10-15 min. Termination reactions were stopped by addition of 4 \( \mu l \) of 95% Formamide, 20 mM EDTA, 0.05% Bromophenol blue, 0.05% Xylene cyanol FF, and stored at -20°C until resolved by polyacrylamide gel electrophoresis.

Radiolabeled DNA fragments were resolved in 6% polyacrylamide gels prepared as a 150 ml solution containing 63 g of urea in 30 ml of 19:1 acrylamide:bisacrylamide in water, 7.5 ml 20x TBE (1 M Tris-base, 1 M Boric acid, 20 mM EDTA, pH 8.0), and adjusted to 150 ml with water. The 6% acrylamide, 7 M urea solution was mixed with 0.96 \( \mu l \) freshly prepared ammonium-persulfate (250 mg/ml) and 0.66 \( \mu l \) TEMED/ml gel solution prior to casting in a 38x50 Bio-Rad Sequi-gen nucleic acid sequencing cell with tapered spacers (0.25 mm to 0.4 mm). Sequencing gels were run in 1x TBE (50 mM
Tris-base, 50 mM Boric acid, 1 mM EDTA, pH 8.0) and prewarmed to 50°C by prerunning at 2800 volts for one hour before loading samples. Sequencing reactions were heat denatured at 90°C for 3 minutes, cooled on ice and 1.6 µl of each was loaded in 0.25 mm wide lanes created with a shark tooth loading comb. Samples were resolved by electrophoresis at 1800 volts/hr for 2-4 hrs. Gels were dried in vacuo at 80°C for 2 hrs (Biorad model 583 Gel Dryer) before exposure to XAR5 X-ray films to visualize resolved sequencing reactions.

Polymerase Chain Reaction (PCR)

Polymerase chain reactions were essentially as described by Maniatis et al. (1989). Briefly, 1 µg plasmid DNA was linearized with an appropriate restriction endonuclease that cleaved outside the area of desired amplification, EtOH precipitated, resuspended in water, and concentration determined by Abs 260 nm. PCR reactions contained 6 µl of 25 mM MgCl₂, 10 µl Tth DNA polymerase reaction buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.8), 10 µl dNTP's 2 mM each, 30 pmol each primer, and 250 ng template DNA in a total volume of 100 µl. PCR reactions were heated to 94°C for 5 min to denature template DNA before addition of 1 µl (1 unit) Tth DNA polymerase. Primer extension reactions were performed with 35 cycles of denaturation (94°C x 1 min.), annealing (55°C x 1 min.), and extension (72°C x 2 min.) followed by one round of denaturation (94°C x 1 min.), annealing (55°C x 1 min.), and extension (72°C x 10 min.). Reactions were kept at 4°C until EtOH precipitation, and analyzed by agarose gel electrophoresis.
Site Directed Mutagenesis

Site-directed mutagenesis was based on the method described by Kunkel, (1985), with some modification. Briefly, uracil containing dsDNA was prepared by plasmid amplification in CJ236, a dut*, ung' bacterial host stain. Following heat denaturation, complimentary mutagenic oligos were hybridized to uracil containing template DNA, extended and ligated with the Klenow fragment of T7 DNA polymerase and T4 DNA ligase respectively. Reaction products were transformed into dut*, ung' competent bacterial host cells which preferentially degrade uracil containing DNA. Clones were screened for mutation by restriction endonuclease digestion.

Radiolabeling of DNA

Radiolabeled DNA was prepared using the Promega Prime-a-Gene random-primed labeling system. Template DNA (25 ng) was heat denatured at 95-100°C for 2 min. and rapidly cooled on ice. Denatured DNA (1-2 μl) was then mixed with 10 μl 5x labeling buffer (250 mM Tris-HCl, pH 8.0, 25 mM MgCl₂, 10 mM DTT, 1 M HEPES, pH 6.6, with 26 A₂₆₀ units random hexadeoxyribonucleotide primers/ml); 2 μl of 500 μM dATP, dTTP, dGTP; 2 μl nuclease-free BSA (400 μg/ml); 5 μl [α-³²P]dCTP (50 μCi, 3000 Ci/mmmole); and 5 units of DNA polymerase I (Klenow fragment). Total volume was adjusted to 50 μl with water before incubation at room temp. for 1 hr. Reactions were terminated by addition of 2 μl 0.5 M EDTA. Unincorporated α-³²P-dCTP radiolabel
was removed using NucTrap push columns prewet with 70 μl 1x STE (100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA). The volume of the radiolabeled DNA solution was adjusted to 70 μl with water before application to the NucTrap column. Radiolabeled DNA was eluted from the column with 70 μl 1x STE and radioactivity determined by scintillation counting to determine labeling efficiency.

**Microautoradiography**

Cultured cells were transfected with radiolabeled DNA as described and examined by microautoradiography at selected times post transfection. Transfected cells were rinsed 2x with 1 ml PBS per well, fixed with 200 μl fixative (0.5 M Na₂PO₄, pH 7.3, 2% paraformaldehyde, 0.2% gluteraldehyde) per well for 3 min. at 4°C, and washed twice with room temperature PBS as described above. Kodak NTB3 nuclear emulsion, 100 μl liquefied in a 45°C water bath, was added under dark room conditions to each 2.0 cm² cell culture well and incubated 1 min. before aspiration of excess emulsion. Emulsion coated wells were then air dried one hour before incubation 4-16 hrs in light-proof containers at 4°C. Silver grains were developed by addition of 400 μl Kodak Dektol Developer to each well for 2 min., rinsing in water (1x 200 μl, 30 sec.), and fixed with 400 μl Kodak fixer for 5 min followed by a second water rinse before air drying.
**Northern Hybridization analysis**

Total RNA was isolated from cultured cells according to Chomczynski and Sacchi, (1987), with some modification. Briefly, cells were plated in 9.6 cm² wells, transfected as described and cultured until RNA harvest. Following culture medium removal, 800 µl of 4 M guanidine isothiocyanate (GITC) solution (100 g GITC in 117 ml water, to which is added 7 ml of 0.75 M sodium citrate, pH 7.0, 10.6 ml 10% sarcosyl, and 0.103 M beta-mercaptoethanol) was added per well and incubated (≤1 min.) at room temperature. Viscous cell lysate was transferred to a 2.0 ml microfuge tube and mixed with 0.1 vol. of 2 M NaOAc, pH 4.1, 1.0 vol. of water saturated phenol, 0.2 vol. of water saturated chloroform:isopentanol (46:1). This mixture was vigorously vortexed for 1 min. and placed on ice for 15-30 min., and centrifuged (10,000 x g, 4°C, 10 min.). The aqueous phase containing the RNA was transferred to a new microcentrifuge tube, mixed with an equal volume of isopropyl alcohol, and stored overnight at -20°C. RNA was then pelleted by centrifugation as above and resuspended in 200 µl of a 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5% SDS solution. RNA was then extracted by vortexing with an equal volume of chloroform followed by phase separation via centrifugation as above. The aqueous phase containing the RNA was then transferred to a new microfuge tube and RNA precipitated by addition of 0.1 vol. 3 M NaOAc, pH 5.4 and 2 vol. absolute EtOH at -20°C overnight.

Following centrifugation as described above, RNA pellets were resuspended in 7 µl of sterile diethyl pyrocarbonate (DEPC)-treated water (Maniatis et al., 1989). RNA
concentrations were determined from the absorbance at 260 and 280 nm of a 1:100 dilution of the RNA solution in water. Depending on the experiment, 20-80 µg total RNA was adjusted to a volume of 5 µl before addition of 25 µl denaturing solution (25 mM HEPES, pH 7.0, 6 mM NaOAc, 1.2 mM EDTA, 60% deionized formaldehyde) and heating at 55°C for 15 min. Denatured RNA was immediately placed on ice and mixed with 10 µl of gel loading buffer (20 mM HEPES, pH 7.0, 10% ficoll, 0.25% bromophenol blue) to a yield a final volume of 40 µl/sample which was stored on ice until resolved in a formaldehyde-agarose gel (1.5% ultrapure agarose, 18% deionized formaldehyde, 20 mM NaPO₄, pH 7.0) submerged in a 22 mM NaPO₄, pH 7.0 buffer. Each gel lane was loaded with 20-80 µg total RNA sample/40 µl, and resolved at 25 volts/hr for 16 hrs or 100 volts/hr for 4 hrs in a SuperSub-HE100 Horizontal electrophoresis chamber. Ethidium bromide (220 µg) was added to the running buffer (2,300 ml) one hour before electrophoresis termination. Gels were then destained by soaking in water at room temp. for 10-15 min. to allow visualization of 18 and 28 S RNA bands under UV illumination.

RNA gels were prepared for transfer to hybridization membranes by sequential submersion for 35 min. at room temp. in denaturation solution (50 mM NaOH, 10 mM NaCl), neutralization solution (0.1 M Tris-HCl, pH 7.5), and 10x SSC (1.5 M NaCl, 150 mM sodium citrate, pH 7.0). RNA was then transferred by capillary action onto GeneScreen plus nylon membranes for 18 hrs (Maniatis et al., 1989). Membranes were then vacuum dried 80°C for 2 hrs. and prehybridized by immersion in hybridization solution (50 ml deionized formamide, 10 ml of 10% SDS, 40 ml autoclaved Blotto
solution [0.5% (w/v) nonfat dry milk, 0.6 vol. 20 X SSC, 250 μl DEPC]) at 42°C for 1-2 hrs. according to Siegel and Bresnick, (1986).

Radiolabeled DNA probes with a specific activity of $1 \times 10^7$ cpm/μg or higher were prepared and denatured as described before addition to prehybridized membranes. Membranes were incubated with radiolabeled probes at 42°C for 18-20 hrs with gentle agitation. Following hybridization, excess probe was removed by washing each membrane twice in succession with: 2x SSC, room temperature, 5 min.; 2x SSC and 1% SDS, 60°C, 30 min.; and 0.1x SSC, room temperature, 30 min. Washed hybridized membranes were then exposed to XAR5 X-ray film.

Hybridized radiolabeled probes were removed from membranes according to Maniatis et al. (1989), by repeat boiling in elution solution (0.05x SSC, 0.01 M EDTA, pH 8.0, 0.1% SDS) for 5-15 min., then rinsed briefly with 0.01x SSC at room temperature. Membranes were then hybridized with an 18S cDNA probe which had been prepared from an 18S ribosomal RNA cDNA plasmid as described previously.

Endotoxin Assay

Lippopolysaccharide (endotoxin) presence in DNA and polycation preparations was quantitated using a Limulus Amebocyte Lysate (Pyrotell) assay from Cape Cod Associates per manufacturer’s instructions. Briefly, 0.1 ml reconstituted Pyrotell was mixed with 0.1 ml of sample in a 10x75 mm depyrogenated flint glass reaction tube and incubated at 37°C ±1°C for 60 ±2 min. At the end of the incubation period, the tube was
removed from the incubator and inverted. If a gel formed and remained intact following tube inversion, the test was considered positive, indicating the concentration of endotoxin in the tube is greater than or equal to the sensitivity of the Pyrotell. If no gel is formed or if it breaks loose from the bottom of the tube, the test is considered negative. Endotoxin levels were quantitated by comparison with intra-assay endotoxin standards.

Endotoxin Removal

Lipopolysaccharide (endotoxin) contamination of plasmid DNA preparations was removed by passage through a polymixin B chromatography column equilibrated with 300 mM NaCl, 50 mM Tris-HCl, pH 7.6, 1 mM EDTA. Endotoxin levels were quantitated by limulus amebocyte lysate assay as described.

In Vivo Methods

In Vivo Bovine and Ovine Mammary Transfection

In vivo mammary transfections utilized transfection solutions as described in methods. Prior to bovine or ovine intramammary infusion of transfection solutions, milk was removed by hand milking. Selected non-pregnant animals were then injected I.M. with 1-5cc oxytocin by and re-milked to maximize removal of residual milk. Prepartum ovine mammary glands were either left untreated, hand milked, or hand milked and washed by intramammary infusion of 0.3M sucrose, 10 mM HEPES, pH 7.4 followed by hand milking to remove wash solution. Transfection solutions of up to 150 ml were
infused into mammary glands with a 50cc syringe and sterile plastic. Larger infusion volumes (>150 ml) required a one liter bottle connected to a syringe and cannula via sterile IV infusion tubing and a three way check valve. Teat ends were sterilized with 70% ETOH prior to cannulation. Transfection solutions were left in the gland until the next scheduled milking interval (≤ 12 hrs.) or until parturition (non-lactating animals). Milk samples were collected daily by manual milking of a 5-10ml aliquot. Milk fat was removed after centrifugation (3000 x g, 6 min.) and the fat-free (skimmed) milk was stored at -80°C until assayed for recombinant gene expression.

In Vivo Guinea Pig Transfection

Transfection complex, solutions prepared as described earlier were infused into 1-2 day prepartum guinea pig mammary glands (2 ml transfection medium per mammary gland) by cannulation of the teat with a blunt end, 27 ga. needle. Teat ends were alcohol sterilized prior to cannulation. Post-parturient milk samples were collected daily by hand milking after 4 hr. pup withdraw. Fresh milk samples were defatted by centrifugation (3000 x g, 6 min.) before storage at -80°C until assayed for hGH content by radioimmunoassay as described. All transfection of guinea pig mammary glands by intramammary infusion was done by I. Mather and J. Hens at the University of Maryland, using transfection polymers and DNA prepared and provided by M. Amstutz as part of a collaborative study.
**Miscellaneous Methods**

**Poly(glutamylhistamineglutamate) Synthesis**

Poly(glutamylhistamineglutamate) (Glu₉·HisN) was synthesized as described by Shen, (1990). Poly-L-glutamic acid (30 mg) (15-50 kDa) was derivatized with histamine dihydrochloride (25 mg) by addition of 40 mg EDAC (10 mg each at 5 min. intervals) in 0.5 M NaPO₄, pH 5.5. Synthesis was continued in an orbital shaker (300 rpm) for 1 hr. at room temperature before adjustment of pH to 7.0. Glu₉·HisN was exhaustively dialyzed (MW cutoff 3500 kDa) in PBS to remove underivatized histamine, filter (0.2µ) sterilized, and stored at 4°C until use.

**Fluorescent Labeling of Polycations and Proteins**

Fluorescently labeled polycations were prepared as described by Baxter Healthcare Corporation technical bulletin (1987). Briefly, 0.2 mg (solid) fluorescein isothiocyanate (FITC), QFITC, or Texas Red was added to 1 mg Orn, Lys, or DEAE in 0.5 M NaHCO₃, pH 9.5 and incubated in the dark for 2 hrs. at room temperature with periodic mixing. Unincorporated fluorescent label was removed by gel filtration using a Sephadex G-25 column (1.5 cm x 5 cm) equilibrated and eluted with 0.1 M NaPO₄, pH 7.2.
Peptide Synthesis and Purification

Synthesis of a 25 amino acid human rhinovirus lytic peptide (HRV2), amino acid sequence NPVENYIDEVLNEVLVPNINSSNC (Zauner et al., 1995), utilized solid-phase peptide synthesis (Merrifield, 1963; Merrifield et al., 1966) with the assistance of Dr. Charles Brooks (The Ohio State University), on an Applied Biosystems city model 431A peptide synthesizer using an Fmoc-Cys(Trt)-NovaSyn® TGA resin as the solid support and fluorenylmethoxycarbonyl-protected (FMOC) amino acids. Synthesized peptides were cleaved from the resin and deprotected with 550 μl of chilled cleavage mixture (100 μl thioanisole, 50 μl water, 50 μl ethanedithiol, and 1.8 ml trifluoroacetic acid) for two hours at room temp. Deprotected peptides were separated from the synthesis resin by filtration through a glass wool column. Peptides were then precipitated (5 times) with 10 ml methyl butyl ether and redissolved in 2 ml 80% acetonitrile, 20% water, 0.1% TFA. The peptide was fully solubilized with 25 μl NH₄OH and lyophilized. Peptides were resuspended in 10 mM HEPES, pH 7.3, and purified by HPLC on a C-18 preparative column equilibrated with 20% isopropanol, 80% water, 0.1% TFA and eluted with a linear gradient (0-100%) of 20% isopropanol, 80% acetonitrile, 0.1% TFA over 60 min. at 5 ml/min. Eluted peptide fractions were lyophilized, redissolved in 10 mM HEPES, pH 7.4, and stored at -80°C until used. Mass spectroscopy of the HRV2 lytic peptide fractions was performed by Dr. Chris Yu of The Ohio State University Campus Chemical Instrument Center.
**Transmission Electron Microscopy**

Incubation of freshly prepared transfection complexes for 4 min. in contact with Formvar-coated electron microscopy grids allowed attachment of transfection complexes to the grids for transmission electron microscopy. Transfection complexes adhered to Formvar-coated grids were negatively stained with 2% uranyl acetate for 1 min. according to Foisner and Wiche, (1985) before examination in a Philips 201C medium resolution transmission electron microscope.

**Dissociation of Radiolabeled DNA from Immobilized Mixed Polvion Transfection Complexes**

Transfection complexes were formed as described above using ^32^P-labeled DNA and applied to empty 2.0 cm² wells for 4 hrs. at 37 °C. Spent transfection medium was aspirated and wells washed 2 x 1 min. with 0.3 M sucrose, 10 mM HEPES, pH 7.4, or HBSS to remove excess ^32^P-labeled DNA not firmly attached in transfection complexes. Wells were then washed 3 x 30 min. with shaking (100 RPM) at 37°C with 0.5 ml of either HBSS; 0.3 M sucrose, 10 mM HEPES, pH 7.4; 50 mM citrate, 10 mM HEPES; 50 mM citrate, 10 mM HEPES, 25 mM NaCl; or 50 mM citrate, 10 mM HEPES, 1 M NaCl. pH of citrate-based wash solutions varied with experimental design and all washes were collected by aspiration and assayed individually for radiolabeled DNA release.
Chapter 4

Development of a charge-shifting, polyion-mediated recombinant
gene transfer method for primary bovine mammary cells in vitro.

The ruminant mammary gland's capacity for large scale protein synthesis and
secretion make it an excellent vehicle for recombinant protein production. Indeed,
transgenic animals have been created that contain tissue-specific regulatory sequences to
direct secretion of biologically active eukaryotic proteins in milk at levels which rival or
exceed endogenous milk protein concentrations, as reviewed by Houdebine (1994),
Bawden et al. (1994), and Wilmut and Whitelaw (1994). Despite this success, the
production of transgenic animals remains costly and time consuming, and has not yet
proven feasible for mammary specific expression in cattle.

As an alternative to transgenesis, Archer et al., (1994) used direct intramammary
infusion of replication-defective recombinant retroviral vectors to achieve (< 60 ng/ml)
mammary-specific transgene expression in the caprine mammary gland. However,
retroviral gene transfer systems are limited by the requirements for high viral titer in the
infused medium, target cell replication, insert size limitations, and safety concerns over
the potential for viral recombination (Smith et al., 1991). Therefore, development of an
efficient non-germline, non-viral, recombinant gene transfer method that would allow
direct transfer into target organs, such as the mammary gland, would facilitate
modification of mammary function and/or milk composition without the time or expense
of transgenic cattle.

The unique anatomy of the mammary gland may allow novel gene transfer
approaches to be utilized. The secretory epithelium of the mammary gland consists of a
ductular tree which begins at the exterior opening of the teat and extends throughout the
gland with ever finer bifurcation, terminating in spherical secretory mammary alveoli.
Each alveolus is lined by a single layer of epithelial cells which vectorially secrete milk
proteins into the alveolar lumen which empties into the ductular tree. The apical surfaces
of secretory epithelial cells lining each alveolus and fine ductule are directly accessible
by intramammary infusion. Thus, unlike most other tissues, the functional cells of the
mammary gland are directly accessible for transfection of recombinant genes by
intramammary infusion, as first proposed by Patton et al., (1984).

Solutions containing recombinant transgenes may be infused into the mammary
gland via teat cannulation and pass through the ductular tree to reach mammary alveoli
which are lined by the apical surfaces of secretory mammary epithelial cells responsible
for milk protein synthesis and secretion. Direct mammary cell transfection by
intramammary infusion of recombinant transgenes should; 1) allow efficient delivery to
most or all target cells, 2) simplify expression vector design by eliminating the need for
mammary specific regulatory elements, since the anatomy of the mammary epithelium
and ducts assures that only mammary epithelial cells will be exposed to transfection components, and 3) be applicable to any animal regardless of genetic background which allows rapid adjustment to constantly changing markets.

Direct transfection of somatic cells in situ requires that DNA: 1) be protected from nuclease attack while in transit to the target cell; 2) be selectively targeted for delivery and uptake by the desired cell type; 3) escape endosome entrapment or degradation after uptake; and 4) be translocated to the nucleus in an expression competent form. Direct transfection of the mammary gland by intramammary infusion would avoid the time lag, difficulty, and expense of creating transgenic dairy animals, and avoid the safety concerns associated with retroviral vectors.

The anatomy, polarity of the mammary epithelium, and motion of the bovine mammary gland preclude the use of gravity-dependent transfection systems (such as calcium phosphate:DNA coprecipitation) in vivo (Keown et al., 1990). Transfection procedures which require short-duration cell shock (e.g., glycerol or DMSO exposure, etc.) by rapid short-term application and exchange of shock medium are also not feasible in the bovine mammary gland due to its size and the consequent difficulty of rapid introduction and complete and rapid removal of fluid by teat drainage or aspiration (Keown et al., 1990; Maliенou-Ngassa et al., 1990). Furthermore, the high cost of selection agents, average size of the mammary gland (~ 50 lbs.) (Schmidt, 1971, and references therein), and limited post-parturient mammary cell replication (Schmidt, 1971) will likely preclude survival selection of transfected cells in the bovine mammary gland.
in vivo (Kaufman, 1990; Keown et al., 1990). Therefore, priority must be placed on the
development of gene transfer systems capable of transfection and expression of
recombinant transgenes in most or all cells of the mammary gland without the need for
selection or cell replication.

Polycations such as poly-L-ornithine (Orn), poly-L-lysine (Lys), and DEAE-
dextran (DEAE) can mediate efficient gene transfer into several mammalian cell lines
(Bond and Wold, 1987; Davis et al., 1986; Farber et al., 1975), including mouse
(Danielson et al., 1989) and bovine (Gorewit et al., 1987) mammary cell lines in vitro.
Gorewit et al. (1987) used DEAE-mediated transfection to produce high levels of bovine
growth hormone (~120 ng/ml) in a bovine mammary myoepithelial cell line in vitro.
However, only DEAE, which is toxic to some cells (Rosenthal, 1987) has been used to
transfect primary mammary cells in vitro (Malienou-Ngassa et al., 1990).

Poly-L-ornithine enhanced the uptake and expression of exogenous DNA more
reproducibly and to a greater extent than did DEAE or poly-L-lysine in Chinese hamster
ovary (Dong et al., 1993) and lung (Farber et al., 1975) cells. Furthermore, polyornithine
can mediate efficient transfection of non-replicating quiescent cells (Dong et al., 1993)
as might be encountered in the mammary gland in vivo.

Transfection of primary cells has proven more difficult than transfection of
established cell lines whose transformed genotype often facilitates extrachromosomal
plasmid replication and concomitant amplification of recombinant gene expression
(Asselbergs and Grand, 1993; de Chasseval and de Villartay, 1992). Nevertheless,
Malienou-Ngassa et al. (1990) transfected primary rabbit mammary cells cultured on floating collagen gels with DEAE (200 µg/ml) and a transgene comprised of the recombinant Simian virus 40 (SV40) promoter and human growth hormone (hGH) reporter which yielded hGH expression (~1 ng hGH/ml) in cell culture medium. Therefore, polycation-mediated recombinant gene transfer into primary mammary cells is clearly feasible in vitro.

The ability to deliver and express recombinant transgenes in most or all mammary cells is critical because recombinant protein expression level and value will likely define potential applications of gene transfer into mammary tissue and recombinant protein expression in milk. Polycations should effectively deliver recombinant transgenes to all mammary epithelial cells via ionic charge interaction with both negatively charged DNA and the anionic cell surface (Farber et al., 1975; Pagano, 1970). Additionally, polycations are easy to prepare, simple to use, inexpensive, non-infectious, and compatible with direct intramammary infusion.

The primary objective of this study was to develop a safe, efficient, non-germline, non-viral recombinant gene transfer method compatible with direct intramammary infusion for the purpose of expressing recombinant genes in bovine mammary epithelia in order to modify mammary function and/or the production, composition, and qualities of milk. Initial mammary cell transfection studies focused on polycations such as DEAE, Lysₙ, or Ornₙ used by others for mammalian cell transfection (Bond and Wold, 1987; Dong et al., 1993; Keown et al., 1990). Such polycations, able to bind both DNA and the
negatively charged cell surface by electrostatic charge interaction, should bind polycation:DNA transfection complexes to the anionic apical mammary cell surfaces exposed to the intraluminal fluid space within the mammary gland.

Screening of candidate transfection systems and vectors for efficacy and cytotoxicity, as measures of their potential for direct gene insertion into the bovine mammary gland in vivo, was done with an in vitro model involving transfection of primary bovine mammary cells capable of milk protein synthesis and secretion in culture (Talhouk et al., 1992). However, due to their slow growth, limited availability and requirement for culture on collagen gels, primary bovine mammary cells were used to corroborate results obtained with a mouse mammary cell line Comma-D (Danielson et al., 1989).

Initial transfection of primary bovine mammary cells with expression vectors containing αs1-casein or whey acidic protein (WAP) promoters and chloramphenicol acetyltransferase (CAT) or WAP reporter genes indicated that stronger promoters would be needed for recombinant gene expression in bovine mammary cells. Therefore, the expression of recombinant transgenes was determined non-destructively and with high sensitivity using an expression plasmid constructed from the pcDNA1 eukaryotic expression vector (Invitrogen, CA) having a cytomegalovirus (CMV) promoter to drive strong expression of the downstream reporter gene independent of cell type, differentiation state or hormone status. hGH was chosen as the reporter gene because of the stability and efficient processing and translation of its mRNA, and secretion of hGH
protein, by most cell types (Selden et al., 1986). This allowed the non-destructive analysis of ongoing expression in transfected cells by radioimmunoassay (RIA) (Nichols Institute) or enzyme-linked immunoadsorbent assay (ELISA) (Talhouk et al., 1990) of hGH secreted into the culture medium.

Early transfection studies indicated that mixtures of polycations may offer transfection and expression advantages over individual polycations alone. To facilitate polycation:DNA dissociation and further increase recombinant gene expression, a novel charge-shifting polyion was synthesized and incorporated into mixed polycation transfection complexes used to transfect primary bovine mammary cells in vitro. Recombinant gene expression was increased further when the order of polycation addition to DNA was controlled to allow polycations with lower charge density to complex first with DNA before addition of higher charge density polycations which facilitate cell targeting.

Described here is the development of an ordered charge-shifting mixed-polyion-based transfection system capable of inducing strong recombinant gene expression for prolonged periods in cultures of primary bovine mammary cells, and which is compatible with anticipated requirements for in situ bovine mammary transfection by direct intramammary infusion.
Methods

Plasmid Construction

Human growth hormone (hGH) eukaryotic expression plasmids purchased from Nichols Institute (San Juan Capistrano) contained the hGH gene under the control of either no promoter (p0/hGH), the mouse metallothionine promoter (pX/hGH5), or the thymidine kinase promoter (pTK/hGH) (Selden et al., 1986) (Fig. 4.1). The complete human growth hormone gene from p0/hGH was ligated into the BamHI/EcoRI restriction endonuclease cleavage sites in the multiple cloning site of the pcDNA1 expression vector (Invitrogen) downstream of the cytomegalovirus (CMV) promoter. The resulting CMV promoter driven hGH expression plasmid designated pcDNA1/hGH (Fig. 4.2) allowed aggressive expression of secreted hGH in transfected mammary cells independent of cell differentiation state, extracellular matrix substratum, or hormone stimulation.
Figure 4.1. Recombinant expression vectors containing the human growth hormone (hGH) gene. Human growth hormone eukaryotic expression plasmids purchased from Nichols Institute (San Juan Capistrano) contained the hGH gene under the control of either no promoter (pO/hGH), the mouse metallothionine promoter (pX/hGH5), or the thymidine kinase promoter (pTK/hGH) (Selden et al., 1986).
Figure 4.2. Construction of pcDNA1/hGH. The complete human growth hormone gene was excised from pO/hGH with BamHI/EcoRI restriction endonucleases and ligated into the corresponding BamHI/EcoRI restriction endonuclease cleavage sites in the multiple cloning site of the pcDNA1 expression vector (Invitrogen) downstream of the cytomegalovirus (CMV) promoter.
Results

Preliminary transfections of primary bovine mammary cells with the pTK/hGH expression vector using either poly-L-ornithine (Orn,) (16.6 μg/ml), or DEAE-dextran (DEAE) (250 μg/ml), yielded no detectable human growth hormone (hGH) expression (results not shown). The difficulty of expression, slower growth in culture, and limited availability of primary bovine mammary cells required an alternative and more sensitive model for screening prospective transfection methods and expression vectors. For convenience, a mouse mammary cell line (Comma-D) capable of milk protein synthesis and secretion was used to determine efficacy of transfection methods and expression vectors prior to their use in primary bovine mammary cells.

Cellular Uptake and subcellular distribution of polycation-transfected radiolabeled plasmid DNA

To assess the cellular uptake and subcellular distribution of transfected plasmid DNA, Comma-D mouse mammary cells were cultured as described in methods and transfected with ³H-thymidine-labeled pTK/hGH plasmid DNA complexed with polycations or HBSS (control). After transfection, cells were trypsinized, lysed with NP-40, and nuclear and cytosol fractions analyzed for radiolabeled plasmid (Ausubel et al., 1994). Parallel sets of Comma-D cells were transfected with unlabeled pTK/hGH and monitored for hGH secretion.
Cellular uptake and subcellular distribution of radiolabeled plasmid DNA in Comma-D cells varied widely with the polycation used. Transfection with an Om:\DNA complex at a 3.4:1 ratio (polycation:DNA, wt:wt) resulted in nearly 100% cellular uptake of radiolabeled plasmid DNA, with greater than 80% of that found in the nuclear fraction (Fig. 4.3A). In contrast, DEAE-mediated transfection delivered ≤ 25% of the radiolabeled-plasmid DNA to Comma-D cells in vitro, with virtually all intracellular radiolabeled-plasmid detected in the nuclear fraction (Fig. 4.3B). As expected, transfections performed in the absence of polycations (HBSS control) resulted in full recovery of radiolabeled-plasmid DNA in the posttransfection medium (Fig. 4.3C).

Secreted recombinant hGH expression did not correlate with radiolabeled-plasmid DNA internalization and nuclear localization. Human growth hormone expression was low (1 ng/ml) from Comma-D cells transfected with Om:\DNA transfection complexes (Fig. 4.3D), higher (> 5 ng/ml) for DEAE-mediated transfection (Fig. 4.3E), and as expected, only marginally detectable (≤ 0.3 ng/ml) for DNA transfection alone (Fig. 4.3F) on day 4 posttransfection. The higher hGH expression (Fig. 4.3E) from the smaller amount of DNA delivered to DEAE-transfected cells (Fig. 4.3B) indicates higher expression per microgram of internalized DNA for DEAE compared to Om:\-mediated transfections (Fig. 4.3D and A). This suggested that Om:\ and DEAE might be combined in transfections since Om:\-mediated transfection provided good cell targeting and nuclear uptake (Fig. 4.3A) but relatively poor hGH expression (Fig. 4.3D), while DEAE-mediated
Figure 4.3. Cellular uptake, subcellular distribution, and expression of plasmid DNA in Comma-D mouse mammary cells following transfection with DNA alone or complexed with DEAE-dextran (DEAE) or poly-L-ornithine (Orn.) Comma-D mouse mammary cells were transfected as described in methods, using $^3$H-thymidine labeled pTK/hGH plasmid DNA (5 µg/ml), (1.0 µg/2.0 cm$^2$ well) complexed with either: poly-L-ornithine (A) (16.6 µg/ml) (Orn.); DEAE-dextran (B) (250 µg/ml) (DEAE); or DNA alone (C) in HBSS. Panels A-C: $^3$H-thymidine-labeled plasmid DNA recovered in pre- and posttransfection medium (Trnfxn medium), supernatant after trypsinization of cells from well (Tryp Sup), cell washes 1 and 2, and total cellular uptake for washed cells (Cell), and in the cytosol (Cyt) and nuclei (Nuc) after 4 hr. transfection. Recombinant hGH expression is shown for cells transfected with Orn. (D); DEAE (E); or DNA alone (F), from Panels A, B, C.
Figure 4.3
transfection gave enhanced expression (Fig. 4.3E) for the lower amount of DNA which it delivered to cells (Fig. 4.3B).

Surprisingly, cellular uptake and nuclear localization of radiolabeled plasmid DNA in Lys$_n$-mediated transfection (Fig. 4.4) resembled DEAE-mediated transfection more closely than Orn$_n$-mediated transfection (Fig. 4.3A and B). Unfortunately, recombinant hGH expression comparisons could not be made as hGH expression from Lys$_n$-transfected cells was not determined in this experiment. Radiolabeled-plasmid DNA was detected in the trypsinization supernatant of cells transfected with Lys$_n$ (Fig. 4.4) but not with DEAE, Orn$_n$, or HBSS (Fig. 4.3A-C), suggesting tryptic removal of Lys$_n$:DNA complexes from the cell surface. Additionally, very little radiolabeled-plasmid DNA ($\leq$5%) was detected in either wash 1 or 2, or in the cytosolic fractions of all transfection methods tested (Fig. 4.3A-C and Fig. 4.4), suggesting that internalized plasmid DNA was rapidly translocated to the nucleus after transfection with all polycations tested.

Effect of mixed polycations in Comma-D transfections

To test whether transfections with mixed polycations might combine benefits for cell targeting and expression efficiency, Comma-D cells were cultured as described in methods and transfected with pX/hGH5 plasmid DNA (containing the more aggressive mouse metallothionine promoter) complexed with mixtures of Orn$_n$ and DEAE at total polycation:DNA ratios (wt:wt) of either 3.4:1, 10:1 or 20:1. The 3.4:1 total
Figure 4.4. Cellular uptake and subcellular distribution of plasmid DNA in Comma-D mouse mammary cells after transfection with Lys₅:DNA. Comma-D mouse mammary cells were transfected as described in methods, using ³H-thymidine labeled pTK/hGH plasmid (5μg/ml), (1.0 μg/2.0 cm² well) complexed with poly-L-lysine (8.3 μg/ml) (Lys₅) in HBSS. Bars represent ³H-thymidine-labeled plasmid DNA recovered in pre- and posttransfection medium, supernatant after trypsinization of cells from well (Tryp Sup), whole cell washes 1 and 2, and total cellular uptake for washed cells (Cell), and in the cytosol (Cyt) and nuclei (Nuc) after 4 hr. transfection.
polycation:DNA ratio as used here and for Figure 4.3A was as for Orn₉-mediated transfection described by Bond and Wold (1987), while the 10:1 and 20:1 polycation:DNA ratios were tested to see if additional polycations either alone or in combination would increase transfection complex targeting to the cell surface as measured by subsequent recombinant gene expression. Within each polycation:DNA ratio, the ratio of Orn₉:DEAE (wt:wt), was also varied from 1:0, 3:1, 1:1, 1:3, 1:5, and 0:1 to determine which (if any) combinations of polycations would enhance recombinant gene expression. Within each total polycation:DNA ratio, variation of the Orn₉:DEAE ratio in the total polycation mixture gave dramatically different expression levels and profiles (Fig. 4.5A-C). As predicted from the results in Figure 4.3, transfections with a mixture of Orn₉ and DEAE gave dramatically higher expression for all total polycation:DNA ratios (Fig. 4.5). A total polycation:DNA ratio of 10:1 with an Orn₉:DEAE ratio of 3:1 gave the highest and most sustained hGH expression in Comma-D cells transfected in vitro (Fig. 4.5B). Further increasing polycation:DNA ratios to 20:1 reduced hGH expression, regardless of Orn₉:DEAE ratio (Fig. 4.5C), but with expression increasing throughout the culture period. Reduced expression with a Orn₉:DNA ratio greater than 10:1 was likely caused by cell injury as indicated by severe vacuolization and cell detachment (results not shown). Cell viability was not determined. These results demonstrate that combinations of polycations can mediate gene transfer and expression much more effectively than single polycations alone.
Figure 4.5. Effect of total polycation:DNA and Orn<p>:DEAE ratios on mixed polycation transfections of Comma-D mouse mammary cells. Comma-D mouse mammary cells were transfected with pX/hGH5 plasmid DNA (5 μg/ml) in HBSS (1.0 μg/2.0 cm² well) containing total polycation:DNA ratios (wt:wt) of either: 3.4:1 (A), 10:1 (B), or 20:1 (C). Within each total polycation:DNA ratio, Orn<p>:DEAE ratios (wt:wt) were varied at 1:0 (O), 3:1 (●), 1:1 (∆), 1:3 (∇), 1:5 (□), and 0:1 (■) as indicated.
Development of pcDNA1/hGH

Early efforts to study polycation-mediated transfection in primary bovine mammary cells using pTK/hGH and pX/hGH gave insufficient hGH expression ($\leq 1 \mu g$ hGH/ml) for reliable interpretation of relative efficacy of treatments. Therefore, development of transfection systems for primary mammary cells required increased expression to improve analytical feedback. Hence, the hGH gene from pO/hGH was ligated into the pcDNA1 eukaryotic expression vector, immediately downstream from the strong human cytomegalovirus (CMV) promoter (Fig. 4.2). Subsequent transfections utilized the pcDNA1/hGH expression vector, in which sufficient hGH expression was driven by the strong human cytomegalovirus (CMV) early promoter/enhancer region to allow transfection studies in primary bovine mammary cells as well as Comma-D mouse mammary cells and other cell lines as required.

Effect of mixed polycations in primary bovine mammary transfections

To confirm that mixed polycation transfections behaved predictably across cell type from different species, primary bovine mammary cells were cultured as described in methods and transfected with OmA and DEAE alone or in combination, using the pcDNA1/hGH expression vector and the polycation ratios determined optimal for Comma-D transfection (10:1 total polycation:DNA; 3:1 OmA:DEAE), in the previous experiment. The slower growth on collagen substratum and limited availability of
primary bovine mammary cells precluded confirmation of all polycation ratios and combinations tested in Comma-D cells of Figure 4.5.

As with Comma-D cells, transfection of primary bovine mammary cells with mixed polycations [Orn₁₉:DEAE:DNA] doubled hGH expression over that for either polycation alone (Fig. 4.6). Recombinant hGH expression from mixed polycation-transfected primary bovine mammary cells steadily increased to 14 ng/ml on day 8 posttransfection (Fig. 4.6). DEAE- transfected primary bovine mammary cells exhibited a similar expression profile, but with lower peak hGH concentrations (7.5 ng/ml) (Fig. 4.6). In contrast, hGH expression from Orn₁₉-mediated transfection peaked at 6.0 ng/ml on day 6 posttransfection, then decreased slightly (Fig. 4.6). Thus, the efficacy of expression with pcDNA1/hGH allowed study of gene transfer in primary bovine mammary cells in vitro. Furthermore, mixed polyions increased recombinant hGH expression (Fig. 4.6) as predicted from Comma-D mouse mammary cell transfections (Fig. 4.5), and enhanced analytical feedback in primary bovine mammary cells.

Rationale for the use of poly(glutamylhistamineglutamate) (Glu₉-HisN) in polyion-mediated transfection

Although Orn₁₉-mediated transfection of Comma-D cells delivered significantly more DNA to the nucleus than did DEAE-mediated transfection (Fig. 4.3A and B), the DNA appears to be largely non-functional, as little hGH was produced (Fig. 4.3D). Conversely, despite it’s inefficient cellular delivery, DEAE-mediated transfection gave
Figure 4.6. Effect of single and mixed polycation-mediated transfection in primary bovine mammary cells in vitro. Primary bovine mammary cells were cultured and transfected as described in methods by addition of 0.2 ml transfection medium containing polycation:DNA complexes to cells in 2.0 cm² wells and left in contact with cells for 4 hrs at 37°C. Transfection complexes were prepared by addition of pcDNA1/hGH plasmid DNA to a final concentration of 5 µg DNA/ml to the following polycation:DNA combinations: Omₙ (■) [Omₙ(16.6 µg/ml), pcDNA1/hGH (5 µg/ml)]; DEAE (▼) [DEAE (250 µg/ml), pcDNA1/hGH (5 µg/ml)]; and mixed polycations (○) [Omₙ (37.5), DEAE (12.5 µg/ml), pcDNA1/hGH (5 µg/ml)] in HBSS to achieve a 10:1 polycation:DNA ratio and 3:1 Omₙ:DEAE ratio.
much higher expression per microgram of DNA in the nucleus (Fig. 4.3B and E). Collectively, these observations led to the hypothesis that differences in the cationic charge-density (interval) within Ornₙ and DEAE polyions may account for the incongruence between the nuclear localization of radiolabeled plasmid DNA and recombinant hGH expression. At physiological pH, each ε-amine (pKa = 10.8) in a poly-L-ornithine or poly-L-lysine polymer is positively charged, whereas DEAE contains about one amine (pKa 9.5) (Pharmacia, 1980) per two or three glucose residues (Borenfreund et al., 1973). Additionally, Shapiro et al. (1969) described the interaction of polylysine with DNA as a 1:1 stoichiometry (one amino acid residue/nucleotide). This suggested that the greater interval between cationic charges of DEAE may result in a lower affinity of DEAE for DNA, which would facilitate polycation:DNA dissociation and exposure of plasmid DNA to transcriptional components in the nucleus. Likewise, the shorter interval between cationic charges of Ornₙ may result in a higher affinity of Ornₙ for DNA, which would inhibit polycation:DNA dissociation. Therefore, incorporation of additional polyions which might destabilize strong DNA:polycation interactions into mixed polycation transfection complexes should facilitate exposure of internalized transfected DNA to the transcriptional apparatus and enhance subsequent recombinant gene expression.

Synthesis of poly(glutamylhistamineglutamate) (Gluₙ-HisN)

A charge-shifting polymer poly(glutamylhistamineglutamate) (Gluₙ-HisN) was synthesized according to Shen, (1990) as outlined in Figure 4.7, by activation of
Synthesis of Charge-Shifting Polylon: poly(glutamylhistamineglutamate)

Figure 4.7. Synthesis of poly(glutamylhistamineglutamate). Poly(glutamylhistamine-g glutamate) (GluN-HisN) was synthesized by carbodiimide (EDAC) activation of the free \( \gamma \)-carboxyl groups of poly-L-glutamic acid (Glu) and subsequent derivatization with histamine dihydrochloride (HisN) as described in methods.
poly-L-glutamic acid (Glu_n) with 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDAC) and partially derivatized with histamine dihydrochloride (3:2 Glu_n:HisN molar ratio), to yield the charge-shifting polypeptide poly(glutamyl-histamineglutamate) (Glu^-HisN) (Fig. 4.7). Amino acid analysis of Glu^-HisN indicated 40% of the glutamic acid residues had been modified by addition of histamine.

**Effect of pH on net charge of poly(glutamylhistamineglutamate) (Glu^-HisN)**

The unique charge-shifting properties predicted for Glu^-HisN (polyanionic at pH > 6.2, neutral at pH < 6.2 > 4.5, and polycationic at pH < 4.5) (Fig. 4.8) can be attributed to the sequential protonation of the imidazole (pKa 6.0) of the derivatizing histamine and unreacted glutamate γ-carboxyl (pKa 4.1) groups. At the pH of transfection complex formation (7.0-7.4), Glu^-HisN is anionic and should be readily incorporated into mixed polycation:DNA transfection complexes [Orn_n:DEAE:DNA] by ionically interacting with both Orn_n and DEAE. Polycation:DNA transfection complexes are internalized by endocytosis (Kabanov and Kabanov, 1995; Marciano-Cabral et al., 1981) resulting in exposure of transfection complexes to mildly acidic (pH ~ 6) conditions (Anderson and Orci, 1988) which should partially protonate the imidazole (pKa 6.0) of the derivatizing histamine in Glu^-HisN to shift the net charge of Glu^-HisN to neutral. Fusion of late endosomes with lysosomes would further expose Glu^-HisN to increasingly acidic conditions (pH 5.0) which should protonate more fully the histamines, and also a portion of the underivatized glutamate γ-carboxyls to give Glu^-HisN an overall cationic charge.
Figure 4.8. Effect of pH on net charge of poly(glutamylhistamineglutamate) Glu\textsuperscript{-}-HisN. The unique charge-shifting properties of Glu\textsuperscript{-}-HisN (polyanionic at pH > 6.2, and polycationic at pH < 4.5) can be attributed to the sequential protonation of the derivatized imidazole (pKa 6.0) and unreacted carboxyl (pKa 4.1) groups. During transfection complex formation at pH ≥ 7, Glu\textsuperscript{-}-HisN should ionically bind polycations. Protonation of the histamine imidazole gives the molecule an overall net neutral charge which should facilitate dissociation of polycations in a mildly acidic environment such as within endosomes (pH 5-6). Further protonation of the carboxyl moiety at acidic pH ≤ 4.5 should allow Glu\textsuperscript{-}-HisN to compete with polycations for DNA interaction.
Effect of pH on Net Charge of poly(glutamylhistamineglutamate)

pH > 6.2
(−)

pH < 6.2 > 4.1
(0)

pH < 4.1
(+)

Glutamate

Histamine

Figure 4.8
Because Gluₙ-HisN is ionically incorporated into mixed polycation transfection complexes, a shift in the overall net charge of Gluₙ-HisN from anionic to neutral or cationic, should weaken or eliminate the interaction of Gluₙ-HisN with both Orn and DEAE, leading to dissociation of the transfection complex and possible exposure of DNA for transcription which should facilitate recombinant gene expression. A pH-dependent polyion:DNA dissociation strategy as outlined above requires exposure of internalized transfection complexes to decreasing pH (5-6) as would be expected during endocytosis.

**Effect of Gluₙ-HisN addition to mixed polycation transfection complexes**

To test the hypothesis that incorporation of additional polyions which might destabilize strong DNA:polycation interactions into mixed polyion transfection complexes and thereby facilitate exposure of internalized transfected DNA to the transcriptional apparatus for enhanced recombinant gene expression, primary bovine mammary cells were transfected with individual polycations, mixed polycations, or mixed polycations containing the charge-shifting Gluₙ-HisN polymer. For additional comparison, primary bovine mammary cells were transfected with the commercially available Lipofectin polycationic-liposome.

As predicted, inclusion of the charge-shifting Gluₙ-HisN polymer in mixed polycation transfections dramatically increased recombinant hGH expression (~5 fold) over that from transfections with mixed polycations or individual polycations alone (DEAE) (Fig. 4.9). Recombinant hGH expression peaked on days 10-12 posttransfection.
Figure 4.9. Effect of Glu-HisN in mixed polycation transfection complexes on recombinant hGH expression from primary bovine mammary cells transfected in vitro. Primary bovine mammary cells were transfected with: cationic lipids (■) [Lipofectin (25 μg/ml), pcDNA1/hGH (5 μg/ml)]; single polycation (▼) [DEAE (250 μg/ml), pcDNA1/hGH (5 μg/ml)]; mixed polycation (●) [Ornₙ (37.5 μg/ml), DEAE (12.5 μg/ml), pcDNA1/hGH (5 μg/ml)]; or charge-shifting polyion (○) [Ornₙ (37.5 μg/ml), Glu-HisN (75 μg/ml), DEAE (12.5 μg/ml), pcDNA1/hGH (5 μg/ml)] complexes formed in HBSS.
at an unprecedented 100 ng/ml for mixed polycation transfections utilizing the charge-shifting Glu$_n$-HisN polymer (Fig. 4.9). hGH expression from mixed polycation- and DEAE-mediated transfections reached only 30 and 8 ng hGH/ml on days 6 and 10 posttransfection, respectively (Fig. 4.9). Surprisingly, transfection of primary bovine mammary cells with Lipofectin gave only marginally detectable hGH expression (< 2 ng/ml). Additionally, incorporation of Glu$_n$-HisN into mixed polycation transfections appeared to extend the expression profile compared to that obtained with mixed polycation transfection which peaked at day 6 posttransfection then steadily declined (Fig. 4.9) suggesting that transfection with charge-shifting polion:DNA complexes may have the potential to prolong expression. Similar but less dramatic increases in recombinant hGH expression were seen when the charge-shifting Glu$_n$-HisN polion was used in mixed polycation transfections of Comma-D cells (results not shown). These results are consistent with the hypothesis that facilitation of polycation:DNA dissociation by perturbation of charge complexes would increase recombinant gene expression.

**Cellular uptake and subcellular distribution of transfected plasmid DNA in mixed polycation transfections with or without the charge-shifting Glu$_n$-HisN polymer**

To determine if increases in recombinant gene expression from mixed polycation transfections with or without Glu$_n$-HisN were related to increased nuclear localization, Comma-D cells were transfected with radiolabeled plasmid DNA, and examined for cellular uptake and subcellular distribution, as previously described. Cellular uptake and
subcellular distribution studies required the use of Comma-D cells, as primary bovine mammary cells cultured on collagen gels showed poor recovery from the well and subcellular fractionation.

As might be expected, the addition of Orn, to DEAE in mixed polycation transfections doubled the relative nuclear localization (55%) (Fig. 4.10) of radiolabeled plasmid DNA over that for DEAE alone (Fig. 4.3B), while retaining DEAE's capacity for high level gene expression (Fig. 4.9) for the transfected DNA delivered to the nucleus. Interestingly, cellular uptake and subcellular distribution of radiolabeled plasmid DNA in Comma-D cells was similar for mixed polycation transfections performed with or without Glu,HisN (Fig. 4.10). As in the earlier subcellular distribution experiment (Fig. 4.3), virtually all intracellular radiolabeled-plasmid was detected in nuclear fractions, with almost none detected in cytosolic fractions or trypsinization supernatants (Fig. 4.10). This suggests that the increased recombinant hGH expression seen in transfections containing mixed polycations and Glu,HisN was not due to increased cellular or nuclear internalization, but instead was due to other mechanism(s), possibly facilitation of polycation:DNA dissociation.

Rationale for polyanion order of addition

The enhanced expression from transfection complexes prepared with polycations in combination compared to single polycations suggested that a reduction in the ionic interactions between the DNA and polycation might facilitate recombinant gene
Figure 4.10. Effect of incorporation of Glu₉-HisN into mixed polycation transfection complexes on cellular uptake and subcellular distribution of ³²P-labeled plasmid DNA. Comma-D cells were cultured as described in methods and transfected with ³²P-labeled plasmid DNA (5μg/ml), (1.0 μg/2.0 cm² well) and analyzed for cellular uptake and subcellular distribution as described if Fig. 4.3. Cells were transfected with either mixed polycations (solid bars) [Omr₉ (37.5 μg/ml), DEAE (12.5 μg/ml), pcDNA1/hGH (5 μg/ml)], or charge-shifting polyions (hatched bars) [Omr₉ (37.5 μg/ml), Glu₉-HisN (75 μg/ml), DEAE (12.5 μg/ml), pcDNA1/hGH (5 μg/ml)] formed in HBSS. Bars indicate percent of ³²P-radiolabeled-plasmid DNA recovered in pre- and posttransfection medium, supernatant after trypsinization of cells from well (Tryp Sup), washed cells (Cells), and for total cellular uptake in the subcellular cytosol (Cyt) and nuclear (Nuc) fractions after 4 hr. transfection.
expression. Incorporation of the charge-shifting Glu$_n$-HisN polymer, predicted to perturb DNA:polycation interaction in acidic environments, dramatically increased recombinant gene expression (Fig. 4.9). Therefore, mechanisms which further weaken or disrupt polycation:DNA ionic interactions would be predicted to facilitate increased recombinant gene expression. Thus, polyion transfection complex formation was controlled for the order of addition of the polyions to the DNA so as to further minimize polycation:DNA ionic interactions by placing weaker polycations (DEAE) adjacent to the DNA to facilitate polycation:DNA dissociation and to thereby increase recombinant gene expression. Previous transfection studies were conducted with polyion transfection complexes formed by the final addition of DNA to solutions of polycations alone, premixed polycations (Orn$_n$:DEAE), or premixed polycations and the charge-shifting polymer (Orn$_n$ :Glu$_n$ - HisN:DEAE). Therefore, the order and proportion of addition of polyions to DNA during transfection complex formation was likely driven by the concentration and affinity of each individual polyion for DNA already in solution. Consequently, despite the controlled order of addition of polyions to the solution before addition of DNA, there was no control over the order in which those polyions would interact with DNA added in the final step of transfection complex formation. Hence, ordered addition of transfection polyions so as to first form a complex between DNA and a weaker polycation (DEAE), with subsequent addition of the anionic (at pH 7.4) Glu$_n$-HisN to form an intermediate charge-shifting polyion layer before addition of the final strong polycation (Orn$_n$) layer should maximize the expression potential for DNA arriving to the nucleus while providing
maximum surface cation charge-density to facilitate cell targeting. Transfection complexes formed by allowing weaker polycations (DEAE) to complex first with DNA, followed by subsequent Glu₃-HisN and Orn₆ addition successively, were termed "ordered" transfection complexes, as opposed to the earlier "unordered" mixed polyion transfection complexes in which order of polyion exposure to DNA was not controlled.

In conjunction with ordered polyion transfection complex development, an isotonic sucrose/HEPES buffered medium (0.3 M sucrose, 10 mM HEPES, pH 7.4), used by others to maintain osmotic and ionic balance in small ruminant intramammary infusion studies (C. Bennett, personal communication, 1993), was tried as the vehicle for transfection complex formation and delivery in hope of further alleviating the limited cell injury suspected for transfections done in HBSS. Isotonic sucrose/HEPES buffered solutions have previously been used for direct gene transfer into mouse muscle in vivo (Wolff et al., 1991).

**Effect of polyion order of addition and transfection medium**

As predicted, transfection of primary bovine mammary cells with ordered charge-shifting mixed polyion transfection complexes [DNA:DEAE:Glu₃-HisN:Orn₆] gave over two-fold higher hGH expression (32 ng/ml on day 17 posttransfection) than did unordered charge-shifting mixed polyion transfection complexes [Orn₆:Glu₃-HisN:DEAE:DNA] (12 ng/ml on day 15 posttransfection) each prepared in HBSS (Fig. 4.11). Similarly, greater recombinant hGH expression was also observed for ordered (63 ng hGH/ml on day 17
Figure 4.11. Effect of polyion order of addition and transfection medium on recombinant hGH expression. Primary bovine mammary cells were cultured as described in methods and transfected with: ordered transfection complexes formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4, (■) [pcDNA1/hGH (5 μg/ml), DEAE (12.5 μg/ml), Glu\textsubscript{n}-HisN (75 μg/ml), Orn\textsubscript{n} (37.5 μg/ml)]; ordered transfection complexes formed in HBSS (●) [pcDNA1/hGH (5 μg/ml), DEAE (12.5 μg/ml), Glu\textsubscript{n}-HisN (75 μg/ml), Orn\textsubscript{n} (37.5 μg/ml)]; unordered transfection complexes formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4, (□) [Orn\textsubscript{n} (37.5 μg/ml), Glu\textsubscript{n}-HisN (75 μg/ml), DEAE (12.5 μg/ml), pcDNA1/hGH (5 μg/ml)]; or unordered transfection complexes formed in HBSS (○) [Orn\textsubscript{n} (37.5 μg/ml), Glu\textsubscript{n}-HisN (75 μg/ml), DEAE (12.5 μg/ml), pcDNA1/hGH (5 μg/ml)]. Polyions and DNA were mixed in the order listed.
posttransfection) compared to unordered (25 ng hGH/ml on day 11 posttransfection) charge-shifting mixed polyion transfection complexes formed and applied in 0.3 M sucrose, 10 mM HEPES, pH 7.4 (Fig. 4.11). More surprising was the substantial increase in expression from transfection complexes prepared in sucrose/HEPES versus those prepared in HBSS (Fig. 4.11). Whether ordered or unordered, charge-shifting mixed polyion transfection complexes formed and applied in sucrose/HEPES transfection medium consistently gave two-fold higher hGH expression than identical transfection complexes formed in HBSS (Fig. 4.11). Unexpectedly, ordered formation of charge-shifting mixed polyion transfection complexes was synergistic with sucrose/HEPES transfection medium, increasing total hGH expression 168% over that predicted from the additive effects (Fig. 4.11). These results are consistent with the hypothesis that factors which should facilitate polycation:DNA dissociation lead to increased recombinant gene expression.

Effect of carbohydrates on ordered charge-shifting mixed polyion transfection

The synergism between ordered formation of charge-shifting mixed polyion transfection complexes and sucrose/HEPES transfection medium was quite unexpected. Therefore, to investigate if the synergism was specific for transfection complex formation in sucrose transfection medium, other mono- and disaccharides were examined for their ability to increase recombinant gene expression. Therefore, a single preliminary study used ordered charge-shifting mixed polyion transfection complexes formed in 10 mM
HEPES, pH 7.4, also containing 0.3 M mono- or disaccharides sucrose, lactose, maltose, mannose or D-glucose to transfect primary bovine mammary cells, as above.

Consistent with previous findings (Fig. 4.11), formation of ordered charge-shifting mixed polyion transfection complexes in sucrose/HEPES dramatically increased recombinant hGH expression (~8 fold) over identical transfection complexes prepared in HBSS (Fig. 4.12). However, recombinant hGH expression from ordered mixed polyion transfection complexes formed in HBSS was much lower than normally observed (Fig. 4.11 vs. Fig. 4.12). Nevertheless, for this single experiment, hGH expression from ordered charge-shifting mixed polyion transfection complexes formed in isotonic carbohydrate-based transfection media was consistently higher than expression from transfection complexes formed and applied in HBSS (Fig. 4.12). Interestingly, transfection complexes formed in sucrose gave markedly higher expression (peak hGH = 38 ng/ml on day 12 posttransfection) than those formed in other carbohydrates (peak hGH ≤ 20 ng/ml) (Fig. 4.12).

Effect of transfection with increasing DNA concentration at constant polycation:
DNA ratios on recombinant hGH expression

To further optimize recombinant hGH expression from ordered charge-shifting mixed polyion transfection complexes formed in sucrose/HEPES, primary bovine mammary cells were transfected with increasing concentrations of DNA in ordered charge-shifting mixed polyion transfection complexes which maintained constant total
Figure 4.12. Effect of different isotonic mono- and disaccharides on hGH expression when added to ordered charge-shifting mixed polyion transfection complexes. Primary bovine mammary cells were cultured as described in methods and transfected with ordered mixed polyion transfection complexes [pcDNA1/hGH (5 µg/ml), DEAE (12.5 µg/ml), Glu-HisN (75 µg/ml), Orn (37.5 µg/ml)] formed and applied to cells in 10 mM HEPES, pH 7.4, made isotonic by addition of 0.3M: sucrose (●), lactose (▼), maltose (■), mannose (□), or D-glucose (○), or in HBSS (▼) without carbohydrates.
polycation:DNA and Orn4:DEAE ratios. For transfections performed with 0, 1, 5, or 10 
µg DNA/ml, hGH expression increased with DNA concentration, with similar temporal 
patterns and expression differences persisting over the 14 day posttransfection period 
(Fig. 4.13), with 10 µg DNA/ml giving maximum expression. However, peak expression 
from transfections performed with 25 µg DNA/ml was relatively lower (27.5 ng hGH/ml) 
on day 14 posttransfection (Fig. 4.13).

Effect of increasing DNA concentration at constant polycation:DNA ratios on 
cumulative recombinant hGH expression

A second, more comprehensive study was done to better define the effects of 
transfecting DNA concentrations from 10-25 µg DNA/ml on recombinant hGH 
expression in primary bovine mammary cells transfected with ordered charge-shifting 
mixed polycation transfection complexes formed in sucrose/HEPES. For all transfecting 
DNA concentrations, ratios of total polycation:DNA (10:1) and Orn4:DEAE (3:1) were 
maintained as before. Cumulative hGH expression is defined as the summation of hGH 
concentrations (ng/ml) in transfection medium at each sampling day without accounting 
for variation in volume of cell culture medium recovered.

As previously seen in Fig. 4.13, primary bovine mammary cells transfected with 
ordered charge-shifting mixed polycation transfection complexes containing 10 µg DNA/ml 
gave the highest total hGH expression with a cumulative total of 450 ng hGH secreted 
through 20 days posttransfection (100%) (Fig. 4.14). Increasing DNA concentrations
Figure 4.13. Effect of increasing DNA concentration at constant polycation:DNA ratios on hGH expression in primary bovine mammary cells transfected in vitro. Primary bovine mammary cells were cultured as described in methods and transfected with ordered mixed polyion transfection complexes containing increasing amounts of DNA and corresponding concentrations of polyions to maintain the 10:1 total polycation:DNA ratio and 3:1 Orn₃:DEAE ratio formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4, consisting of: untransfected control (○); [pcDNA1/hGH (1 μg/ml), DEAE (2.5 μg/ml), Glu₅-HisN (15 μg/ml), Orn₃ (7.5 μg/ml)] (■); [pcDNA1/hGH (5 μg/ml), DEAE (12.5 μg/ml), Glu₅-HisN (75 μg/ml), Orn₃ (37.5 μg/ml)] (▲); [pcDNA1/hGH (10 μg/ml), DEAE (25 μg/ml), Glu₅-HisN (150 μg/ml), Orn₃ (75 μg/ml)] (○); or [pcDNA1/hGH (25 μg/ml), DEAE (62.5 μg/ml), Glu₅-HisN (375 μg/ml), Orn₃ (187.5 μg/ml)] (□).
Figure 4.14. Effect of increasing DNA concentration at constant polycation:DNA ratios on cumulative hGH expression in primary bovine mammary cells transfected in vitro. Primary bovine mammary cells were transfected with ordered charge-shifting mixed polyion transfection complexes containing increasing amounts of DNA and corresponding concentrations of polyions to maintain the 10:1 total polycation:DNA ratio and 3:1 Orn<sub>a</sub>:DEAE ratio. Complexes were formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4, and consisted of: untransfected control; [pcDNA1/hGH (1 μg/ml), DEAE (2.5 μg/ml), Glu<sub>n</sub>-HisN (15 μg/ml), Orn<sub>a</sub> (7.5 μg/ml)]; [pcDNA1/hGH (5 μg/ml), DEAE (12.5 μg/ml), Glu<sub>n</sub>-HisN (75 μg/ml), Orn<sub>a</sub> (37.5 μg/ml)]; [pcDNA1/hGH (10 μg/ml), DEAE (25 μg/ml), Glu<sub>n</sub>-HisN (150 μg/ml), Orn<sub>a</sub> (75 μg/ml)]; [pcDNA1/hGH (15 μg/ml), DEAE (37.5 μg/ml), Glu<sub>n</sub>-HisN (225 μg/ml), Orn<sub>a</sub> (112.5 μg/ml)]; [pcDNA1/hGH (20 μg/ml), DEAE (50 μg/ml), Glu<sub>n</sub>-HisN (300 μg/ml), Orn<sub>a</sub> (150 μg/ml)]; or [pcDNA1/hGH (25 μg/ml), DEAE (62.5 μg/ml), Glu<sub>n</sub>-HisN (375 μg/ml), Orn<sub>a</sub> (187.5 μg/ml)].
above 10 μg DNA/ml decreased hGH expression as DNA concentration increased (Fig. 4.14). Transfection with 15 μg DNA/ml gave 375 ng total hGH (roughly 80%), while transfections with 0, 1, 5, 20, or 25 μg DNA/ml yielded less than 50% of the total hGH from transfections with 10 μg DNA/ml (Fig. 4.14). These dose/response relationships and optimum DNA concentration are consistent with the previous experiment (Fig. 4.13) and confirm that 10 μg DNA/ml is optimal for in vitro transfection of primary bovine mammary cells with ordered charge-shifting mixed polyion transfection complexes formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4.

**Effect of free poly-L-glutamic acid or free histamine on transfection**

To demonstrate that the increase in recombinant hGH expression from ordered charge-shifting mixed polyion transfection complexes was a result of the histamine derivatization of poly-L-glutamic acid and was not due to either underivatized Glu, or histamine alone, primary bovine mammary cells were transfected with ordered mixed polyion transfection complexes containing either poly(glutamylhistamineglutamate) (Glu-HisN), underivatized Glu, free histamine, or a combination of underivatized Glu, and free histamine each added in the same order as was Glu-HisN to form transfection complexes in sucrose/HEPES. Incorporation of the charge-shifting polyion Glu-HisN in mixed polycation transfection dramatically increased peak recombinant hGH expression (85 ng hGH/ml) over that from transfection with mixed polycations alone (17 ng hGH/ml) (Fig. 4.15) similar to previous results (Fig. 4.9). However, inclusion of free
Figure 4.15. Effect on recombinant hGH expression of transfection complexes in which histamine and underivitized Gluₙ were substituted for Gluₙ-HisN in ordered charge-shifting mixed polyion transfection of primary bovine mammary cells in vitro. Primary bovine mammary cells were transfected with ordered mixed polyion transfection complexes formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4, as follows: [pcDNA1/hGH (10 μg/ml), DEAE (25 μg/ml), Gluₙ-HisN (150 μg/ml), Ornₙ (75 μg/ml)] (●); [pcDNA1/hGH (10 μg/ml), DEAE (25 μg/ml), Ornₙ (75 μg/ml)] (■); [pcDNA1/hGH (10 μg/ml), DEAE (25 μg/ml), histamine (94 μg/ml), Ornₙ (75 μg/ml)] (□); [pcDNA1/hGH (10 μg/ml), DEAE (25 μg/ml), poly-L-glutamic acid (150 μg/ml), Ornₙ (75 μg/ml)] (▲); [pcDNA1/hGH (10 μg/ml), DEAE (25 μg/ml), poly-L-glutamic acid (150 μg/ml), histamine (94 μg/ml), Ornₙ (75 μg/ml)] (○).
histamine in ordered charge-shifting mixed polyion transfection complexes at 0.75 times the molar histamine concentration provided by Glu\textsubscript{n}-HisN in ordered charge-shifting mixed polyion transfection complexes gave recombinant hGH expression similar to that from transfections with mixed polycations without Glu\textsubscript{n}-HisN (Fig. 4.15). Surprisingly, the addition of underivatized Glu\textsubscript{n} at equimolar concentrations of Glu\textsubscript{n} provided by Glu\textsubscript{n}-HisN in ordered charge-shifting mixed polyion transfection complexes, with or without free histamine, to mixed polycation transfection complexes virtually abolished recombinant hGH expression. From this, histamine derivatization of Glu\textsubscript{n} is required for proper Glu\textsubscript{n}-HisN function in mixed polyion transfection complexes (Fig. 4.15). Neither histamine or Glu\textsubscript{n} alone or combined could mimic the ability of Glu\textsubscript{n}-HisN to enhance expression from mixed polycation transfections.

**Preliminary comparison of varied polyion levels in ordered charge-shifting mixed polyion transfection complexes**

To determine whether addition of Glu\textsubscript{n}-HisN altered optimal polycation ratios in mixed polycation transfection complexes, particularly following the development of ordered polyion addition and transfection in 0.3 M sucrose, 10 mM HEPES, pH 7.4, primary bovine mammary cells were transfected with ordered charge-shifting polyion transfection complexes formed in sucrose/HEPES in which polyion levels were independently varied.
Not surprisingly, dose response curves for the effects of each polyion on recombinant hGH expression showed a shift in optimal polyion ratios in those with Glu$_n$-HisN in the transfection complex (Fig. 4.16A-C). Response of hGH expression to varied DEAE concentrations in transfection complexes was somewhat erratic, but complexes containing 10 to 17.5 µg DEAE/ml gave slightly better expression, with 17.5 µg DEAE/ml appearing slightly superior with over 400 ng hGH total expression through day 20 posttransfection (Fig. 4.16A). This optimum DEAE concentration was 40% greater than that used in previous mixed polycation transfection complexes (12.5 µg DEAE/ml). Interestingly, variation of Glu$_n$-HisN from 10-175 µg/ml gave a clear optimum at 100 µg Glu$_n$-HisN/ml with cumulative hGH expression of over 500 ng to day 20 posttransfection (Fig. 4.16B). This represents a 33% increase of Glu$_n$-HisN concentration in transfection complexes over that used in previous experiments (75 µg Glu$_n$-HisN/ml). Finally, Orn$_n$ concentrations between 10 and 25 µg Orn$_n$/ml transfection medium showed slightly elevated hGH expression (Fig. 4.16C). The highest recombinant gene expression (> 400 ng hGH total) was obtained with 20 µg Orn$_n$/ml, a 46% decrease in Orn$_n$ from the 37.5 µg/ml found optimal for mixed polycation transfections alone (Fig. 4.16C).

Owing to the extensive reiteration which would have been required for complete optimization of charge-shifting mixed polyion transfection, and the limited availability of primary bovine mammary cells, this is the first time polyion effects had been examined since the addition of the charge-shifting polymer, with the exception of limited study to determine effective Glu$_n$-HisN concentrations.
Figure 4.16. Effect of independent polyion variation in ordered mixed polyion transfection complexes on recombinant hGH expression in primary bovine mammary cells transfected in vitro. Primary bovine mammary cells were transfected with ordered charge-shifting mixed polyion transfection complexes formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4, consisting of: [pcDNA1/hGH (5 μg/ml), DEAE (2.5-20 μg/ml), Glu−HisN (75 μg/ml), Orn (37.5 μg/ml)] (Panel A); [pcDNA1/hGH (5 μg/ml), DEAE (12.5 μg/ml), Glu−HisN (10-175 μg/ml), Orn (37.5 μg/ml)] (Panel B); or [pcDNA1/hGH (5 μg/ml), DEAE (12.5 μg/ml), Glu−HisN (75 μg/ml), Orn (5-50 μg/ml)] (Panel C).
Discussion

In an effort to develop a non-viral gene transfer system suitable for transfection of bovine mammary cells in vivo, various polycations were first examined for their ability to facilitate radiolabeled plasmid DNA uptake and nuclear localization in Comma-D mouse mammary cells in vitro. Both DEAE- (Fig. 4.3B) and Lysₙ-mediated transfection (Fig. 4.4) facilitated cellular uptake of only a small portion of the total radiolabeled-plasmid DNA (≤25%). In contrast, Ornₙ-mediated transfection (Fig. 4.3A) gave 100% cellular uptake of radiolabeled-plasmid DNA. These differences may be due in part to either the lower cation charge density (interval) of DEAE (Borenfreund et al., 1973) or that DEAE may facilitate DNA entry into cells without entering the cell itself as described by Borenfreund et al. (1973). A lower charge-density polymer such as DEAE may not bind to the negatively charged cell surface or to DNA as efficiently, or with the affinity of a high charge-density polycation such as Ornₙ or Lysₙ. However, polymer charge-density cannot account for the decreased internalization of radiolabeled-plasmid DNA from Lysₙ-mediated transfection which would be predicted to be similar or identical to Ornₙ-mediated transfection. Both Ornₙ and Lysₙ presumably facilitate recombinant gene transfer by ionic interaction with both the negatively charged plasmid DNA and the negatively charged cell surface (Farber et al., 1975; Pagano, 1970). However, this depends on sufficient excess polycation in DNA transfection complexes to give a strong net positive charge on the surface of the complex. Shapiro et al. (1969) provide evidence that poly-L-lysine binds DNA with 1:1 stoichiometry, (one amino acid...
residue/nucleotide) forming spherical condensates (torroids) or rods, reminiscent of DNA packaging in bacteriophage heads (Eickbush and Moudrianakis, 1978), which are of appropriate size (40-100 nm) (Shapiro et al., 1969) to be internalized by pinocytotic vesicles (Steinman et al., 1983). DEAE caused precipitation or colloid formation when mixed with nucleic acids (Maes et al., 1967), and therefore appears to behave similarly to polylysine in its interaction with DNA.

The significant amount of radiolabeled DNA (≥30%) detected in the trypsinization supernatant from Lys₉⁻transfected cells (Fig. 4.4) suggested that Lys₉⁻complexed DNA may have bound to the cell surface but was released by subsequent trypsinization. This also suggests that a significant portion of the Lys₉⁻:DNA transfection complexes bound to the cell exterior were not internalized. Farber et al. (1975) reported similar inconsistencies in Lys₉⁻-mediated transfection of Chinese hamster lung cells and concluded that Orn₉⁻-mediated transfection was consistently more effective, which is supported by these results (Fig. 4.3 and Fig. 4.4).

Regardless of the polycation used for transfection complex formation, most (≥85%) of the intracellular radiolabeled plasmid DNA detected in transfected Comma-D cells was found in the nuclear fraction, indicating efficient nuclear delivery of that DNA which was internalized after transfection (Fig. 4.3A, B and Fig. 4.4). This is consistent with Farber et al. (1975), who found that greater than 95% of Orn₉⁻-transfected DNA found in Chinese hamster lung cells was detectable in the nuclear fraction within 15 minutes posttransfection. Such rapid and complete plasmid nuclear localization suggests
that polycations may mimic nuclear localization signals, often identified as short tracts of basic amino acids in proteins destined for nuclear localization (Davis, 1992). Consensus sequences for nuclear localization signals are not highly conserved, and can consist of repeated but non-contiguous regions of basic amino acids (Davis, 1992, and references therein). Additionally, for all polycation transfections tested in these studies, the absence of radiolabeled DNA in cytosolic fractions suggested that internalized plasmid is either rapidly translocated to the nucleus (Fig. 4.3A, B and Fig. 4.4) (Farber et al., 1975) or recycled to the cell exterior by exocytosis (Friend et al., 1996) but does not remain long in the cytosolic endosomes. Therefore, unlike for other gene transfer systems (Friend et al., 1996; Zauner et al., 1995), endosome escape of transfection complexes into the cytosol does not appear to be rate-limiting for polycation-based transfections.

Surprisingly, recombinant hGH expression did not correlate with radiolabeled-plasmid DNA internalization and nuclear localization (Fig. 4.3A-F). In Comma-D cells transfected with unlabeled pTK/hGH, the only hGH expression plasmid available at the time, DEAE-mediated transfection gave hGH expression levels (~6 ng/ml) (Fig. 4.3B) similar to that from DEAE-mediated transfection of primary rabbit mammary cells (1 ng/ml) (Malienou-Ngassa et al., 1990). The higher hGH expression from the smaller amount of DNA delivered to DEAE-transfected cells (Fig. 4.3E and B) indicates higher expression per microgram of internalized DNA for DEAE compared to Om^n^-transfected cells (Fig. 4.3D and A). Again, these observations suggest a relationship of expression to the cation charge density of the polycation directly complexed to the transfected DNA.
Polycations with a lower charge-density, such as DEAE (Borenfreund et al., 1973), would be expected to have a lower affinity for DNA which would facilitate polycation:DNA dissociation and exposure of plasmid DNA to the transcriptional apparatus in the nucleus for increased recombinant protein expression. In contrast, high charge-density polycations such as Orn would be predicted to have a higher affinity for DNA and therefore not dissociate as readily, effectively preventing DNA interaction with transcriptional components. Modulation of DNA condensation and exposure to transcriptional apparatus in the nucleus by interaction with basic polypeptides is not without precedent. Histones, highly basic nuclear proteins, bind and condense DNA in a manner similar to polycations (Isenberg, 1979), but dissociate from DNA to allow its exposure for transcriptional activation (Yaniv and Cereghini, 1986) upon acetylation (Wu et al., 1986). These combined observations suggested that Orn and DEAE might be combined in transfections since Orn-mediated transfection provided good cell targeting and nuclear uptake but relatively poor hGH expression, while DEAE-mediated transfection gave enhanced hGH expression from a lower amount of DNA which it delivered to cells. Subsequent transfection of Comma-D cells with transfection complexes comprised of mixtures of polycations (Orn and DEAE) substantially increased recombinant gene expression over transfections with individual polycations alone at total polycation:DNA ratios of 10:1 and 20:1 (Fig. 4.5). A total polycation:DNA ratio of 10:1 with an Orn:DEAE ratio of 3:1 proved optimal for recombinant gene expression in Comma-D cells, giving expression which increased through day 8 posttransfection (Fig.
4.5). This is the first description of transfection with combined polycations in any cell type, and of the advantages of mixed polycations.

Construction of the pcDNA1/hGH expression vector with its stronger CMV promoter gave sufficient hGH expression in polycation-transfected primary bovine mammary cells to allow assessment of relative efficacy of transfection complexes and polycations. As with Comma-D cells, mixed polycation transfections of primary bovine mammary cells with pcDNA1/hGH gave greater recombinant hGH expression than transfections performed with either polycation alone (Fig. 4.6). Thus, the polycation transfection mixtures developed in this study cooperate to capitalize on the ability of Orn to efficiently deliver plasmid DNA to the cell and nucleus, and on the ability of DEAE to facilitate recombinant gene expression. This is the first description of mixed polycation-mediated transfection in primary bovine mammary cells.

It was hypothesized that polycation:DNA affinity, as determined by polycation charge-density, may control intracellular polycation:DNA dissociation and subsequent access to essential transcriptional apparatus, thereby influencing recombinant gene expression. Therefore, addition of polyions which might perturb polycation:DNA interactions to mixed polycation transfections was predicted to facilitate plasmid exposure and subsequent recombinant gene expression. Hence, a novel polyion, poly(glutamyhistamineglutamate) (Glu—HisN), designed by Shen (1990) to facilitate drug release from carrier molecules in mildly acidic endosomes has unique charge-shifting zwitterionic character (polyanionic at pH > 6.2, neutral at pH < 6.2 >4.5, and polycationic
at pH < 4.5) (Fig. 4.8) which might be used to perturb polycation:DNA interactions. Therefore, the Glu^-HisN charge shifting polion was synthesized (Fig. 4.7) and incorporated into mixed polycation transfection complexes and tested for its ability to enhance expression in transfected primary bovine mammary cells in vitro.

As predicted, mixed polycation transfection complexes containing the charge-shifting Glu^-HisN polion produced almost 5 times as much hGH as mixed polycation transfection complexes alone (Fig. 4.9). These results with Glu^-HisN were consistent with the hypothesis that perturbation of polycation:DNA interactions could facilitate recombinant gene expression.

A commercially available cationic lipid (Lipofectin) was also tested in primary bovine mammary cells for comparison with polycation-mediated transfection. Lipofectin-mediated gene transfer in primary bovine mammary cells gave only low level (~2 ng/ml) hGH expression (Fig. 4.9), in contrast to mixed polycation transfection complexes containing Glu^-HisN (Fig. 4.9), and contrary to the high recombinant gene expression in many cell lines transfected with cationic lipid complexes (Felgner et al., 1987; Murray, 1991). Interestingly, Lipofectin- and mixed polycation-mediated transfection gave similar expression of 30 and 35 ng hGH/ml, respectively, in Comma-D mouse mammary cells (results not shown). Therefore, the low level of expression directed by Lipofectin transfection in primary bovine mammary cells was not due to improper complex formation or nonfunctional DNA.
To determine if increased recombinant gene expression obtained from inclusion of the charge-shifting polymer was due to increased nuclear localization, the previous uptake and subcellular localization study was repeated using mixed polycations with and without the charge-shifting Glu$_n$-HisN polymer. These studies had to be performed on Comma-D cells, as primary bovine mammary cells required culture on contractible collagen gels for differentiation, making cell harvest and dissociation difficult. Interestingly, there were no significant differences in either cellular uptake or subcellular distribution of radiolabeled DNA transfected with or without the charge-shifting polymer (Fig. 4.10). However, addition of Orn$_n$ to DEAE to form mixed polycation transfection complexes doubled the amount of radiolabeled plasmid DNA localized to the nucleus (Fig. 4.10) over that for DEAE transfection alone (Fig. 4.3B) and increased hGH expression (Fig. 4.6), supporting the concept that Orn$_n$ enhanced nuclear delivery and DEAE enhanced expression of transfected DNA. Additionally, these results suggest that the charge-shifting Glu$_n$-HisN polymer does not increase gene expression by increasing cellular internalization or nuclear localization of transfected plasmid DNA, but rather exerts it's action by another mechanism(s).

Assuming that polycation:DNA affinity is proportional to intermolecular ionic interactions, then disruption of such interactions should facilitate dissociation of DNA and polycations and subsequent recombinant gene expression. The increased expression observed for transfection complexes which incorporated the charge-shifting Glu$_n$-HisN polyion together with mixed polycations is consistent with a role for Glu$_n$-HisN as a
perturbant of the primary polycation:DNA interaction. However addition of DNA to a preformed solution of all polyions allowed no control over which polycation formed the primary complex with DNA. Involvement of weaker polycations in the primary DNA:polycation complex should also facilitate dissociation of polycation from DNA and exposure of DNA for transcription. Hence ordered addition of transfection polyions so as to first form a complex between DNA and a weaker polycation (DEAE), with subsequent addition of the anionic (at pH 7.4) Glu$_n$-HisN to form an intermediate layer of charge-shifting polyion before addition of the final strong polycation (Orn$_n$) layer should maximize expression potential for DNA arriving to the nucleus while providing maximum surface cation charge-density to facilitate cell targeting. Indeed, ordered charge-shifting polyion complexes [DNA:DEAE:Glu$_n$-HisN:Orn$_n$] formed as described gave maximum expression (Fig. 4.9).

In contrast, the basis for the enhancement of expression by transfection complexes made in isotonic sucrose (0.3 M), HEPES (10 mM), pH 7.4, is less predictable. Isotonic sucrose/HEPES buffered medium, commonly used to maintain osmotic and ionic balance in intramammary infusion (C. Bennett, personal communication), was tried as the vehicle for transfection complex formation and delivery to further minimize even the limited cell injury suspected for transfections done in HBSS. Isotonic sucrose/HEPES has also been used in direct gene transfer into mouse muscle in vivo (Wolff et al., 1990; Wolff et al., 1991). The enhancement of expression for transfections done in sucrose/HEPES medium was even stronger than expected, and applied to both ordered and unordered
charge-shifting transfection complexes (Fig. 4.11). The single experiment to test effects of other mono and disaccharides for this effect showed that it was unique to sucrose, the only non-reducing sugar tested. Although the low expression from transfections in HBSS suggest caution in conclusion from this single pilot experiment, the effect appears related to the nonreducing disaccharide sucrose, and suggests either that other nonreducing sugars would have similar nonspecific effects, or that the specificity for sucrose might be due to receptor-mediated specificity and events, probably at intracellular levels. Manipulation of intracellular and endosomal osmotic events enhanced transfection in avian erythrocytes (Lieber et al., 1987). Therefore, examination of nonreducing carbohydrates other than sucrose is warranted, as well as inclusion of lipids in ordered charge-shifting mixed polycation transfection complexes, as Gao and Huang (1996) recently demonstrated an increase in recombinant gene expression when polycations are added to polycationic liposome-mediated transfections.

Examination of the temporal and cumulative effects of increasing DNA concentration on recombinant gene expression with constant total polycation:DNA and Orn₄:DEAE ratios indicated that 10 μg DNA/ml was optimal for ordered charge-shifting mixed polycation-mediated transfection of primary bovine mammary cells in vitro (Fig. 4.14). These high DNA requirements will likely need to be reduced for in vivo transfection of the bovine mammary gland (i.e., infusion of one liter transfection solution per quarter at 10 μg DNA/ml would require 40 mg DNA per animal)!
Substitution of underivatized Glu\textsubscript{n}, free histamine, or a combination thereof for Glu\textsubscript{n}-HisN in ordered charge-shifting mixed polyion transfection complexes confirmed that the unique charge-shifting character of the Glu\textsubscript{n}-HisN polyion was responsible for the increased recombinant hGH expression seen in primary bovine mammary cells and was not attributable to individual transfection components. The reduction in recombinant hGH expression with addition of underivatized Glu\textsubscript{n} to ordered mixed polyion transfection complexes (Fig. 4.15) may be due to ionic interaction between either DEAE or Orn\textsubscript{n} and Glu\textsubscript{n}, since underivatized Glu\textsubscript{n} has a higher charge density than Glu\textsubscript{n}-HisN. Increased ionic interaction between DEAE and Glu\textsubscript{n} may coat the DNA:DEAE complex so tightly with free Glu\textsubscript{n} and subsequent Orn\textsubscript{n} that the complex cannot readily dissociate within the intracellular pH range (pH 5-7). Alternatively, increased Orn\textsubscript{n};Glu\textsubscript{n} interaction could disrupt efficient cell targeting by neutralizing Orn\textsubscript{n} charge to a point where it no longer efficiently interacts with the negatively charged cell surface. Regardless, these results indicate that increases in recombinant hGH expression levels are dependent on the histamine derivatized Glu\textsubscript{n}-HisN and its unique charge-shifting character.

To determine whether addition of Glu\textsubscript{n}-HisN to mixed polycation transfection complexes altered optimal polycation ratios, particularly after the development of ordered polion addition and transfection in 0.3 M sucrose, 10 mM HEPES, pH 7.4, primary bovine mammary cells were transfected with ordered charge-shifting mixed polyion transfection complexes formed in sucrose/HEPES, and in which polion levels were independently varied (Fig. 4.16). These transfections identified new optimal
concentrations for individual polyions; DEAE (17.5 µg/ml), Glu-HisN (100 µg/ml), and Orn (20 µg/ml) (Fig. 4.16) in ordered charge-shifting mixed polyion transfections. However, complete optimization would have extensive reiteration which was not feasible in this investigation.

Described here is the development of a novel ordered charge-shifting mixed polyion-mediated transfection method capable of driving high level and prolonged recombinant gene expression in primary bovine mammary cells in vitro. The transfection components and conditions are anticipated to be compatible with in vivo transfection of the bovine mammary gland by direct intramammary infusion. Such polycationic complexes should be efficiently delivered to all mammary epithelial cells via non-specific ionic interaction following direct intramammary infusion, and therefore should allow transfection of most or all mammary cells in vivo without the need for survival selection.

Single polycation:DNA transfection complexes gave only low level (≤ 8 ng hGH/ml) expression in primary bovine mammary cells transfected in vitro (Fig. 4.6). Transfection of primary bovine mammary cells with mixed polycation transfection complexes (Fig. 4.6) using polycation concentrations optimized in Comma-D cells (Fig. 4.5) doubled expression over single polycation:DNA transfection complexes alone (Fig. 4.6). Addition of the novel charge-shifting Glu-HisN polymer to mixed polycation transfections (Fig. 4.9) increased recombinant hGH expression five fold over mixed polycations in primary bovine mammary cells (Fig. 4.6). Ordered charge-shifting mixed polyion transfection complexes formed and applied in sucrose/HEPES (Fig. 4.11) further
increased recombinant gene expression in primary bovine mammary cells two-fold over those formed and applied in HBSS (Fig. 4.11). An additional increase in hGH expression of 40% or more was attained when primary bovine mammary cells were transfected with 10 μg DNA/ml (Figs. 4.13 and 4.14) as opposed to the standard 5 μg DNA/ml (Figs. 4.13 and 4.14). However, use of 10 μg DNA/ml for transfection of bovine mammary glands in vivo will likely not be economically feasible. Therefore, further studies should focus on identification of polyions that might facilitate polycation:DNA dissociation, either by reduced charge-interaction (synthetic polymers with controlled charge intervals) or self-hydrolyzation. Furthermore, identification of the intracellular fate of ordered charge-shifting mixed polyion transfection complexes and the proportion of cells expressing transfected recombinant genes should help develop rational strategies to further increase recombinant gene expression level and efficiency by focusing on either increasing delivery and/or exposure of plasmid DNA in the nucleus or delivery of transfection complexes to more cells.
Chapter 5

Investigation of Limitations to Polyion-Mediated Transfection

The previous section (Chapter 4), described the development of a non-viral charge-shifting mixed polyion recombinant gene transfer method for the transfection of primary bovine mammary cells in vitro. This system produced high and sustained recombinant gene expression from primary bovine mammary cells in vitro with transfection conditions anticipated to be compatible with transfection of the bovine mammary gland in vivo. However, little is known of the mechanisms or details of polycation-mediated recombinant gene transfer and expression, particularly for DNA internalization, the intracellular trafficking and localization, transgene fate, expression within the nucleus, or limitations to gene expression.

Subcellular fractionation studies conducted in Chapter 4 showed virtually all internalized DNA was present in nuclear fractions, yet recombinant hGH expression was incongruent with nuclear localization (Fig. 4.3). Additionally, how did addition of the charge-shifting polyion, Glu^-HisN, reported to disrupt endosomes (Shen, 1990) increase recombinant gene expression if internalized DNA is localized to the nucleus? These inconsistencies suggest a better understanding is needed for intracellular trafficking and fate of transfection. Moreover, the well known inefficiency of most gene transfer
methods (Kabanov and Kabanov, 1995) suggests that further understanding of intracellular mechanisms for gene transfer and expression will be required in order to increase efficacy and efficiency for expression of transfected recombinant genes both in vitro and in vivo. Identification of the intracellular fate of ordered charge-shifting mixed polyion transfection complexes and the number of cells expressing transfected recombinant genes should help develop rational strategies to further increase polyion-mediated recombinant gene expression by focusing on either increasing delivery and/or exposure of plasmid DNA in the nucleus or delivery of transfection complexes to more cells.

However, for transfected genes, a distinction must be made between transfection efficiency, defined here as the number of cells receiving intracellular recombinant transgenes, and expression efficiency, defined as the proportion of transfected cells which actually express the transfected transgene. Few studies of cell transfection systems have distinguished between the two. Many transfection methods which claim to increase transfection efficiency cannot distinguish between actual increased transfection efficiency (cell targeting) and increased expression efficiency, since the number of cells receiving transfected transgenes was not quantified (Alberti and Fornaro, 1990; Gonzalez and Joly, 1995).

Zabner et al. (1995), in the first detailed study of the intracellular fate of transfected DNA, concluded that a better understanding of the cellular and molecular mechanisms involved in gene transfer is imperative for the development of rational
strategies to overcome current impediments to gene transfer. Zabner et al. (1995), identified five general barriers to successful recombinant gene transfer as: 1) delivery of recombinant DNA to target cells, 2) entry of DNA into cells, 3) escape of DNA from endosomes, 4) cytosolic translocation and nuclear entry of DNA, and 5) dissociation of the DNA:cationic lipid complex. Barriers to successful recombinant gene transfer vary with cell type (Kukowska-Latallo et al., 1996) and transfection method (Cotten et al., 1992; Zabner et al., 1995).

DNA may be delivered to cells by viruses (Smith et al., 1991), specific receptor-ligand interactions (Chowdhury et al., 1993; Cotten et al., 1993), and non-specific ionic interactions (Bond and Wold, 1987; Kukowska-Latallo et al., 1996). DNA may enter cells in a variety of ways including direct microinjection (Gordon et al., 1980), viral insertion (Jahner et al., 1985), electrophoresis (Xie and Tsong, 1993), physical disruption of cell membranes (Fechheimer et al., 1987), or endocytosis (Loyter et al., 1982; Rosenkranz et al., 1992). In fact, endocytosis is thought to be the predominate mechanism of DNA internalization for a number of gene transfer methods both in vitro (Loyter et al., 1982; Rosenkranz et al., 1992) and in vivo (Wu and Wu, 1988).

DNA internalized by endocytosis must subsequently escape the endosome, translocate through the cytosol, and then enter the nucleus to be available for transcription. Fusion of internalized liposomes with endosome vesicle membranes following endocytosis accounts for endosomal exit of polycationic lipid-transfected DNA
Exactly how DNA escapes from endosomes prior to their fusion with lysosomes is largely unknown.

Zauner et al. (1995) and Cotten et al. (1992) have shown that inactivated viral particles or viral lytic peptides incorporated into ligand-targeted transfections facilitated endosome lysis and DNA release. Others suggest that transfected DNA does not escape from endosomes, but instead escapes from lysosomes (Boussif et al., 1995; Mislick et al., 1995). Boussif et al. (1995) proposed that the cationic polymer polyethylenimine acts as a "proton sponge" in the lysosome providing extensive buffering which results in lysosomal swelling and rupture to release transfected DNA into the cytoplasm. The lysosomotropic agent chloroquine is presumed to facilitate endosome/lysosome escape in similar fashion (Kukowska-Latallo et al., 1996; Mislick et al., 1995).

Following endosome/lysosome escape, DNA must translocate from the cytosol into the nucleus. In addition, it must also dissociate from transfection components, if still complexed, either while still in the cytosol or once in the nucleus. Very little is known of how DNA translocates through the cytosol to enter the nucleus and how or when it dissociates from transfection components. Zabner et al. (1995) show that DNA:cationic lipid dissociation is a requirement for transfected transgene expression. By whatever mechanism(s), DNA eventually makes its way to the nucleus as evidenced by the expression of the recombinant gene.

Zabner et al. (1995) showed that only a small proportion of polycationic lipid-transfected DNA becomes internalized during transfection and that only a small subset
of that fraction ever exits the cytosol into the nucleus. Polycationic lipid-mediated transfection of 1.3 ug DNA/ml was estimated by Zabner et al. (1995) to introduce an average of $2.95 \times 10^5$ plasmid molecules per cell, which is similar to the calculated $1.46 \times 10^5$ plasmids expected to be delivered per cell by the mixed polyion-mediated transfection system described in Chapter 4, suggesting that the rate-limiting step for expression of transfected DNA is localization and exposure or availability for expression rather than cellular internalization (Zabner et al., 1995).

Upon internalization into the nucleus, recombinant transgenes become subject to the same transcriptional and translational constraints as endogenous gene expression. Furthermore, heterologous transgene expression may be subject to other regulatory constraints. For instance, in vitro heterologous transgene expression can be inhibited by DNA promoter methylation CMV (Prosch et al., 1996) and SV40 (Muiznieks and Doerfler, 1994) or methylation within the transcribed region of the reporter gene (Komura et al., 1995).

Recombinant transgene association with nuclear DNA binding proteins or chromatin structure may also be important for recombinant transgene expression. Chromatin is less condensed in areas that are transcriptionally active (Yaniv and Cereghini, 1986). Presumably, condensation prevents interaction with transcription factors and thereby inhibits transcription. Polyamines have also been shown to condense DNA (Leng and Felsenfeld, 1966) and likely will also have to dissociate from DNA prior
to initiation of transcription. Zabner et al. (1995) showed that DNA:cationic liposome dissociation was required for efficient transgene expression in COS cells in vitro.

Additionally, cDNAs appear to be expressed less efficiently than complete gene sequences containing introns. The presence of an intron, even a heterologous intron, located near the 5' end of the gene appears to be advantageous for expression (Brinster et al., 1988). Intervening intron sequences are reported to increase the efficiency of RNA 3' splicing (Huang and Gorman, 1990), thereby leading to an increase in the levels of both nuclear and cytoplasmic poly(A)+ RNA (Buchman and Berg, 1988).

Chapter 4, described the development of a non-viral charge-shifting mixed polyion recombinant gene transfer method for the transfection of primary bovine mammary cells in vitro which produced high and sustained recombinant gene expression from primary bovine mammary cells in vitro with transfection conditions anticipated to be compatible with transfection of the bovine mammary gland in vivo. The additional studies described here were conducted to determine if the high level recombinant gene expression reported in Chapter 4 was due to broad low level expression from a large number of cells or high level expression from very few cells, and to identify limitations to in vitro transfection with ordered charge-shifting mixed polyion:DNA complexes which might allow development of strategies to further increase recombinant gene expression.

Initial evaluation of polyion-mediated recombinant gene expression was accomplished by visualization of beta-galactosidase (β-gal) reporter gene expression in fixed transfected Comma-D mouse mammary and primary bovine mammary cells.
Subsequent transfections utilized the green fluorescent protein (GFP) reporter, developed by Chalfie et al. (1994), which allows nondestructive visualization of recombinant gene expression in living cells. Transfection of primary bovine mammary cells, Comma-D mouse mammary cells, Adenovirus type 5 transformed primary human embryonic kidney cells, (293 cells); SV40-transformed African green monkey kidney cells, (COS-1 cells); and human hepatocellular carcinoma cells, (Hep G2) with unordered charge-shifting mixed polyion:DNA transfection complexes indicated that very few cells actually expressed transfected transgenes as determined by β-gal and GFP visualization.

Therefore, to determine the proportion of cells exposed to mixed polyion transfection complexes that actually received transfected transgenes, fluorescently-labeled polycations or radiolabeled-DNA were incorporated into mixed polyion transfection complexes formed with and without the charge-shifting Glu₉-HisN polymer to examine transgene delivery and subcellular distribution. Transfections performed with quinolizino-substituted fluorescein isothiocyanate (QFITC) or Texas Red (TR)-labeled polycations and subsequent DAPI counterstaining of nuclei allowed determination of the subcellular distribution of fluorescently labeled transfection complex. Comparison of the subcellular distribution of transfection complexes in expressing and non-expressing cells was possible when a GFP-encoding expression vector was also used in conjunction with fluorescently-labeled polycations in transfection complexes and DAPI nuclear staining.

Studies of the subcellular distribution of transfection complexes suggested that endosome escape, nuclear entry, or both were potential rate-limiting steps for efficient
expression from ordered charge-shifting mixed polyion-mediated transfection. Transmission electron microscopy was used to examine the structure of mixed polyion transfection complexes and to gauge their approximate sizes. In addition, a viral endosomalolytic peptide (Zauner et al., 1995) was synthesized and incorporated into ordered charge-shifting mixed polyion transfection complexes in an effort to facilitate endosome escape and increase subsequent recombinant gene expression.

Ordered charge-shifting mixed polyion transfection complexes formed with fluorescently-labeled polycations could be seen attached to the exposed plastic cell culture dish surface in regions not covered by cells. This serendipitous observation allowed the development of an in vitro polycation:DNA dissociation assay with transfection complexes immobilized on plastic vessels which indicated that protonation of glutamatic acid γ-carboxyl moieties was responsible for dissociation of polycation:DNA complexes in vitro. This in vitro dissociation assay can also be used to rapidly identify potential polycation:DNA perturbants which might facilitate polycation:DNA dissociation and subsequent recombinant gene expression.

This study describes the transfection and expression efficiency and potential rate-limiting steps for ordered charge-shifting mixed polyion-mediated transfection. Also reported here are efforts to circumnavigate some of the rate-limiting steps identified and the development of an in vitro dissociation assay which may be used to rapidly identify polycation:DNA perturbants which might facilitate recombinant gene expression.
**Results**

**Efficiency of recombinant transgene expression in mammary cells.**

Polycation-mediated transfection should deliver recombinant transgenes to all cells indiscriminately via ionic charge interactions, giving each cell equal opportunity to express recombinant transgenes (Farber et al., 1975; Pagano, 1970). To determine if the high level recombinant gene expression reported in Chapter 4 was due to broad low level expression from a large number of cells or high level expression from very few cells, Comma-D mouse mammary and primary bovine mammary cells were transfected with unordered charge-shifting mixed polycation transfection complexes containing pCMV/β (Fig. 5.1), a plasmid capable of beta-galactosidase (β-gal) expression from the CMV promoter. Only a small percentage of cells showed β-gal transgene expression after unordered charge-shifting mixed polycation transfection of pCMV/β into Comma-D mouse (Fig. 5.2A and B) or primary bovine (Fig. 5.3) mammary cells. Transfection with DNA:polycion complexes composed of individual polycations [DEAE:DNA], [Orn_n:DNA], or mixed polycations [Orn_n:DEAE:DNA] produced similar results (not shown). Similarly, very few β-gal expressing cells were evident in transfected cell lines (COS-1, Hep G2, and 293) capable of SV40 ori-dependent extrachromosomal plasmid replication and high level recombinant gene expression (Asselbergs and Grand, 1993) (Fig. 5.4A-C). Although the relative β-gal expression efficiency varied in these cell lines (293 > COS > HepG2) (Fig. 5.4A-C) they contained cells which exhibited both
Figure 5.1. pCMVβ, a eukaryotic expression vector encoding the β-galactosidase gene driven from the CMV promoter.
Figure 5.2. β-galactosidase expression in Comma-D mouse mammary cells. Comma-D mouse mammary cells transfected with unordered charge-shifting mixed polyelectrolyte complexes [Orn\textsubscript{n} (37.5 µg/ml), Glu\textsubscript{n}-HisN (75 µg/ml), (DEAE (12.5 µg/ml), pCMVβ (5 µg/ml)] formed in HBSS. Cells were fixed and stained for cytosolic β-gal expression on day 11 posttransfection as described in methods. Panel A shows β-gal expression in cells magnified at 40x; Panel B, β-gal expression at 100x Magnification of field from within Panel A.
Figure 5.3. β-galactosidase expression in primary bovine mammary cells. Primary bovine mammary cells transfected with unordered charge-shifting mixed polyion complexes [Orn$_n$ (37.5 μg/ml), Glu$_n$-HisN (75 μg/ml), (DEAE (12.5 μg/ml), pCMVβ (5 μg/ml)] formed in HBSS. Cells were fixed and stained for cytosolic β-gal expression on day 4 posttransfection as described in methods. Note sparse β-gal expressing cells (arrows) (Magnification 40x).
Figure 5.4. β-galactosidase expression in COS-1, 293, and HepG2 cells. COS-1, 293, and HepG2 cells transfected with unordered charge-shifting mixed polyion complexes [Orr, (37.5 μg/ml), Glu-HisN (75 μg/ml), (DEAE (12.5 μg/ml), pCMVβ (5 μg/ml)] formed in HBSS. Cells were fixed on day 4 posttransfection before staining for cytosolic β-gal expression. Strong (filled arrows) and weak (open arrows) β-gal expression in COS (Panel A), 293 (Panel B), and HepG2 (Panel C) cells at 200x Magnification.
weak (open arrows) and strong (filled arrows) β-gal expression (Fig. 5.4A-C). Furthermore, neither the proportion of cells expressing β-gal nor the intensity of β-gal expression appear substantially different in plasmid replication competent cell lines (Fig. 5.4A-C) and Comma-D mouse mammary cells (Fig. 5.2), but both proportion and intensity seemed slightly greater than for primary bovine mammary cells (Fig 5.3). These results suggested that the high level recombinant gene expression from transfected primary bovine mammary cells seen in Chapter 4 was likely due to high level expression from very few expressing cells rather than low level expression from a large number of cells.

**Cellular uptake and subcellular distribution of FITC-labeled polycations in the absence of DNA.**

The absence of detectable recombinant β-gal gene expression in the majority of cells exposed to unordered charge-shifting mixed polyion transfection complexes (Fig. 5.2-5.4) necessitated confirmation of the initial premise, that polycations (and therefore mixed polyion transfection complexes) would bind to and transfect all cells indiscriminately. To confirm the extent of interaction of polycations and polyion transfection complexes with the cell type of primary interest, primary bovine mammary cells, FITC-labeled polycations were examined for their cellular uptake and subcellular distribution in the absence of DNA. For these experiments requiring a uniform layer of cells for photomicrography, primary bovine mammary cells were cultured on plastic at
39°C and transfected as described in methods with FITC-labeled polycations, since their irregular growth morphology on collagen gels precluded photomicrography and their growth at 39°C on plastic was previously shown to induce high \( \alpha_\text{S1}-\text{casein} \) and lactoferrin expression (Denise Beaulieu, personal communication, 1994).

As expected, FITC-labeled polycations were rapidly bound, internalized, and localized to the nucleus by nearly all primary bovine mammary cells exposed to F-Orn\(_n\) (Fig. 5.5A and B), F-Lys\(_n\) (Fig. 5.5C), or F-DEAE (Fig. 5.5D) in the absence of DNA. FITC-labeled polycations localized to the nucleus rapidly, as indicated by appearance of prominent nuclear fluorescence by 6 hrs. posttransfection (Fig. 5.5C and D). Little FITC-labeled polycation was visible in the cytosol of FITC-labeled polycation-transfected primary bovine mammary cells (Fig. 5.5A-D). These results show that free polycations not complexed with DNA ionically interacted with virtually all cells and were rapidly internalized regardless of polycation type. Interestingly, nearly all internalized polycation appears to have been translocated into the nucleus within the 6-24 hr. time frame of this study, and as expected if the polycation mimicked a cationic nuclear localization signal (Davis, 1992). Similar results were seen with Comma-D cells (results not shown).

**Cellular uptake and subcellular distribution of FITC-labeled Orn\(_n\) incorporated into transfection complexes containing DNA.**

To determine if FITC-labeled polycation internalization and subcellular localization was altered when FITC-labeled polycations were complexed with DNA,
Figure 5.5. Rapid uptake and nuclear localization of FITC-labeled polycations by primary bovine mammary cells in the absence of DNA. Primary bovine mammary cells were cultured on plastic at 39°C as described in methods and exposed to fluorescein-labeled (F) polycations as follows: [F-Orn, (143 μg/ml)] (Panels A and B), [F-Lys, (37.5 μg/ml)] (Panel C), or [F-DEAE (665 μg/ml)] (Panel D) in HBSS. Panels A and B: F-Orn localization at 24 and 16 hours posttransfection, respectively; Panel C: F-Lys localization at 6 hours posttransfection; Panel D: F-DEAE localization at 6 hours posttransfection. (Magnification A, C, D 200x; B 400x).
primary bovine mammary cells were cultured on plastic at 39°C and transfected with polycation:DNA transfection complexes containing FITC-labeled Orn$_n$ (F-Orn$_n$) alone (Fig. 5.6A and B) [F-Orn$_n$;DNA]; unordered charge-shifting mixed polyion complexes (Fig. 5.6C and D) [F-Orn$_n$;Glu$_n$-HisN:DEAE:DNA]; or ordered charge-shifting mixed polyion complexes (Fig. 5.6E and F) [DNA:DEAE:Glu$_n$-HisN:F-Orn$_n$] all formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4.

Within 4 hrs. posttransfection virtually all primary bovine mammary cells exposed to DNA:FITC-labeled polycation transfection complexes showed strong intracellular fluorescence (Fig. 5.6A-F). The high proportion of primary bovine mammary cells showing uptake and internalization of FITC-labeled polycation:DNA transfection complexes (Fig. 5.6A-F) appeared similar to that for FITC-labeled polycations alone (Fig. 5.5A-D), but the subcellular distribution of FITC-labeled polycation transfection complexes was dramatically different. Posttransfection localization of transfection complexes composed solely of F-Orn$_n$:DNA showed foci of closely packed small particles concentrated in the nuclear or perinuclear region of most cells (Fig. 5.6B). However, DNA:FITC-labeled charge-shifting mixed polyion transfection complexes, whether unordered [F-Orn$_n$:Glu$_n$-HisN:DEAE:DNA] (Fig 5.6D) or ordered [DNA:DEAE:Glu$_n$-HisN:F-Orn$_n$] (Fig. 5.6F), showed internalized complexes which appeared as foci of larger and more punctate FITC-labeled particles, a pattern which differed subtly from that for cells transfected with F-Orn$_n$ complexes as in Fig. 5.6B. Internalized FITC-labeled unordered charge-shifting mixed polyion transfection complexes (Fig 5.5D) appeared as
Figure 5.6. Uptake and subcellular distribution of FITC-labeled polyion transfection complexes by primary bovine mammary cells. Primary bovine mammary cells were cultured on plastic at 39°C and transfected with F-Orn$_n$:DNA [F-Orn$_n$ (37.5 µg/ml), DNA (5 µg/ml)] (Panels A, B); FITC-labeled unordered charge-shifting mixed polyion:DNA complexes [F-Orn$_n$ (37.5 µg/ml), Glu$_n$-HisN (75 µg/ml), DEAE (12.5 µg/ml), DNA (5 µg/ml)] (Panels C, D); or FITC-labeled ordered charge-shifting mixed polyion:DNA complexes [DNA (5 µg/ml), DEAE (12.5 µg/ml), Glu$_n$-HisN (75 µg/ml), F-Orn$_n$ (37.5 µg/ml)] (Panels E, F) all formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4. Panels A, B show DNA:F-Orn$_n$ transfection complexes in the nuclear (open arrows) and perinuclear (filled arrows) regions. Panel F (arrowheads) shows DNA:FITC-labeled ordered charge-shifting mixed polyion transfection complexes adhered to the plastic cell culture plate where cells are absent. Panels A, C, and E are phase contrast photomicrographs of B, D, and F fluorescent photomicrographs, respectively. All photomicrographs were taken 12-18 hours posttransfection. (Magnification 200x).
Figure 5.6
small clusters of fluorescent foci, while FITC-labeled ordered charge-shifting mixed polyion transfection complexes (Fig 5.6F) appeared as slightly larger aggregates of fluorescent foci. Additionally, DNA:FITC-labeled charge-shifting mixed polyion transfection complexes, whether unordered (Fig 5.6D) or ordered (Fig 5.6F), showed distinct aggregation of fluorescent foci.

Transfection complexes containing DNA and FITC-labeled polyions (Fig. 5.6B, D, and F) did not show the distinct intranuclear localization seen with FITC-labeled polyions alone (Fig. 5.5A-D). However, the available resolution of the light microscope did not allow determination of whether FITC-labeled ordered (Fig. 5.6F) or unordered (Fig. 5.6D) charge-shifting mixed polyion transfection complexes were truly localized in the nucleus or instead concentrated in the perinuclear region. Nevertheless, DNA:F-Orn\textsubscript{n} transfection complexes appeared to be both in the nucleus (Fig. 5.6A and B filled arrows), and in the perinuclear region (Fig. 5.6A and B open arrows). Interestingly, DNA:FITC-labeled charge-shifting mixed polyion transfection complexes can be seen adhered to the plastic of the cell culture plate in areas where cells were absent (Fig. 5.6F arrowheads). These attached transfection complexes visibly persisted for at least 20 days posttransfection, suggesting they were strongly immobilized.

While these results suggest that virtually all cells internalize DNA:FITC-labeled polycation transfection complexes, the subcellular distribution appears dramatically different for FITC-labeled polycations complexed with DNA compared to that for polycations alone. Additionally, incorporation of Glu\textsubscript{n}-HisN into DNA:FITC-labeled
mixed polyion transfection complexes showed intracellular localization characterized by aggregates of larger and distinct fluorescent foci or particles.

**Effect of FITC-Ornₙ labeling on recombinant hGH expression in primary bovine mammary cells.**

To confirm that the DNA:FITC-labeled polyion transfection complexes shown in Figure 5.6 were functional (expressible) and therefore that the observed subcellular localizations were representative of unlabeled transfection complexes, and also to determine whether the observed differences in subcellular distribution correlated with recombinant gene expression levels, primary bovine mammary cells were cultured on collagen gels at 39°C and transfected with the pcDNA1/hGH eukaryotic expression vector in polycation:DNA complexes also containing FITC-labeled Ornₙ as for Figure 5.6, as follows: F-Ornₙ polycation:DNA [F-Ornₙ;DNA]; mixed polycation:DNA [F-Ornₙ;DEAE :DNA]; unordered charge-shifting polyion:DNA [F-Ornₙ;Gluₙ-HisN:DEAE:DNA]; and ordered charge-shifting polyion:DNA [DNA:DEAE:Gluₙ-HisN:F-Ornₙ] all formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4 (Fig. 5.7).

Primary bovine mammary cells transfected with polycation:DNA [DNA:F-Ornₙ] complexes gave low level (≤ 1.5 ng/ml) recombinant hGH expression at day 16 post transfection (Fig. 5.7), slightly lower than previously reported expression (Fig. 4.6) with unlabeled Ornₙ. Transfection with mixed polycation:DNA complexes [F-Ornₙ;DEAE:DNA] gave higher expression (~5 ng hGH/ml) than F-Ornₙ alone, and
Figure 5.7. Effect of incorporating FITC-labeled Orn into polyion transfection complexes on hGH expression in primary bovine mammary cells transfected in vitro. Primary bovine mammary cells were cultured on collagen gels at 39°C and transfected with transfection complexes prepared with FITC-labeled Orn in 0.3 M sucrose, 10 mM HEPES, pH 7.4, as follows: untransfected control (○); polycation:DNA (▲) [F-Orn (37.5 μg/ml), pcDNA1/hGH (5 μg/ml)]; mixed polycation (▼) [F-Orn (37.5 μg/ml), DEAE (12.5 μg/ml), pcDNA1/hGH (5 μg/ml)]; unordered charge-shifting mixed polyion:DNA (●) [F-Orn (37.5 μg/ml), Glu-HisN (75 μg/ml), DEAE (12.5 μg/ml), pcDNA1/hGH (5 μg/ml)]; or ordered charge-shifting mixed polyion:DNA (●) [pcDNA1/hGH (5 μg/ml), DEAE (12.5 μg/ml), Glu-HisN (75 μg/ml), F-Orn (37.5 μg/ml)] complexes.
showed expression which persisted through 20 days posttransfection (Fig. 5.7), but with expression also slightly lower than previously described for this transfection complex containing unlabeled-Orn (Fig. 4.6). Transfection with ordered [DNA:DEAE:Glu -HisN:F-Orn] or unordered [F-Orn :Glu -HisN:DEAE:DNA] charge-shifting mixed polyion transfection complexes containing F-Orn gave peak hGH expression levels of 44 and 17 ng hGH/ml, respectively (Fig. 5.7), which again was slightly below previously reported expression levels with unlabeled-Orn (Fig 4.11). Therefore, inclusion of Glu^-HisN in either ordered or unordered charge-shifting mixed polyion transfection complexes containing F-Orn increased recombinant hGH expression over mixed polycations alone [F-Orn:DEAE:DNA], consistent with previous results using unlabeled orn (Fig 4.9). Similarly, ordered charge-shifting mixed polyion transfection complexes containing F-Orn increased recombinant hGH expression (~ 3 fold) over unordered transfection complexes (Fig. 5.7), also consistent with previous findings (Fig. 4.11).

Although hGH expression levels were lower for F-Orn-containing transfection complexes used to transfect primary bovine mammary cells (Fig. 5.7), they were well within the normal range of expression obtained with transfection complexes containing unlabeled Orn (Figs. 4.6, 4.9, and 4.11). More importantly, treatment effects were consistent with previous transfections. These results suggest that FITC-labeled Orn incorporation into mixed polyion transfection complexes did not substantially alter cellular internalization, subcellular distribution, or expressibility of transfected plasmid DNA, as evidenced by similar recombinant gene expression levels.
Microautoradiographic assessment of cellular uptake and subcellular distribution of transfected radiolabeled-plasmid DNA.

To confirm that transfected plasmid DNA, like FITC-labeled polycations, is internalized by most or all cells following polyion-mediated transfection, and to investigate subcellular distribution of DNA following internalization, primary bovine mammary cells were cultured on plastic at 37°C and transfected with ordered charge-shifting mixed polyion transfection complexes [DNA:DEAE:Glu-HisN:Orn] containing radiolabeled DNA. Distribution of transfected radiolabeled plasmid DNA was determined by nuclear emulsion overlay (Fig 5.8A), with nuclei visualized by staining with propidium iodide (Fig 5.8B).

As expected from the predicted ionic interactions between polycations and DNA, transfected radiolabeled plasmid DNA was internalized by almost every cell (Fig. 5.8A) and appeared to localize in the nuclear or perinuclear region (Fig. 5.8), much like the subcellular distribution noted with transfection of FITC-labeled polyion complexes (Fig. 5.6). Unfortunately, microscopic resolution was not adequate for a clear determination of whether DNA localization was actually intranuclear or instead concentrated in the perinuclear region. Similar results were obtained with [Orn:DNA] alone or [Orn:DEAE:DNA] mixtures in both Comma-D and primary bovine mammary cells (results not shown). This study confirmed that transfected plasmid DNA, like FITC-labeled polycations, is internalized by virtually every cell and localized to the nuclear or
Figure 5.8. Intracellular fate of $^{33}$P-radiolabeled plasmid DNA incorporated into ordered mixed polyion transfection complexes in primary bovine mammary cells. Primary bovine mammary cells were cultured on plastic at 37°C and transfected with ordered charge-shifting mixed polyion transfection complexes [$^{33}$P-radiolabeled-pcDNA1/hGH plasmid DNA (5 μg/ml), DEAE (12.5 μg/ml), Glu-HisN (75 μg/ml), Orn (37.5 μg/ml)] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4. Panel A, intracellular distribution of $^{33}$P-radiolabeled-pcDNA1/hGH; Panel B, propidium iodide-stained nuclei; and Panel C, photographic superimposition of A and B. Panels A-C, concordance of $^{33}$P-radiolabeled plasmid DNA and propidium iodide stained nuclei (Magnification 200x).
perinuclear region. Therefore, cellular internalization of transfected plasmid DNA does not appear rate-limiting for polyion-mediated transfection.

Concordant increases in cellular uptake and nuclear localization of radiolabeled plasmid DNA and FITC-labeled polycations, and cumulative recombinant hGH expression.

To further examine the relationship between cellular uptake of radiolabeled plasmid DNA and FITC-labeled polycations and subsequent recombinant gene expression, parallel cultures of Comma-D mouse mammary cells were transfected with ordered charge-shifting mixed polyion transfection complexes [DNA:DEAE:Glu₉-HisN:Orn₉] containing either radiolabeled plasmid DNA to monitor nuclear uptake of transfected DNA as determined by centrifugal subcellular fractionation, F-Orn₉ to allow visualization of internalized transfection complexes, or unlabeled Orn₉ to allow monitoring of subsequent hGH expression. Nuclear uptake of transfected radiolabeled plasmid DNA, determined by centrifugal subcellular fractionation, increased nearly linearly over the four hour transfection period, and was closely paralleled by cumulative hGH expression to day 12 posttransfection (Fig. 5.9). Similarly, intracellular accumulation of FITC-labeled transfection complexes also increased over the four hour transfection period and also showed localization in the nuclear/perinuclear region with time (Fig 5.9B). This showed that recombinant hGH expression was directly proportional to the length of time cells were exposed to transfection complexes, possibly indicating a
Figure 5.9. Concordant increases in cumulative hGH expression, nuclear uptake of transfected $^{32}$P-radiolabeled DNA, and cellular binding and focal localization of FITC-labeled transfection complexes with increasing transfection time in Comma-D mouse mammary cells. Comma-D mouse mammary cells were cultured as described in methods and exposed for 0, 0.5, 1, 2, 3, or 4 hr., to ordered charge-shifting mixed polyion:DNA transfection complexes [DNA (5 μg/ml), DEAE (12.5 μg/ml), Glu$_n$-HisN (75 μg/ml), Om$_n$ (37.5 μg/ml)] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4, and prepared with pcDNA1/hGH DNA either unlabeled (■) or $^{32}$P-radiolabeled (●) (Panel A); or with FITC-Orn$_n$ [pcDNA1/hGH (5 μg/ml), DEAE (12.5 μg/ml), Glu$_n$-HisN (75 μg/ml), F-Orn$_n$ (37.5 μg/ml)] (Panel B). Panel A: nuclear uptake of $^{32}$P-radiolabeled plasmid DNA (●) as determined by centrifugal subcellular fractionation following exposure of cells to transfection complexes for 0, 0.5, 1, 2, 3, or 4 hrs., and cumulative hGH expression (■) to day 12 posttransfection for transfections performed for 0-4 hrs. Cumulative hGH expression was determined as defined in Chapter 4. Panel B: FITC-Orn$_n$ distribution in Comma-D cells at 0.5, 1, 2, and 4 hrs. after addition of FITC-labeled transfection complexes. (Magnification 200x).
Figure 5.9

Cumulative hGH Secretion (ng)
to Day 12 Post Transfection

% Transfected DNA in Nucleus

Transfection Time (Hrs)
time-dependent internalization process. It further showed that hGH expression was paralleled by nuclear uptake of radiolabeled DNA, as determined by subcellular fractionation, and that time-dependent apparent nuclear localization of radiolabeled plasmid DNA was paralleled by microscopic visualization of cell uptake and intracellular localization of FITC-labeled transfection complexes.

**Comparison of the relative nuclear subcellular distribution of fluorescently-labeled transfection complexes in cells expressing or not expressing recombinant transgenes.**

To examine the relationship between subcellular distribution of DNA:polyion transfection complexes, particularly for intranuclear or perinuclear localization, in cells with and without recombinant transgene expression, COS-1 cells capable of high level recombinant gene expression were transfected with pEGFP-N1 eukaryotic expression vector plasmid DNA encoding the *Aequorea victoria* green fluorescent protein (GFP) as the reporter gene, incorporated into polyion transfection complexes made with red fluorescent Texas Red (TR) labeled Orn (TR-Orn) to allow fluorescent visualization of internalized complexes.

Nuclei were visualized by DAPI fluorescence staining (blue), while individual cells expressing the GFP reporter gene were evident from the green fluorescence in their cytosol. Hence, simultaneous viewing of all these labels allowed assessment of cellular uptake and intracellular localization of transfection complexes (red fluorescing Texas Red-Orn), relative to the nucleus of each cell (blue DAPI fluorescence), and which cells
were also expressing GFP (green cytosolic fluorescence). It was necessary to use a cell line capable of high recombinant transgene expression such as COS-1 for these studies because GFP expression was not readily detectable in either Comma-D mouse mammary or primary bovine mammary cells transfected with mixed polyions.

Internalized DNA:TR-Ornₙ labeled polyion transfection complexes (arrows) were observed as punctate fluorescent foci immediately adjacent to virtually every DAPI stained nucleus (arrowheads) (Fig 5.10A), similar to the perinuclear localization patterns seen after transfection of mammary cells with FITC-Ornₙ transfection complexes (Fig. 5.6B, D, F). Transfection efficiency, determined as the percent of cells showing cytosolic or perinuclear fluorescent TR-Ornₙ particles were very high for polycation:DNA [TR-Ornₙ:DNA] alone (99.3%); mixed polycation [TR-Ornₙ:DEAE:DNA] (99%), unordered charge-shifting mixed polyion [TR-Ornₙ:Glu₉-HisN:DEAE:DNA] (99.6%), and ordered charge-shifting mixed polyion [DNA:DEAE:Glu₉-HisN:TR-Ornₙ] (98.7%) transfection complexes. Regardless of the polyion composition of the transfection complex, very few of the cells exposed to TR-Ornₙ-labeled transfection complexes showed nuclei without closely associated TR-Ornₙ. Most TR-Ornₙ fluorescent foci were detected in the perinuclear region and did not appear to be within the nucleus (Fig. 5.10A and Fig 5.11A). Careful examination of TR-Ornₙ-labeled transfection complexes which initially appeared to be in the nucleus showed most to be in a different focal plane above or below the nucleus. However, intranuclear localization of TR-Ornₙ-labeled transfection
Figure 5.10. Fluorescence photomicrographs showing subcellular localization of polyion transfection complexes containing TR-Orn$_n$ and DAPI-stained nuclei in COS-1 cells. COS-1 cells were transfected with polycation:DNA complexes [TR-Orn$_n$ (37.5 μg/ml), pEGFP-N1 DNA (5 μg/ml)] formed in HBSS. Panel A, dual fluorescence photomicrograph taken on day 2 posttransfection of TR-Orn$_n$-labeled transfection complexes (arrows) were concentrated in the perinuclear region of DAPI-stained nuclei (arrowheads) of virtually all COS-1 cells. Panel B, corresponding phase contrast photomicrograph showing cell boundaries and nuclei for the same field shown in Panel A. (Magnification 400x).
Figure 5.11. High Magnification dual wavelength fluorescent photomicrograph showing perinuclear localization of TR-Orn₉-labeled polyion transfection complexes, and concordance of TR-Orn, and DAPI-stained transfected plasmid DNA. COS-1 cells were transfected with unordered charge-shifting mixed polyion transfection complexes [TR-Orn₉ (37.5 µg/ml), Glu₉-HisN (75 µg/ml), DEAE (12.5 µg/ml), pEGFP-N1 DNA (5 µg/ml)] formed in HBSS. Photomicrographs showing concordant localization of TR-Orn₉-labeled polyion transfection complexes (Panel A, arrow) and DAPI-stained transfected plasmid DNA (Panel C, arrow) immediately posttransfection in COS-1 cells as shown by: Panel A, dual fluorescence illumination for both TR-Orn₉ and DAPI; Panel B, phase contrast for cell boundaries and nuclei; and Panel C, DAPI fluorescence. (Magnification 600x).
Figure 5.11
complexes could not be confirmed with this optical resolution. Similar perinuclear localization of TR-Ornₙ-labeled transfection complexes was seen with all polyion transfection complexes tested (results not shown).

Figure 5.11 is a representative series of photomicrographs showing perinuclear localization of TR-Ornₙ-labeled unordered charge-shifting polyion transfection complexes and DAPI stained DNA in transfected COS-1 cells with phase contrast (Fig. 5.11A), dual fluorescence (Fig. 5.11B), and DAPI wavelength fluorescence (Fig. 5.11C). Interestingly, following DAPI staining for nuclear visualization, small punctate fluorescent foci (arrow) were visible in the cytosol of many cells (Fig 5.11C) coincident with TR-Ornₙ localization (arrow) (Fig 5.11A). This confirmed the colocalization of transfected plasmid DNA and polycation of the transfection complex that was suggested earlier from the separate localizations of radiolabeled plasmid DNA (Fig. 5.8) and FITC-Ornₙ:DNA complexes (Fig. 5.6).

Interestingly, after transfection of cells with fluorescently-labeled transfection complexes, areas of the cell culture plates lacking cells (Fig. 5.12A) showed evenly distributed and uniformly sized fluorescent particles (arrowheads) apparently attached to the exposed plastic (Fig. 5.12B and C), similar to that described earlier with FITC-Ornₙ incorporated into DNA:polyion transfection complexes (Fig. 5.6F, arrowheads).

To further define the relationship between subcellular distribution of DNA:polyion transfection complexes relative to the nucleus in recombinant transgene expressing and non-expressing cells, COS-1 cells were transfected with ordered charge-shifting mixed
Figure 5.12. Apparent immobilization of TR-Orn$_n$-labeled polyion transfection complexes on plastic cell culture plates in areas lacking cell growth. COS-1 cells were transfected with unordered charge-shifting mixed polyion transfection complexes containing Texas Red-labeled Orn$_n$[TR-Orn$_n$(37.5 μg/ml), Glu$_n$-HisN (75 μg/ml), DEAE (12.5 μg/ml), pEGFP-N1 (5 μg/ml)] formed in HBSS. Panel A, phase contrast photomicrograph showing cell boundaries; Panel B, DAPI stained nuclei (arrows); and Panel C, dual fluorescent illumination showing TR-Orn$_n$-labeled polyion transfection complexes (arrowheads) immobilized on exposed plastic surfaces of cell culture wells in areas lacking cell growth. (Magnification 400x).
Figure 5.12
polyion transfection complexes [pEGFP-N1 DNA:DEAE:Glu₆-HisN:TR-Orn₆] formed in HBSS, and examined by triple fluorescent label microscopy with optical Z-plane sectioning and refinement of images by reiterative deconvolution (Vaytek, Inc.). Virtually all cells contained TR-Orn₆ labeled polyion transfection complexes localized in the perinuclear region, but predominately to one side of most nuclei (arrowheads) (Fig. 5.13B). Furthermore, the internalized fluorescently labeled transfection complexes also contained DAPI stained DNA (Fig. 5.13C). However, intranuclear detection of TR-Orn₆ labeled transfection complexes did not always correlate with GFP expression in transfected cells, as some cells contained TR-Orn₆-labeled fluorescent transfection complex particles in the nucleus but lacked detectable GFP expression (Fig. 5.13A).

Interestingly, an enlargement of the GFP-expressing cell in Figure 5.13A showed an array of scattered TR-Orn₆-labeled polyion transfection complexes extending from the cell periphery to the concentration of transfection complexes in the perinuclear region, and enclosed within what appeared to be vacuoles which excluded recombinant GFP in this 0.5 μm optical section (Fig. 5.14). This strongly suggested that DNA:polyion transfection complexes were internalized in membrane bound vesicles (endosomes ?) and transported to the perinuclear region where they either fail to exit the vesicle or are too large for nuclear internalization. It is uncertain whether the apparent cytosolic vesicles containing the transfection complexes are actually endosomes. The lack of nuclear internalization of transfection complexes probably accounts for the low efficiency of recombinant transgene expression after transfection of COS-1 cells, and as shown earlier
Figure 5.13. Subcellular distribution of TR-Ornₙ-labeled polyion transfection complexes in a GFP expressing cell and in non-GFP expressing cells. COS-1 cells were transfected with ordered charge-shifting mixed polyion transfection complexes containing TR-Ornₙ and expression plasmid for GFP [pEGFP-N1 DNA (5 μg/ml), DEAE (12.5 μg/ml), Glu₉-HisN (75 μg/ml), TR-Ornₙ (37.5 μg/ml)] in HBSS. Panel A, fluorescent visualization of cytosolic GFP expression in one cell (arrow), internalized TR-Ornₙ-labeled polyion transfection complexes (arrowheads); and Panel B, DAPI stained DNA in nuclei (arrowhead) and transfection complexes (arrows) with green channel removed in COS-1 cells on day 2 posttransfection. Photomicrographs were acquired with Z-plane optical sectioning (0.5 μm) and refined with reiterative deconvolution (Vaytek, Inc.). (Magnification 600x).
Figure 5.14. Subcellular distribution of TR-Orn$_n$-labeled polyion transfection complexes in a GFP expressing cell. COS-1 cells were transfected with ordered charge-shifting mixed polyion transfection complexes containing TR-Orn$_n$ and expression plasmid for GFP [pEGFP-N1 DNA (5 µg/ml), DEAE (12.5 µg/ml), Glu$_n$-HisN (75 µg/ml), TR-Orn$_n$ (37.5 µg/ml)] in HBSS. Enlargement and color enhancement of photomicrograph in Figure 5.13A showing a GFP-expressing cell containing an array of scattered internalized TR-Orn$_n$-labeled polyion transfection complexes (arrows) extending from the cell periphery to the concentration of transfection complexes in the perinuclear region, enclosed within what appeared to be vacuoles or membrane bound compartments which excluded recombinant GFP. Photomicrograph was acquired with Z-plane optical sectioning (0.5 µm) and refined with reiterative deconvolution (Vaytek, Inc.).
in Figures 5.2-5.4, as virtually all cells, regardless of cell type or transfection complex composition tested, seem to contain internalized DNA:polyion transfection complexes.

**Transmission electron microscopy of DNA:polyion transfection complexes.**

Transfections with fluorescently-labeled polycations suggested that nuclear entry of polyion transfection complexes may be a major limitation to efficient recombinant transgene expression from polyion-mediated transfection. This could potentially be due to polyion transfection complex size. Therefore, to examine the size and structure of DNA:polyion transfection complexes, DNA:polyion transfection complexes were formed in HBSS and allowed to attach to Formvar-coated transmission electron microscopy grids. Attached DNA:polyion transfection complexes were then visualized by uranyl acetate negative staining and transmission electron microscopy.

Attached DEAE:DNA [DEAE:DNA] polycation transfection complexes appeared as evenly distributed and uniformly sized (~235 nm) spheres with irregular surfaces (Fig. 5.15A and B) and an occasional large aggregate (Fig. 5.15B). Attached Orn₉:DNA [Orn₉:DNA] polycation transfection complexes appeared as uniformly sized (~240 nm) toroids (Fig. 5.16A and B) similar to those described by Laemmli (1975) and Shapiro et al. (1969), while mixed polycation [Orn₉:DEAE:DNA] transfection complexes were characterized by a mixture of free plasmid (Fig. 5.17A and B, filled arrows), condensed rods (Fig. 5.17A and B, open arrows), and amorphous aggregates (Fig 5.17A arrowheads, C, and D). Ordered (Fig. 5.18) and unordered (not shown) charge-shifting mixed polyion
Figure 5.15. Transmission electron photomicrograph of [DEAE:DNA] polycation transfection complexes formed in HBSS. Transfection complexes [DEAE (250 µg/ml), DNA (5 µg/ml)] were formed in contact with Formvar-coated transmission electron microscopy grids for 4 min. prior to negative staining with 2% uranyl acetate for 1 min. Magnification: Panel A, 10,000x; Panel B, 45,000x.
Figure 5.16. Transmission electron photomicrographs of [Orn<sub>n</sub>:DNA] polycation transfection complexes formed in HBSS. Transfection complexes [Orn<sub>n</sub> (37.5 µg/ml), DNA (5 µg/ml)] were formed in contact with Formvar-coated transmission electron microscopy grids for 4 min. prior to negative staining with 2% uranyl acetate for 1 min. Magnification: Panel A, 10,000x; Panel B, 45,000x.
Figure 5.17. Transmission electron photomicrographs of mixed polycation [Orn_{5}:DEAE:DNA] transfection complexes formed in HBSS. Transfection complexes [Orn_{5} (37.5 μg/ml), DEAE (12.5 μg/ml), DNA (5 μg/ml)] were formed in contact with Formvar-coated transmission electron microscopy grids for 4 min. prior to negative staining with 2% uranyl acetate for 1 min. Magnification: Panel A, 10,000x; Panels B-D, 45,000x. Panels A and B show free plasmid DNA (filled arrows) and rods (open arrows). Large amorphous aggregates are shown in Panels A (arrowheads), C, and D.
Figure 5.18. Transmission electron photomicrograph of ordered charge-shifting mixed polyion [DNA;DEAE;Glu-HisN;Orn] transfection complexes formed in HBSS. Ordered charge-shifting mixed polyion transfection complexes [DNA (5 μg/ml), DEAE (12.5 μg/ml), Glu-HisN (75 μg/ml), Orn (37.5 μg/ml)] were formed in contact with Formvar-coated transmission electron microscopy grids for 4 min. prior to negative staining with 2% uranyl acetate for 1 min. (Magnification 45,000x).
transfection complexes appeared predominately as large irregular aggregates of smaller rough-edged spherical individual particles of similar diameter (~90 nm). This suggested that incorporation of the Glu–HisN charge-shifting polymer into mixed polyion transfection complexes might promote formation of smaller transfection complexes which subsequently aggregate, similar to the aggregation of Orn–DNA complexes described by Marciano-Cabral et al. (1981).

Effect of an endosomolytic peptide on polyion transfection complex subcellular distribution and recombinant hGH expression.

Wagner et al. (1992) and Zauner et al. (1995) added synthetic pH sensitive viral lytic peptides to transfection complexes to facilitate recombinant transgene release from intracellular endosomes with an increase in recombinant transgene expression of ~500 fold, in cultured cell lines transfected with transferrin-Lys₄:DNA complexes. The previous polycation transfection studies described here (Fig. 5.10-5.13) indicated that fluorescently-labeled polyion transfection complexes were internalized and concentrated in the perinuclear region but failed to enter the nucleus, which is consistent with endosome entrapment of transfection complexes as suggested by Zabner et al. (1995), Wagner et al. (1992), and Zauner et al. (1995). Therefore, in an attempt to facilitate endosome release of ordered charge-shifting mixed polyion transfection complexes, a 25 amino acid human rhinovirus lytic peptide (HRV2 LP) (sequence 1-NPVENYIDEVLN EVLVVPNINSSNC-25) (Zauner et al., 1995) was synthesized and incorporated into
ordered charge-shifting mixed polyion transfection complexes. The HRV2 LP should be
directly incorporated into the DNA:polyion transfection complexes due to its net negative
charge, and therefore should ionically interact with both DEAE and Orn, at pH 7.4 used
for formation of transfection complexes. Disruption of endosomes by incorporation of
HRV2 into TR-Orn,-labeled polyion transfection complexes should be reflected by
altered subcellular distribution of TR-Orn,-labeled polyion transfection complexes and
enhanced recombinant hGH expression compared to that for cells transfected with the
same polyion transfection complexes lacking HRV2 LP.

Initial primary bovine mammary cell transfections with ordered charge-shifting
mixed polyion transfection complexes formed with incorporation of crude unfractionated
HRV2 LP synthesis mixture showed that recombinant hGH expression increased more
and with greater reproducibility when the crude HRV2 LP mixture was added prior to
addition of Orn, to the transfection complex [DNA:DEAE:Glu,-HisN:HRV2 LP:Orn,] (Fig. 5.19A), compared with addition of the crude HRV2 LP mixture after the addition
of Orn, to the transfection complex [DNA:DEAE:Glu,-HisN:Orn,:HRV2 LP] (Fig. 5.19B).

Purification of the HRV2 LP from the crude peptide synthesis reaction mixture
by high pressure liquid chromatography (HPLC) yielded three peptide fractions, a main
peak (Fraction II) with leading (Fraction I) and trailing (Fraction III) shoulders, which
were tested individually for their ability to facilitate increased recombinant hGH
expression by transfection of parallel primary bovine mammary cell cultures with
Figure 5.19. Effect of crude HRV2 lytic peptide synthesis reaction mixture on recombinant hGH expression in transfected primary bovine mammary cells. Primary bovine mammary cells were cultured on floating collagen gels at 37°C and transfected with ordered charge-shifting mixed polyion transfection complexes [DNA (5 µg/ml), DEAE (12.5 µg/ml), Glu₅·HisN (75 µg/ml), Orn₅ (37.5 µg/ml)] containing 0, 0.1, 0.5, 1, 5, 10, 50, or 100 µg crude HRV2 lytic peptide mixture/ml transfection solution added prior to Orn₅ addition (Panel A) or after Orn₅ addition (Panel B).
increasing amounts of the HRV2 LP fractions added prior to addition of Orn in ordered charge-shifting mixed polyion transfection complexes formed as follows: [pcDNA1/hGH DNA:DEAE:Glu-HisN:HRV2 LP fraction I, II, or III, Orn] in 0.3 M sucrose, 10 mM HEPES, pH 7.4. These transfections used complexes formed with 10 μg DNA/ml, which had just been determined as the optimal DNA concentration for ordered charge-shifting mixed polyion transfections (Figs. 4.13 and 4.14). Total polycation:DNA (10:1) and Orn:DEAE (3:1) ratios were maintained as before.

Generally, there was a trend toward increasing recombinant hGH expression (Fig. 5.20) with addition of increasing amounts of HRV2 LP fractions I and III to ordered charge-shifting polyion transfection complexes prior to the addition of Orn. In contrast, there was no clearly discernable response when HRV2 LP fraction II was added to ordered charge-shifting polyion transfection complexes prior to the addition of Orn. In general, HRV2 LP fractions I and III increased recombinant hGH expression most when incorporated into polyion transfection complexes at 2-50 μg lytic peptide/ml transfection medium (Fig. 5.20).

The effect of HRV2 LP fraction III on subcellular distribution of polyion transfection complexes was subsequently examined by transfecting COS-1 cells with either 1 (Fig. 5.21A and B) or 10 (Fig. 5.21C and D) μg lytic peptide/ml incorporated into ordered charge-shifting mixed polyion transfection complexes containing TR-Orn. HRV2 LP fraction III was used for this study because it gave the largest increase in hGH expression in transfected primary bovine mammary cells (Fig. 5.20A-C).
Figure 5.20. Dose response curves of three synthetic HRV2 lytic peptide fractions added to ordered charge-shifting mixed polyion transfections. Primary bovine mammary cells were cultured on floating collagen gels at 37°C and transfected with ordered charge-shifting mixed polyion transfection complexes [DNA (5 μg/ml), DEAE (12.5 μg/ml), Glu-HisN (75 μg/ml), HRV2 LP Fraction x (x μg/ml), Orn (37.5 μg/ml)] containing either 0, 0.01, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, or 100 μg HRV2 lytic peptide/ml added prior to the addition of Orn. Panel A, dose response curve of HRV2 fraction I; Panel B, dose response curve of HRV2 fraction II; and Panel C, dose response curve of HRV2 fraction III.
Ordered charge-shifting mixed polyion transfection complexes containing TR-Orn, and either 1 (Fig. 5.21A and B) or 10 (Fig. 5.21C and D) µg HRV2 LP/ml showed slightly different subcellular localization of polyion transfection complexes compared with that for cells transfected with ordered charge-shifting mixed polyion transfection complexes alone (Fig. 5.13). HRV2 lytic peptide incorporation at 1 (Fig. 5.21A and B) or 10 (Fig. 5.21C and D) µg HRV2 LP/ml, into ordered charge-shifting mixed polyion transfection complexes fluorescently labeled with TR-Orn, appeared to increase the perinuclear localization of transfection complexes (arrows) and also appeared to cause transfection complex aggregation at 10 µg lytic peptide/ml (Fig. 5.21C). Inclusion of HRV2 LP did not have a dramatic effect on subcellular distribution of transfection complexes and did not appear to facilitate increased nuclear internalization when incorporated into ordered charge-shifting mixed polyion transfection complexes in transfected COS-1 cells.

Mass spectroscopic analysis of HRV2 lytic peptide fractions, performed after completion of the study of HRV2 effects on transfection, indicated that fraction I contained two major peptides, comprising Val16-Cys25 and Val17-Cys25, while fractions II and III contained peptides of undeterminable sequence. These results were not entirely surprising given the difficulty of synthesis for this peptide. The first attempt at HRV2 lytic peptide synthesis appeared to fail at Asp9, as estimated from monitoring of the carbamate salt conductivity during the FMOC cleavage reaction of the peptide synthesis cycles. This was presumed to be due to peptide secondary structure or peptide folding.
Figure 5.21. Subcellular distribution of TR-Orn, labeled ordered charge-shifting mixed polyion transfection complexes containing HRV2 lytic peptide fractions. COS-1 cells were transfected with ordered charge-shifting mixed polyion transfection complexes [pEGFP-N1 DNA (5 µg/ml), DEAE (12.5 µg/ml), Glu-HisN (75 µg/ml), HRV2 lytic peptide (1 µg/ml), Orn (37.5 µg/ml)] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4, with HRV2 peptide fractions added at 1 (Panels A and B) or 10 (Panels C and D) µg/ml. Panels A and C, dual fluorescent microscopy shows TR-Orn, labeled transfection complexes (arrows) and DAPI stained nuclei (arrowheads), with corresponding phase contrast photomicrographs, (Panels B and D), respectively. (Magnification 400x).
into the resin, thus obscuring the peptide N-terminus from further elongation. Therefore to maximize N-terminal peptide residue exposure and prevent premature intrapeptide folding and blockage of further synthesis, a second attempt at HRV2 LP synthesis utilized a NovaSyn TG synthesis resin with a lower C-terminal amino acid derivatization density of 0.1-0.3 pmole Cysteine/gm resin as opposed to the previous and normal 1 mM Cys/gm resin. NovaSyn TG synthesis resins are reported to have improved physico-chemical properties, including improved chemical efficiency of coupling (Bayer et al., 1991). Carbamate salt conductivity during the second attempt to synthesize the HRV2 LP suggested that peptide synthesis progressed to completion. Thus, biological activity studies were performed prior to the completion of mass spectrophotometric analysis.

It is clear, however, that despite the apparent incomplete HRV2 lytic peptide synthesis, addition of even the partial HRV2 lytic peptide (Val_{16}-Cys_{25}, or Val_{17}-Cys_{25}) to charge-shifting polyion transfections increased recombinant hGH expression approximately two fold. However, that was only a fraction of the ~ 500 fold increase in luciferase expression reported by Zauner et al. (1995) after addition of a complete HRV2 LP to DNA:polylysine-transferrin transfections. This suggests that addition of a complete HRV2 LP to ordered charge-shifting mixed polyion transfections yet may have dramatic potential to facilitate recombinant gene expression in primary bovine mammary cells.
Effect of pH and salt concentration on dissociation of radiolabeled DNA from immobilized charge-shifting polyion transfection complexes.

Incorporation of fluorescently-labeled Orn into ordered charge-shifting mixed polyion transfection complexes revealed an even distribution of fluorescently labeled transfection complexes attached to the plastic surface in areas not covered by cells (Fig. 5.6F, arrow and Fig. 5.12A-C arrows). These fluorescent complexes were of consistent size and shape and their persistence for 20 days or more in culture suggested that they were firmly attached to the cell culture plate. Hence, formation and attachment of charge-shifting mixed polyion transfection complexes to empty cell culture wells should provide an in vitro model for testing the dissociation of DNA:polyion complexes.

Therefore, \(^{32}\text{P}\)-random primed radiolabeled plasmid DNA was used to form ordered charge-shifting mixed polyion transfection complexes \([\text{DNA:DEAE:Glu}^-\text{HisN:Orn}_n]\) in either HBSS or 0.3 M sucrose, 10 mM HEPES, pH 7.4. Transfection complexes were then added to empty 2 cm\(^2\) cell culture wells and allowed to attach to the plastic cell culture plates over a four hour simulated transfection period. Transfection medium was then removed and wells were washed twice (one minute per wash) with the transfection medium buffer to remove unattached immobilized transfection complexes or unincorporated radiolabeled DNA. To test the effects of pH or ionic strength on dissociation of radiolabeled plasmid DNA from immobilized transfection complexes, wells were washed three times (with a 30 minute incubation at 37°C per each wash) with 10 mM HEPES, 50 mM citrate, adjusted with 0.1 M HCL or NaOH to either pH 3.5 or
pH 7.0, and containing either 0, 25, or 1 M NaCl. Control wells were washed with either HBSS or 0.3 M sucrose, 10 mM HEPES, pH 7.4. Dissociation of immobilized transfection complexes was determined by release of radiolabeled plasmid DNA into aspirated wash solutions from complexes bound to plastic.

As predicted, dissociation of radiolabeled plasmid DNA from immobilized transfection complexes was pH- and ionic strength-dependent for complexes formed in both HBSS (Fig. 5.22A) and 0.3 M sucrose, 10 mM HEPES, pH 7.4 (Fig. 5.22B). Radiolabeled DNA dissociation from immobilized transfection complexes was greater than background only when complexes were exposed to pH 3.5 or when the salt concentration was 1 M (Fig. 5.22A and B). Surprisingly, pH- or salt-dependent dissociation was 2-4 fold greater for transfection complexes formed in HBSS (Fig. 5.22A) as opposed to sucrose/HEPES (Fig. 5.22B). This preliminary experiment suggested that immobilized charge-shifting mixed polyion transfection complexes would be useful for characterizing polycation:DNA dissociation.

**Effect of pH on radiolabeled plasmid DNA dissociation from immobilized ordered charge-shifting mixed polyion transfection complexes.**

To more closely examine the effects of pH on radiolabeled plasmid DNA dissociation from immobilized polyion transfection complexes, the pH of the 10 mM HEPES, 50 mM citrate, 25 mM NaCl wash solution was varied from pH 7.0 to pH 2.0. Furthermore, to demonstrate that the pH sensitivity seen in Figure 5.22A and B was
Figure 5.22. Effect of pH and salt concentration on dissociation of $^{32}$P-radiolabeled DNA from immobilized ordered charge-shifting mixed polyion transfection complexes. Ordered charge-shifting mixed polyion transfection complexes [pcDNA1/hGH DNA (5 μg/ml), DEAE (12.5 μg/ml), Glu-HisN (75 μg/ml), Orn (37.5 μg/ml)] were formed in HBSS (A) or 0.3 M sucrose, 10 mM HEPES, pH 7.4, (B) and allowed to attach to plastic cell culture plates for 4 hrs. at 37°C. Following attachment of transfection complexes, wells were washed twice with either HBSS (A) or 0.3 M sucrose, 10 mM HEPES, pH 7.4, (B) to remove unincorporated radiolabeled-DNA or unattached transfection complexes. Wells containing immobilized transfection complexes were then washed three times (with incubation for 30 min./wash at 37°C) with 0.5 ml of either HBSS (open bar) (A); 0.3 M sucrose, 10 mM HEPES, pH 7.4 (open bar) (B); 50 mM citrate, 10 mM HEPES, adjusted to either pH 7.0 (filled bars) (A,B) or pH 3.5 (hatched bars) (A,B), and containing either 0, 25, or 1000 mM NaCl.
attributable to the Glu-n-HisN polymer, the pH-dependent dissociation of DNA from immobilized ordered charge-shifting polion transfection complexes [DNA:DEAE:Glu-n-HisN:Orn_n] was compared with the pH-dependent DNA dissociation of immobilized mixed polion transfection complexes lacking the charge-shifting Glu-n-HisN polion [Orn_n:DEAE:DNA] and formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4. Transfection complex formation and wash technique was performed as described earlier (Fig. 5.22).

Radiolabeled plasmid DNA dissociation from mixed polycation transfections lacking Glu-n-HisN [Orn_n:DEAE:DNA] was not pH-dependent, as shown by failure of DNA dissociation with the decrease of pH from 7.0 to 2.0 (Fig. 5.23). Interestingly, immobilized ordered charge-shifting polion transfection complexes [DNA:DEAE:Glu-n-HisN:Orn_n] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4, had higher intrinsic dissociation of radiolabeled DNA than did mixed polycation transfection complexes [Orn_n:DEAE:DNA] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4, which lack Glu-n-HisN, as shown by the higher recovery of radiolabeled plasmid DNA at pH 6.0 and pH 7.0 (Fig. 5.23). For ordered charge-shifting mixed polion transfection complexes, most of the release of radiolabeled plasmid DNA began at pH 5.0 and was nearly complete with the decrease of pH to 3.0 (Fig. 5.23). The dramatic radiolabeled DNA dissociation from ordered charge-shifting mixed polion transfection complexes below pH 5.0 was not expected, given the linearity of the Glu_n-HisN titration curve (Fig. 5.24) which would predict a linear release of radiolabeled plasmid DNA between pH 7.0 and pH 3.0 for polion transfection complexes containing the charge-shifting Glu_n-HisN polymer. The
Figure 5.23. Effect of pH on $^{32}$P-radiolabeled DNA dissociation from ordered charge-shifting mixed polyion transfection complexes. Ordered charge-shifting mixed polyion transfection complexes (■) [pcDNA1/hGH (5 μg/ml), DEAE (12.5 μg/ml), Glu$_n$-HisN (75 μg/ml), Omg$_n$ (37.5 μg/ml)] and mixed polycation transfection complexes (○) [Omg$_n$ (37.5 μg/ml), DEAE (12.5 μg/ml), pcDNA1/hGH (5 μg/ml)] were formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4, and allowed to attach to plastic cell culture plates for 4 hrs. at 37°C. Following transfection complex attachment and rinsing to remove unbound transfection complexes and excess radiolabeled DNA, wells containing immobilized transfection complexes were washed three times (with incubation for 30 min./wash at 37°C) with 0.5 ml of 50 mM citrate, 10 mM HEPES, 25 mM NaCl at pH 7.0, 6.0, 5.0, 4.0, 3.0, or 2.0.
Figure 5.24. Titration curve of Gluₙ-HisN. Gluₙ-HisN was exhaustively dialyzed against water and pH was adjusted to 3.0 with 0.1 M HCl prior to titration with 0.01 M NaOH to pH 9.0 at 37°C. Gluₙ-HisN exhibited extensive buffering capacity between pH 9.0 and pH 3.0 and did not show predicted imidazole (6.1) or γ-carboxyl (4.1) pKa shifts probably due to proximal imidazole-γ-carboxyl interactions.
abrupt dissociation of radiolabeled plasmid DNA over the pH 5.0 to pH 3.0 range suggests a critical involvement of glutamate γ-carboxyl groups (pKa ~ 4.1), but not histamine imidazole groups (pKa ~ 6.1) in transfection complex formation or dissociation.

**Effect of immobilized polion transfection complex composition on pH-dependent radiolabeled DNA dissociation.**

To further confirm that the pH sensitivity of DNA dissociation from transfection complexes (Fig. 5.23) was attributable to the charge-shifting Gluⁿ-HisN polymer and not to either the parent Gluⁿ or Histamine above, immobilized polion transfection complexes containing either DNA alone [DNA], DEAE [DEAE:DNA], Ornⁿ [Ornⁿ:DNA], mixed polycations [Ornⁿ:DEAE:DNA], or mixed polycation transfection complexes containing free histamine substituted for Gluⁿ-HisN at 0.75 molar equivalence for the amount of HisN estimated to be in the Gluⁿ-HisN polymer [DNA:DEAE: histamine:Ornⁿ] formed in HBSS and washed as described with 10 mM HEPES, 50 mM citrate, pH 3.5, showed no pH-sensitive dissociation of radiolabeled DNA (Fig. 5.25).

In contrast, immobilized mixed polycation transfection complexes containing free poly-L-glutamic acid (Gluⁿ) substituted for Gluⁿ-HisN at equimolar concentrations for the amount of Gluⁿ estimated to be in the Gluⁿ-HisN polymer [DNA:DEAE:Gluⁿ :Ornⁿ ] displayed pH-sensitive radiolabeled DNA dissociation (Fig. 5.25), as did ordered charge-shifting mixed polion [DNA:DEAE:Gluⁿ-HisN:Ornⁿ], and unordered charge-shifting mixed polion [Ornⁿ:Gluⁿ-HisN:DEAE:Ornⁿ] transfection complexes.
Figure 5.25. Effect of polyion transfection complex composition on $^{32}$P-radiolabeled DNA dissociation from immobilized transfection complexes. Transfection complexes consisting of: A) DNA alone [DNA (5 µg/ml)]; B) DEAE [DEAE, (12.5 µg/ml), DNA (5 µg/ml)]; C) Orn [Orn (37.5 µg/ml), DNA (5 µg/ml)]; D) mixed polycations [Orn (37.5 µg/ml), DEAE, (12.5 µg/ml), DNA (5 µg/ml)]; E) ordered charge-shifting mixed polyions with HisN substituted for Glu^-HisN [DNA (5 µg/ml), DEAE, (12.5 µg/ml), Histamine (94 µg/ml), Orn (37.5 µg/ml)]; F) ordered charge-shifting mixed polyions with Glu substituted for Glu^-HisN [DNA (5 µg/ml), DEAE, (12.5 µg/ml), Glu (150 µg/ml), Orn (37.5 µg/ml)]; G) ordered charge-shifting mixed polyions with Glu and Histamine substituted for Glu^-HisN [DNA (5 µg/ml), DEAE, (12.5 µg/ml), Glu (150 µg/ml), Histamine (94 µg/ml), Orn (37.5 µg/ml)]; H) unordered charge-shifting mixed polyions [Orn (37.5 µg/ml), Glu^-HisN (75 µg/ml), DEAE, (12.5 µg/ml), DNA (5 µg/ml)]; I) ordered charge-shifting mixed polyions [DNA (5 µg/ml), DEAE, (12.5 µg/ml), Glu^-HisN (75 µg/ml), Orn (37.5 µg/ml)]; or J) ordered charge-shifting mixed polyions containing the HRV2 lytic peptide [DNA (5 µg/ml), DEAE, (12.5 µg/ml), Glu^-HisN (75 µg/ml), HRV2 LP Fractions III (10 µg/ml), Orn (37.5 µg/ml)] were formed in HBSS as and allowed to attach to plastic cell culture plates for 4 hrs. at 37°C. Following transfection complex attachment, wells were washed as previously described with 0.5 ml of 50 mM citrate, 10 mM HEPES, 25 mM NaCl, pH 3.5, and aspirated wash solutions were measured for released radiolabeled DNA.
Figure 5.25
Interestingly, incorporation of free poly-L-glutamic acid (Glu\textsubscript{\textgamma}) into mixed polycation transfection complexes [Orn\textsubscript{\textalpha}:Glu\textsubscript{\textgamma}:DEAE:DNA] in place of Glu\textsubscript{\textgamma}-HisN facilitated radiolabeled DNA dissociation as well as did Glu\textsubscript{\textgamma}-HisN incorporated into unordered mixed polyion transfection complexes [Orn\textsubscript{\textgamma}:Glu\textsubscript{\textgamma}-HisN:DEAE:Orn\textsubscript{\textgamma}] (Fig. 5.25). However, the Glu\textsubscript{\textgamma}-mediated increase in radiolabeled DNA dissociation was moderately inhibited by addition of free histamine to mixed polycation transfection complexes containing free Glu\textsubscript{\textgamma} [Orn\textsubscript{\textalpha}:Glu\textsubscript{\textgamma}:histamine:DEAE:DNA] (Fig. 5.25). Radiolabeled DNA dissociation was 32\% greater for ordered charge-shifting mixed polyion transfection complexes [DNA:DEAE:Glu\textsubscript{\textgamma}-HisN:Orn\textsubscript{\textalpha}] than for unordered [Orn\textsubscript{\textgamma}:Glu\textsubscript{\textgamma}-HisN:DEAE:DNA] charge-shifting mixed polyion transfection complexes (Fig. 5.25). Furthermore, incorporation of the HRV2 lytic peptide into immobilized ordered charge-shifting polyion transfection complexes [DNA:DEAE:Glu\textsubscript{\textgamma}-HisN:HRV2 LP:Orn\textsubscript{\textgamma}] increased radiolabeled DNA dissociation 16\% over that for ordered charge-shifting mixed polyion transfection complexes without HRV2 peptide [DNA:DEAE:Glu\textsubscript{\textgamma}-HisN:Orn\textsubscript{\textalpha}] (Fig. 5.25).

Collectively, these results suggest that protonation of the \textgamma-carboxyl of poly-L-glutamic acid or Glu\textsubscript{\textgamma}-HisN is responsible for the pH-dependent dissociation of DNA from charge-shifting polyion:DNA complexes seen in this in vitro assay. Additionally, the small increase in DNA dissociation seen with addition of the HRV2 lytic peptide suggests that the lytic peptide might aid Glu\textsubscript{\textgamma}-HisN in destabilizing polycation:DNA interactions.
Transfection and expression efficiency of TR-Orn<sub>a</sub>-labeled polyion transfection complexes in COS-1 cells in vitro

Transfection and expression efficiency of polyion-mediated transfection of COS-1 cells was determined from the photodocumentation obtained in experiments utilizing TR-Orn<sub>a</sub>-labeled polyion transfection complexes and the GFP reporter gene. Polyion transfection treatments examined included: Orn<sub>a</sub> alone [TR-Orn<sub>a</sub> :pEGFP-N1 DNA]; mixed polycations [TR-Orn<sub>a</sub>:DEAE:pEGFP-N1 DNA]; unordered charge-shifting mixed polyions [TR-Orn<sub>a</sub>:Glu<sub>a</sub>-HisN:DEAE:pEGFP-N1 DNA]; ordered charge-shifting mixed polyions [pEGFP-N1:DEAE:Glu<sub>a</sub>-HisN:TR-Orn<sub>a</sub>]; and ordered charge-shifting mixed polyions with the HRV2 peptide [pEGFP-N1:DEAE:Glu<sub>a</sub>-HisN:HRV2 LP Fraction III:TR-Orn<sub>a</sub>] (Table 5.1). COS-1 cells were considered transfected if they contained TR-Orn<sub>a</sub>-labeled transfection complexes adjacent to DAPI-stained nuclei, and were considered to be expressing the recombinant transgene if GFP fluorescence could be visualized within the cell.

Consistent with the hypothesis that non-specific ionic charge interaction is responsible for the association of cationic transfection complexes with anionic cell surfaces, polyion transfection resulted in greater than 99% transfection efficiency for all polyion-mediated transfection complexes examined (Table 5.1). This indicated that recombinant transgene delivery into cells was not a major limitation for polyion-mediated transfection.
<table>
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Table 5.1. Transfection and expression efficiency of COS-1 cells transfected with polycation:DNA transfection complexes. Photomicrographs of COS-1 cells transfected with: Orn_n alone [TR-Orn_n (37.5 μg/ml), pEGFP-N1 DNA (5 μg/ml)]; mixed polycations [TR-Orn_n (37.5 μg/ml), DEAE (12.5 μg/ml), pEGFP-N1 DNA (5 μg/ml)]; unordered charge-shifting polyions [TR-Orn_n (37.5 μg/ml), Glu_n-HisN (75 μg/ml), DEAE (12.5 μg/ml), pEGFP-N1 DNA (5 μg/ml)]; ordered charge-shifting polyions [pEGFP-N1 DNA (5 μg/ml), DEAE (12.5 μg/ml), Glu_n-HisN (75 μg/ml), TR-Orn_n (37.5 μg/ml)]; and ordered charge-shifting polyions with the HRV2 LP [pEGFP-N1 DNA (5 μg/ml), DEAE (12.5 μg/ml), Glu_n-HisN (75 μg/ml), HRV2 LP (0.1 μg/ml), TR-Orn_n (37.5 μg/ml)] were examined to give an estimation of transfection and expression efficiency. The number of transfected cells was determined by counting the number of DAPI stained nuclei associated with TR-Orn_n-labeled transfection complexes. Transfection efficiency was calculated as the percentage of total cells that were transfected. Expression efficiency was calculated as the percentage of transfected cells in which GFP expression could be visualized by fluorescent microscopy on day 2 posttransfection. Some photomicrographs taken shortly after transfection when no GFP expression would be expected, and were not included in the calculation of expression efficiency.
However, despite the extremely high transfection efficiency, recombinant transgene expression efficiency in transfected COS-1 cells was disappointingly low (≤ 6.5%) on day 2 posttransfection (Table 5.1). Nevertheless, the high level of recombinant gene expression from the few expressing cells suggests a tremendous potential for dramatic improvement in recombinant gene expression levels if most or all cells could be induced to express recombinant proteins at similar levels. Taken collectively, these results suggest that DNA escape from endosomes and nuclear entry of transfected DNA may be the major limitations to efficient polyion-mediated recombinant gene transfer and expression.
Discussion

The previous chapter described the development of an ordered charge-shifting mixed polycation-transfection system for recombinant gene transfer into primary bovine mammary cells. This transfection system is capable of high level sustained recombinant gene expression in vitro, under conditions compatible with those anticipated as required for direct intramammary infusion and gene transfer in vivo. To identify rational strategies to further increase recombinant gene expression, the first step was to identify rate-limiting steps for efficient polycation-mediated gene transfer with the novel ordered charge-shifting mixed polycation-based transfection system.

Polycations are known to interact ionically with negatively charged plasmid DNA and should likewise interact with the negatively charged cell surface (Farber et al., 1975; Pagano, 1970). Therefore, polycations should effectively deliver transfected DNA with near 100% transfection efficiency to all cells indiscriminately. With this premise in mind, the expression efficiency of Comma-D and primary bovine mammary cells was examined by transfection with mixed polycation transfection complexes containing pCMVβ, a eukaryotic expression vector encoding the β-galactosidase gene (β-gal) which can be visualized following cell fixation. Regardless of cell type or polycation transfection complex composition, expression efficiency was extremely low (≤ 10%). This included cell lines (COS-1, Hep G2, and 293) capable of extrachromosomal plasmid replication and high level recombinant gene expression. This low expression efficiency was similar to those
for other gene transfer systems (Furth et al., 1995; Wolff et al., 1990; Zabner et al., 1995).
This suggests that the high level recombinant gene expression seen in cell lines capable of extrachromosomal plasmid replication and in mammary cells likely arises from high level expression in very few cells as opposed to low level expression from a large number of cells. Therefore, tremendous potential exists for dramatic improvement in recombinant gene expression levels if most or all cells could produce recombinant proteins at the similar apparently high levels observed for the few individual cells.

To confirm the initial premise that polycations would deliver DNA to all cells indiscriminately, fluorescently labeled polycations were applied to Comma-D and primary bovine mammary cells in vitro. FITC-labeled polycations were rapidly internalized and concentrated in the nucleus of virtually every mammary cell exposed to transfection polyions. Polyamine internalization is presumed to occur via nonspecific endocytosis (Kabanov and Kabanov, 1995). However, it is likely that the extensive cationic charge of polyamines is responsible for their nuclear localization. Nuclear localization signals (NLS) direct protein transport through the nuclear pore (Feldherr and Akin, 1990), and typically consist of short tracts of 5-10 basic amino acids (Davis, 1992) whose sequence and lengths are not highly conserved (Ryser and Shen, 1978). Thus, polycations such as Orn\textunderscore m, composed entirely of basic amino acids, might be expected to mimic nuclear localization signals.

Alternatively, Holter et al. (1961) demonstrated that simple addition of proteins to the extracellular environment can stimulate ameobas to selectively internalize proteins.
by endocytosis. Support for polycation endocytosis comes from Marciano-Cabral et al. (1981) who observed that DNA:Ornₙ complexes are concentrated in membrane bound vesicles following Ornₙ-mediated transfection. Furthermore, conjugation of proteins (albumin and horseradish peroxidase) to polyornithine (Ryser and Hancock, 1965; Shen and Ryser, 1978) or conjugation of methotrexate to polylysine (Ryser and Shen, 1978) has been reported to enhance protein/drug uptake by endocytosis in cultured mammalian cells. Cellular uptake and subcellular distribution of mixed polyion transfection complexes containing DNA was examined by incorporating FITC-labeled Ornₙ into mixed polyion transfection complexes used to transfect Comma-D mouse and primary bovine mammary cells. Cellular internalization of FITC-labeled polyion transfection complexes was rapid and nearly linear over the four hour transfection period (Fig 5.9), however, subcellular distribution was dramatically different than transfection with polycations alone. When complexed with DNA, FITC-labeled Ornₙ localized to the nuclear or perinuclear regions of virtually all cells. Mixed FITC-labeled polycation transfection complexes containing Gluₙ-HisN also showed perinuclear localization but with punctate fluorescent foci (Fig. 5.6D and F). Thus, whether alone or complexed with DNA, polycations are rapidly internalized and transported to the nucleus or the perinuclear region, suggesting that cellular entry is not rate-limiting for polyamine-mediated gene transfer into mammary cells in vitro.

Zabner et al. (1995) noted that cellular entry was not rate-limiting for polycationic liposome-mediated transfection of COS-1 cells, and although most cells internalized
plasmid DNA, less than 50% expressed the transfected transgene. Additionally, transfected plasmid DNA stained with ethidium bromide accumulated within 24 hours in discrete foci in the perinuclear region, much like the FITC-labeled polycation:DNA complexes of this study.

To demonstrate that these intracellular localization studies were not confounded by incorporation of FITC-labeled Omₙ into transfection complexes, primary bovine and Comma-D mouse mammary cells were transfected with FITC-labeled polycations. Subsequent expression of recombinant hGH was low (Fig. 5.7) but within the range of expression observed with primary bovine mammary cells transfected with unlabeled polycations. More significantly, treatment effects were consistent with transfections utilizing unlabeled Omₙ (Fig. 4.9 and Fig. 4.11). Thus, it appears that FITC-labeling of polycations had little or no effect on polycation localization or polycation-mediated transfection.

Comparison of the intracellular localization of radiolabeled DNA, determined by nuclear emulsion overlay of mammary cells transfected with radiolabeled plasmid DNA, and of fluorescently-labeled polycation in transfection complexes, suggested colocalization of intracellular transfected plasmid (Fig. 5.8) and FITC-labeled polycations (Fig. 5.6). However, because mixed polycation transfections contained a 10:1 (wt:wt) polycation:DNA ratio, it was a concern that the subcellular fluorescence seen in Figure 5.6 was attributable to free FITC-labeled polycations alone.
Further elucidation of polycation intracellular localization and confirmation of fluorescently labeled polycation colocalization with transfected DNA used fluorescent microscopy capable of simultaneous visualization of three fluors (red, green, and blue). Triple fluorescent labeling made it dramatically clear that virtually all cells received fluorescently labeled transfection complexes and concentrated them asymmetrically in the perinuclear region, but not in the nucleus (Figs 5.10, 5.11, and 5.13). Internalized TR-Ornₙ could be visualized in intracellular vesicles apparently en route to the nuclear/perinuclear region in the single cell strongly expressing GFP in Figure 5.14. Additionally, all cells expressing the recombinant GFP transgene had detectable TR-labeled polycations in the nucleus (Fig. 5.13). However, not all cells containing TR-labeled polycations in their nuclei showed GFP expression (Fig. 5.13A). It is unlikely that fluorescent foci represent previously dissociated TR-Ornₙ or undissociated polycation-DNA complexes, since free FITC-labeled polycations were rapidly concentrated in the nucleus (Fig. 5.5) and were virtually absent from the perinuclear region after exposure to cells.

Dual wavelength fluorescence clearly confirmed colocalization of DAPI-stained transfected plasmid DNA and TR-Ornₙ (Fig. 5.11 and Fig. 5.13). The predominant perinuclear localization of fluorescently labeled transfection complexes suggests that endosome escape or nuclear entry, or both, may be rate-limiting for polycation-mediated transfection.
Nuclear entry was determined to be rate limiting for polycationic liposome transfection of COS-1 cells (Zabner et al., 1995) and for polycation:DNA complexes directly injected into the cytosol of rat adrenal pheochromocytoma (PC12) cells (Malecki and Skowron, 1995). Malecki and Skowron (1995) overcame this limitation by incorporating the SV40 T antigen nuclear localization signal (Colledge et al., 1986; Kalderon et al., 1984) into a polycation:DNA complex, suggesting that mixed polycation transfection complexes might also be targeted to the nucleus in similar fashion. The polycationic character of polyamines may mimic the polycationic nuclear localization signal (Davis, 1992) to direct the transport of DNA:polycation complexes into the nucleus.

The size and structure of mixed polycation transfection complexes was examined by formation of polycation:DNA complexes in contact with transmission electron microscopy grids. Transmission electron microscopy showed DEAE:DNA condensates to be spherical with a 230-240 nm average diameter (Fig. 5.15). Orn:DNA condensates (Fig. 5.16) formed torroids of similar size, and were larger than those previously described by Wagner et al. (1991), Laemmli (1975), and Arscott et al. (1990) as 50-100 nm avg. diameter, but smaller than those described by Shapiro et al. (1969) as 340 nm avg. diameter. The morphology of mixed polycation transfection complexes of this study consisted of free plasmid, condensed rods, and amorphous aggregates (Fig. 5.17) like those described for other polycations (Eickbush and Moudrianakis, 1978) and polycationic liposomes (Zabner et al., 1995). Mixed polycation transfection complexes
containing the charge-shifting \( \text{Glu}_{n}-\text{HisN} \) polymer consisted almost entirely of large amorphous aggregates ranging in size from 100 to 2000 nm (Fig. 5.18).

The diameter of newly formed endocytic vesicles in amoebae and tissue-culture cells may range from several hundred to several thousand nanometers (Holter, 1961) indicating that endocytic vesicles can accommodate large particles and aggregates such as those formed by DNA:polyion complexes. Zabner et al. (1995) clearly show endocytosis of aggregated polycationic-liposome-DNA complexes in membrane bound vesicles having diameters of up to 700 nm. Thus, endocytosis should provide an efficient mechanism for internalization of polyion:DNA complexes. However, nuclear pores are about 9 nm in diameter (Alberts et al., 1989, and references therein) and therefore even the smallest DNA:polycation complex will likely not be able to cross nuclear membranes unaided.

The ability to control and minimize the size of polyion:DNA complexes may facilitate nuclear entry of transfected DNA. This might be accomplished by forming DNA:polyion complexes comprised of a single plasmid molecule as opposed to the multimolecular (13-26 plasmid molecules per torroid or rod) complexes previously described by Arscott et al. (1990) which were smaller than those seen in Figures 5.15 and 5.16 or by size fractionation to purify polycation:DNA complexes of a select size.

The earlier studies of cellular uptake and subcellular distribution of radiolabeled plasmid DNA, as determined by subcellular fractionation, showing complete nuclear localization of polycation-transfected DNA (Fig. 4.3 and 4.10) are inconsistent with the
localization of fluorescently-labeled polycation transfection complexes in the perinuclear region (Figs. 5.0, 5.11, and 5.13). Additionally, Marciano-Cabral et al. (1981) reported accumulation of large cytoplasmic \( \text{Orm}_n \cdot \text{DNA} \) condensates in membrane-bound bodies. Collectively, these data suggest that studies of the subcellular fate of transfection complexes or transfected DNA by cell lysis and centrifugal subcellular fractionation may be inaccurate due to cosedimentation of polycation transfection complexes with nuclear fractions, due to similarities in their probable specific gravity (as a peptide:DNA complex versus nucleoprotein:DNA complex) size, proximity to the nucleus, and aggregative properties.

Endosome escape has been identified as a major limitation to polycationic liposome (Zabner et al., 1995) and ligand-mediated gene transfer (Zauner et al., 1995). Polycation-DNA complexes have also been reported to accumulate in intracellular membrane bound vesicles (Marciano-Cabral et al., 1981). In this study, charge-shifting polyion transfection complexes were localized in perinuclear aggregates of particles consistent with entrapment of most in endosomes. Therefore, to examine whether endosome escape was rate-limiting for mixed polycation-mediated gene transfer, an HRV2 lytic peptide was synthesized as described by Zauner et al. (1995) and incorporated into mixed polycation transfection complexes used to transfect primary bovine mammary cells. Recombinant hGH expression levels increased over two fold from primary bovine mammary cells transfected with mixed polycations containing the lytic peptide (Fig. 5.20), suggesting that facilitating DNA escape from endosomes may be of considerable
value in increasing transgene expression efficiency. Unfortunately, mass spectral analysis of the synthesized HRV2 lytic peptide indicated that its synthesis was incomplete and had stopped at either Val₁₆ or Val₁₇ to give only the C-terminal 9 or 10 amino acid peptides. However, even this incomplete HRV2 lytic peptide appeared to stimulate recombinant gene expression over two-fold, suggesting substantial potential for further increases in recombinant gene expression if a full length lytic peptide were incorporated into ordered charge-shifting mixed polyion transfection complexes.

Interestingly, no subcellular distribution differences were detected in TR-Ornₙ-labeled transfection complexes containing the HRV2 lytic peptide (Fig. 5.21). Thus, it appears that mixed polycation-mediated gene transfer may be limited by both endosomal exit and nuclear entry. However, nuclear entry was not rate-limiting for FITC-labeled polycations alone. Therefore, polycation condensation of DNA apparently either disrupts charge or structural parameters essential for nuclear internalization, or forms DNA:polyion complexes too large for transit through the nuclear pore.

As seen previously, mixtures of polycations (Fig. 4.6) and addition of the charge-shifting Gluₙ-HisN polymer (Fig. 4.9) strongly suggested that DNA-polycation dissociation also may be a major limitation to successful recombinant gene expression. Indeed, it is widely accepted that transcriptionally active DNA is free from cationic histones and is exposed for transcription as evidenced by hypermethylation and nuclease sensitivity studies (Yaniv and Cereghini, 1986). Additionally, Zabner et al. (1995) found that direct intranuclear injection of naked DNA gave higher recombinant gene expression
than intranuclear injection of polycationic liposome:DNA complexes. Therefore, an in vitro model to test molecules for their ability to facilitate DNA:polycation dissociation would dramatically facilitate screening of candidate polycation perturbants.

The serendipitous observation that mixed polycation transfection complexes containing fluorescently-labeled polycations were visible as uniformly distributed particles of similar diameter in open areas of cell culture plates, and the fact that these particles persisted in culture suggested that these "immobilized" transfection complexes could be used to test for pH-sensitive DNA:polycation dissociation. Dissociation was measured by the release of radiolabeled DNA into the wash medium which varied in pH and salt concentration. High salt (1M) and low pH (3.5) readily dissociated radiolabeled plasmid DNA from immobilized transfection complexes formed in either HBSS or 0.3 M sucrose, 10 mM HEPES, pH 7.4 (Fig. 5.22). Radiolabeled DNA dissociation was approximately two-fold higher in HBSS than in sucrose/HEPES (Fig. 5.22), possibly due to divalent cations in HBSS which weakened the DNA:polycation interactions. This may indicate cooperative dissociation since polycations have been shown to bind DNA in cooperative fashion (Shapiro et al., 1969 and references therein).

Only mixed polycation transfection complexes containing Glu\textsuperscript{n}-HisN or free Glu\textsuperscript{n} showed pH-sensitive dissociation of radiolabeled plasmid DNA from immobilized transfection complexes (Fig. 5.25), yet previous expression studies (Fig. 4.15) confirm that histamine derivatization of Glu\textsuperscript{n} is required for high level recombinant gene expression. Dissociation of radiolabeled DNA from polycations between pH 7.0 and pH
2.0 was not linear (Fig. 5.23) as might have been predicted from the Glu^-HisN titration curve (Fig. 5.24). It had previously been hypothesized that pH sensitive DNA:polycation dissociation was a result of derivatized histamine protonation, since endosome pH has been reported to reach a minimum pH of ~6 (Alberts et al., 1989). However, significant radiolabeled DNA dissociation did not begin until pH 5.0, and facilitation of DNA:polycation dissociation by underivatized Glu suggests protonation of underivatized γ-carboxyl moities of Glu^-HisN and Glu as responsible for pH-sensitive dissociation of transfection complexes. However, this would be most likely to occur in lysosomes which are more acidic (pH ~5) than endosomes (pH ~6) (Alberts et al., 1989). Lysosomal exit of polycation:DNA transfection complexes containing Glu^-HisN is not entirely infeasible, in view of evidence (Boussif et al., 1995) and rationale (Mislick et al., 1995) for lysosomal exit of transfection components, and fits well with the buffering capacity (Fig. 5.24) and subcellular localization (Fig. 5.11) of the mixed polyion Glu^-HisN transfection system.

Like polyethylenimine, Glu^-HisN could extensively buffer endosomes or lysosomes leading to osmotic swelling and lysis as proposed by Mislick et al. (1995). Increased buffering capacity in endosomes or lysosomes is thought to lead to hyperosmotic ion concentrations which in turn leads to vesicle swelling and eventual lysis. Hence, Glu^-HisN may perform a dual role in mixed polyion gene transfer through 1) facilitation of endosome/lysosome exit by exhaustive buffering, and 2) promoting DNA:polycation dissociation upon protonation. Since the pH sensitive recovery of DNA
from transfection complexes in vitro indicated large-scale dissociation below pH 5.0, then
the dramatic increase in recombinant gene expression with inclusion of Glu₉-HisN in
transfection complexes may be attributable to acidification of endosomes or lysosomes
to pH 5.0. If so, this would suggest substantial potential for improvement in recombinant
gene expression by further acidification of the endosome/lysosome.

If transfection complexes dissociate in endosomes or lysosomes, then why is TR-
Orn₉ visible in the nuclei of GFP expressing cells, and how are free nucleic acids
transported into the nucleus? The most likely explanation is that either the polyion:DNA
complex has dissociated and only TR-Orn₉ is being visualized, or the complex did not
dissociate and was imported into the nucleus intact. Further studies using fluorescently-
labeled DNA and high resolution triple fluorescent microscopy should resolve this
question.

This study shows that free polycations and polycation:DNA complexes are rapidly
internalized by virtually all cells and that free polycations are concentrated in the nucleus,
whereas polycation-DNA complexes are localized to the perinuclear region.
Incorporation of a prematurely terminated rhinovirus lytic peptide increased recombinant
gene expression 2-3 fold but did not change fluorescently-labeled polycation transfection
complex distribution. Although it did not increase recombinant gene expression 500-fold
as described by Zauner et al. (1995), the increased recombinant gene expression obtained
with the synthesized small and incomplete lytic peptide fragment suggests further
increases in recombinant gene expression might be obtainable with the complete 25 amino acid peptide.

In vitro dissociation experiments indicated that the γ-carboxyl of Glu_n in Glu_n-HisN was probably responsible for pH-dependent polycation:DNA dissociation. However, the intrinsic requirement for histamine derivatization of Glu_n for high level recombinant gene expression was previously demonstrated (Fig. 4.15) suggesting there is a cooperative interaction between the γ-carboxyl of Glu_n and the histamine imidazole in Glu_n-HisN. Therefore, sequential protonation of the histamine imidazole and γ-carboxyl of Glu_n in Glu_n-HisN is likely responsible for mediating the large increase in recombinant gene expression when Glu_n-HisN is incorporated into mixed polycation transfection complexes. These results are consistent with the hypothesis that polyions which facilitate DNA:polycation dissociation would result in increased recombinant gene expression. These studies have further identified endosomal exit, nuclear entry, and DNA:polycation dissociation as major limitations to efficient mixed polyion-mediated gene transfer in mammary cells in vitro. Further increases in recombinant gene expression can likely be achieved with strategies designed to overcome these gene transfer limitations.
Chapter 6

Gene expression systems to enhance recombinant

gene expression level and duration in mammary cells

The mixed polyion-based gene transfer system described in Chapter 4 was shown to be capable of inducing high level sustained recombinant gene expression in primary bovine mammary cells transfected in vitro, and should be amenable to direct intramammary infusion and gene transfer in vivo. However, further increases in recombinant gene expression levels will likely be needed for modification of bulk milk composition in vivo. Additionally, sustained recombinant gene expression would eliminate the need for repeated intramammary infusions and reduce production costs. Therefore, alternative recombinant gene expression systems were investigated for their ability to increase or prolong recombinant gene expression in mammary epithelial cells transfected with mixed polyions in vitro.

T7 RNA polymerase-dependent transgene expression

The use of prokaryotic regulatory elements for recombinant gene expression in mammalian cells has recently attracted much interest for their potential to amplify gene

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expression, since gene expression by prokaryotic promoters should not be restricted by the availability of host cell transcription factors and RNA polymerase, and should be independent of eukaryotic gene regulation. Furthermore, recombinant gene expression in mammalian cells from prokaryotic-dependent expression systems has been reported to be greater than that observed with conventional transient expression vectors which are dependent on transcription by the host RNA polymerase II (Elroy-stein and Moss, 1990; Zhou et al., 1990) and within the range of expression obtained with the strong Rous sarcoma virus (RSV) and cytomegalovirus (CMV) promoters (Lieber et al., 1993). Bacteriophage T7 RNA polymerase-dependent mRNA transcript production has been reported to comprise up to 30% of total cytoplasmic RNA in CV-1 cells (Elroy-stein and Moss, 1990). Furthermore, Lieber et al. (1993) reported stable, T7 RNA polymerase-dependent, expression of human growth hormone at levels of 20-30 μg/ml/10^6 cells/day, while Deng et al. (1991) showed that recombinant luciferase levels approached 20% of total cellular protein in T7 RNA polymerase-dependent mammalian gene expression systems. Similarly, Fuerst et al. (1986) showed that CV-1 cells infected with vaccinia virus expressing T7 RNA polymerase produced 400-600 times more CAT than that observed with conventional mammalian transient expression systems regulated by the Rous sarcoma virus long (RSV) terminal repeat or the simian virus (SV40) promoter. Therefore, prokaryotic gene expression systems appear to offer tremendous potential for dramatically increasing recombinant gene expression in mammalian cells.
The bacteriophage T3 (McGraw et al., 1985) and T7 (Dunn and Studier, 1983) RNA polymerases have been cloned and their respective promoters have been characterized (Adhya et al., 1981; Bailey and McAllister, 1980; Oakley and Coleman, 1977). Although both eukaryotic RNA polymerase II and bacteriophage T7 RNA polymerase have high catalytic activity, T7 RNA polymerase differs in that it is a single-subunit 100 kDa enzyme (Chamberlin et al., 1970) and possesses stringent specificity for its 17 bp promoter (Dunn and Studier, 1983), which is of sufficient length that it is unlikely to occur by chance in eukaryotic cells (Dunn and Studier, 1983). Moreover, low level T7 RNA polymerase expression in its native host, Eschericia coli (E. coli), is sufficient to drive high level gene expression from bacteriophage T7 DNA (Studier and Moffatt, 1986). Additionally, use of prokaryotic regulatory elements for transcription in eukaryotic cells has the advantage of avoiding competition with eukaryotic cellular or viral promoters for transcription factors necessary for high level gene expression (Wilk et al., 1992). Thus, a T7 RNA polymerase-dependent gene expression system should allow high level expression of specific recombinant transgenes in mammalian cells independent of the availability of eukaryotic transcription factors and would only require expression of one additional protein, the T7 RNA polymerase itself.

These attributes provided the basis for development of bacteriophage RNA polymerase gene expression systems in E. coli (Studier and Moffatt, 1986; Tabor and Richardson, 1985) capable of driving exceptionally high expression of recombinant genes, recombinant vaccinia virus-infected eukaryotic cells (Fuerst et al., 1986), and
more recently in eukaryotic cells constitutively expressing T7 RNA polymerase (Deng et al., 1991; Elroy-stein and Moss, 1990).

However, the use of prokaryotic gene expression components in eukaryotic cells also poses several challenges. First, prokaryotic proteins do not contain nuclear localization signals (NLS) to direct their transport into the nucleus, and T7 RNA polymerase is too large (100 kDa) (Chamberlin et al., 1970) to freely diffuse through nuclear pores (5.5-9 nm diameter) (Lang et al., 1986; Paine et al., 1975). Thus, they are unable to enter the nucleus and catalyze transcription therein. Therefore, eukaryotic gene expression systems reliant on prokaryotic promoter elements either must be functional in the cytoplasm or be modified by addition of nuclear localization signals to the prokaryotic RNA polymerase to allow its entry into the nucleus. Secondly, mRNA transcripts produced by bacteriophage RNA polymerases are not efficiently translated in eukaryotic cells, presumably because they lack a 7-methylguanosine cap (Deuschle et al., 1989) on their 5' terminus which is essential for ribosome binding to and translation of eukaryotic mRNAs. The eukaryotic 5' mRNA cap has also been implicated in mRNA half-life regulation, targeting of mRNA to spliceosomes, nuclear export of mRNA, and subcellular localization of mRNA (Banerjee, 1980; Wilk et al., 1992). Thus, the use of bacteriophage polymerase/promoter systems in eukaryotic cells may yield reporter gene mRNA species which are inadequately processed and incapable of directing protein translation.

To be fully functional, eukaryotic mRNAs usually require extensive posttranscriptional processing in the nucleus, such as splicing, capping, methylation, and
polyadenylation. Thus, for T7 RNA polymerase to be useful as a gene expression system in eukaryotic cells, it would have to be either transported from its site of synthesis in the cytosol into the nucleus of the cell, or else the eukaryotic enzymes responsible for RNA-modification would have to be functional in the cytoplasm. Furthermore, transcription and nuclear processing of T7 RNA polymerase-dependent reporter genes would require nuclear localization of T7 RNA polymerase (Lieber et al., 1993). Nuclear localization of functional T7 RNA polymerase has been accomplished both by fusion of a nuclear localization signal (NLS) to the amino terminus of the T7 RNA polymerase protein (Dunn et al., 1988), or by substituting the NLS for the N-terminal 5% of the polymerase (Lieber et al., 1993) to allow chloramphenicol acetyl transferase (CAT) expression in mouse L cells (Ltk−) in vitro (Lieber et al., 1993).

Zhou et al. (1990) and Elroy-stein (1990) circumvented the requirement for a 5' 7-methylguanosine cap for mRNA translation by fusing 5' nontranslated leader sequences from the encephalomyocarditis virus (EMC) to the upstream end of target gene coding regions to allow cap-independent translation of T3 or T7 produced transcripts in mouse cell lines constitutively expressing T3 and T7 RNA polymerases. The 5' untranslated region of the EMC virus functions as an internal ribosome entry site (IRES) to allow 5' mRNA cap-independent binding of ribosomes and translation from uncapped mRNAs in both in vitro transcription assays (Jang et al., 1988) and eukaryotic cell culture (Elroy-stein and Moss, 1990; Zhou et al., 1990).
However, of the T7 RNA polymerase-dependent expression studies reporting increased mRNA (Elroy-stein and Moss, 1990) or increased recombinant transgene expression (Deng et al., 1991; Fuerst et al., 1986; Lieber et al., 1989; Lieber et al., 1993), none utilized both an IRES in the T7-dependent reporter gene transcript and a nuclear targeted T7 RNA polymerase. Rather, these results were obtained with cell lines constitutively expressing an NLS-modified T7 RNA polymerase in conjunction with either an IRES incorporated into the T7-dependent reporter gene transcript (Deng and Wolff, 1994; Elroy-stein and Moss, 1990) or selection agents to guarantee T7-dependent reporter gene expression (Lieber et al., 1989; Lieber et al., 1993). Alternatively, Fuerst et al. (1986) infected cells with vaccinia viruses encoding recombinant T7 RNA polymerase. Viral enzymes are then presumed to facilitate T7 RNA polymerase-dependent gene expression in the cytosol (Fuerst et al., 1986, and references therein), eliminating the requirement for both a nuclear localization and an IRES. Thus, cotransfection of mammalian cells with expression vectors encoding: 1) an NLS-modified T7 RNA polymerase, and 2) a T7 promoter-driven reporter gene flanked at it's 5' end by an IRES likely has the potential to dramatically increase recombinant gene expression.

Episomal replication

To complement increased recombinant gene expression levels, it is also desirable to increase expression duration. The previously described (Chapter 4) high level recombinant gene expression in mammary cells transfected with mixed polyions in vitro
persisted for 10-14 days posttransfection and then declined, presumably because transfected plasmid DNA becomes hypermethylated or degraded over time in the nucleus, thus leading to decreased expression (Clough et al., 1982; Razin and Riggs, 1980). Hence, one way to increase the duration of expression might be to replenish expression vector DNA through plasmid replication.

Viral genomes such as Simian virus 40 (SV40), Epstein-Barr virus (EBV), and bovine papilloma virus (BPV), may persist intracellularly as circular plasmids capable of extrachromosomal replication in appropriate host cells (Gluzman, 1982; Nonoyama and Pagano, 1972; Sarver et al., 1981). However, SV40 expression is normally limited to certain primate cells and often culminates in cell death (De Benedetti and Rhoads, 1991). Although EBV-based plasmid replication had been reported in human and primate cell lines (Yates et al., 1985), it was not known whether bovine mammary cells would permit EBV-based extrachromosomal plasmid replication. The only known requirements for EBV-based plasmid replication are expression of the EBNA1 protein in host cells and the oriP sequence from the EBV genome (Lupton and Levine, 1985; Yates et al., 1984; Yates et al., 1985). EBNA1 binding within oriP causes distortions in DNA secondary structure both in vitro and in vivo (Hsieh et al., 1993), and is both necessary and sufficient for episomal replication of EBV-based plasmids in human and primate cell lines (Yates et al., 1985). Because purified EBNA1 protein lacks helicase activity (Frappier and O'Donnell, 1991; Middleton and Sugden, 1992) and does not appear to possess enzymatic activity (Yates and Camiolo, 1988), EBNA1-dependent extrachromosomal plasmid replication
is thought to be dependent on cellular DNA replication machinery (Wysokenski and
Yates, 1989) which limits plasmid replication to once per cell cycle (Yates and Guan,
1991). Nevertheless, Sugden et al. (1979) reported that EBV-infected human
lymphocytes carried anywhere from 10 to several hundred plasmid copies per cell.
Extrachromosomal replication of EBV based plasmids in primary bovine mammary cells
transfected with EBNA1/oriP would be another approach to further extend the duration
of the high level recombinant gene expression described in Chapter 4.

Green Fluorescent Protein Secretion as a non-hGH reporter gene.

Expression of a reporter protein other than hGH in the milk of transfected
mammary glands may be desirable for transfection studies since both bovine (Devolder
et al., 1993) and rabbit (Postel-Vinay et al., 1991) milk have been reported to contain
human growth hormone-specific binding proteins which may interfere with conventional
ELISA assays by masking essential antigenic epitopes.

Cloning and sequencing of the Aequorea victoria green fluorescent protein (GFP)
gene (Prasher et al., 1992) has allowed real time visualization of recombinant transgene
expression in living cells both in vitro (Kaether and Gerdes, 1995; Muldoon et al., 1997)
and in vivo (Higgs et al., 1996). GFP expression is highly resistant to photobleaching,
requires no additional gene products, substrates, or cofactors, and is reported to occur in
a species independent fashion (Kain and Ganguly, 1995). Moreover, GFP expression
does not appear to be toxic to either bacteria or eukaryotic cells (Kain and Ganguly, 1995, and references therein).

However, in the jellyfish (*A. victoria*), GFP is a cytosolic protein and is not secreted. Thus, as a tool with which to study protein secretion, Kaether and Gerdes (1995) created a human chromogranin/B-GFP fusion protein and documented its progression through the secretory pathway and into the culture medium of transfected HeLa cells. The hGH secretion signal, which is sufficient for high level secretion of hGH from many cell types (Selden et al., 1986) including primary bovine mammary cells (Chapter 4), should likewise be capable of directing efficient GFP secretion if attached to the N-terminus of GFP. A secretable form of GFP should allow rapid, sensitive, non-destructive analysis of recombinant gene expression and provide an alternative reporter protein to monitor recombinant gene expression in the milk of transfected mammary glands.

Described here are efforts to increase and prolong recombinant gene expression in primary bovine mammary cells, based on construction of a bacteriophage T7 RNA polymerase-driven dual plasmid gene expression system and EBV-based episomal plasmid replication, respectively. Cotransfection of plasmids encoding: 1) CMV-driven expression of T7 RNA polymerase having an NLS added to its 5'-end, and 2) addition of an IRES to the 5'-end of the T7 polymerase-dependent transcript produced from the T7 promoter may allow amplification of gene expression over that attainable with the CMV promoter alone. An EBV-based episomal plasmid replication system was also tested for
its ability to extend the duration of high level hGH gene expression driven from the CMV promoter in primary bovine mammary cells transfected in vitro. Lastly described are efforts to develop a secretable version of the GFP reporter gene for use as a non-destructive, fluorescently-detectable reporter gene not vulnerable to the potential interference in its assay from the hGH binding proteins reported in some milk.
METHODS

New Expression Vector Constructions

Construction of pCEP4/hGH

The human growth hormone gene (hGH) was excised from the eukaryotic expression vector pcDNA1/hGH (bases 2196-4401) (described previously) with HindIII, XhoI restriction endonucleases and subcloned into the pCEP4 plasmid (Invitrogen) (Fig. 6.1) at the corresponding restriction endonuclease sites in the multiple cloning site immediately downstream of the CMV promoter. The newly formed eukaryotic expression vector pCEP4/hGH contains both the coding sequence for the EBNA1 protein driven by its endogenous EBV-promoter and the origin of replication (oriP) from Epstein-Barr virus, which is sufficient for episomal replication in primate and canine cells.

Construction of pEGFPss

The construction of GFP with an N-terminal secretion signal was accomplished as follows (Fig. 6.2). pcDNA1/hGH nucleotide 2022-2636 encompassing the hGH secretion signal and eight amino acids of the mature hGH protein were PCR amplified as described in general methods using synthetic oligonucleotide primers pcDNA1/hGH 2041 (5'-GGCACCAAAATCAACGGGAC-3') and hGH2615 (5'-GGGCCCGCGGCCTGGATAGGAATGGTTGG-3') which contained a KspI restriction endonuclease site (bold). The resultant 614 bp PCR product was digested with HindIII and KspI, purified
Figure 6.1. Construction of pCEP4/hGH, a eukaryotic cell episomal replication vector for CMV-dependent expression of hGH. The hGH gene excised from pcDNA1/hGH with HindIII and XhoI, was ligated into the same restriction endonuclease sites within the multiple cloning site downstream of the CMV promoter in the pCEP4 EBNA-1-dependent episomal replication vector.
Figure 6.2. Construction of pEGFPss encoding a secretable version of GFP comprised of the 26 amino acid secretion signal (and 1st intron) and first eight amino acids of the mature protein from hGH fused to the N-terminus of GFP for expression of the fusion reporter gene from the CMV promoter.
by agarose gel electrophoresis, and ligated into the corresponding restriction endonuclease sites in pEGFP-N1 to give pEGFPss (Fig. 6.2). The resultant fusion mRNA contains nucleotides from the hGH gene which encode the first exon, the intervening first intron, and 31 amino acids of the second exon up to Arg of the mature hGH protein (Fig. 6.3A). The expressed fusion protein (following signal peptide cleavage) should be comprised of eight amino acids of the mature hGH protein joined by a 10 amino acid linker to the N-terminus of the GFP protein (Fig. 6.3C).

It was necessary to include the first intron and a portion of exon two from hGH because the hGH secretion signal spans exons one and two (DeNoto et al., 1981). Furthermore, the presence of an intron, even a heterologous intron, located near the 5' end of the transgene is advantageous for gene expression (Brinster et al., 1988), presumably by increasing the efficiency of mRNA 3' splicing (Huang and Gorman, 1990) and thereby leading to an increase in the levels of both nuclear and cytoplasmic poly(A)+ mRNA (Buchman and Berg, 1988). Additionally, inclusion of the first eight amino acids of the mature hGH protein should provide a region sufficient for proper signal peptide cleavage while avoiding the following hGH alpha-helical secondary structure which might interfere with GFP folding and subsequent fluorescence.

The pEGFP-N1 expression vector for GFP used in these studies encoded a version of GFP which had been optimized by conversion to human codons, and contained the S65T mutation to enhance fluorescence, but lacked the F64L mutation now in
Figure 6.3. Schematic diagram of the pEGFPss expression vector encoding the human growth hormone secretion signal-GFP fusion protein. Panel A, unprocessed mRNA encoding the hGH secretion signal-GFP fusion protein; Panel B, processed mRNA; and Panel C, mature modified GFP protein containing the hGH secretion signal.
Figure 6.3
commercially available pEGFP-N1 and which enhances GFP fluorescence another 3-5 fold.

Construction of phGHGFP

A eukaryotic expression vector encoding an hGH-GFP fusion protein was created as follows (Fig. 6.4). The entire hGH gene (pcDNA1/hGH nucleotides 2022-3737), with the exception of the stop codon, was amplified by PCR as described in methods with synthetic oligonucleotides pcDNA1/hGH 2022 (described previously) and hGHfull (5'-GGGCCCAGCGGGAAGCCACAGCTGCCCTCCAC-3') which was complementary to pcDNA1/hGH nucleotides 3717-3737 (underlined) and contained a KspI restriction endonuclease cleavage site (bold) in a 5' non-complementary tail. The resultant PCR product was digested with HindIII and KspI, and ligated into the corresponding HindIII and KspI restriction endonuclease sites in pEGFP-N1 to give phGHGFP (Fig. 6.4). The resultant fusion mRNA contains nucleotides encoding the complete hGH gene, with exception of the stop codon, a 10 amino acid linker, and the GFP cDNA (Fig. 6.5 A). The mature fusion protein (phGHGFP) (following signal peptide cleavage) contains the mature hGH protein joined by a 10 amino acid linker to the N-terminus of the GFP protein (Fig. 6.5B).

Construction of pBS/hGH

To generate a T7 RNA polymerase-dependent hGH eukaryotic expression vector, the hGH gene was excised from pcDNA1/hGH (bases 2196-4401) with HindIII and XhoI
Figure 6.4. Construction of phGHGFP, a full length human growth hormone-GFP chimera comprised of the entire hGH gene, with exception of the stop codon, fused to the N-terminus of GFP for expression of the fusion reporter gene from the CMV promoter.
Figure 6.5. Schematic diagram of the phGHGFP expression vector encoding the full length human growth hormone-GFP fusion protein. Panel A, unprocessed mRNA encoding the hGH-GFP fusion protein; Panel B, processed mRNA; and Panel C, mature hGH-GFP fusion protein.
Figure 6.5
and subcloned into the corresponding restriction endonuclease sites in the multiple
cloning site of pBluescript II KS+ (pBSKS+) to yield pBS/hGH (Fig. 6.6). pBSKS+
contains the T3 and T7 RNA polymerase promoters in opposing orientations flanking the
insertion site. Therefore, cloning directionality dictates that recombinant hGH expression
will be driven by the T7 RNA polymerase from the T7 RNA polymerase promoter.

Construction of pcDNA1/T7A T7 RNA Polymerase Expression Vector

To construct a eukaryotic expression vector for expression of T7 RNA polymerase
driven from the strong cytomegalovirus (CMV) promoter, the T7 RNA polymerase gene
was excised from pGP1-2 with SacI and BamHI and subcloned into the corresponding
restriction endonuclease sites in pcDNA1 to give pcDNA1/T7A (Fig. 6.7). This cloning
strategy eliminated three 3'-terminal base pairs of the CMV promoter and the T7 RNA
polymerase promoter located immediately 5' to the multiple cloning site in pcDNA1,
which therefore abrogates T7 RNA polymerase self-amplification (Fig. 6.7). However,
this cloning protocol also transferred the lambda phage pL promoter that was located
immediately 5' to the T7 RNA polymerase cDNA in pGP1-2 (Fig. 6.7).

Construction of pcDNA1/T7B and pcDNA1/T7C T7 RNA Polymerase Expression
Vectors

Construction of pcDNA1/T7B, which differs from pcDNA1/T7A only in that the
three 3'-terminal base pairs of the CMV promoter were restored, was a multistep process
Figure 6.6. Construction of pBS/hGH, a T7 RNA polymerase-dependent hGH expression vector. pBS/hGH contains the complete hGH gene downstream of the T7 RNA polymerase promoter.
Figure 6.7. Construction of pcDNA1/T7A, a T7 RNA Polymerase Expression Vector. The T7 RNA polymerase gene and 5'-flanking lambda phage pL promoter were excised from pGP1-2 with ScaI and BamHI and ligated into the same restriction endonuclease sites in the multiple cloning site of pcDNA1.
that began with the elimination of the SacI site (2208 bp) in the multiple cloning site of pcDNA1. This was necessary for subsequent cloning into a second SacI site (2121) for removal of the T7 RNA polymerase promoter. The SacI site (2208 bp) was eliminated by HindIII, EcoRI digestion of pcDNA1 and subsequent subcloning of HindIII, EcoRI digested pGEM vector into the corresponding restriction endonuclease sites to yield pcDNA1pGEM (Fig. 6.8).

The T7 RNA polymerase promoter (bases 2170-2190) and 6 additional bases between the promoter and the start of the multiple cloning site were then deleted from pcDNA1pGEM to prevent T7 RNA polymerase self-amplification. This was accomplished by PCR amplification of pcDNA1 bases 2021-2169 using primers pcDNA1-2022 (described previously) and pcDNA1-2152HIN (5'-CCTGAAAGCTTAATTTGATAAGCCAGTTA-3'), which contained a HindIII restriction enzyme recognition sequence (bold) in the 5' non-complementary tail (underlined). Following PCR amplification, the PCR product was cleaved with SacI and HindIII and the 44 bp insert was subcloned into SacI and HindIII digested pcDNA1pGEM to give pcpGOT7 (Fig. 6.9).

To facilitate T7 RNA polymerase subcloning, the T7 RNA polymerase gene was excised from pcDNA1/T7A with SacI and BamHI, and ligated into SacI, BamHI digested pSE380 superlinker vector to give pSE380/T7 (Fig. 6.10). The T7 RNA polymerase gene was then HindIII, EcoRI excised from pSE380/T7 and ligated into both HindIII and EcoRI digested pcpGOT7 and pcDNA1pGEM to yield pcDNA1/T7B (Fig. 6.11) and
Figure 6.8. Construction of pcDNA1pGEM. The SacI site (2208) in pcDNA1 was eliminated as shown to allow subsequent cloning into a second SacI site (2021) in the newly created pcDNA1pGEM vector.
Figure 6.9. Construction of pcpGOT7. The T7 RNA polymerase promoter was deleted from pcDNA1 as indicated to prevent T7 RNA polymerase self-amplification upon subsequent subcloning of the T7 RNA polymerase gene into pcpGOT7.
Figure 6.10. Construction of pSE380T7. The T7 RNA polymerase gene was subcloned into the pSE380 superlinker vector containing a large multiple cloning site to facilitate subsequent cloning operations.
pcDNA1/T7C (Fig. 6.11) respectively. Both pcDNA1/T7B and pcDNA1/T7C contain the T7 RNA polymerase gene under control of the CMV promoter, but pcDNA1/T7C also permits T7 RNA polymerase self amplification, due to retention of the T7 RNA polymerase promoter from pcDNA1pGEM. pcDNA1/T7B differs from pcDNA1/T7A in that the cloning strategy used to create pcDNA1/T7A removed 3 bp of the 3'-CMV promoter. The lack of hGH expression in initial transfection studies with pcDNA1/T7A suggested these bases may be essential for CMV function.

**Construction of pcDNA1/T7D and pcDNA1/T7E Modified T7 RNA Polymerase Expression Vectors**

The incorporation of a nuclear localization signal into T7 RNA polymerase was accomplished as described by Dunn et al. (1988). The T7 RNA polymerase gene was excised from pSE380/T7 with HindIII and BamHI and subcloned into the corresponding restriction endonuclease sites in the multiple cloning region of pBSKS+ to give pBSKS+/T7 (Fig. 6.12) which then could be used to generate single-stranded uracil-containing DNA for site-directed mutagenesis. The T7 RNA polymerase native sequence GAC-TTC (codons 10-11) (Asp-Phe) allows the conservative C→A transversion which would result in a conservative Asp to Glu change in the amino acid sequence and create a unique EcoRI site. The mutation was incorporated by hybridization of a mutagenic oligonucleotide CTAAGAACGAATTC TCTGACATCG to single-stranded uracil-containing pBSKS+/T7 DNA and subsequent extension and ligation to yield
Figure 6.11. Construction of pcDNA1/T7B and pcDNA1/T7C T7 RNA Polymerase expression vectors. T7 RNA polymerase expression is driven from the CMV promoter in both pcDNA1/T7B and pcDNA1/T7C, but pcDNA1/T7C also contains the T7 promoter to allow T7 RNA polymerase self-amplification.
Figure 6.12. Construction of pBSKS+/T7. The T7 RNA polymerase gene along with the lambda phage pL promoter was excised from the pSE380T7 vector with HindIII and BamHI and subcloned into the corresponding restriction endonuclease sited in pBSKS+ to allow production of single-stranded uracil-containing DNA for site directed mutagenesis.
pBSKS+/T7Eco (Fig. 6.13). Overlapping oligonucleotides T7NLS 111894 (5'-AATTCCTCGAGCCTCCAAAAAAGAAGAGAAAGGTCG-3') and T7NLSCOMP (5'-AATTTCGACCTTTCTCTTTTTTGAGGCTCGAGG-3') encoding complementary EcoRI overhangs and the nuclear localization signal (NLS) of the SV40 large T antigen (Pro Lys Lys Lys Arg Lys Val Glu) (Kalderon et al., 1984) plus five extra amino acids specified by linker sequences (Fig. 6.14) were ligated into the unique EcoRI site of pBSKS+/T7Eco to yield pBST7NLS (Fig. 6.14). A unique XhoI site (bold) was incorporated into one end of the linker to serve as a unique marker for determining insert orientation.

The T7 RNA polymerase gene with the nuclear localization signal was excised from pBST7NLS with HindIII and NotI, and subcloned into HindIII, NotI digested pcpGOT7 and pcDNA1/hGH to give plasmids pcDNA1/T7D (Fig. 6.15) and pcDNA1/T7E (Fig. 6.15), respectively. Plasmids pcDNA1/T7D and pcDNA1/T7E are identical to pcDNA1/T7B and pcDNA1/T7C except that they contain a NLS in the T7 RNA polymerase gene between native codons 10 and 11.

Construction of pcDNA1/T7F and pcDNA1/T7G Modified T7 RNA Polymerase Expression Vectors

The pGP1-2 expression vector developed by Tabor and Richardson (1985) contained the inducible lambda phage $P_L$ promoter 5' to the T7 RNA polymerase gene. Examination of the nucleotide sequence in this region revealed 6 false AUG start sites.
Figure 6.13. Construction of pBSKS+/T7Eco. An oligonucleotide primer complementary to pBSKS+/T7 containing a one base pair mutation to generate a unique EcoRI site between codons 10 and 11 in the T7 RNA polymerase gene was annealed to single stranded uracil-containing pBSKS+/T7 DNA, extended with T7 DNA polymerase, and ligated with T4 DNA ligase. The hybrid double stranded DNA (pBSKS+/T7Eco) was transformed into NM522 E. coli which preferentially degrade uracil-containing DNA, leaving the mutagenic strand to be replicated by the host.
Overlapping complementary oligonucleotides encoding the SV40 large T Antigen NLS (Bold)

Glu{Phe Leu Glu Pro Pro Lys Lys Lys Arg Lys Val Glu} Phe
5' - AA TTC CTC GAG CCT CCA AAA AAG AAG AGA AAG GTC G -3'
3' - _____G GAG CTC GGA GGT TTT TTC TTC TCT TTC TCT TCT CAG CTT AA -5'

EcoRI Xhol EcoRI

Figure 6.14. Construction of pBST7NLS. pBSKS+/T7Eco was digested with EcoRI and two overlapping oligonucleotides encoding the SV40 large-T antigen nuclear localization signal (bold) were ligated into the EcoRI restriction endonuclease cleavage site between codons 10 and 11 of the T7 RNA polymerase gene.
Figure 6.15. Construction of pcDNA1/T7D and pcDNA1/T7E, NLS-modified T7 RNA polymerase expression vectors. Both pcDNA1/T7D and pcDNA1/T7E contain the T7 RNA polymerase gene with a nuclear localization signal under control of the CMV promoter. pcDNA1/T7E also contains the T7 promoter 5' of the T7 RNA polymerase gene to permit T7 RNA polymerase self-amplification.
5' to the proper translation initiation site for T7 RNA polymerase (Fig. 6.16), and would likely impair translation (Kozak, 1994). Therefore, the region containing false AUG’s (pBST7NLS bases 2778 to 3279) was removed by PCR amplification of pBST7NLS (bases 2522-2777) with oligos pBST7NLS 2522 (5'-CGGAAGCG TGCTTCACCCAT-3') and pBST7NLS 2777Hin (5'-CCTGAAGCTTTTATCTTAGGTC ATCTG-3'), (synthetic HindIII site in bold). The NruI, HindIII digested PCR product was subcloned into the corresponding restriction endonuclease sites in HindIII, NruI digested pBST7NLS to give pBST7COMP (Fig. 6.17).

The NLS-modified T7 RNA polymerase gene lacking the 6 false AUG’s was HindIII, NotI excised from pBST7COMP and subcloned into the corresponding restriction endonuclease sites in pcDNA1/T7D and pcDNA1/T7E to give pcDNA1/T7F (Fig. 6.18) and pcDNA1/T7G (Fig. 6.18) respectively.

**Construction of pCIT4B/hGH**

To generate a T7 RNA polymerase-dependent hGH expression construct containing an internal ribosome entry site (IRES) within the 5' terminus of the hGH mRNA transcript, the hGH gene was excised from pcDNA1/hGH with BamHI, XhoI and subcloned into the corresponding restriction endonuclease cleavage sites in the multiple cloning site of pCITE-4B to give pCIT4B/hGH (Fig. 6.19).
mRNA transcript initiation site

GGCTAACTAG AGAACCCACT CTTAAGCTGG CTTATCGAAA TTAATACGAC
TCACTATAGG GAGACCGGAA GCTTAAAGTTG CACGGCCCACT GTGGCACACTA
GTACTTCTCG AGCTGCCCGG GGGATCTCTC ACTACCAGAA CAAATGCCCCC
CTGCAAAAAA TAAAATTCTAT TAAAAAACAT ACTAGACATT ACTGCTCGTG
ATAAATTATC TCTTGCGGTT TGGACTAAAA TACCAGTGTC GGTGATCGTG
AGCACCAGCAG CAGGAGCGAC TGGACCATCA TGGTTTTTTA TGGTATTACTG
GTTACGATCG AAGAAGGGCA GCAGGCCAAGCAGAAGGCTT TGGGTGTGTG
GATAGCAACA AACAGTTCATG CCGTAAGTGC GATCCGGAT AATCTgCACAAGA
TGTTCCAATC GCCGGGGTT TTTGTTCAAGG ACTACAACGT CCAACACCAA
CCAAAGCTCA CTCAGAGGAG AATCCAGATG AATCCACGAG TGGTTGGGGT
GGCCTGAATA GGTACGATTT ACTAACGTTT GAGGCACTA AATGAACACG

T7 RNA polymerase
start codon

Figure 6.16. Lambda phage sequence encoding the pL promoter region. Lambda phage bases 35715 to 35259 encoding the pL promoter, and which contains six false AUG start sites (underlined) which were eliminated by PCR deletion.
Figure 6.17. Construction of pBST7comp. The lambda phage pL promoter region containing the false translation initiation sites 5' of the T7 RNA polymerase gene was eliminated as indicated to allow proper initiation of translation at the T7 RNA polymerase start codon.
Figure 6.18. Construction of pcDNA1/T7F and pcDNA1/T7G NLS-modified T7 RNA polymerase expression vectors with the false AUG’s removed. Both pcDNA1/T7F and pcDNA1/T7G have the lambda phage pL promoter sequence removed and contain an NLS-modified T7 RNA polymerase gene under control of the CMV promoter. pcDNA1/T7G also contains the T7 promoter 5' of the T7 RNA polymerase gene to allow T7 RNA polymerase self-amplification.
Figure 6.19. Construction of pCIT4B/hGH, a T7 RNA polymerase-dependent hGH expression construct containing a 5'-flanking internal ribosome entry site (IRES). pCIT4B/hGH should allow T7 RNA polymerase to generate an hGH mRNA transcript containing an internal ribosome entry site 5' to the hGH gene, which should facilitate translation.
Within pCIT4B/hGH, the IRES viral translation initiation start site is 164 base pairs 5' of the hGH translation initiation start site. Therefore, to make the hGH and viral translation initiation start sites coincident, and to improve translatability, a second T7 RNA polymerase-dependent hGH expression construct containing an IRES was created as follows. pcDNA1/hGH nucleotides 2279-2840 were PCR amplified with synthetic oligonucleotides complementary to bp 2279-2300 (2821 Hin) (5'-GTCAAAGCTT CATGTCTACAGGTAAGCGCCCCCTAAA-3') and 2821-2840 (pcDNA1/hGH 2821) (5'-AGCGTGTGCTCATCTGCCT-3'). The 5' oligo (2821 Hin) contained a non-complementary 5' tail (underlined) and encoded HindIII and AccI restriction endonuclease sites (bold). Following PCR amplification, the PCR product was digested with HindIII and SacI and ligated into the corresponding restriction endonuclease sites of pBSKS+ to give pBSpcr3i (Fig. 6.20). pBSpcr3i was digested with AccI, treated with DNA polymerase I large fragment (Klenow) under conditions optimized for 3'-5' exonuclease activity to remove the 2 bp 3' overhang, and subsequently digested with SacI. This fragment was then ligated into MluNI, SacI digested pCITE-4B to give pCITpcr3 (Fig. 6.21). The remainder of the hGH gene was then excised from pcDNA1/hGH with SacI, XmalIII and subcloned into the corresponding SacI, XmalIII sites in pCITpcr3 to give pCIT4/hGH2 (Fig. 6.22). pCIT4/hGH2 is a T7 RNA polymerase-dependent hGH expression vector containing an IRES to facilitate ribosome binding to the uncapped hGH transcript, and coincident viral IRES and hGH translation initiation start sites.
Figure 6.20. Construction of pBSpcr3i. The first and second exons of the hGH gene were amplified by polymerase chain reaction beginning at the hGH start codon and subcloned into pBSKS+ digested with HindIII and SacI restriction endonucleases to allow plasmid amplification for future subcloning into pCITE4B. This would allow the hGH start codon and the IRES translation initiation start codon to be coincident.
Figure 6.21. Construction of pCITpcr3. The hGH and IRES viral translation initiation start sites were made coincident by excising the hGH polymerase chain reaction fragment (exons 1 and 2) from pBSpcr3i with AccI and Sacl, filling the AccI 5'-overhang with DNA polymerase I (Klenow) to create a blunt end, and subcloning the resultant fragment into pCITE4B at the MluNI and XmaIII restriction endonuclease sites.
Figure 6.22. Construction of pCIT4/hGH2, a T7 RNA polymerase-dependent hGH expression vector with coincident EMC viral IRES and hGH translation initiation sites. pCIT4/hGH2 should allow T7 RNA polymerase to produce an uncapped hGH mRNA transcript which should be translated more efficiently due to the viral IRES and coincident EMC viral IRES and hGH translation initiation sites.
All constructions were confirmed correct by dideoxynucleotide sequencing as described in methods.
Results

Transfection of primary bovine and Comma-D mouse mammary cells with an episomal-replication competent eukaryotic expression vector (pCEP4/hGH)

To determine if EBV-based plasmid replication could increase the duration of recombinant transgene expression in bovine mammary cells, primary bovine and Comma-D mouse mammary cells were cultured as described in methods and transfected with unordered charge-shifting mixed polycation transfection complexes [Orn<sub>n</sub>:Glu<sub>n</sub>-HisN:DEAE:plasmid DNA] formed in HBSS. Transfections utilized either pcDNA1/hGH or the episomal-replication competent eukaryotic expression vector pCEP4/hGH, both driven by the CMV early promoter.

Comma-D (Fig. 6.23) and primary bovine mammary cells (Fig. 6.24) transfected with pCEP4/hGH gave substantially less recombinant hGH expression than those transfected with pcDNA1/hGH. Comma-D cells transfected with pCEP4/hGH consistently gave only 2-3 ng hGH/ml through day 12 posttransfection, while those transfected with pcDNA1/hGH produced > 30 ng hGH/ml by day 8 posttransfection (Fig. 6.23). Similarly, recombinant gene expression from primary bovine mammary cells transfected with pCEP4/hGH peaked at 7.5 ng hGH/ml on day 6 posttransfection, while expression from those transfected with pcDNA1/hGH peaked at 44 ng hGH/ml on day 10 posttransfection (Fig. 6.24). The differences in hGH expression levels between
Figure 6.23. Transfection of Comma-D mouse mammary cells with episomal-replication competent (pCEP4/hGH) or incompetent (pcDNA1/hGH) eukaryotic expression vectors. Comma-D mouse mammary cells were transfected with unordered charge-shifting mixed polyion transfection complexes [Orn (37.5 μg/ml), Glu-HisN (75 μg/ml), DEAE (12.5 μg/ml), DNA (5 μg/ml)] formed in HBSS. Transfections utilized either pcDNA1/hGH (▲) or the episomal-replication competent eukaryotic expression vector pCEP4/hGH (□), both driven by the CMV early promoter.
Figure 6.24. Transfection of primary bovine mammary cells with episomal-replication competent (pCEP4/hGH) or incompetent (pcDNA1/hGH) eukaryotic expression vectors. Primary bovine mammary cells were transfected with unordered charge-shifting mixed polyanion transfection complexes [Orn⁺ (37.5 µg/ml), Glu⁻-HisN (75 µg/ml), DEAE (12.5 µg/ml), DNA (5 µg/ml)] formed in HBSS. Transfections utilized either pcDNA1/hGH (▲) or the episomal-replication competent eukaryotic expression vector pCEP4/hGH (□), both driven by the CMV early promoter.
expression vectors pcDNA1/hGH and pCEP4/hGH was unexpected since both vectors rely on CMV-driven hGH expression.

hGH expression from primary bovine mammary cells transfected with pCEP4/hGH and pcDNA1/hGH increased to days 6 and 10 posttransfection, respectively, then decreased over time but was still detectable at 50 days posttransfection. pCEP4/hGH gave slightly higher hGH expression (−0.5 ng/ml) at day 50 posttransfection than did pcDNA1/hGH (−0.25 ng/ml) (Fig. 6.24 inset). Because primary bovine mammary cells transfected with pCEP4/hGH did not produce substantial levels of hGH nor show prolonged expression at sufficient levels, no attempt was made to document episomal replication.

**Effect of a hGH secretion signal or hGH-GFP chimera on GFP secretion in transfected COS-1 cells**

Secretion of a fluorescent reporter protein should allow rapid, non-destructive analysis of recombinant gene expression both in vitro and in vivo. The cloning and sequencing of the *Aequorea victoria* green fluorescent protein (GFP) gene (Prasher et al., 1992) has allowed visualization of recombinant gene expression in living cells independent of species and without the need for expression or presence of cofactors (Kain and Ganguly, 1995, and references therein). Although GFP is a cytosolic protein, Wang and Hazelrigg (1994) demonstrated that N- and C-terminal GFP fusion proteins retain the fluorescent properties of native GFP, suggesting that mammalian secretion signal motifs
could be fused to GFP to create a secreted fluorescent reporter protein, as demonstrated by Kaether and Geredes (1995) who created a human chromagranin B-GFP fusion protein. Fusion of the hGH secretion signal, known to direct secretion of high levels of recombinant hGH in many cells and particularly in primary bovine mammary cells, to the N-terminus of GFP should allow secretion of GFP from transfected cells.

Two simultaneous approaches were undertaken to generate a secreted form of GFP. The first was the simple addition of the hGH secretion signal to the N-terminus of GFP (Fig. 6.2). The second approach was to fuse the entire hGH gene (minus the stop codon) to the N-terminus of GFP as described in Figure 6.4 to give the phGHGFP fusion protein. Synthesis of a full length hGH-GFP chimera would allow comparison of single and multiple intron effects on recombinant protein production and would be useful for growth hormone synthesis and secretion studies. Transfection with either pEGFPss or phGHGFP should therefore direct GFP secretion into the extracellular medium.

Because native GFP fluorescence could not be visualized in transfected primary bovine or Comma-D mouse mammary cells, COS-1 cells were cultured as described in methods and transfected with plasmids to express either native GFP, secretion signal-GFP fusion protein, or a full length hGH-GFP chimera in a DEAE:plasmid DNA transfection complex. Subsequent GFP expression and secretion was monitored by fluorescence microscopy and ELISA of spent culture medium.
COS-1 cells transfected with pEGFP-N1 expressing native non-secreted GFP (Fig. 6.25A-C) displayed more intense cytoplasmic GFP fluorescence through day 6 posttransfection than those transfected with either pEGFPss expressing the hGH secretion signal-GFP fusion peptide (Fig. 6.25D-F) or phGHGFP expressing the complete hGH/GFP fusion protein (Fig. 6.25G-I). The intracellular and cytoplasmic GFP fluorescence observed in COS-1 cells transfected with either pEGFPss (Fig. 6.25D-F) or phGHGFP (Fig. 6.25G-I) was quite unexpected since both pEGFPss and phGHGFP should direct GFP secretion into the extracellular culture medium. Interestingly, COS-1 cells transfected with pEGFPss (Fig. 6.25D-F) showed more intense cytoplasmic GFP fluorescence than COS-1 cells transfected with phGHGFP (Fig. 6.25G-I). Regardless of the expression vector transfected, cytoplasmic GFP fluorescence intensity increased with time posttransfection (Fig. 6.25A-I).

To determine whether 1) pEGFPss and phGHGFP could direct GFP secretion, and 2) the decreased cytoplasmic GFP fluorescence intensity from COS-1 cells transfected with pEGFPss (Fig. 6.25D-F) and phGHGFP (Fig. 6.25G-I) was correlated with GFP secretion, spent culture medium from the cells seen in Figure 6.25 A-I was assayed by ELISA for GFP. Surprisingly more GFP was detected in spent cell culture medium from COS-1 cells transfected with native pEGFP-N1 expressing non-secretable GFP (100 ng GFP/ml) than from COS-1 cells transfected with either pEGFPss (20 ng GFP/ml) (secretion signal-GFP), or phGHGFP (10 ng GFP/ml) (hGH-GFP chimera) (Fig 6.26). Contrary to expectation, GFP expression in spent cell culture medium reflected the
Figure 6.25. GFP and modified-GFP expression in COS-1 cells transfected with pEGFP-N1, pEGFPss, or phGHGFP. COS-1 cells were cultured as described in methods and transfected with either native GFP [DEAE (250 µg/ml), pEGFP-N1 (5 µg/ml)] (Panels A-C), a secretion signal-GFP fusion protein [DEAE (250 µg/ml), pEGFPss (5 µg/ml)] (Panels D-F), or a full length hGH-GFP chimera [DEAE (250 µg/ml), phGHGFP (5 µg/ml)] (Panels G-I). GFP expression was monitored by fluorescence microscopy on Days 2 (A-C), 4 (D-F), and 6 (G-I) posttransfection.
Figure 6.26. GFP and modified GFP secretion from transfected COS-1 cells. COS-1 cells were cultured as described in methods and transfected with either: native GFP (●) [DEAE (250 µg/ml), pEGFP-N1 (5 µg/ml)], a secretion signal-GFP fusion protein (■) [DEAE (250 µg/ml), pEGFPss (5 µg/ml)], or a full length hGH-GFP chimera (▲) [DEAE (250 µg/ml), phGHGFP (5 µg/ml)]. Recombinant GFP expression was detected by ELISA using anti-GFP antisera.
amount and intensity of cytoplasmic GFP fluorescence seen in COS-1 cells expressing GFP (Figs. 6.25A-I). The number and relative fluorescence intensity of GFP-expressing COS-1 cells appeared to decrease with increasing GFP protein modification (pEGFP-N1 (Fig. 6.25A-C) > pEGFPss (Fig. 6.25D-F) > phGHGFP (Fig. 6.25G-I)), as did GFP expression in the medium (Fig. 6.26) indicating that pEGFPss and phGHGFP were not directing efficient GFP secretion as predicted. The hGH-GFP chimera encoded by the phGHGFP expression vector could be detected in spent COS-1 cell culture medium with either anti-hGH, anti-GFP, or a combination of both polyclonal antibodies in a double sandwich ELISA, indicating that the hGH-GFP chimera contained antigenic epitopes from both hGH and GFP, as expected from the construction.

Northern Hybridization analysis of specific mRNA transcribed in COS-1 cells transfected with pEGFP-N1, pEGFPss, or phGHGFP

The cytoplasmic GFP fluorescence present in COS-1 cells transfected with either pEGFPss (Fig. 6.25D-F) or phGHGFP (Fig. 6.25G-I) confirmed GFP synthesis, yet ELISA analysis (Fig. 6.26) indicated that GFP was secreted only at low levels compared to COS-1 cells transfected with native pEGFP-N1. Therefore, to confirm that modified GFP-mRNA transcripts produced in COS-1 cells transfected with pEGFPss and phGHGFP were of proper size to encode the hGH secretion signal and the complete hGH gene, respectively, and also to investigate whether mRNA transcript production correlated with cytoplasmic fluorescence intensity, total mRNA was purified from the COS-1 cells
transfected with either pEGFP-N1, pEGFPss or phGHGFP (expression seen in Fig. 6.26). Total mRNA was then separated by denaturing agarose gel electrophoresis and hybridized with a radiolabeled 741 bp BamHI, NotI fragment (bases 660-1400) from pEGFP-N1 containing the complete GFP cDNA.

GFP and modified-GFP mRNA transcripts were readily detectable in RNA purified from COS-1 cells transfected with pEGFP-N1, pEGFPss, or phGHGFP, but not from untransfected COS-1 cells (Fig. 6.27). Observed and predicted mRNA sizes for the native GFP (1570 nt vs. 978 nt), secretion signal GFP (1750 nt vs. 1132 nt), and hGH-GFP chimera (2020 nt vs. 1681 nt) showed similar differences of 592, 618, and 339 nt, respectively, suggestive of polyadenylation and were of appropriate size for each mRNA (Fig. 6.27). Observed mRNA transcript sizes increased 180 nt and 450 nt over native GFP following addition of the secretion signal and complete hGH gene, compared with the predicted increases of 154 and 703 nts, respectively. Additionally, estimation of relative mRNA quantity from hybridization band intensity and size (Fig. 6.27), suggested that mRNA transcript levels correlated with the amount of cytoplasmic GFP fluorescence seen in Figure 6.25. Although the modified-GFP mRNA transcripts for pEGFPss and phGHGFP appeared to be of appropriate size, neither the hGH secretion signal nor the entire hGH gene fused to GFP seemed to be capable of directing efficient or significant GFP secretion.
Figure 6.27. Northern hybridization analysis of GFP and modified GFP mRNA in transfected COS-1 cells. COS-1 cells were cultured as described in methods and left untransfected (Lane 1); or transfected with either: a full length hGH-GFP chimera (Lane 2) [DEAE (250 µg/ml), phGHGFP (5 µg/ml)]; an hGH secretion signal-GFP fusion protein (Lane 3) [DEAE (250 µg/ml), pEGFPss (5 µg/ml)]; or native GFP (Lane 4) [DEAE (250 µg/ml), pEGFP-N1 (5 µg/ml)]. GFP and modified-GFP mRNA transcripts were detected by hybridization with a radiolabeled 741 bp BamHI, NotI fragment containing the complete GFP gene (bases 660-1400) from pEGFP-N1.
Development of a T7 RNA polymerase-dependent hGH expression system

To test whether a T7 RNA polymerase-dependent gene expression system could give high level recombinant gene expression in primary bovine mammary cells, the complete T7 RNA polymerase gene was subcloned from pGP1-2 into pcDNA1/hGH to give the pcDNA1/T7A expression vector (Fig. 6.6). pcDNA1/T7A should produce high levels of T7 RNA polymerase since expression is driven from the strong CMV promoter, previously shown to drive strong expression of hGH (Chapter 4) and GFP (Chapters 5 and 6) in transfections. Unfortunately, the restriction endonuclease sites available did not allow convenient insertion of T7 RNA polymerase into the pcDNA1 multiple cloning site, and subcloning into the SacI site (2121) (Fig. 6.7) eliminated the last three base pairs of the 3'-CMV promoter region, and was 13 base pairs 5' of the putative CMV transcriptional start site (Fig. 6.28), which could possibly inhibit efficient transcription.

To test whether this (pcDNA1/T7A) T7 RNA polymerase vector could drive hGH expression when cotransfected a T7-dependent hGH expression vector lacking an IRES (pBS/hGH) (Fig. 6.28), Comma-D mouse and primary bovine mammary cells were cotransfected with pcDNA1/T7A and pBS/hGH. The T7 RNA polymerase produced from the CMV promoter of pcDNA1/T7A should bind the T7 promoter in pBS/hGH and produce an uncapped hGH mRNA transcript which may not be efficiently translated, but may result in low level hGH production. However, if the last three base pairs of the CMV promoter are essential for strong CVM promoter activity, little or no T7 RNA polymerase would be produced. High level hGH expression would not be expected
Figure 6.28. Schematic Diagram of T7 RNA polymerase expression vectors and T7 RNA polymerase-dependent hGH expression constructs.
since the T7 RNA polymerase in pcDNA1/T7A lacks an NLS and the hGH reporter gene in pBS/hGH is not flanked (5') by an IRES (Fig. 6.28).

Not surprisingly, cotransfection of primary bovine or Comma-D mouse mammary cells with pcDNA1/T7A (2.5 μg/ml) and pBS/hGH (2.5 μg/ml) with unordered charge-shifting mixed polyion complexes [Orn₅::Glu₅::HisN::DEAE::total DNA] gave no detectable recombinant hGH expression (results not shown). The lack of expression was presumably due to combined detrimental effects. First, the removal of the 3 base pairs from the 3' end of the CMV promoter region consequent to cloning of T7 RNA polymerase into the SacI site (2121) may inactivate the CMV promoter (Fig. 6.7). Secondly, there is a probable requirement for nuclear localization of T7 RNA polymerase to enable T7 RNA polymerase-dependent transcription. Finally, the reporter gene construct lacked an IRES to facilitate ribosome binding and translation of the uncapped hGH mRNA produced by T7 RNA polymerase (Fig. 6.28). Therefore, a series of recombinant expression vectors were constructed with modifications to correct these predicted deficiencies.

**T7 RNA polymerase expression vectors**

Restoration of the 3 base pairs of CMV 3' promoter region previously deleted in pcDNA1/T7A and simultaneous elimination of the T7 RNA polymerase promoter in pcDNA1 to prevent T7 self-amplification was a multistep process (Figs. 6.7-6.10). The resultant expression vectors, pcDNA1/T7B and pcDNA1/T7C, have the 3' end of the CMV promoter restored and contain the T7 RNA polymerase gene downstream of the
Lambda phage pL promoter which was acquired upon excision of the T7 RNA polymerase cDNA from pGP1-2 (Fig. 6.28). pcDNA1/T7B and pcDNA1/T7C differ in that pcDNA1/T7C contains the T7 promoter which would permit T7 RNA polymerase self-amplification (Fig. 6.28).

A nuclear localization signal from the SV40 large-T antigen encoding the amino acid sequence (Pro Lys Lys Lys Arg Lys Val Glu) (Colledge et al., 1986; Kalderon et al., 1984) was incorporated into the N-terminus of the T7 RNA polymerase cDNA essentially as described by Dunn et al. (1988) and depicted in Figures 6.11-6.15. pcDNA1/T7D and pcDNA1/T7E (Fig. 6.28) are essentially identical to pcDNA1/T7B and pcDNA1/T7C (Fig. 6.28), respectively, except the former contain the newly incorporated NLS and should direct expressed T7 RNA polymerase to the nucleus of the cell.

During the course of the construction of the T7 RNA polymerase expression vector it was noted that the lambda phage pL promoter, acquired with T7 RNA polymerase excision from pGP1-2 and which would have constituted the 5' end of the T7 RNA polymerase transcript from the CMV promoter, contained 6 false AUG translation initiation sites 5' to the proper T7 RNA polymerase translation initiation start site (Fig. 6.16). Thus, this region was removed by PCR deletion as described in Figures 6.17 and 6.18 to give pcDNA1/T7F and pcDNA1/T7G, which are identical to pcDNA1/T7D and pcDNA1/T7E except that the former have the lambda phage pL promoter region containing the 6 false AUG translation start sites removed (Fig. 6.28) to allow proper initiation of translation at the T7 RNA polymerase start site. Putative CMV transcription
initiation starts 12 bp into the lamda phage pL promoter sequence in pcDNA1/T7A, 34 base pairs 5' of the subcloned lamda phage pL promoter/T7 RNA polymerase cDNA sequence in pcDNA1/T7 B and D, 33 base pairs 5' of the T7 promoter in pcDNA1/T7 C, E, and G, and 38 bp 5' of the NLS-modified T7 RNA polymerase cDNA sequence in pcDNA1/T7F (Fig. 6.28).

Incorporation of an IRES into reporter constructs

Two T7 RNA polymerase-dependent reporter constructs pCITE4B/hGH and pCIT4/hGH2 containing identical IRES elements to allow cap-independent mRNA translation were developed as described in Figure 6.19 and Figures 6.20-6.22, respectively. pCIT4/hGH2 differs from pCITE4B/hGH in that the EMC viral and hGH translation initiation sites are coincident in pCIT4/hGH2 to allow more efficient translation. To remove the S-Tag sequence used for protein purification (not shown) and to make the hGH translation initiation codon coincident with the EMC viral IRES translation initiation codon, for presumably greater translation efficiency, the intervening region between the viral translation initiation codon and the conventional eukaryotic cell translation start site of hGH in pCITE4B/hGH was eliminated by PCR deletion as described in Figures 6.20-6.22. Thus, both reporter vectors contain the hGH reporter gene 3' of an IRES to allow cap-independent translation of mRNA produced under control of the T7 RNA polymerase promoter (Fig. 6.28B).
Lack of T7 RNA polymerase-dependent hGH expression in cotransfected primary bovine mammary cells

Although expression and reporter constructs were tested as they were created, results reported are from representative comparative experiments utilizing COS-1 and primary bovine mammary cells.

Ordered charge-shifting mixed polyion cotransfection [total plasmid DNA:DEAE:Glu$_n$-His$_n$:Orn$_n$] of primary bovine mammary cells with any of the T7 RNA polymerase expression vectors (pcDNA1/T7 B through G) (Fig. 6.28A) and pBS/hGH which lacked an IRES (Fig. 6.28B) failed to give detectable recombinant hGH expression (Fig. 6.29A). There also was no expression when either pCIT4B/hGH which contains an IRES (Fig. 6.28B) or pCIT4/hGH2 which contains an IRES and coincident viral and hGH translation initiation start sites (Fig. 6.28B) was substituted for pBS/hGH (Fig. 6.29 B and C) in T7 RNA polymerase cotransfections. In contrast, primary bovine mammary cells transfected with pcDNA1/hGH (alone) [pcDNA1/hGH:DEAE:Glu$_n$-His$_n$:Orn$_n$] produced peak hGH expression levels ranging from 60-150 ng/ml (Fig. 6.29A-C).

In contrast, DEAE-cotransfection [DEAE:total DNA] of COS-1 cells with any of the pcDNA1/T7 B through G expression vectors and pBS/hGH, which lacks an IRES, gave low level (≤ 20 ng hGH/ml) recombinant hGH expression (Fig. 6.30B). Furthermore, there were no substantial differences in the recombinant gene expression levels or profiles obtained from the various pcDNA1/T7 expression vectors, regardless of the incorporation of a nuclear localization signal (pcDNA1/T7 D-G), removal of false
Figure 6.29. Cotransfection of primary bovine mammary cells with T7 RNA polymerase and T7 RNA polymerase-dependent hGH expression vectors. Primary bovine mammary cells in culture were either left untransfected (○), transfected with pcDNA1/hGH (■), or cotransfected with pcDNA1/T7B (Ø), pcDNA1/T7C (●), pcDNA1/T7D (Δ), pcDNA1/T7E (▲), pcDNA1/T7F (□), pcDNA1/T7G (♦) and pBSKS+/hGH (Panel A); pCIT4B/hGH (Panel B); or pCIT4/hGH2 (Panel C). All transfections were done with ordered charge-shifting mixed polion transfection complexes composed of: [total plasmid DNA (5 µg/ml), DEAE (12.5 µg/ml), Glu^-HisN (75 µg/ml), Orn^- (37.5 µg/ml)] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4. Cotransfections utilized 2.5 µg/ml of each respective plasmid.
Figure 6.30. Cotransfection of COS-1 cells with T7 RNA polymerase-dependent pBSKS+/hGH and T7 RNA polymerase expression vectors. COS-1 cells in culture were either left untransfected (○), transfected with pcDNA1/hGH (■), or cotransfected with pBSKS+/hGH and pcDNA1/T7B (○), pcDNA1/T7C (●), pcDNA1/T7D (▲), pcDNA1/T7E (△), pcDNA1/T7F (□), or pcDNA1/T7G (♦) (Panels A,B). All transfections were done with ordered charge-shifting mixed polyion transfection complexes composed of: [total plasmid DNA (5 μg/ml), DEAE (12.5 μg/ml), Glu+HisN (75 μg/ml), Orn+ (37.5 μg/ml)] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4. Cotransfections utilized 2.5 μg/ml of each respective plasmid. Panel B, an expanded view of expression in Panel A.
AUG's (pcDNA1/T7 F and G) or ability to self-amplify (T7 promoter) (pcDNA1/T7 C, E, and G) (Fig. 6.28A). In comparison, COS-1 cells transfected with pcDNA1/hGH alone [DEAE:pcDNA1/hGH], produced 800 ng hGH/ml on day 6 posttransfection (Fig. 6.30A). Similar low hGH expression was obtained when pCIT4/hGH2, containing an IRES at the 5' end of the hGH transcript and with coincident viral and hGH translation initiation start sites, was substituted for pBS/hGH (Fig. 6.31A and B). However, when pCIT4B/hGH containing an IRES with non-coincident viral and hGH translation initiation start sites was substituted for pBS/hGH, recombinant hGH expression levels were considerably lower (1-4 ng hGH/ml) (Fig. 6.32A and B). Thus, for unknown reason(s), cotransfections with pcDNA1/T7 expression vectors and T7-dependent reporter constructs failed to give high level recombinant hGH expression in either primary bovine or COS-1 cells as expected.

Northern hybridization analysis of specific mRNA transcribed in COS-1 cells cotransfected with T7 RNA polymerase expression vectors and T7-dependent hGH reporter constructs

To determine if the low level T7 RNA polymerase-dependent hGH expression seen in cotransfected COS-1 cells might be due to limited production or rapid degradation of mRNA transcripts for either T7 RNA polymerase or hGH, COS-1 cells were cotransfected with either pcDNA1/T7E or pcDNA1/T7G and pBS/hGH, pCIT4B/hGH, and pCIT4/hGH2 and examined for T7 RNA polymerase or hGH mRNA transcript
Figure 6.31. Cotransfection of COS-1 cells with T7 RNA polymerase-dependent pCIT4B/hGH and T7 RNA polymerase expression vectors. COS-1 cells in culture were either left untransfected (○), transfected with pcDNA1/hGH (■), or cotransfected with pCIT4/hGH2 and pcDNA1/T7B (▲), pcDNA1/T7C (●), pcDNA1/T7D (Δ), pcDNA1/T7E (▲), pcDNA1/T7F (□), or pcDNA1/T7G (♦) (Panels A,B). All transfections were done with ordered charge-shifting mixed polyion transfection complexes composed of: [total plasmid DNA (5 μg/ml), DEAE (12.5 μg/ml), Glu₆-HisN (75 μg/ml), Omt₆ (37.5 μg/ml)] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4. Cotransfections utilized 2.5 μg/ml of each respective plasmid. Panel B, an expanded view of expression in Panel A.
Figure 6.32. Cotransfection of COS-1 cells with T7 RNA polymerase-dependent pCIT4/hGH2 and T7 RNA polymerase expression vectors. COS-1 cells in culture were either left untransfected (○), transfected with pcDNA1/hGH (■), or cotransfected with pCIT4B/hGH and pcDNA1/T7B (⊙), pcDNA1/T7C (●), pcDNA1/T7D (▲), pcDNA1/T7E (●), pcDNA1/T7F (□), or pcDNA1/T7G (♦) (Panels A, B). All transfections were done with ordered charge-shifting mixed polycation transfection complexes composed of: [total plasmid DNA (5 µg/ml), DEAE (12.5 µg/ml), Glu \text{\textsubscript{H}}-HisN (75 µg/ml), Orn \text{\textsubscript{H}} (37.5 µg/ml)] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4. Cotransfections utilized 2.5 µg/ml of each respective plasmid. Panel B, an expanded view of expression in Panel A.
production. T7 RNA polymerase expression vectors pcDNA1/T7E and pcDNA1/T7G (Fig. 6.28A), lacking the lambda phage pL promoter region that contained the false AUG’s and also containing an NLS incorporated into the T7 RNA polymerase transcript were utilized since they were expected to be most capable of driving both T7 RNA polymerase and secondary hGH expression. The three reporter constructs were chosen in order to compare between T7 RNA polymerase-derived hGH mRNA transcripts from vectors with (pCIT4B/hGH, pCIT4/hGH2) or without (pBS/hGH) an IRES, or with an IRES and coincident viral and hGH translation initiation start sites (pCIT4/hGH2) (Fig. 6.28B). COS-1 cells transfected with pcDNA1/hGH served as a positive control.

Spent COS-1 cell culture medium from cells prepared for Northern hybridization was collected and assayed for secreted hGH (Fig. 6.33). As previously described (Figs. 6.30-6.32), COS-1 cells cotransfected with T7 expression vectors pcDNA1/T7E or pcDNA1/T7G and T7-dependent hGH expression vectors pBS/hGH, pCIT4B/hGH, or pCIT4/hGH2 gave low level recombinant gene expression (≤ 15 ng hGH/ml) compared to pcDNA1/hGH-transfected COS-1 cells (~ 300 ng hGH/ml) (Fig. 6.34A and B).

Total mRNA was purified from transfected cells on day 6 posttransfection and separated by denaturing electrophoresis as described in methods. Specific mRNA transcripts were then hybridized with radiolabeled a) pcDNA1/hGH plasmid, b) purified complete T7 RNA polymerase gene fragment (2644 bp) excised from pcDNA1/T7G with EcoRI and BamHI, or c) an 80 bp highly conserved region of 18S rRNA (pT7 RNA 18S).
Figure 6.33. hGH expression from COS-1 cells used for Northern hybridization analysis. Cultured COS-1 cells were either left untransfected (♦), transfected with pcDNA1/hGH (■), or cotransfected with either pcDNA1/T7E and pBSKS+/hGH (□), pCIT4B/hGH (●), and pCIT4/hGH2 (○) or pcDNA1/T7G and pBSKS+/hGH (▲), pCIT4B/hGH (Δ), and pCIT4/hGH2 (φ). All transfections consisted of ordered charge-shifting mixed polycation transfection complexes composed of: [total plasmid DNA (5 μg/ml), DEAE (12.5 μg/ml), Glu↑↓HisN (75 μg/ml), Orn↑↓ (37.5 μg/ml)] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4. Cotransfections utilized 2.5 μg/ml of each respective plasmid.
Figure 6.34. Northern hybridization analysis of hGH and T7 RNA polymerase-dependent hGH mRNA from transfected COS-1 cells. Total RNA was prepared from COS-1 cells left untransfected, transfected with pcDNA1/hGH, or cotransfected with either pcDNA1/T7E and pBSKS+/hGH, pCIT4B/hGH, and pCIT4/hGH2 or pcDNA1/T7G and pBSKS+/hGH, pCIT4B/hGH, and pCIT4/hGH2. 80 μg RNA/lane was then separated by denaturing agarose gel electrophoresis as described in methods and hGH or 18s rRNA specific mRNA transcripts were then hybridized with radiolabeled pcDNA1/hGH plasmid and an 80 bp highly conserved region of 18S rRNA (pT7 RNA 18S, Ambion).
No T7 RNA polymerase mRNA transcripts or T7 RNA polymerase-dependent hGH mRNA transcripts were detectable with total cellular RNA loaded at 20 μg RNA/lane (results not shown). Furthermore, no T7 RNA polymerase-dependent hGH mRNA transcripts were detectable when gels were loaded with 80 μg RNA/lane (Fig. 6.35). In contrast, total cellular RNA from control cells transfected with pcDNA1/hGH showed hGH mRNA (750 nts) at both 20 (not shown) and 80 μg total RNA/lane (Fig. 6.34) which was slightly smaller than the expected 880 nt hGH transcript size.

Several bands of varied size hybridized with the T7 RNA polymerase probe in each lane when the gel was loaded with 80 μg total cellular RNA/lane (Fig. 6.35). Total cellular RNA from COS-1 cells transfected with either pcDNA1/T7B, pcDNA1/T7C, pcDNA1/T7D, or pcDNA1/T7E hybridized with the T7 RNA polymerase probe all showed multiple hybridization bands of ~4800 nt (filled arrow), 4350 nt (open arrow), or 2830 nt (arrowhead), compared with the predicted T7 RNA polymerase mRNA transcript sizes of 4166 nt (pcDNA1/T7B), 4191 nt (pcDNA1/T7C), 4134 nt (pcDNA1/T7D), and 4159 nt (pcDNA1/T7E). Similarly, total cellular RNA from COS-1 transfected with pcDNA1/T7F and pcDNA1/T7G, hybridized with the T7 RNA polymerase probe showed multiple hybridization bands of ~4800, 4250, and 2380 nt, and 4850, 4250, 2380 nt, respectively, compared with the predicted T7 RNA polymerase mRNA transcript sizes of 3488 nt (pcDNA1/T7F), and 3513 nt (pcDNA1/T7G) (Fig. 6.35). Although several hybridized bands were detected in each lane, the band at ~4250-4350 appears closest to predicted T7 RNA polymerase transcript sizes (Fig. 6.35).
Figure 6.35. Northern hybridization analysis of T7 RNA polymerase mRNA from transfected COS-1 cells. Total RNA was prepared from COS-1 cells left untransfected (lane 1), transfected with pcDNA1/T7B (lane 2), pcDNA1/T7C (lane 3), pcDNA1/T7D (lane 4), pcDNA1/T7E (lane 5), pcDNA1/T7F (lane 6), pcDNA1/T7G (lane 7), or cotransfected with either: pcDNA1/T7E and pBSKS+/hGH (lane 8); pcDNA1/T7E and pCIT4B/hGH (lane 9); pcDNA1/T7E and pCIT4/hGH2 (lane 10); or pcDNA1/T7G and pBSKS+/hGH (lane 11); pcDNA1/T7G and pCIT4B/hGH (lane 12); pcDNA1/T7G and pCIT4/hGH2 (lane 13). Total RNA loaded at 80 μg RNA/lane was then separated by denaturing agarose gel electrophoresis as described in methods and T7 RNA polymerase-specific mRNA transcripts were then hybridized with a purified full length radiolabeled T7 RNA polymerase (2644 bp EcoRI, BamHI fragment from pcDNA1/T7G), and an 80 bp highly conserved region of 18S rRNA (pT7 RNA 18S). Hybridization bands of ~4800 nt (filled arrow), 4300 nt (open arrow), and 2830 nt (arrowhead).
Cotransfection of COS-1 cells with pcDNA1/T7E and either pBS/hGH, pCIT4B/hGH, or pCIT4/hGH2, yielded hybridization bands like that for pcDNA1/T7E transfection alone (4350 and 2830 nt), but also gave mRNA transcripts too large to accurately measure (pcDNA1/T7E, pCIT4B/hGH) and (pcDNA1/T7E, pCIT4/hGH2) (Fig. 6.35). Similarly, transfection with pcDNA1/T7G and either pBS/hGH, pCIT4B/hGH, or pCIT4/hGH2, yielded mRNA transcripts like pcDNA1/T7G transfection alone (4250 and 2830 nt), but also gave mRNA transcripts too large to accurately measure (pcDNA1/T7G, pBS/hGH) and (pcDNA1/T7E, pCIT4/hGH2) (Fig. 6.35).

It is not known why the T7 RNA polymerase probe hybridized with so many mRNA transcripts, particularly those > 4800 bp and those < 3500 bp. It is clear however that no mRNA transcripts were detectable with the T7 RNA polymerase probe in untransfected COS-1 cells (Fig. 6.35) indicating that hybridization was specific for COS-1 cells transfected with T7 RNA polymerase expression constructs. Interestingly, although present only at low levels, the larger transcripts (> 4000 bp) (filled and open arrows) appeared stable, in contrast to the smaller transcript(s) (> 3000 bp) which appeared to be undergoing degradation (arrowhead) (Fig. 6.35). Unexpectedly, removal of the lambda phage pL promoter (~450 bp) from pcDNA1/T7D and pcDNA1/T7E yielded mRNA transcripts of only about 100 nt shorter (from 4350 to 4250 nt) in COS-1 cells transfected with pcDNA1/T7F and pcDNA1/T7G (Fig. 6.35).
The apparent low or absent expression of T7 RNA polymerase mRNA (Fig. 6.35) and/or instability of the transcripts likely accounts for the low level of T7-dependent hGH expression observed in cotransfected COS-1 cells (Figs. 6.30-6.32).

The low level hGH expression seen in Figures 6.30-6.32 may be attributable to effects of undefined negative elements within the mRNA of T7 RNA polymerase or within the 5' or 3' flanking cloning sequences. Over the course of this investigation it was noted that different recombinant reporter genes gave dramatically different expression levels when transfected under identical conditions with expression driven from the same CMV promoter, regardless of cell type, suggesting that intragenic sequences may affect mRNA production and/or stability. Thus, despite mRNA transcript production from the same CMV promoter, expression of reporter genes (to day 6 posttransfection) varied up to 50 fold relative to hGH expression (100%) in COS-1 cells, transfected with either bovine α-lactalbumin (2%), or bovine lactoferrin (25%). Similar results were noted in Comma-D mouse mammary cells transfected with hGH (100%), human transferrin (8%), and whey acidic protein (WAP) (19%), and in primary bovine mammary cells transfected with hGH (100%) or human transferrin (16%). Expression levels for secreted reporter genes or cDNAs other than hGH but all driven from the CMV promoter ranged from 0 to 25% of the expression levels for recombinant hGH. However, the differences in expression cannot be attributed to the use of cDNAs versus complete gene expression since WAP gene expression only amounted to 19% of that for hGH in Comma-D cells. Schanbacher et al. (1997) showed that the half-life of bovine lactoferrin (bLf) mRNA, the
level of bLf mRNA, and bLf peptide expression are all affected by 3'-untranslated regions (UTR) of bLF. Therefore, further examination of reporter gene sequences may reveal opportunities for increasing recombinant gene expression levels.
Discussion

To complement the high level recombinant hGH expression described in Chapter 4, an EBV-based expression plasmid, pCEP4/hGH, known to be capable of autonomous episomal replication in primate and canine but not rodent cells, was examined for its ability to prolong the duration of high level expression in primary bovine mammary cells transfected in vitro. Comma-D mouse (Figs 6.23) and primary bovine mammary cells (Fig. 6.24) transfected with pCEP4/hGH produced only one-fifth the recombinant hGH expression of mammary cells transfected with pcDNA1/hGH. The decrease in hGH expression with pCEP4/hGH was surprising since recombinant hGH expression is driven from the same CMV promoter in both pCEP4/hGH and pcDNA1/hGH plasmids. However, the pCEP4/hGH plasmid with episomal replication potential due to the encoded EBV EBNA1 gene and the EBV oriP origin of replication is an additional 6000 base pairs larger that pcDNA1/hGH.

The inability of pCEP4/hGH to prolong high level hGH expression in Comma-D cells was not surprising since rodent cells do not permit EBV-based plasmid replication (Wysokenski and Yates, 1989; Yates et al., 1985). However, EBV-based plasmid replication had not previously been examined in bovine cells. Interestingly, in spite of the low peak expression, primary bovine mammary cells transfected with pCEP4/hGH gave hGH expression that appeared to stabilize at ≤ 1 ng/ml at 45-50 days posttransfection (Fig. 6.24), suggesting plasmid persistence consistent with episomal
replication. However, it was clear from this experiment that even if EBV-based expression vectors had replicated in primary bovine mammary cells they did not extend the duration of high level recombinant hGH expression. Nonetheless, autonomous episomal replication may be responsible for the low level persistent expression (45-50 days posttransfection) seen in primary bovine mammary cells transfected with pCEP4/hGH (Fig. 6.24). While EBV-based episomal replication is reported to maintain 10-100 plasmid copies/cell (Sugden et al., 1979), ligand- (Chowdhury et al., 1993) and cationic liposome-mediated transfection (Zabner et al., 1995) delivered large numbers of plasmid molecules per cell ($1.2 \times 10^4 - 3.3 \times 10^5$). From the near total uptake of plasmid DNA by transfected bovine mammary cells (Fig. 4.3), transfection at 1 μg DNA/0.2 ml would deliver nearly $1.5 \times 10^5$ plasmid molecules per cell. Thus, if polycation-mediated transfection also delivers such large amounts of plasmid/cell then the high level expression seen to day 20 posttransfection (Fig. 6.24) may be a result of gene expression from the large plasmid pool, whereas persistent but low expression ($> 20$ days posttransfection) may arise from depletion of the large pool of transfected plasmid to a point where newly replicated plasmid produces persistent recombinant transgene expression from a lower number of plasmid molecules. Because primary bovine mammary cells transfected with pCEP4/hGH did not produce high levels of recombinant hGH, and persistent expression was at extremely low levels, and mammary cells cannot be replated to verify episomal replication, no further attempt was made to document actual episomal replication.
Episomal replication may also exhibit species specificity based on the requirement for cellular replication machinery (Wysokenski and Yates, 1989). Murakami et al. (1986) demonstrated that the DNA polymerase α-primase complex isolated from HeLa or monkey cells could restore replication in replication-depleted extracts in vitro, whereas DNA polymerase α-primase complexes isolated from either mouse or calf thymus could not. Therefore, EBV replication may be limited by species specific interactions between cellular replication machinery and the EBNA1 protein which preclude replication in rodent and bovine cells. Consistent with such species limitations, Henkel et al. (1994) subsequently showed that EBV-based vectors are unable to replicate episomally in bovine cells. Thus, EBV-based extrachromosomal plasmid replication will likely not be capable of extending the duration of high level recombinant gene expression in bovine mammary cells.

Uncertainties in the immunoassays for hGH in mammary secretions, which might arise from hGH binding proteins present in milk (Devolder et al., 1993; Postel-Vinay et al., 1991), warrant development of another reporter that is easily analyzed and is non-destructive. Development of a secretable fluorescent reporter protein should provide such an alternative to the hGH reporter gene expression used here in transfected mammary cells, and also allow rapid and sensitive non-destructive analysis of recombinant gene expression which should not be subject to interference from the hGH binding proteins in bovine and rabbit milk (Devolder et al., 1993; Postel-Vinay et al., 1991) which may interfere with conventional ELISA or RIA immunoassays by masking antigenic epitopes.
Green fluorescent protein (GFP) is now a commonly used fluorescent reporter protein that has been cloned and optimized for codon use, translation efficiency, and fluorescence yield (Kain and Ganguly, 1995; Prasher et al., 1992) but is retained intracellularly and is not secreted (Prasher et al., 1992). To generate a secretable version of GFP that might be analyzed non-destructively, the secretion signal (1st exon, 1st intron and a portion of the second exon terminated before α-helix 1) from hGH was ligated onto the N-terminus coding region of GFP to create a GFP fusion protein with a bona fide secretion signal. Secondly, a full length hGH-GFP chimera was constructed to allow comparison of single and multiple intron effects on recombinant protein production and secretion.

Surprisingly, GFP secretion from COS-1 cells was not enhanced by inclusion of the hGH secretion signal (pEGFPss) or the full length hGH-GFP chimera (phGHGFP) (Fig. 6.25). In view of the lack of a secretion signal peptide and the known cytosolic intracellular localization of GFP, a surprising amount of GFP was detected in the extracellular medium following transfection with native pEGFP-N1 (Fig. 6.26), and was likely due to release of intracellular GFP with the lysis of COS-1 cells expressing high levels of cytoplasmic GFP, since such strongly fluorescent cells were observed to round up and detach from cell culture plates. Interestingly, GFP expression from the secretion-modified GFP vectors, pEGFPss or phGHGFP, was visualized intracellularly (Fig. 6.25), in spite of both clones having hGH secretion signals sufficient for high level hGH secretion (Chapter 4). Furthermore, in transfected COS-1 cells the mRNAs for native GFP (pEGFP-N1), GFP with an hGH secretion signal (pEGFPss), and a full length
hGH-GFP chimera (phGHGFP) (Fig. 6.27) were of the appropriate size. Hence, failure of secretion was not likely due to absence of the secretion signal. From this, inclusion of the hGH secretion signal either alone or as a full length hGH chimera is not sufficient to direct secretion of some cytosolic proteins such as GFP.

Although possible, it is highly unlikely that translation was initiated primarily at the GFP start codon, since ribosomal subunits are thought to bind eukaryotic mRNAs at the 5'-GTP cap of the mRNA and “scan” the mRNA until finding an initiator (AUG) codon (Kozak, 1994). Furthermore, ELISA analysis of spent COS-1 cell culture medium indicated that the hGH-GFP chimera was detectable with both hGH and GFP antibodies indicating the presence of hGH antigenic epitopes, suggesting synthesis of the full length hGH-GFP chimera.

There has been only one other report of recombinant GFP secretion. Kaether and Gerdes (1995) created a C-terminal human chromogranin B-GFP fusion protein and monitored GFP chimera secretion in HeLa cells by temperature arrest. Unfortunately, no attempt was made by them to quantitate secreted GFP in this experiment. However, others have had difficulties similar to those described here in generating a securable form of GFP (Henry Rascoff, personal communication, 1996). The difficulty of GFP secretion may be related to three large hydrophobic regions (Phe$_8$ to Gly$_{20}$), (Leu$_{42}$ to Phe$_{71}$), and (Val$_{219}$ to His$_{233}$) predicted within GFP from independent Kyte-Doolittle, Hoop-Woods, and Eisenberg hydropathy analysis. Large hydrophobic protein regions, particularly those regions predicted to form $\alpha$-helices or $\beta$-sheets flanked by charged amino acids such as...
is present in GFP, can sometimes either integrate or associate with lipid membranes (Yeagle, 1989). Thus, one or more of the hydrophobic regions of GFP may insert into or associate itself with a lipid membrane to impede secretion. These results have strong implications for protein localization studies utilizing GFP fusion proteins.

Further increases in recombinant gene expression might be achievable with gene expression systems not dependent on host cell transcriptional apparatus and which are independent of eukaryotic gene regulation. Therefore, bacteriophage T7 RNA polymerase was investigated for its ability to direct recombinant gene expression in mammary cells since it had been reported to increase recombinant gene expression over 500 fold in other eukaryotic cells (Deng et al., 1991; Lieber et al., 1993).

Native T7 RNA polymerase-dependent gene expression was expected to be low due to its lack of an NLS and because T7 RNA polymerase produces uncapped mRNA. A dual plasmid expression system was constructed wherein one plasmid had CMV-driven expression of an NLS-modified T7 RNA polymerase and a second plasmid was designed to produce a T7 RNA polymerase-dependent hGH transcript modified at the 5' end with an IRES.

Cotransfection of primary bovine mammary cells using T7 RNA polymerase-dependent hGH expression vectors failed to give detectable levels of recombinant hGH (Fig. 6.29), although the same transfection system gave only very low level (10 ng/ml) hGH expression in COS-1 cells (Fig. 6.30-6.32). The difficulty of expressing hGH was unexpected since T7 RNA polymerase-dependent gene expression had been demonstrated
in other mammalian cells (Deng et al., 1991; Elroy-stein and Moss, 1990; Lieber et al., 1989). However, this was the first attempt at cotransfection of a NLS-modified T7 RNA polymerase in conjunction with an IRES-containing reporter gene.

T7 RNA polymerase and T7 RNA polymerase-dependent reporter gene mRNA production was evaluated to provide insight toward the limitations of T7 RNA polymerase-dependent hGH expression. Disappointingly, northern hybridization analysis indicated that very little T7 RNA polymerase mRNA was present since T7 RNA polymerase mRNA was not detectable when denaturing agarose gels were loaded with the standard 20 μg RNA/lane (not shown), and were only weakly detectable when loaded at 80 μg RNA/lane (Fig. 6.35). In contrast, GFP mRNA (or hGH, not shown) transcripts expressed from the CMV promoter of pEGFP-N1, pEGFPss, and phGHGPF were easily detectable at 20 μg RNA/lane (Fig. 6.27). The inability to demonstrate T7 RNA polymerase mRNA was unexpected since the same CMV promoter responsible for high level hGH mRNA transcript production (Fig. 6.34) also directed T7 RNA polymerase mRNA production (Fig. 6.35). Nonetheless, it appears likely that T7 RNA polymerase-dependent hGH expression was first limited by the low level of T7 RNA polymerase mRNA production regardless of whether it might also have been limited by inefficient translation of uncapped hGH mRNA transcripts. Alternatively, the low level hGH expression observed in cotransfected COS-1 cells (Figs. 6.30-6.32) may be a result of host cell polymerases driving expression from the T7 RNA polymerase promoter since Lieber et al. (1993) showed that the T7 promoter can be transcribed by a cellular polymerase.
The low level of T7 RNA polymerase mRNA transcript production and its instability may be due to detrimental influences on transcript processing, stability, or translation by intragenic nucleotide sequences of the T7 RNA polymerase gene, or the 5' and 3' untranslated regions acquired during subcloning for the CMV expressed recombinant T7 RNA polymerase, since native prokaryotic genes do not inherently encode 5' and 3' UTR's like eukaryotic genes. Intragenic sequences as well as 5' and 3' untranslated regions of bovine lactoferrin were found to act individually and to interact to dramatically impact mRNA transcript production level and half-life (Schanbacher et al., 1997). Furthermore, Schanbacher et al. (1997) showed that these mRNA regions may interact to influence mRNA stability and mRNA level.

In conclusion, development of a secretable fluorescent reporter gene was not straightforward as anticipated. Neither incorporation of the hGH secretion signal nor a full length hGH-GFP chimera was sufficient to direct GFP secretion in COS-1 cells. However, it is worth noting that hGH is efficiently secreted in COS-1 cells and therefore, the difficulty of secreting the secretion signal-GFP fusion protein and the hGH-GFP chimera is probably inherent to GFP itself. Hence, identification and elimination of the element(s) within GFP which inhibit secretion should allow development of a secretable fluorescent reporter protein to allow rapid, non-destructive analysis of recombinant gene expression in living cells.

Episomal replication of EBV or SV40 vectors will not likely be possible in bovine mammary cells due to the fundamental species incompatibility at the DNA polymerase
level as suggested by Henkel et al. (1994). However, the design of episomal replication vectors based on bovine episomal replicating plasmids, such as bovine papilloma virus (BPV), may be feasible and beneficial, but would require much more work from a much weaker base of understanding of the BPV virus.

Lastly, and disappointingly, T7 RNA polymerase was unable to direct recombinant gene expression in primary bovine mammary cells, and directed only weak expression in COS-1 cells, apparently because of low level T7 mRNA production. However, given the success of T7 RNA polymerase-dependent gene expression in other systems (Deng et al., 1991; Deng and Wolff, 1994; Lieber et al., 1993), the amplification of expression in bovine mammary cells with T7 RNA polymerase should be feasible, and data suggests that further modification of T7 RNA polymerase to stabilize mRNA and enhance its processing may be required.
Chapter 7

In vivo mammary transfection and recombinant protein secretion in milk.

A major goal of recombinant gene expression studies is to produce recombinant human proteins in either blood or milk which could be used for human gene therapy. While numerous transgenic animals secrete recombinant human proteins in milk (Bawden et al., 1994 and references therein) most either do not produce substantial amounts of recombinant proteins or are smaller mammals with milk yields insufficient for large scale recombinant protein production needs. Moreover, the production of transgenic livestock which possess the capacity to produce large amounts of recombinant proteins in milk is inhibited by high cost and extended generation time.

Alternatively, only two non-transgenic gene transfer systems have resulted in successful recombinant protein expression in milk. Archer et al. (1994) used direct intramammary infusion of replication-defective retroviral vectors to express hGH during lactation in hormonally induced goat mammary glands and milk. Multiple intramammary infusions were performed in conjunction with hormonal induction of mammary gland development to facilitate transgene integration. hGH expression peaked at 118 ng hGH/ml on day one of lactation, but rapidly declined to 12 ng/ml by day three of
lactation. Furthermore, hGH expression could not be directly attributed to mammary cell secretion, since the mammary infusate contained both viral particles and helper packaging cells, the latter of which is also capable of producing and secreting hGH (Archer et al., 1994). Nevertheless, this is the only report of recombinant gene expression in milk resulting from direct intramammary infusion as first proposed by Patton et al. (1984a).

For reasons outlined earlier (Chapter 2), non-germline, non-viral gene transfer methods are becoming increasingly popular for in vitro recombinant gene expression studies. However, the only report of non-germline, non-viral recombinant gene expression in mammary tissue with subsequent secretion in milk comes from Kerr et al. (1996) who produced low level recombinant hGH (1 ng/ml) in ovine milk following jet injection of an hGH transgene construct. However, jet injection does not rely on intramammary infusion and probably will not be feasible for large scale recombinant gene expression in the mammary gland or milk of dairy cattle due to its limited gene delivery area, tissue damage, and risk of infection.

Therefore, development of an efficient non-germline, non-viral recombinant gene transfer method that would allow direct gene transfer into target organs such as the mammary gland would facilitate modification of mammary function and/or milk composition without the time or expense required for production of transgenic cattle, and the safety concerns associated with the use of retroviral vectors.

The unique anatomy of the mammary gland should allow solutions containing recombinant transgene to be infused into the mammary gland via teat cannulation and
passage through the ductular tree to reach mammary alveoli which are lined by the apical surfaces of secretory mammary epithelial cells responsible for milk protein synthesis and secretion. Direct mammary cell transfection by intramammary infusion of recombinant transgenes should: 1) allow efficient delivery of DNA to most or all mammary cells; 2) simplify expression vector design by eliminating the need for mammary specific regulatory elements, since the anatomy of the mammary epithelium and ducts assures that only mammary epithelial cells will be exposed to transfection components; and 3) be applicable to any animal regardless of genetic background which allows rapid adjustment to constantly changing markets.

The anatomy, polarity of the mammary epithelium, and motion of the bovine mammary gland preclude the use of gravity-dependent transfection systems (such as calcium phosphate:DNA coprecipitation) in vivo (Keown et al., 1990). Transfection procedures which require short-duration cell shock (e.g., glycerol or DMSO exposure, etc.) by rapid short-term application and exchange of shock medium are also not feasible in the bovine mammary gland due to its size and the consequent difficulty of rapid introduction and complete and rapid removal of fluid by teat drainage or aspiration (Keown et al., 1990; Malienou-Ngassa et al., 1990). Furthermore, the high cost of selection agents, average size of the mammary gland (~ 50 lbs.) (Schmidt, 1971, and references therein), and limited post-parturient mammary cell replication (Schmidt, 1971) will likely preclude survival selection of transfected cells in the bovine mammary gland in vivo (Kaufman, 1990; Keown et al., 1990). Therefore, priority must be placed on the
development of gene transfer systems capable of transfection and expression of recombinant transgenes in most or all cells of the mammary gland without the need for selection or cell replication.

The ability to deliver and express recombinant transgenes in most or all mammary cells is critical because recombinant protein expression level and value will likely define potential applications of gene transfer into mammary tissue and recombinant protein expression in milk. Polycations able to bind both DNA and the negatively charged cell surface by electrostatic charge interaction, should bind polycation:DNA transfection complexes to the anionic apical surfaces of all mammary cells exposed to the intraluminal fluid space within the mammary gland. Furthermore, polycations are easy to prepare, simple to use, inexpensive, and non-infectious.

A previous section (Chapter 4), described the use of a primary bovine mammary cell culture system (Talhouk et al., 1990; Talhouk et al., 1992) to develop a novel charge-shifting polyion-mediated gene transfer system within the constraint of the anticipated or perceived requirements for transfection of secretory mammary epithelial cells in the bovine mammary gland in vivo. This gene transfer system produced high and sustained recombinant gene expression from primary bovine mammary cells transfected in vitro. Described herein are efforts to express recombinant proteins in milk following direct intramammary infusion of the charge-shifting polyion-mediated gene transfer system described in Chapter 4.
RESULTS

Intramammary infusion and transfection considerations.

Transfection of the mammary gland in vivo presents additional challenges for transfection not encountered with transfection of mammary cells in vitro. For example, mammary secretion composition may influence the timing of in vivo mammary gland transfection. Prepartum developing bovine mammary glands produce viscous proteinaceous secretions with protein concentrations ranging from 100-300 mg total protein/ml as opposed to lactating bovine mammary gland secretions containing protein concentrations typically ranging from 30-40 mg total protein/ml (Hoppe, 1976; Schanbacher and Smith, 1975). Thus, because the recombinant gene transfer system described in Chapter 4 is ionically based and likely will interact non-specifically with both the anionic cell surface and anionic milk proteins, the transfer of recombinant genes into mammary epithelial cells in vivo with polycationic transfection complexes may be infeasible during the prepartum period due to the high viscosity and concentration of anionic protein in prepartum mammary secretions. Therefore, initial in vivo mammary transfections were performed in the lactating bovine gland where protein concentrations are substantially lower, and most (>85%) secretion can be removed by milking.

Secondly, the production of large quantities of purified plasmid DNA required to infuse each bovine mammary gland (5-10 mg/transfected quarter) was time consuming, relatively expensive, and limited the number of in vivo bovine mammary transfections.
which could be performed. Plasmid DNA utilized for in vivo mammary transfections also required purification to remove bacterial lipopolysaccharides (endotoxins), released upon bacterial lysis during plasmid purification, since even low level (10 μg) endotoxin infusion in the bovine mammary gland can suppress milk production and cause inflammation (Hogan et al., 1993; Shuster et al., 1991). Endotoxin contamination of plasmid DNA preparations has also been reported to reduce transfection efficiency in vitro (Weber et al., 1995). Unpurified DNA preparations typically contained 8-10 μg endotoxin/5 mg DNA (the amount to be infused in one quarter). Therefore, bacterial endotoxins were removed by passage of the purified plasmid preparation through a polymyxin B column as described in methods to yield “endotoxin-free” DNA which typically contained <500 ng endotoxin/5 mg DNA. Thus, all in vivo bovine mammary transfections were performed with endotoxin-free DNA during lactation where the majority (>85%) of milk proteins could be removed by milking.

**Intramammary infusion and transfection of the lactating bovine mammary gland.**

To investigate the ability of charge-shifting polyion-mediated gene transfer to facilitate recombinant protein production in milk following intramammary infusion, a single multiparous Holstein-Friesian (cow No. 3485) in midlactation (238 days in milk) was completely milked by machine followed by further removal of residual milk by hand milking for maximum removal of milk from the glands before transfection. Following milk removal, three glands were infused with unordered mixed polyion transfection
complexes [Orn\textsubscript{n Гlu\textsubscript{n ГHisN:DEAE:DNA}], while the fourth gland was left uninfused as a control. The capacity of individual mammary gland, as determined by complete milk removal, ranged from 2-3 liters per quarter. Therefore, one liter of transfection solution (HBSS) containing unordered mixed polyions [Orn\textsubscript{n Гlu\textsubscript{n ГHisN:DEAE: and DNA (pcDNA1/hGH, pCEP4/hGH or pO/hGH)] was infused per quarter. Intramammary infusions took approximately seven minutes per quarter and mammary glands were hand massaged following infusion to ensure complete and even distribution of transfection solution throughout the gland. Since one liter of transfection solution was less than the volume of milk removed, it would not be expected to induce engorgement and adversely affect the gland, and should provide ample solution volume for transfection complexes to reach most or all peripheral mammary tissue. At this time these studies were started, the effects of infusing such volumes into the mammary gland were unknown, and infusion of larger volumes would have required production of even more plasmid DNA which was the limiting component for in vivo bovine mammary transfections.

Three different plasmids were utilized in this in vivo study: pO/hGH as a negative plasmid control since it contains no promoter to drive hGH expression; pcDNA1/hGH which gave the highest expression in primary bovine mammary cells transfected in vitro, and pCEP4/hGH an episomal replication competent eukaryotic expression vector. Although pCEP4/hGH showed lower hGH expression than pcDNA1/hGH in vitro (Fig. 6.24), it was tested here to investigate whether mammary epithelial cells in vivo might provide essential components able to support episomal replication of pCEP4/hGH in vivo.
but which were lacking in cultured mammary epithelial cells. Furthermore, it was unknown at the time what effect the infusion of polycations would have on the mammary gland. If polycation-mediated transfection damages the mammary gland, pCEP4/hGH may be able to replicate during the brief period of cell division during the recovery from mammary trauma induced by intramammary infection (mastitis) (Schanbacher and Smith, 1975). For in vivo transfection studies, all transfection reagents and DNA preparations were confirmed functional by first transfecting primary bovine mammary cells in vitro prior to in vivo transfection.

Although recombinant hGH expression was undetectable in milk from all transfected or control mammary glands for the 12 days immediately posttransfection which were assayed (Fig. 7.1), intramammary infusion of mixed polyions appeared benign, and did not appear to elicit a fever, inflammatory response, or cause noticeable mammary irritation. Furthermore, milk synthesis and dry matter intake were suppressed only briefly for 24-48 hrs after intramammary infusion, and rapidly recovered to near normal levels (Fig. 7.1), suggesting that any effects of intramammary infusion of charge-shifting polion transfection complexes on animal and mammary health were temporary and relatively minor. The very bottom of the infused mammary gland became firm for 1-2 days post-infusion, possibly due to casein precipitation by polycations since limited amounts of coagulated proteins (casein micelles?) could be expressed by hand from the gland, but all attempts to culture mastitis-causing coliform bacteria were unsuccessful.
Figure 7.1.  Temporary suppression of milk secretion and dry matter intake following intramammary infusion of charge-shifting polyanion transfection complexes. Individual mammary glands of a multiparous midlactation (256 days in milk) Holstein-Friesian cow were infused with unordered charge-shifting mixed polyanions: [Orn (37.5 µg/ml), Glu-HisN (75 µg/ml), DEAE (12.5 µg/ml), DNA (5 µg/ml)] formed in HBSS. One liter of the above polyanion transfection mixture containing either pO/GH (●), pcDNA1/hGH (▽), or pCEP4/hGH (◆) was infused in each of three quarters, while one quarter remained as an uninfused control (□). Effect of intramammary infusion of charge-shifting polyanion transfection complexes on dialy milk production (●) and dry matter intake (○)
Despite the lack of hGH expression, these results were very encouraging for two reasons. First, the infusion of volumes as large as one liter, which had not been previously reported in the literature, had no significant long lasting adverse effect on the mammary gland. Secondly, the fact that polycation infusion did not traumatize the gland was encouraging since it was unknown what effect polycations would have on the mammary gland.

The difficulty of hGH expression in the first in vivo mammary transfection was hypothesized to be a result of non-specific ionic interaction between residual anionic milk proteins and cationic transfection complexes. This interaction was presumed to preclude transfection complexes from reaching mammary cell apical surfaces. Thus, for subsequent intramammary transfections, the following steps were undertaken to reduce the amount of residual anionic milk protein in the mammary gland while maximizing cationic transfection complex presence to increase the likelihood of cationic transfection complex:mammary apical cell surface interaction. One mammary gland of cow 3485 was completely milked and subsequently infused with one liter of sterile HBSS to “flush” the gland of any residual anionic milk proteins. Thirty minutes later the cow was intraveinously injected with 2.5 ml oxytocin to stimulate complete milk letdown and allow exhaustive removal of the HBSS-milk mixture by hand milking. To further optimize the cationic transfection complex:anionic milk protein ratio, the concentration of transfected DNA and corresponding polyions was doubled [DNA (10 μg/ml), DEAE (25 μg/ml), Glu₉-HisN (150 μg/ml), Orn₈ (75 μg/ml)]. A second intramammary infusion
was then performed using the same cow (#3485) now in late lactation (304 days in milk). In vivo mammary transfections were performed in the same animal to conserve on carcass disposal, since animals infused with recombinant transgenes and their milk, were removed from the food chain, as a precautionary safety measure.

Following milk removal (as described above), one liter of transfection solution (HBSS) containing unordered mixed polyion transfection complexes [Orn\(_n\):Glu\(_n\)-HisN:DEAE:DNA (pcDNA1/hGH)] at twice the normal concentration was infused into one quarter of the mammary gland. The right front quarter which had not been infused during the first in vivo transfection was again utilized as the uninfused control. However, no recombinant hGH expression was detectable by RIA in the milk of the transfected quarter for the 10 days immediately after transfection (results not shown). Once again, no mammary irritation or inflammation was noted during or following infusion of charge-shifting mixed polyion transfection complexes even at double the previous concentration. However, approximately eight hours after polyion transfection complex infusion the cow produced a fever which peaked at 106.2°F. Total milk production and dry matter intake profiles paralleled the results shown in Figure 7.1 with transient depression of milk production on day 1-2 posttransfection. The lack of expression from these in vivo mammary transfections suggested further improvement in transfection efficiency and/or expression efficiency would be required for successful bovine mammary transfection in vivo.
Subsequent transfection of primary bovine mammary cells in vitro indicated that controlling the ordered addition of polyions to the DNA during transfection complex formation, and formation of transfection complexes in isotonic 0.3 M sucrose, 10 mM HEPES, pH 7.4, dramatically increased recombinant gene expression (Fig. 4.11) over unordered transfection complexes formed in HBSS used in the previous in vivo transfections (Fig. 7.1).

Furthermore, because the previous attempts at in vivo transfection failed to yield detectable recombinant hGH expression, it was hypothesized that polyion:DNA transfection complexes may be ionically interacting with anionic casein micelles remaining in any residual milk which could not be removed from the gland and therefore were not targeting to the mammary apical cell surface properly. In anticipation that a ligand-targeted transfection complex might be necessary to avoid interaction with residual milk proteins, a charge-shifting polyion transfection complex containing mannosylated-Omₙ and concanavalin A (con-A), a mannose-binding plant lectin, had been previously tested in primary bovine mammary cells in vitro (Fig. 7.2). Con-A was previously shown by Patton et al. (1984b) to deliver marker molecules to the apical cell surface and be internalized after intramammary infusion in rats.

Substitution of mannoslyated-Omₙ for a portion of underivatized Omₙ should allow tetravalent con-A to bind polyion transfection complexes via interaction with mannose, and also allow targeting of lactating mammary apical cell surfaces.
Figure 7.2. Effect of incorporation of mannosylated-Orn and concanavalin A in charge-shifting polycation transfection complexes on hGH expression. Cultured primary bovine mammary cells were transfected with unordered charge-shifting mixed polycation transfection complexes without mannosylated-Orn and con A targeting: [Orn_37.5 (37.5 µg/ml), Glu_n-HisN (75 µg/ml), DEAE (12.5 µg/ml), DNA (5 µg/ml)] (●), or with mannosylated-Orn and concanavalin A targeting: [Orn_21.5 (21.5 µg/ml), mannosylated-Orn_16 (16 µg/ml), Glu_n-HisN (75 µg/ml), DEAE (12.5 µg/ml), concanavalin A (20 µg/ml), DNA (5 µg/ml)] (○), both formed in HBSS. Ratios for total polycation:DNA (10:1) and Orn_n:DEAE (3:1) were maintained as before.
Recombinant hGH expression levels decreased ~ 50% when con-A and mannosylated-Orn$_n$ were incorporated into unordered charge-shifting mixed polyion transfection complexes (Fig. 7.2). Nevertheless, incorporation of con-A and mannosylated-Orn$_n$ should allow targeting to mammary epithelia by binding apical cell surface mannosyl- or glucosyl carbohydrate moieties (Patton et al., 1984b), even in the presence of high concentrations of anionic soluble milk proteins.

Therefore, to test the effects of: (1) controlled order of polyion addition and formation of transfection complexes in isotonic sucrose/HEPES medium, and (2) inclusion of the con-A ligand targeting transfection system in vivo, cow 3485 was transfected for a third time, late (317 days in milk) in her subsequent lactation. The right front mammary gland was left as an uninfused control, while the left front mammary gland was infused with unordered charge-shifting polyion transfection complexes formed in HBSS [Orn$_n$:Glu$_n$:HisN:DEAE:pcDNA1/hGH DNA]. The left rear mammary gland was infused with ordered charge-shifting polyion transfection complexes formed in sucrose/HEPES [pcDNA1/hGH DNA:DEAE:Glu$_n$:HisN:Orn$_n$]. The right rear mammary gland was infused with a modified unordered charge-shifting polyion treatment designed to specifically target binding of the mannosyl-derivatized transfection complex to the mammary epithelial cell apical surface with con-A, similar to Patton et al. (1984b). Mannosyl-derivatized Orn$_n$ was partially substituted for Orn$_n$ (1:3 wt:wt) to allow binding of transfection complex by con-A. Thus, the modified unordered charge-shifting polyion treatment consisted of: [Orn$_n$:mannosylated-Orn$_n$:Glu$_n$:HisN:DEAE:concanavalin.
A:pcDNA1/hGH DNA] formed in HBSS. Consistent with previous intramammary infusions, all transfection solutions were left undisturbed in the gland until the next regular milking interval (10-12 hours).

Skimmed milk samples collected for 12 days immediately posttransfection were analyzed for recombinant hGH secretion by ELISA as described in methods. Initial ELISA analysis indicated low (< 7 ng/ml) expression of hGH in milk from mammary glands transfected with all versions of the charge-shifting polyion transfection system (Fig. 7.3A). Interestingly, the left front mammary gland transfected with unordered charge-shifting polyions [Omn:GlUn-HisN:DEAE:pcDNA1/hGH DNA] appeared to give the highest hGH expression peaking at 6.6 ng hGH/ml on day 2 posttransfection (Fig. 7.3A). By comparison, the con-A targeted [Omn:mannosylated-Omn:Glu:-HisN:DEAE:concanavalin A:pcDNA1/hGH DNA] and ordered charge-shifting polyion transfection [pcDNA1/hGH DNA:DEAE:Glu:-HisN:Omn] appeared to peak at 2 ng hGH/ml on day 2 posttransfection (Fig. 7.3A). hGH expression from all transfections declined to < 1 ng hGH/ml by day 6 posttransfection (Fig. 7.3A). Unfortunately, the uninfused negative control quarter also showed low (1 ng hGH/ml) recombinant hGH expression (Fig. 7.3A).

The concentration of heat treated rabbit serum (HTRS) was increased from 1% to 10% in the sandwich ELISA assay in an attempt to correct for potential interference and nonspecific binding (Masseyeff, 1978) suspected as responsible for the apparent presence of hGH in the control gland and potentially contributing to false positive analysis for hGH.
Figure 7.3. Equivocal recombinant hGH expression in bovine milk following transfection with charge-shifting mixed polycations. ELISA analysis of apparent hGH expression in bovine milk from individual mammary glands following either no intramammary infusion (O); or intramammary infusion of unordered charge-shifting polycations [Orn\textsubscript{n} (37.5 μg/ml), Glu\textsubscript{n}-HisN (75 μg/ml), DEAE (12.5 μg/ml), pcDNA1/hGH DNA (5 μg/ml)] formed in HBSS (▲); or ordered charge-shifting polycations [pcDNA1/hGH DNA (5 μg/ml), DEAE (12.5 μg/ml), Glu\textsubscript{n}-HisN (75 μg/ml), Orn\textsubscript{n} (37.5 μg/ml)] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4 (●); or with con-A targeted unordered charge-shifting polycations [Orn\textsubscript{n} (21.5 μg/ml), mannosylated-Orn\textsubscript{n} (16 μg/ml), Glu\textsubscript{n}-HisN (75 μg/ml), DEAE (12.5 μg/ml), concanavalin-A (20 μg/ml), pcDNA1/hGH DNA (5 μg/ml)] formed in HBSS (■). Panel A; normal ELISA with non-specific binding of IgG blocked in milk from tested mammary glands. Inclusion of 10% HTRS decreased apparent hGH by 1% heat treated rabbit serum (HTRS). Panel B; ELISA run with 10% HTRS to minimize non-specific binding of IgG.
expression in the control quarter from 1 ng/ml to 0.5 ng /ml, but did not completely eliminate what is believed to be nonspecific IgG binding (Fig. 7.3B). hGH expression from all transfected mammary glands decreased similarly with addition of 10% HTRS to the ELISA, with maximum apparent hGH reduced to \( \leq 2 \) ng hGH/ml on day 2 post transfection (Fig. 7.3B).

Serum proteins such as rheumatoid factor are known to be especially prevalent in ruminant serum, and can cause nonspecific interference or binding with this double-sandwich antibody ELISA system resulting in false positive readings (Masseyeff, 1978). The same milk samples showing apparent hGH expression by ELISA analysis were then analyzed by commercial hGH RIA analysis kits which had shown no evidence of false positive assays of hGH expression in cell culture medium or milk. RIA analysis showed no detectable recombinant hGH in milk from any mammary gland (results not shown).

These RIA results are presumed to indicate that these transfections did not yield hGH expression, in spite of the apparent hGH found in milk by ELISA. The basis for the false positive hGH ELISA analysis in milk could not be defined, in spite of efforts to do so with internal standards. Consistent with previously reported results (Fig. 7.1), intramammary infusion of polyion transfection complexes did not appear to irritate the mammary gland or elicit an inflammatory response despite repeated polyion infusions. Furthermore, none of the seven infused bovine mammary glands, each of which received one liter of transfection medium, developed an intramammary infection (mastitis).
In vivo transfection of the guinea pig mammary gland by intramammary infusion of charge-shifting polyion transfection complexes.

The difficulty of hGH expression in the bovine mammary gland necessitated the development of an alternative in vivo model for testing transfection methods since the preparation of such large quantities of purified DNA was quite costly and time consuming. Coincidentally, colleagues (Dr. Ian Mather and Ms. Julie Hens) at the University of Maryland were unsuccessful in efforts to express recombinant proteins in guinea pig milk using a commercially available cationic lipid (Lipofectamine) gene transfer system for mammary transfection by intramammary infusion (I. Mather, personal communication, 1995). Hence, they were amenable to a proposed collaborative study involving transfection of guinea pig mammary glands with the ordered charge-shifting polyion transfection procedure. Thus, for these collaborative in vivo transfection studies, all transfection components except the sucrose/HEPES solution were synthesized and packaged ready for use and shipped on ice to the University of Maryland for in vivo guinea pig mammary gland transfection.

From their (I. Mather and J. Hens) previous intramammary infusion of cationic lipids, the optimum infusion volume had been established as two milliliters. Infusion of transfection complexes into the guinea pig mammary gland was accomplished through a blunt end 27 gauge needle inserted through the teat canal of a recumbant guinea pig anesthetized with ketamine (40 mg/kg), and Rompin (5 mg/kg) (Harkness and Wagner, 1983). Transfected guinea pigs were hand milked daily after a 3 hr. period of pup
removal to allow milk accumulation. Milk samples were centrifuged as above and stored at -80°C until assayed by RIA for hGH expression in milk. Figures 7.4, 7.5, and 7.6 illustrate the expression attained with charge-shifting polyion-mediated transfection of the guinea pig mammary gland in vivo in studies by Mather and Hens.

The initial guinea pig transfection was performed two days prepartum by intramammary infusion of ordered charge-shifting polyion transfection complexes [pcDNA1/hGH:DEAE:Glu₅-His₉:Ornₙ] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4. The same DNA (5 µg/ml) and polyion ratios and concentrations were utilized as described previously for transfection studies of primary bovine mammary cells in vitro (Chapter 4). Interestingly, recombinant hGH expression was low, but readily detectable (< 5 ng/ml) in milk throughout lactation from the transfected gland, but not from the sham transfected contralateral mammary gland infused with polyions lacking DNA (Fig. 7.4). Moreover, hGH expression increased over the duration of the lactation, peaking at day 12-13 of lactation at nearly 4 ng hGH/ml milk (Fig. 7.4). Furthermore, no mammary irritation or inflammatory response was observed in the transfected gland (I. Mather and J. Hens, personal communication, 1996). The lack of inflammation or irritation in the infused guinea pig mammary gland was consistent with the lack of mammary trauma after mixed polyion transfection by intramammary infusions in dairy cows.
Figure 7.4. hGH expression in guinea pig milk following transfection with ordered charge-shifting mixed polyions. One half of a guinea pig mammary gland was infused with 2.0 ml transfection solution (0.3 M sucrose, 10 mM HEPES, pH 7.4) containing ordered charge-shifting mixed polyions [pcDNA1/hGH (5 μg/ml), DEAE (12.5 μg/ml), Glu$_n$-HisN (75 μg/ml), Orn$_n$ (37.5 μg/ml)] (filled bars), while the contralateral gland was infused with ordered charge-shifting mixed polyions without DNA [DEAE (12.5 μg/ml), Glu$_n$-HisN (75 μg/ml), Orn$_n$ (37.5 μg/ml)] (shaded bars). Experimental design, intramammary infusion, and hGH analysis provided by I. Mather and J. Hens. Transfection reagents provided by M. Amstutz.
Effect of increasing concentrations of transfection complex in intramammary infusion and transfection in the guinea pig.

To further optimize transfection of guinea pig mammary glands, guinea pig mammary glands were infused with 2.0 ml of transfection solution containing 2.5, 5, 25 or 100 µg DNA/ml complexed with ordered charge-shifting polion:DNA complexes [pcDNA1/hGH:DEAE:Glu-:HisN:Orn] formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4, at the standard (10:1, wt/wt) total polycation:DNA and (3:1, wt/wt) Orn:DEAE ratios as used for in vitro transfections (Fig. 4.5).

Recombinant hGH expression was detected in milk from all transfected mammary glands (Fig. 7.5), but was highest (~ 40 ng hGH/ml) in milk from guinea pig mammary glands transfected with ordered charge-shifting polion transfection complexes [pcDNA1/hGH:DEAE:Glu-:HisN:Orn] containing 25 µg DNA/ml (Fig. 7.5). Recombinant hGH expression was detectable in milk as early as two days postpartum and steadily increased throughout the duration of a normal lactation (typically 14-20 days for guinea pigs) (Fig. 7.6) (Harkness and Wagner, 1983). hGH expression peaked on day 14 of lactation at 40 ng hGH/ml milk (Fig. 7.6). No recombinant hGH expression was detectable by RIA in milk from mammary glands of sham transfected controls [DEAE:Glu-:HisN:Orn] (Fig. 7.6).

J. Hens and I. Mather continue to use the polions and DNA (provided by M. Amstutz and F. Schanbacher) to further define and optimize guinea pig mammary transfection in vivo for: 1) the stage of transfection (gestation vs lactation); 2) whether
Figure 7.5. Effect of increasing DNA and corresponding polyion concentrations on hGH expression in milk of transfected guinea pigs. One mammary gland per guinea pig was infused with 2.0 ml transfection solution (0.3 M sucrose, 10 mM HEPES, pH 7.4) containing increasing amounts of DNA and ordered charge-shifting mixed polyions as follows: [pcDNA1/hGH (2.5 µg/ml), DEAE (6.25 µg/ml), Glu₅-HisN (37.5 µg/ml), Orn₇ (18.75 µg/ml)]; [pcDNA1/hGH (5 µg/ml), DEAE (12.5 µg/ml), Glu₅-HisN (75 µg/ml), Orn₇ (37.5 µg/ml)]; [pcDNA1/hGH (25 µg/ml), DEAE (62.5 µg/ml), Glu₅-HisN (375 µg/ml), Orn₇ (187.5 µg/ml)]; or [pcDNA1/hGH (100 µg/ml), DEAE (250 µg/ml), Glu₅-HisN (1500 µg/ml), Orn₇ (750 µg/ml)]. Corresponding contralateral glands were infused with polyions alone (no DNA) as a negative control (not shown). Experimental design, intramammary infusion, and hGH analysis provided by I. Mather and J. Hens. Transfection reagents provided by M. Amstutz.
Figure 7.6. Typical profile of hGH expression in guinea pig milk following transfection with ordered charge-shifting mixed polyions. One half of a guinea pig mammary gland was infused with 2.0 ml transfection solution (0.3 M sucrose, 10 mM HEPES, pH 7.4) containing ordered charge-shifting mixed polyions at the optimized concentrations [pcDNA1/hGH (25 μg/ml), DEAE (62.5 μg/ml), Glu₅-HisN (375 μg/ml), Orn₅ (187.5 μg/ml)] (●), while the contralateral gland was infused with ordered charge-shifting mixed polyions without DNA [DEAE (62.5 μg/ml), Glu₅-HisN (375 μg/ml), Orn₅ (187.5 μg/ml)] (■). Experimental design, intramammary infusion, and hGH analysis provided by I. Mather and J. Hens. Transfection reagents provided by M. Amstutz.
expression persists into a subsequent lactation; and 3) definition of cell types expressing recombinant transgenes in the mammary gland following intramammary infusion. Preliminary results indicated that guinea pig mammary glands transfected with ordered charge-shifting polyions [pcDNA1/hGH:DEAE:Glu\textsubscript{n}-HisN:Orn\textsubscript{n}] at the optimal 25 μg DNA/ml during mid gestation produced little (< 5 ng hGH/ml milk) hGH expression, while those transfected 1-2 days into lactation gave erratic results with one guinea pig expressing < 5 ng hGH/ml milk and a second expressing > 100 ng hGH/ml milk. I. Mather and J. Hens (personal communication, 1997) observed that guinea pig mammary glands transfected two days prepartum seem to give the most consistent hGH expression.

**Effect of polyion transfection complex composition on hGH expression in guinea pig milk.**

To determine whether transfection of the guinea pig mammary gland in vivo reflected bovine mammary cell transfection in vitro, and to determine the validity of primary bovine mammary cells as a model for in vivo mammary gland transfection, guinea pig mammary glands were transfected with the series of DNA:polyion transfection complexes represented in the early development of the procedure (Chapter 4): 1) DEAE [DEAE:pcDNA1/hGH]; 2) Orn\textsubscript{n} [Orn\textsubscript{n} :pcDNA1/hGH]; 3) mixed polycation [Orn\textsubscript{n} : DEAE: pcDNA1/hGH]; 4) unordered charge-shifting polyion [Orn\textsubscript{n}:Glu\textsubscript{n}-HisN: DEAE: pcDNA1/hGH]; or 5) ordered charge-shifting polyion [pcDNA1/hGH:DEAE:Glu\textsubscript{n}-HisN:Orn\textsubscript{n}], all formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4. Two guinea pigs were
utilized per treatment, with three of the four mammary glands infused with the test transfection complex containing DNA while the fourth mammary gland was infused with polyions alone (no DNA) as a sham transfection negative control.

As predicted from transfection of primary bovine mammary cells in vitro, transfection of guinea pig mammary glands with Om gave low level (5 ng hGH/ml milk) expression which also persisted for the duration of lactation (14 days) (Fig. 7.7). Expression profiles from the two guinea pigs transfected with Om differed in that one guinea pig reached peak hGH expression (5 ng hGH/ml) on day 2 of lactation while the second peaked at 5.5 ng hGH/ml on day 8 of lactation (not shown). In contrast to the enhanced expression obtained with mixed polycation [Om:DEAE] transfection of primary bovine mammary cells in vitro, mixed polycation transfection of guinea pig mammary glands in vivo did not increase recombinant hGH expression (6-8 ng hGH/ml) over that for Om alone (Fig. 7.7). However, like transfection with polycations alone, mixed polycation transfection gave hGH expression which persisted for the duration of lactation (14 days) (Fig. 7.7). Also surprising and unlike in vitro transfection of primary bovine mammary cells, addition of the charge-shifting polymer Glu-HisN to mixed polycation transfections did not increase maximum recombinant hGH expression levels (6 ng hGH/ml) in guinea pig milk (Fig. 7.7). However, transfection of guinea pig mammary glands with ordered charge-shifting mixed polyions containing Glu-HisN dramatically increased recombinant hGH expression levels to > 75 ng hGH/ml milk on day 12 of lactation with high level hGH expression noted throughout lactation (Fig. 7.7).
Figure 7.7. Effect of polycation transfection complex composition on hGH expression in guinea pig milk. Guinea pig mammary glands were transfected by infusion of 2.0 ml of DNA:polycation transfection complex composed of either: DEAE [DEAE (1.25 mg/ml), pcDNAI/hGH (25 µg/ml)] (▼); Omₙ [Omₙ (187.5 µg/ml), pcDNAI/hGH (25 µg/ml)] (□); mixed polycations [Omₙ (187.5 µg/ml), DEAE (62.5 µg/ml), pcDNAI/hGH (25 µg/ml)] (▲); unordered charge-shifting polycations [Omₙ (187.5 µg/ml), Gluₙ-HisN (375 µg/ml), DEAE (62.5 µg/ml), pcDNAI/hGH (25 µg/ml)] (○); or ordered charge-shifting polycations [pcDNAI/hGH (25 µg/ml), DEAE (62.5 µg/ml), Gluₙ-HisN (375 µg/ml), Omₙ (187.5 µg/ml)] (●) all formed in 0.3 M sucrose, 10 mM HEPES, pH 7.4. Two guinea pigs were utilized per transfection treatment, with the average expression of the three transfected mammary glands shown. The fourth mammary gland was infused with polycation transfection complexes lacking DNA as a sham transfection negative control. None of the negative control glands showed any detectable hGH expression in milk (not shown). Intramammary infusion, and hGH analysis provided by I. Mather and J. Hens. Experimental design and transfection reagents provided by M. Amstutz.
The increase in hGH expression upon ordered addition of the charge-shifting polymer was consistent with transfection of primary bovine mammary cells in vitro (Fig. 4.11), and similar to previously reported hGH expression in guinea pig milk (Fig. 7.6). Hence, as for in vitro transfection of bovine mammary cells, inclusion of charge shifting polynomials in an ordered polyion transfection complex dramatically improved expression efficiency.

Unexpectedly, guinea pig mammary glands transfected with DEAE:DNA transfection complexes gave very high (300-400 ng hGH/ml) expression in milk which persisted for the duration of lactation (14 days) (Fig. 7.7). In fact, one mammary gland exceeded 500 ng hGH/ml milk on day 6 of lactation! The high level of hGH expression in the milk of guinea pigs transfected with DEAE was quite unexpected for several reasons. First, Malienou-Ngassa et al. (1990) found that DEAE-mediated transfection of primary rabbit mammary cells in vitro gave only 1 ng hGH/ml. Second, primary bovine mammary cells transfected with DEAE in vitro also gave only low level hGH expression (5-10 ng/ml) (Fig. 4.6 and 4.9). Finally, polyion:DNA transfection complexes containing high polycation concentrations (>100 ug/ml) caused extensive cell injury and vacuolization in Comma-D mouse and primary bovine mammary cells transfected in vitro (M. Amstutz, personal observation). The in vitro polycation concentrations (>100 ug/ml) which caused extensive cell injury were much lower than those used here in the guinea pig mammary gland in vivo (1.25 mg/ml).
Although not shown, substantial differences were observed in recombinant hGH expression levels between guinea pigs and within the two glands of the same guinea pig transfected with the same transfection complex.

The lack of hGH expression in the bovine mammary gland could not be attributed to poor quality or degraded DNA since the same DNA preparations were utilized for both bovine and guinea pig transfections. Furthermore, agarose gel electrophoresis of plasmid DNA used for in vivo transfections shows the DNA to consist mainly of supercoiled (Fig. 7.8, filled arrow) and open circular (Fig. 7.8, open arrow) DNA, with a minimal amount of linearized plasmid DNA (Fig. 7.8, arrowhead).

Effect of intramammary infusion of charge-shifting polyanion transfection complexes in the ovine mammary gland on recombinant gene expression in milk.

Following the determination in guinea pigs that prepartum mammary transfection was more consistent and gave higher hGH expression in milk than transfection during lactation, prepartum transfection was attempted in the ruminant mammary gland. However, to conserve DNA and allow multiple transfections, ovine mammary glands were utilized for this experiment. It should be noted that all ovine transfections were performed prior to the realization that DEAE could mediate high level hGH expression in guinea pig milk.

One mammary gland on each of two pregnant nulliparous ewes was hand milked to remove prepartum secretions. Milked glands were then infused with ordered
Figure 7.8. pcDNA1/hGH plasmid DNA used for in vivo bovine and guinea pig mammary gland transfections. pcDNA1/hGH plasmid DNA integrity was assessed by agarose gel electrophoresis of one µg of 1 Kb Gibco BRL DNA molecular weight ladder (lanes 1 and 6), pcDNA1/hGH (as infused) (lanes 2 and 3), EcoRI restriction endonuclease linearized pcDNA1/hGH (lane 4); BamHI and EcoRI restriction endonuclease digested pcDNA1/hGH (lane 5) to show excision of the hGH gene (2154 bp). Supercoiled (filled arrow), open circular (open arrow), and linearized (arrowhead) plasmid DNA.
charge-shifting mixed polyions [pcDNA1/hGH:DEAE:Gluₙ-HisN:Ornₙ] formed in sucrose/HEPES, pH 7.4, at concentrations optimized for in vivo guinea pig transfection. Mammary glands were infused to capacity, determined by engorgement and distension of the gland with 115 and 150 ml of transfection solution, respectively. Contralateral glands remained uninfused as controls.

A third nulliparous pregnant ewe was hand milked as above, infused with 100 ml of 0.3 M sucrose, 10 mM HEPEs, pH 7.4, which was allowed to remain in the gland for 10 min. before removal as completely as possible by hand milking. The gland was then infused to capacity with 85 milliliters of ordered charge-shifting mixed polyions [pcDNA1/hGH:DEAE:Gluₙ-HisN:Ornₙ] formed in 0.3 M sucrose, 10 mM HEPEs, pH 7.4, as above. Mammary gland “flushing” was done to remove viscous prepartum secretions in the gland which contain high concentrations of IgG and other proteins which may interfere with efficient gene delivery. Recovery of flushing solution was incomplete; only about 50% of the infusate was recovered by subsequent hand milking. Oxytocin could not be utilized to facilitate wash solution recovery since it would have induced premature parturition. Infused ewes lambed 2-7 days post infusion, with the variation due to uncertainty of actual breeding dates and normal variation in length of gestation.

No recombinant hGH expression was detectable by ELISA in milk samples from transfected ovine mammary glands for the first 10 days of lactation (results not shown). However, like previous infusions in bovine and guinea pig mammary glands, infusion of mixed polyions in the ovine mammary gland caused no significant irritation, swelling or
visible discomfort to the mammary glands or animals. Although no adverse effects were noted in transfected mammary glands or the lambs nursing from them, lambs were observed to preferentially nurse from untransfected glands.

In a subsequent experiment, one mammary gland of a fourth nulliparous ewe was flushed with isotonic sucrose/HEPES for three consecutive days in an effort to further aid in the removal of viscous prepartum secretions. This mammary gland was then infused (prepartum) with the same ordered charge-shifting transfection complex as the previous three ewes. However, no hGH was detectable in the milk of this ewe either (results not shown).

**Distribution of FITC-Orn<sub>n</sub> incorporated into ordered charge-shifting mixed polyions infused in the ovine mammary gland.**

To assess whether intramammary infusion of ordered charge-shifting mixed polyions delivered transfection complexes to most or all mammary epithelial cells, a prepartum ovine mammary gland was infused with ordered charge-shifting mixed polyions containing fluorescently labeled Orn<sub>n</sub> [pEGFP-N1 DNA:DEAE:Glu<sub>n</sub>-HisN:F-Orn<sub>n</sub>]. Intramammary infusion was performed 10 days prior to the expected parturition date since the ewe was being utilized in another study and was to be sacrificed 2 days perpartum. Mammary tissue samples were obtained upon sacrifice of the animal (2 days perpartum), fixed in 10% zinc formalin, sectioned, and sections were either stained with
hematoxylin/eosin or left unstained to allow visualization of mammary alveoli and fluorescent transfection complexes, respectively.

Lipid-engorged mammary alveoli, typical of prepartum mammary glands, were observed in hematoxylin/eosin stained parenchymal sections (Fig. 7.9A). The alveolar lumen were observed to contain numerous small spheres resembling lipid droplets (Fig. 7.9A, arrows), and larger spheres which appeared to fluoresce (Fig. 7.9A, arrowheads). Unstained mammary sections showed the alveolar lumen to contain large spheres which fluoresced intensely (Fig. 7.9B, arrowhead) and many secretory epithelial apical cell surfaces which appeared to be coated with fluorescent label (Fig. 7.9B, filled arrow), suggesting transfection complex binding to the mammary apical cell surface. There were also mammary apical cell surfaces which did not appear to be coated with fluorescently labeled transfection complexes (Fig. 7.9B, open arrows) suggesting that not all cells were binding transfection complexes.

Due to the limited availability of timed off-seasonally bred animals, there was not an uninfused control for this experiment to rule out autofluorescence since the contralateral gland was infused with TR-Orn incorporated into ordered charge-shifting mixed polycation transfection complexes containing pEGFP-N1 to allow covisualization of transfection complex distribution and GFP expression. TR-Orn-labeled transfection complexes (not shown) showed the same pattern of distribution as FITC-labeled transfection complexes (Fig. 7.9B). Additionally, no GFP fluorescence was observed.
Figure 7.9. Intramammary distribution of fluorescently labeled Orn incorporated into ordered charge-shifting mixed polyion transfection complexes. A 10 day prepartum ovine mammary gland was infused with ordered charge-shifting mixed polyions containing fluorescently labeled Orn. Mammary tissue was obtained two days prepartum (8 days post-infusion), sectioned, and stained with hematoxylin/eosin (Panel A) or left unstained (Panel B) for visualization of fluorescently labeled transfection complexes. Panel A: lipid-engorged alveoli with lumen containing small spheres resembling lipid droplets (arrow) and larger spheres which appeared to fluoresce (arrowhead). Panel B: alveolar lumen containing large spheres which fluoresced intensely (arrowheads) and secretory epithelial cell surfaces which appeared coated (filled arrow) or uncoated (open arrow) by fluorescently labeled transfection complexes. (Magnification 200x).
The infusion of fluorescently labeled transfection complexes in the ovine mammary gland suggests that some, but not all mammary epithelial apical cell surfaces are targeted by transfection complexes (Fig. 7.9B). Furthermore, it appears that transfection complexes aggregated into large fluorescent spheres within the alveolar lumen (Fig. 7.9B) which likely precluded their interaction with the apical cell surface.
DISCUSSION

Initial attempts at transfecting bovine mammary glands in vivo failed to give detectable recombinant gene expression, but infusion of mixed polyions did not impair appetite, nor result in significant mammary gland swelling or irritation. Although not specifically tested, there was no evidence of inflammation or white cell influx indicative of an immune response to intramammary infused transfection components, even after repeated infusion of complexes in the same animal. Furthermore, although milk production was temporarily inhibited (Fig. 7.1), it recovered to normal levels within 48 hours. Similar observations of the non-inflammatory, non-toxic, and non-immunogenic nature of polyion infusion were made in both sheep and guinea pigs. Therefore, infusion of mixed polyions does not appear to unduly irritate mammary tissue or trigger systemic inflammatory reactions and will likely prove safe for use in food animals.

The only apparent effect on animal health was a fever of 106.2° F following one intramammary infusion of cow 3485 accompanied by mild lethargy. This febrile response after the second of three infusions, was only noted following this one infusion and may have been a result of low residual endotoxin contamination in the purified DNA.

An attempt to target polyion transfection complexes to mammary epithelial cells with concanavalin A also appeared ineffective in vivo in that no hGH expression was observed in milk following intramammary infusion of transfection complexes containing con-A and mannosylated Orn$_n$. 

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The initial transfection of a guinea pig mammary gland with ordered mixed polyions gave low level (≤5 ng hGH/ml) but readily detectable recombinant gene expression (Fig. 7.4) clearly distinguishable from controls (Fig. 7.4). This was the first demonstration of non-viral-mediated recombinant gene expression in milk following direct intramammary infusion of recombinant transgenes in any species. Archer et al. (1994) previously reported hGH expression in caprine milk following intramammary infusion of replication-deficient retroviral vectors. However hGH expression peaked on day one of lactation (118 ng hGH/ml) and rapidly declined to 12 ng/ml by day three of lactation. In contrast hGH expression in guinea pig milk persisted (and often increased) for the duration of lactation (14 days) (Fig. 7.4).

Optimal polyion and DNA concentrations for transfection of the guinea pig mammary gland in vivo (Fig. 7.5) were five-fold higher than for optimum transfection of primary bovine mammary cells in vitro (Fig. 4.12 and 4.13), and substantially increased hGH expression in milk to 40 ng/ml (Fig. 7.5). Although not directly demonstrated, the increase in transfection complex concentration required for optimal in vivo expression may be due to cationic transfection complexes interacting with anionically charged milk proteins, which precludes transfection complex interaction with the mammary apical cell surface and subsequent transfection. Therefore higher concentrations of transfection complexes are required to saturate the anionic proteins present in milk prior to transfection complex binding to the mammary cell surface.
Transfection of guinea pig mammary glands in vivo with polycations alone [DEAE:DNA], [Orn\_n:DNA]; mixed polycation [Orn\_n : DEAE:DNA]; or unordered [Orn\_n:Glu\_n-HisN:DEAE:DNA] and ordered charge-shifting mixed polyion [DNA:DEAE:Glu\_n-HisN:Orn\_n] transfection complexes indicated that the primary bovine mammary cell culture system was an effective yet incomplete model for the development of techniques to transfect guinea pig mammary glands in vivo. The failure of mixed polycations and unordered charge-shifting mixed polyions to give high level recombinant hGH expression in vivo (Fig. 7.7), coupled with the high level hGH expression seen with either DEAE alone or ordered charge-shifting mixed polyions in vivo (Fig. 7.7), suggests formation of transfection complexes with DEAE as the innermost component of transfection complexes is essential for efficient polycation:DNA dissociation and exposure of DNA for transcription.

Glu\_n-HisN appears to be essential for high level recombinant gene in vivo, just as for primary bovine mammary cells transfected in vitro since mixed polycations alone failed to give high level expression in transfected guinea pig mammary glands in vivo (Fig. 7.7). Surprisingly, transfection of the prepartum guinea pig mammary glands with all polyion complexes tested gave recombinant hGH concentrations in milk that persisted for the duration of a normal guinea pig lactation (Fig. 7.7). Interestingly, additional studies showed that every guinea pig (>16) transfected with mixed polyions by direct intramammary infusion gave readily detectable or even high levels of recombinant hGH expression in milk, regardless of whether they were infused mid-pregnancy,
pre-partum, or early post-partum (I. Mather and J. Hens personal communication, 1996). However, more recombinant hGH expression was detectable in the milk of guinea pigs transfected 2 days prepartum than those infused at any other time. This may be due to rapid proliferation of mammary epithelium coincident with parturition in guinea pigs (Larson, 1985), as opposed to the longer period of mammary development during gestation in sheep and cattle. Alternatively, the lack of hGH expression in milk of transfected sheep and cattle may be related to differences in total protein concentrations in prepartum mammary secretions. Pre-transfection flushing of the bovine and ovine mammary gland did not facilitate recombinant hGH expression in milk. Either flushing with sucrose/HEPES or HBSS was inadequate to remove prepartum secretions, or other factors such as the rate of mammary development are more critical for in vivo mammary transfection. Prepartum ovine and bovine mammary secretions are very viscous due to high protein content and may prevent mixed polyanion transfection complex access to the target mammary epithelium. In interesting contrast, autopsy of a 5-7 day prepartum guinea pig revealed no visible secretion in the mammary tissue (M. Amstutz, personal observation).

Regardless, successful hGH expression in the guinea pig mammary gland in vivo now provides an excellent model with which to study in vivo mammary transfection, including transfection and expression efficiency as well as intramammary fate of infused mixed polyanion complexes. Initial examination of the intracellular fate of fluorescently labeled transfection complexes in the ovine mammary gland suggests that some but not
all mammary apical cell surfaces have bound transfection complexes. Transfection complexes also appeared to aggregate within the alveolar lumen, which when coupled with the incomplete transfection complex binding to the mammary cell apical surface, suggests that inadequate cell targeting may be responsible for the failure of expression in bovine and ovine mammary glands.

Studies are continuing in an effort to determine intramammary and intracellular fate of fluorescently labeled transfection complexes following in vivo guinea pig mammary gland transfections. Additionally, in vivo transfections utilizing the fluorescent GFP reporter protein are also being conducted to determine the number and types of cells responsible for recombinant protein production in milk. These studies should help identify limitations to polion-mediated in vivo transfection. Rational strategies can then be designed to facilitate more effective polion-mediated intramammary gene transfer.

Of particular concern is the large amount of DNA used for in vivo polion-mediated transfection. The optimized guinea pig transfection protocol utilizes 50 μg DNA/gland (2 ml), which when adjusted to one liter, the amount required to infuse one bovine mammary gland equates to 25 mg DNA/quarter or 1 gram/cow! Although large (mg) quantities of DNA have been prepared here for transfection of bovine mammary glands, the production of larger amounts of DNA will likely only be feasible for expression of highly valuable recombinant pharmaceutical proteins.

In conclusion, this is the first successful demonstration that mammary tissue can be transfected in vivo by direct intramammary infusion of recombinant expression vectors.
with production of recombinant proteins in milk without the use of retroviral vectors. Although this study was unable to demonstrate successful recombinant gene expression in the milk of large ruminants, the infusion of charge-shifting mixed polyions into the mammary glands of all animals tested appeared relatively benign and did not elicit undue irritation or inflammation. Additionally, every guinea pig transfected with polyions gave readily detectable levels of recombinant gene expression in milk postpartum, with some doing so when transfected several weeks prepartum. This contrasts dramatically with the lack of expression after intramammary transfection with a commercially available cationic lipid transfection method (Lipofectamine) which proved totally ineffective in the guinea pig mammary gland in vivo (I. Mathers and J. Hens, personal communication, 1995). Furthermore, the transfection components described in these studies are stable enough to withstand shipment and the procedures are simple enough to be implemented by others with minimal instruction, as would be required for field applications. Most importantly, this study described successful polycation-mediated transfection of the guinea pig mammary gland in vivo which should facilitate the development of even more efficacious intramammary gene transfer methods for large animals.
Chapter 8

DISCUSSION

This study describes the development of the first non-viral recombinant gene transfer method to produce recombinant proteins in milk by direct intramammary infusion of transfection complexes containing recombinant gene expression vectors. Recombinant transgene delivery and expression was accomplished first by a mixed polycation:DNA transfection complex, and later by a novel charge-shifting polyion:plasmid DNA transfection complex, developed in vitro using a mouse mammary cell line (Comma-D) (Danielson et al., 1989) and a primary bovine mammary cell culture system (Talhouk et al., 1990; Talhouk et al., 1992). The polyion transfection system developed here has proven to be the most effective means for transfection of primary mammary cells in vitro. Recombinant gene expression level was markedly affected by polyion transfection complex composition both in vitro (Chapter 4) and in vivo (Fig. 7.7). This simple and inexpensive polyion transfection system showed the highest recombinant protein production in milk of any non-transgenic transfected animal reported to date. However, recombinant protein expression in milk was limited to the rodent mammary gland. The limitations to polyion-mediated transfection of ruminant mammary glands are profound and not understood. Neither is it clear why virtually all cells internalize transfection
complexes yet only very few cells actually express the transfected transgenes. The limitations to polyion-mediated transfection appear to differ in vitro and in vivo. Polyion-mediated transfection in vitro appears limited by escape of DNA from endosomes, nuclear entry of DNA, or both, while in vivo polyion-mediated gene transfer appears limited by inefficient transgene delivery to mammary epithelial secretory cells.

Recombinant gene expression levels can likely be improved in vitro by facilitating polycation:DNA dissociation within the cell, including endosomolytic agents to facilitate DNA escape from endosomes, and episomal replication competent plasmid systems. In vivo transfection will also likely benefit from the aforementioned considerations, but first appears to be limited by inefficient transgene delivery which may be overcome with ligand-specific targeting.

In vitro development of polyion-mediated transfection

Transfection of cultured primary mammary cells with individual polycations did not produce high levels of recombinant gene expression (Figs. 4.3 and 4.6), consistent with the results of others (Malienou-Ngassa et al., 1990). However, the subcellular distribution of plasmid DNA transfected with polycations suggested that individual polycations possess unique properties for cell targeting and gene expression which when combined might lead to increased recombinant gene expression (Fig. 4.3). Combinations of polycations [Orrin:DEAE] doubled recombinant gene expression in primary bovine mammary cells over expression from individual polycations alone (Fig. 4.6). The use
of mixtures of polycations, which appear to retain individual polycation characteristics, to facilitate recombinant gene expression has not been previously reported in the literature.

A novel charge-shifting polyion poly(glutamylhistamineglutamate) Glu\textsubscript{n}-HisN, described by Shen (1990) to enhance ligand targeted toxin delivery and efficacy to cells in vitro, was added to mixed polycation transfection complexes to facilitate dissociation of strong polycation:DNA interaction, and increased recombinant gene expression in primary bovine mammary cells five fold over mixed polycation transfection alone (Fig. 4.9). The unique charge-shifting character of Glu\textsubscript{n}-HisN (polyanionic at pH > 6.2, neutral at pH < 6.2>4.5, and polycationic at pH < 4.5) should disrupt polycation:DNA interactions upon sequential protonation of the derivatized imidazole of histamine and the free γ-carboxyl of Glu\textsubscript{n} in acidifying intracellular endosomes and/or lysosomes. This effect was specific to Glu\textsubscript{n}-HisN since substitution of underivatized poly-L-glutamic acid or free histamine for Glu\textsubscript{n}-HisN did not increase recombinant hGH expression over mixed polycations alone (Fig. 4.15). However, centrifugal subcellular fractionation studies indicated that Glu\textsubscript{n}-HisN did not increase recombinant gene expression by facilitating internalization of plasmid DNA into the nucleus (Fig. 4.10), and therefore must be acting by some other mechanism(s). The buffering capacity of Glu\textsubscript{n}-HisN (Fig. 5.24) may contribute to enhanced gene expression by protecting internalized plasmid DNA from degradation by intracellular nucleases within acidifying endosomes or
lysosomes. A similar endosomal buffering effect has been described for the cationic polyethylenimine (Abdallah et al., 1995; Behr, 1994).

Further controlling the order of polyion addition to the DNA and formation of polyion:DNA transfection complexes in 0.3 M sucrose, 10 mM HEPES, pH 7.4, increased expression another two fold over that from unordered charge-shifting polyions alone (Fig. 4.11). Controlling the order of polyion addition allowed placement of a weaker polycation (DEAE) next to the plasmid DNA, followed by a layer of Glu-N-HisN which should facilitate polycation:DNA dissociation, and a final outer layer of Orn to provide efficient cellular targeting. Thus, it appears that specific transfection complexes can be formulated, possibly even for tissue specific transfections, by controlling the order of polyion addition to the DNA, effectively controlling transfection complex formation.

Moreover, a synergistic effect was noted when ordered charge-shifting polyion transfection complexes were formed in isotonic sucrose/HEPES as opposed to HBSS (Fig. 4.11). The increase in hGH expression from formation of transfection complexes was specific to sucrose, the only nonreducing sugar tested (Fig. 4.12), suggesting either that other nonreducing sugars may have similar nonspecific effects, or that the specificity for sucrose might be due to receptor-mediated specificity and events, probably at intracellular levels. Lieber et al. (1987) showed that manipulation of intracellular and endosomal events can enhance transfection in avian erythrocytes.

Despite the high level of recombinant gene expression attained with the charge-shifting mixed polyion transfection complexes in mammary cells, very few cells were
found to actually express a recombinant β-galactosidase transgene (Figs. 5.2, 5.3). Similar results were found in cell lines capable of extrachromosomal plasmid replication and high level gene expression (Fig. 5.4). Collectively, these results suggested that the high level expression observed following polyion-mediated transfection is probably a result of very few cells expressing extremely high levels of recombinant protein as opposed to broad low level expression from a larger number of cells. Since all cells were found to contain transfection complexes, but only a few showed expression, tremendous potential thus exists for extremely high expression if all cells could be made to express recombinant proteins at similar high levels.

Investigation of the limitations to polyion-mediated transfection

Investigation of the limitations to polyion-mediated transfection in vitro with fluorescently labeled polycations indicated that free polycations are rapidly internalized by virtually all mammary cells and preferentially concentrated in the nucleus (Fig. 5.5). The mode of polycation entry into cells is still unknown. However, the generally accepted method of polycation entry into cells is via nonspecific endocytosis (Kabanov and Kabanov, 1995; Marciano-Cabral et al., 1981). Although the mechanism of polycation escape from endosomes also remains elusive, polycations are known to promote fusion of lipid membranes under appropriate (acidic) conditions, such as might be found in intracellular endosomes (Walter et al., 1986) which may account for their exit from intracellular endosomes.
Nuclear localization signals (NLS) which direct protein transport into the nucleus (Feldherr and Akin, 1990) typically consist of short tracts of 5-10 basic amino acids (Davis, 1992). Therefore, polymers such as Om\textsubscript{n} composed entirely of basic amino acids, might be expected to mimic a nuclear localization signal. The rapid internalization and nuclear localization of polycations suggested that polycations should efficiently deliver recombinant transgenes to the nuclei of all mammary epithelial cells.

Internalization of fluorescently labeled polyion-DNA transfection complexes did not show the same definitive nuclear localization (Fig. 5.6) seen with fluorescently labeled polycations alone (Fig. 5.5). Instead, transfection complexes appeared to aggregate into clusters of small fluorescent foci which concentrated in the nuclear or perinuclear cytoplasmic regions of virtually all cells (Fig. 5.6). Similar aggregation of internalized polycation-DNA transfection complexes in intracellular membrane bound vesicles has been reported previously by others (Marciano-Cabral et al., 1981). Although light microscopy did not permit sufficient resolution for definitive distinction between intranuclear and perinuclear localization, transfection complex aggregates appeared predominately perinuclear, which is consistent with observations by Marciano-Cabral et al. (1981) and with descriptions of endosome entrapment of internalized DNA (Zabner et al., 1995). Nevertheless, the fact that virtually all cells internalized fluorescently labeled transfection complexes indicated that transgene delivery to cells was not limiting for transfection and expression in vitro. However, it remains a puzzle as to why very few cells express the transfected transgene if virtually all cells can internalize transfected
transgenes and localize them to the perinuclear region. The subcellular localization of internalized fluorescently labeled transfection complexes in conjunction with DAPI stained DNA to allow visualization of nuclei indicated internalized transfection complexes aggregated asymmetrical in the perinuclear region of most cells (Figs. 5.10 and 5.13). Thus, it appeared that internalized transfection complexes had either become trapped in endosomes, or were too large to enter the nucleus by passage through the nuclear pore.

Examination of charge-shifting mixed polyion transfection complexes with an electron microscope indicated that charge-shifting polyion complexes were amorphous and ranged in size up to 200 nm in diameter. Transfection complexes of such large size would not be expected to enter the nucleus since the nuclear pore can only accommodate molecules of about 25 nm in diameter (Feldherr and Akin, 1990) depending on the number and effectiveness of the specific nuclear localization signals.

Synthesis and addition of a human rhinovirus lytic peptide (HRV2 LP), known to facilitate escape of ligand transfected DNA from endosomes and enhance recombinant gene expression 500 fold (Zauner et al., 1995), to ordered charge-shifting mixed polyions increased recombinant hGH expression two fold over that from charge-shifting mixed polyions alone in primary bovine mammary cells transfected in vitro (Fig. 5.20). However, mass spectral analysis indicated that the synthetic peptide was incomplete and consisted of only 8 or 9 amino acids from the C-terminus of the full 25 amino acid peptide (Chapter 5), suggesting previously unsuspected failure of peptide synthesis.
Nevertheless, the doubling in recombinant gene expression from the addition of even an incomplete lytic peptide suggests that DNA escape from endosomes is probably a significant limitation to polyion-mediated transfection in vitro, and that synthesis and incorporation of a full length lytic peptide has the potential to dramatically increase recombinant gene expression from polyion-mediated transfection of mammary cells. Thus, it appears the major limitations to polyion mediated transfection and expression of recombinant transgenes in mammary cells in vitro are DNA escape from endosomes and entry of DNA into the nucleus.

The visualization of internalized fluorescently labeled transfection complexes also gave rise to an interesting paradox. Centrifugal subcellular fractionation studies (Fig. 4.3) showed that internalized radiolabeled plasmid DNA was predominately localized in the nucleus. In contrast, fluorescently labeled transfection complexes concentrated in the perinuclear region of most cells (Fig. 5.6) suggested that plasmid DNA was not entering the nucleus. Furthermore, microrautoradiography (Fig. 5.8) supported the subcellular distribution seen with fluorescently labeled transfection complexes (Fig. 5.6), suggesting that internalized DNA never entered the nucleus. The lack of nuclear internalization is also consistent with the observation that very few cells expressed transfected transgenes (β-galactosidase) (Figs. 5.4), even though virtually all cells show internalized transfection complexes (Figs. 5.10 and 5.13). The incongruity is probably best explained by the aggregation properties of polyion:DNA transfection complexes as described above and by Marciano-Cabral et al. (1981), the size of the aggregates, and their probable specific
gravity and proximity to the nucleus. Collectively, these data suggest that studies of the subcellular fate of transfection complexes or transfected DNA by cell lysis and centrifugal subcellular fractionation may be inaccurate due to cosedimentation of polycation transfection complexes and cell nuclei.

An even distribution of fluorescently labeled transfection complexes was also visualized attached to plastic cell culture plates in areas lacking cell growth. This observation allowed the development of an in vitro polycation:DNA dissociation assay. This in vitro dissociation assay was used to show: 1) polycation:DNA dissociation was pH- and salt dependent; and 2) protonation of the $\gamma$-carboxyl of Glu$_n$ or Glu$_n$-HisN was responsible for the pH-dependent polycation:DNA dissociation. Interestingly, although protonation of the $\gamma$-carboxyl of Glu$_n$ facilitated polycation:DNA dissociation in vitro (Fig. 5.25), histamine derivatization of Glu$_n$ was required for increased recombinant gene expression in primary bovine mammary cells (Fig. 4.15), suggesting an essential cooperative intramolecular interaction between the histamine imidazole and the $\gamma$-carboxyl of Glu$_n$.

**Gene expression systems to enhance recombinant protein production.**

A eukaryotic expression vector (pCEP4/hGH) encoding the EBNA1 protein and EBNA oriP, capable of episomal replication in primate and canine cells (Yates and Guan, 1991; Yates et al., 1985), was unable to extend the duration of high level recombinant gene expression in bovine mammary cells transfected in vitro (Fig. 6.24) and in vivo (Fig.
7.1). Interestingly, hGH expression from pCEP4/hGH was roughly half that from pcDNA1/hGH in vitro, despite expression being driven from the same CMV promoter (Fig. 6.24). Because primary bovine mammary cells transfected with pCEP4/hGH did not produce high levels of recombinant hGH, and persistent expression was at extremely low levels, and mammary cells cannot be replated to verify episomal replication, no further attempt was made to document episomal replication for that plasmid.

Episomal replication may exhibit species specificity for cellular replication machinery (Wysokenski and Yates, 1989). Murakami et al. (1986) demonstrated that the DNA polymerase α-primase complex isolated from HeLa or monkey cells could restore replication in replication-depleted extracts in vitro, whereas DNA polymerase α-primase complexes isolated from either mouse or calf thymus could not. Therefore, EBV replication may be limited by species specific interactions between cellular replication machinery and the EBNA1 protein which preclude replication in rodent and bovine cells. Consistent with such species limitations, Henkel et al. (1994) subsequently showed that EBV-based vectors are unable to replicate episomally in bovine cells. Thus, EBV-based extrachromosomal plasmid replication will likely not be capable of extending the duration of high level recombinant gene expression in bovine mammary cells.

Contrary to expectation, the creation of a secretable fluorescent reporter protein as an alternative to hGH was not straightforward. Neither an hGH secretion signal-GFP fusion protein nor a full length hGH-GFP chimera was sufficient for high level secretion of GFP (Fig. 6.26). The lack of GFP secretion was intriguing since the hGH secretion
signal is sufficient for high level hGH secretion in a number of cell types (Chapters 4 and 5). Although higher levels of GFP were detected in the medium of COS-1 cells transfected with native pEGFP-N1 which lacks a secretion signal (Fig. 6.26), this extracellular GFP likely was not actually secreted, but instead was probably sequestered cytoplasmic GFP released upon spontaneous lysis of GFP-expressing cells since COS-1 cells containing large amounts of GFP were observed to round up and detach from cell culture plates (Chapter 6).

Northern hybridization analysis of GFP and modified-GFP mRNA indicated that mRNA transcripts were of appropriate size to encode an hGH secretion signal-GFP fusion protein and a full length hGH-GFP chimera (Fig. 6.27). Additionally, ELISA analysis of spent COS-1 cell culture medium indicated that the hGH-GFP chimera was detectable with both anti-hGH and anti-GFP antibodies, indicating the presence of both hGH and GFP antigenic epitopes suggesting cellular synthesis of the full length hGH-GFP chimera. Thus, the low amount of modified GFP that was secreted did not appear to be from improper cloning construction or translation initiation at the downstream GFP start codon.

There has been only one other report of recombinant GFP secretion. Kaether and Gerdes (1995) created a C-terminal human chromogranin B-GFP fusion protein and monitored GFP chimera secretion in HeLa cells by temperature arrest. Others have had difficulties similar to those described here in generating a secretable form of GFP (Henry Rascoff, personal communication, 1996). The difficulty of GFP secretion may be related to three large hydrophobic regions (Phe_8 to Gly_20), (Leu_42 to Phe_71), and (Val_219 to His_231)
predicted within GFP from independent Kyte-Doolittle, Hoop-Woods, and Eisenberg hydropathy analysis. Large hydrophobic protein regions, particularly those predicted to form \(\alpha\)-helices or \(\beta\)-sheets flanked by charged amino acids such as found in GFP, can sometimes either integrate or associate with lipid membranes (Yeagle, 1989). Thus, one or more of the hydrophobic regions of GFP may insert into or associate itself with a lipid membrane to impede secretion.

Attempts to increase recombinant gene expression by prokaryotic amplification of expression, which should not be subject to eukaryotic gene regulation, appeared limited by the amount and stability of T7 RNA polymerase produced. Cotransfection of expression vectors encoding an NLS-modified T7 RNA polymerase driven from the CMV promoter, and a T7-dependent hGH reporter construct containing an internal ribosome entry site (IRES) were not sufficient for high level expression in either primary bovine or COS-1 cells (Chapter 6). Furthermore, the low level expression observed in COS-1 cells may not have been attributable to T7 RNA polymerase at all since Lieber et al. (1993) showed that host cell polymerases (probably RNA polymerase II) can drive expression from the T7 promoter. Northern hybridization analysis indicated that only low levels of T7 RNA polymerase mRNA (only detectable at excessive loading of mRNA) were produced in COS-1 cells transfected with T7 RNA polymerase expression vectors. The low level of T7 RNA polymerase mRNA transcript production may be due to detrimental influences of nucleotide sequences within the T7 RNA polymerase gene, or the 5' and 3' untranslated regions acquired during subcloning, since native prokaryotic
genes do not inherently encode 5' and 3' untranslated regions (UTR's) like eukaryotic genes. Intragenic sequences as well as 5' and 3' UTRs of bovine lactoferrin were found to act individually and to interact to dramatically impact mRNA transcript production level and half-life (Schanbacher et al., 1997). Furthermore, Schanbacher et al. (1997) showed that these mRNA regions may interact to influence mRNA stability and mRNA level.

Thus, it appears likely that T7 RNA polymerase-dependent hGH expression was first limited by the low level of T7 RNA polymerase mRNA production regardless of whether it might also have been limited by inefficient translation of uncapped hGH mRNA transcripts.

Direct intramammary infusion of polyion:DNA transfection complexes

Intramammary infusion of charge-shifting mixed polyion transfection complexes into the bovine mammary gland failed to give recombinant hGH expression in milk (Fig. 7.1 and 7.3). The type of transfection complex formed (ordered, unordered, or ligand targeted with concanavalin A) and the type of transfection solution (HBSS or sucrose/HEPES) had no effect on expression (Fig. 7.1 and 7.3). However, these intramammary infusions demonstrated that large volumes of isotonic solutions could be infused into the mammary gland without adverse effects and that polycations did not irritate the mammary gland or elicit a noticeable inflammatory response. The difficulty of hGH expression in milk from the bovine mammary gland coupled with the large DNA
requirements for bovine intramammary infusions compelled consideration of alternatives for an in vivo model for development of intramammary gene transfer methods.

Guinea pig mammary glands transfected with ordered charge-shifting mixed polyion transfection complexes gave readily detectable hGH expression in milk which persisted for the duration of a normal guinea pig lactation (14 days) (Fig. 7.4, 7.6, and 7.7). Optimal hGH expression in milk required transfection complex concentrations five times higher than that found optimal for in vitro primary bovine mammary cell transfection (Fig. 7.5), and also required infusion of transfection complexes about two days prepartum. Although prepartum intramammary infusions gave the most consistent expression in milk, all transfected guinea pigs, whether infused mid-pregnancy, two days prepartum, or in lactation, gave detectable recombinant hGH expression in milk which routinely persisted for the duration of lactation (I. Mather and J. Hens, personal communication, 1996). The persistence of hGH expression in vivo (Fig. 7.6 and 7.7) is consistent with polycation-mediated persistent hGH expression in vitro (Fig. 4.9), since polyion-mediated transfection did not give the typical transient expression profile, but rather extended expression to 10-14 days post transfection in vitro.

Ordered charge-shifting mixed polyion transfection gave higher recombinant hGH expression in primary bovine mammary cells in vitro (Fig. 4.9) and the guinea pig mammary gland in vivo (Chapter 7) than transfection with commercially available cationic lipids Lipofectin, and Lipofectamine, respectively.
Comparison of the effect of varying the polyion composition in transfection complexes on relative expression efficiency in primary bovine mammary cells in vitro versus guinea pig mammary glands in vivo showed that only DEAE and the ordered charge-shifting polyion complex gave high level recombinant hGH expression in vivo (Fig. 7.7). By comparison, Orm₆ alone, mixed polycations, and an unordered charge-shifting polyion complex all gave low level expression in vivo (Fig. 7.7). The effect of DEAE was entirely unpredictable since transfection of cultured primary rabbit (Malienou-Ngassa et al., 1990) and primary bovine mammary cells (Fig. 4.9) with DEAE gave only low level hGH expression. Furthermore, transfection of Comma-D mouse and primary bovine mammary cells in vitro with high polycation concentrations (>100 µg/ml) which were lower than those used for the guinea pig mammary gland in vivo (1.25 mg/ml) caused extensive cell injury, vacuolization, and probable cell death, since cells rapidly detached from the culture plate. Higher concentrations of DEAE, such as used here in vivo, may saturate the anionic milk proteins present in the mammary gland and still leave enough excess transfection complexes in solution not complexed with anionic milk proteins for charge-attraction to and transfection of mammary epithelia.

Unlike prepartum transfection of guinea pig mammary glands, prepartum transfection of ovine mammary glands did not produce recombinant hGH in milk (Chapter 7). Furthermore, flushing of the ruminant mammary gland with HBSS (bovine) or sucrose/HEPES (ovine) either did not remove sufficient anionic milk proteins to prevent competitive interference with targeting of complexes to cell surfaces, or suggests
that anionic milk proteins are not responsible for the lack of hGH expression in ruminant milk. Instead, other factors such as the rapid prepartum proliferation of the guinea pig mammary gland compared to the much slower prepartum proliferation of ruminant mammary glands, may be important for in vivo transfection and expression. The guinea pig mammary gland undergoes rapid proliferation in late gestation and early lactation (Larson, 1985), coincident with the optimal transfection time period (Chapter 7). Unlike the guinea pig, bovine and ovine mammary glands develop over a longer period of time during gestation with little mammary epithelial cell proliferation postpartum (Schmidt, 1971).

It is quite possible that cell division is required for efficient recombinant transgene expression following polyion-mediated transfection. Polyion:DNA complexes were shown here (Figs. 5.15-5.18) and by others (Eickbush and Moudrianakis, 1978; Laemmli, 1975; Shapiro et al., 1969) to compact DNA into torroids or rods small enough to be internalized in endosomes. These DNA:polycation complexes are much to large to pass through the nuclear pore. However, it is quite possible that cell division, which results in temporary nuclear membrane disruption, is required for passage of larger polycation:DNA transfection complexes into the nucleus. Cell division is reported to greatly enhance or be required for nuclear deposition of foreign DNA following viral infection (Humphries and Temin, 1974; Miller et al., 1992). This hypothesis is consistent with the low number of mammary cells expressing detectable β-galactosidase (Fig. 5.2 and 5.3), and the low level of mammary epithelial cell proliferation in vitro (Talhouk et
Furthermore, cell lines which divide rapidly (COS-1, Comma-D) showed more β-galactosidase and GFP expressing cells than slow proliferating primary bovine mammary cells.

The proposed requirement for mammary proliferation should be testable in the ruminant mammary gland in vivo by hormonal induction of the mammary gland (Schanbacher and Smith, 1973) to stimulate mammary epithelial cell proliferation in conjunction with intramammary infusion of polyion:DNA transfection complexes.

Initial examination of the intramammary distribution of fluorescently labeled polyion transfection complexes indicated that in vivo mammary transfection may be limited by inefficient cell targeting since large aggregates of fluorescently labeled transfection complexes were observed in the alveolar lumen. Nevertheless, fluorescently labeled transfection complexes were observed attached to some mammary epithelial apical cell surfaces. Future studies utilizing fluorescently labeled polyion transfection complexes in conjunction with the cytosolic GFP reporter protein should allow determination not only of the fate of intramammary transfection complexes following infusion, but also the transfection complex subcellular distribution, and determination of the number and types of cells expressing recombinant proteins.

These studies have also shown that intramammary infusion of polyion:DNA transfection complexes does not impair appetite, nor produce significant mammary gland swelling or irritation, or incite obvious allergenic responses in transfected bovine, ovine, or guinea pig mammary glands. Although not specifically tested, it is important to note...
that infusion of polyion transfection complexes did not appear to elicit an immune response, despite repeated infusion of transfection complexes into the same animal. This suggests that intramammary infusion of polyion:DNA transfection complexes should be safe for use in food animals.

In conclusion, this study described the development of the first non-viral recombinant gene transfer method to produce recombinant proteins in milk following direct intramammary infusion of recombinant expression vectors. The charge-shifting polyion transfection system developed here is stable enough to withstand shipment, simple enough to be used with minimal instruction, and has proven to be the most effective means for transfection of mammary cells in vitro and in vivo. The in vitro and in vivo limitations to polyion-mediated transfection appear to be different. In vitro polyion-mediated transfection appears limited by escape of DNA from endosomes, nuclear entry of DNA, or both, while in vivo polyion-mediated gene transfer appears limited by inefficient transgene delivery to mammary epithelial secretory cells. Neither is it clear why virtually all cells receive transfection complexes yet very few cells actually express the transfected transgenes. Further development of the in vivo guinea pig expression model should provide additional opportunities for more precise definition of the limitations of polyion-mediated transfection in vivo. Identification of in vivo gene transfer limitations should allow development of rational strategies to overcome these barriers and further increase recombinant gene expression in milk and allow expression in the ruminant mammary gland and milk. The dramatic 10-fold elevation of gene
expression after transfection of DEAE:DNA complexes, together with the very high expression of recombinant GFP in the few cells actively expressing, suggests dramatic potential for this system to be improved even more to achieve high level expression in milk sufficient to be economically important to the dairy industry, or for gene therapy.
LIST OF REFERENCES


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