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Characterization of an oncofetal protein (OFP) and cloning of its cDNA

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The Ohio State University, 1991
CHARACTERIZATION OF AN ONCOFETAL PROTEIN (OFP) AND CLONING OF ITS cDNA

DISSERTATION

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

By

Steven William Runge, B.S.

The Ohio State University
1991

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Dissertation
Dept. of Medical Biochemistry
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1991
To all the members of my family
ACKNOWLEDGEMENTS

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LIST OF VECTORS AND BACTERIAL STRAINS

DH5αF'  F sup E44 Δ lac U169 (φ80 lac Z ΔM15)
         hsd R17 rec A1 end A1 gyr A96 thi -1 rel A1

λgt11  λlac 5 Δ shn dIIIλ2-3 srIλ 3°
        cItS 857 sr Iλ4° nin 5 sr Iλ5° Sam100

Y1089  ara D139 Δlac U169 pro A+ Δlon rps L
        hfl A150[chr :: tn10 (tetR)] pMC9

Y1090R- sup F hsd R ara D139 Δlon Δ lac U169
         rps L trp C22 :: Tn10 (tetR) pMC9

pUC19  (see figure 3.5)
LIST OF ABBREVIATIONS

#; number
% ; percent
(NH₄)₂SO₄ ; ammonium sulfate
2-D ; two dimensional
3' ; three prime
³H ; tritium
5' ; five prime
5' -> 3' ; five prime to three prime
16-mer ; 16 base oligonucleotide
17-mer ; 17 base oligonucleotide
24-mer ; 24 base oligonucleotide
³²P ; Phosphorus 32
[α-³²P]dATP ; alpha-phosphorus-32, deoxyadenosine triphosphate
[α-³⁵S]dATP ; alpha-sulfur-35, deoxyadenosine triphosphate
[γ-³²P]ATP ; gamma-phosphorus-32, adenosine triphosphate
[IgG(H+L)]-HRP ; Immunoglobulin G (heavy + light chains) - Horseradish peroxidase conjugate

A ; Absorbance
ADP-ribose ; Adenosine diphosphate - ribose
AFP ; Alphafetoprotein
AIDS ; Acquired Immunodeficiency Syndrome
ala ; Alanine
amp ; Amperage
Anti-OFP ; Anti-oncofetal protein antibody
arg ; Arginine
ASN ; Asparagine
asn ; Asparagine
ASP ; Aspartic acid
ATP ; Adenosine triphosphate
avidin-HRP ; Avidin-Horseradish peroxidase conjugate
BRL ; Bethesda Research Laboratories, Inc.
BSA ; Bovine Serum Albumin
BSL-1 ; Bandeiraea simplifolia Lectin 1
C ; Cytosine
CaCl₂ ; Calcium Chloride
GTP ; Guanosine triphosphate
H₂O ; Water
H₂O₂ ; Hydrogen Peroxide
H₃PO₄ ; Phosphoric Acid
H7777 ; Hepatoma 7777
hCG ; Human chorionic gonadotropin
his ; Histidine
HIV-I ; Human immunodeficiency virus-I
HPRT ; Hypoxanthine phosphoribosyl transferase
HRP ; Horseradish peroxidase
i.e. ; in essences
I.V. ; Intravenous
IEF ; Isoelectric focusing
IGF-I ; Insulin-like growth factor-I
IgG ; Immunoglobulin G
IgG₁ ; Immunoglobulin G one
IPTG ; Isopropylthio-β-D-galactoside
kb ; Kilobase
KCl ; Potassium Chloride
KCl-TMK ; Potassium Chloride-Tris, Magnesium, Potassium buffer
kD ; Kilodaltons
kg ; Kilograms
KH₂PO₄ ; Potassium phosphate monobasic
LB ; Luria-Bertani Medium
LCA ; Lens culinaris agglutinin
LEU ; Leucine
leu ; Leucine
LiCl ; Lithium Chloride
lys ; Lysine
M ; Mole/molar solution
mA ; Milliamps
μCi ; MicroCurie
MEM-HEPES ; Minimal essential medium ; N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid
MgCl₂ ; Magnesium chloride
MgSO₄ ; Magnesium sulfate
ml ; Milliliter
μl ; Microliter
mM ; Millimolar
mm ; Millimeter
MMLV ; Moloney Murine Leukemia Virus
MnCl$_2$; Manganese chloride
MOFP; Monoclonal antibody against Oncofetal protein
MOFP-A; Monoclonal antibody A against OFP
MOFP-B; Monoclonal antibody B against OFP
MOFP-C; Monoclonal antibody C against OFP
MOFP-D; Monoclonal antibody D against OFP
MOFP-E; Monoclonal antibody E against OFP
MOFP-F; Monoclonal antibody F against OFP
MOPS; 3-[N-Morpholino] propanesulfonic acid
mRNA; Messenger ribonucleic acid
Mw; Molecular weight
N$_2$; Nitrogen
Na$_2$HCO$_3$; Sodium bicarbonate
Na$_2$HPO$_4$; Sodium phosphate dibasic
NaCl; Sodium Chloride
NaOH; Sodium Hydroxide
ND; Not detectable
nd; Not detectable
ng; Nanogram
NH$_4$OAc; Ammonium acetate
NP-40; Nonidet P-40 detergent
OCH; Ocher
OD; Optical density
OD$_{260}$; OD at 260 nanometers
OD$_{600}$; OD at 600 nanometers
OFP; Oncofetal protein
oligo-dT; Oligo-deoxythymidylate
OPA; Opal
PBS; Phosphate buffered saline
PCR; Polymerase chain reaction
PDGF; Platelet derived growth factor
PEG; Polyethylene glycol
PEP; Phospho-enol-pyruvate
pfu; Plaque-forming unit
pH; Log of Hydrogen ion concentration
PHE; Phenylalanine
pmol; Picomoles
PMSF; Phenylmethylsulfonylflouride
poly A; Polyadenylic acid
poly A$^+$; polyadenylic acid positive
pro; proline
PSA; Pisum sativum agglutinin
psi; pounds per square inch
PTH; Phenyl isothiocyanate
PVDF; Polyvinylidene difluoride
ρ; Density
RIA; Radioimmuno assay
RNA; Ribonucleic acid
RNase; Ribonuclease
RNase A; Ribonuclease A
RNase H; Ribonuclease H
rpm; Revolutions per minute
S.D.; Standard deviation
SBA; Soybean agglutinin
SDS; Sodium dodecyl sulfate
SDS-PAGE; SDS-polyacrylamide gel electrophoresis
SER; Serine
ser; Serine
SJA; Sophora japonica
SWGA; Succinylated wheat germ agglutinin
T; Thymine
TBS; Tris-buffered saline
TCA; Trichloroacetic acid
THR; Threonine
thr; Threonine
TMK; Tris-magnesium chloride; potassium chloride buffer
Tris; Tris [hydroxymethyl]-aminomethane
Tris-HCl; Tris [hydroxymethyl]-aminomethane hydrochloride
TTBS; Tween-20 containing tris-buffered saline
Tween-20; Polyoxyethylene sorbitan monolaurate
UEA-1; Ulex europaeus agglutinin I
UV; Ultraviolet
V; Volts
v/v; Volume/volume
VAL; Valine
val; Valine
w/v; Weight/volume
WB; Wash buffer
WGA; Wheat germ agglutinin
X-gal; 5-bromo-4-chloro-3-indolyl-β-galactoside
xg; Times gravity
°C; Degree centigrade
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General Introduction

The processes of eukaryotic ribonucleic acid (RNA) synthesis, processing, and degradation are well known while the molecular mechanisms controlling these events are still poorly understood. Regulation of gene expression begins with transcription. For structural genes, the use of alternate promoters for determining multiple sites of transcription initiation has been demonstrated (1, 2). The initial transcripts are then processed in a number of ways. The use of alternate splicing (3) can include or exclude specific exons during the excision of intron containing sequences, significantly effecting the primary structure of the final product. All eukaryotic mRNA's are modified by the addition of a methylated cap at the 5' end and most have a stretch of approximately 200 adenine residues attached to the 3' end. The most notable exceptions to the addition of poly A tails are the histone proteins messenger RNA's (4). A subset of RNA's are retained in the nucleus until the proper export signals are obtained or are later degraded (5). Messages destined to be translated must be exported from the nucleus to the cytoplasm, the location of the translational machinery.

The mechanism of action of any putative RNA transport factor has not yet been elucidated. It is known that the RNA is exported from the nucleus via the nuclear pore and that anti-pore antibodies can inhibit this process (6). The production of an anti-nucleus
antibody was shown to inhibit nucleocytoplasmic RNA transport \textit{in vivo} (7). This study also demonstrated that the accumulation in the nucleus of non-histone chromosomal proteins was not inhibited by the antibody, suggesting that the mechanism involved is not simply a blockade of the nuclear pores. Several laboratories, including our own, have demonstrated nuclear membrane independence of RNA transport by denuding the nuclei with detergents prior to \textit{in vitro} transport (8, 9, 10) and by the lack of non-specific RNA efflux with intentional nuclear envelope damage (11).

Several laboratories have also reported the energy dependence of the RNA transport process (12, 13, 14). More, recently it has been reported that RNA helicase activity may be necessary (15) possibly for conversion of the RNA molecule to a configuration accessible to the cytoplasmic binding proteins and ultimately the translational machinery.

As demonstrated initially in the simian virus (SV 40) (16) and more recently with human immunodeficiency virus type 1 (HIV-1) system (17), sequence elements in the RNA molecule may be necessary for transport. In both cases, the punitive response element is located in an intronic sequence. This does not preclude such sequences being present in exonic sequences also. In the case of the HIV-1 env mRNA, a 210 nucleotide element has been identified for which a very stable stem and loop secondary structure has been predicted. This element can be relocated to other intron or exon sequences of the env mRNA and transport is unaffected unless
its 5' to 3' orientation is reversed. This mRNA is transported from
the nucleus of infected cells only in the presence of the virally
encoded transport protein Rev. In the absence of Rev, these
unprocessed RNA's are retained in the nucleus. The implication is
that the Rev protein is interacting directly with the env mRNA
response element during transport.

In order to gain insight into the process of nuclear restriction of
RNA and nucleocytoplasmic RNA transport, an in vitro assay system
was developed. This system, first reported in 1972 (18, 19) is
essentially rat liver nuclei, containing radiolabelled RNA, suspended
in a surrogate cytoplasm. At low protein concentrations, nuclei are
stable, but the system is limiting in RNA transport activity. Thus it is
sensitive to the addition of exogenous transport factors. This
"bioassay" was first used for the investigation of macromolecules
critical to RNA transport in cytosolic fractions from normal rat livers
or rat hepatomas (20, 21, 22). The hepatoma cytosol was found to
have an elevated level of RNA transport activity on a per milligram
protein basis. RNA transport activity was subsequently determined
to be present in the blood plasma of rats and, again, elevated levels
were detected in animals carrying tumors (23, 24). This elevated
level of nucleocytoplasmic RNA transport in the blood plasma was
shown to be induced by treating animals with known chemical
carcinogens, but not by non-carcinogenic analogs (25). Further
investigations demonstrated this activity to be present in the plasma
of human cancer patients (26).
In order to determine the utility of this assayable activity as a tumor marker, plasma samples from patients having a wide range of disease conditions were tested (27). In contrast to other known tumor markers [e.g. carcinoembryonic antigen (CEA); γ-glutamyl transpeptidase; alpha fetoprotein (AFP)] this activity was shown to be associated with every human tumor type tested. It was not detected in the blood plasma of patients (surgical and non-surgical) with non-malignant conditions. Subsequently, the activity was detected in amniotic fluid and rat fetal tissues, but never in maternal circulation (28).

An initial characterization of the propagator of this as yet undefined biological activity was obtained by fractionating the blood plasma (and also tissue and tumor cytosols) from normal and tumor-bearing rats by size exclusion chromatography on a Sepharose CL-6B column. Each fraction was tested for RNA transport activity in the bioassay. For the first time, it was demonstrated that a unique factor of approximately 60 kilodaltons was responsible for the elevated nucleocytoplasmic RNA transport observed under malignant and fetal conditions (28, 29). By this analysis, the same factor, or at least a factor of roughly the same molecular weight, was acting in both the rat and human systems. The RNA transport activity present in all normal tissues and blood plasma was shown to be associated with a 35 kilodalton factor in rat and a 25 kD factor in humans. Due to the presence of the 60 kD protein in malignant and fetal systems, and its apparent absence from normal tissues, it was defined as an oncofetal protein and named OFP.
To assess whether the normal and tumor factors were general RNA transport proteins or if they acted upon different classes of RNA in terms of transport, cDNA:RNA hybridization kinetic analysis was undertaken (30, 31). For this set of experiments, total cDNA from rat liver or rat hepatoma 7777 was hybridized with the RNA transported in response to either the normal or tumor factor. Hybrid melting curves demonstrated conclusively that the two RNA transport factors were inducing the release of different subclasses of RNA from the normal rat liver nuclei. Specificity of messenger RNA transport was evaluated by the dot blot hybridization of the transport RNA's with albumin and $\alpha$-2$\mu$-globulin cDNA's. The 35 kD normal factor transported more of both of the mRNA's as compared to the tumor factor, again demonstrating differential specificity or at least different rates of turnover. The limitation of this study was that the 35 kD factor was present in both set of the transport assay. The base transport assay contained a low level of normal liver cytosol which provided a necessary nuclear stabilizing factor. The cytosol also contained a small amount of the 35kD transport factor. High levels of the tumor and normal factors were then added to the assay above this limiting amount of cytosol. Thus a conclusive interpretation that the two factors affect the transport of distinct subsets of RNA could not be made.

The significance of the differential RNA specificity or rates of transport, became apparent when the initial point of OFP induction in carcinogenesis was determined. Experiments designed to investigate the time course expression of the tumor associated RNA transport
during a rat model carcinogenic protocol were performed. It was shown that the RNA transport activity rapidly increased in the blood plasma of rats having a 2/3 partial hepatectomy followed, 24 hours later, by administration of a single dose of diethylnitrosamine (DENA). The level of RNA transport activity peaked in the blood plasma at 21 days post-carcinogen treatment and then sharply declined to near non-detectable levels before slowly rising again. In contrast, the levels in the liver cytosol rose slowly to a plateau, never exhibiting the decrease shown in the plasma. This response was observed in other carcinogenesis systems and appears to be independent of carcinogen, carcinogen dose or target organ (32). The initial interpretation from these data was that there was an immune clearance of OFP from the blood plasma. This was confirmed by Western blot analysis of plasma proteins from a tumor-bearing rat, using 21 day serum from a DENA treated rat as the primary antibody. Two bands of molecular weight 50-60 kD were detected (32). This demonstrated that OFP was eliciting an auto-immune response, not uncommon for oncofetal proteins (33).

Evidence that the tumor is the source of the OFP in the blood plasma was obtained from studies in which hepatoma 7777 cells were transplanted into the hind leg of Buffalo strain rats. The levels of OFP transport activity were monitored in the plasma as the tumors grew, as well as after the tumors were surgically removed. OFP activity rose in an almost linear fashion following inoculation. This activity was rapidly cleared from the plasma subsequent to surgical
removal of the solid tumor. The biological half-life of the OFP was determined to be approximately 4 days \textit{in vivo} (34).

The rat tumor plasma form of OFP was purified to high specific activity by ammonium sulfate fractionation followed by Sepharose CL-6B and CM-Affi-gel Blue chromatography (28). This protein preparation was used as an immunogen to raise rabbit anti-OFP polyclonal antibodies (35). These antibodies were initially used in an ELISA assay to detect the presence of OFP in the blood plasma of carcinogen treated rats. Later experiments revealed their greatest utility to be in their ability to immuno-precipitate OFP.

Experiments involving immuno-precipitation of OFP from rat tumor plasma, rat fetal cytosol and rat amniotic fluid followed by analysis of the supernatant in the bioassay demonstrated the gross immunological identity between each of these forms of OFP. The bulk of the RNA transport activity could usually be recovered in the precipitated fractions by washing the pellet and resuspending in buffer prior to analysis in the bioassay. The polyclonal antibody was also used to show, for the first time, a post-translational modification of OFP. Blood plasma was collected from tumor-bearing rats 3 hours after the administration of $^{32}$P-orthophosphate. The OFP was partially purified by gel exclusion chromatography and the proteins immuno-precipitated. Following polyacrylamide gel electrophoresis and autoradiography, a single band was detected in the 55-60 kD range. This strongly indicated that OFP was a phosphoprotein. Autophosphorylation as a mechanism of this result was ruled out, at least \textit{in vitro}, as the 55-60 kD phosphorylated band was detected
only in labeling reactions with [γ-32P]ATP when the catalytic subunit of protein kinase from bovine heart was included in the reaction mix (28). Further analysis of OFP using this antibody preparation was limited as analysis of impure protein preparations using the standard Western blot technique determined that the antibody contained significant cross-reactivity with other proteins in the rat plasma (T.E. Webb, personal communication).

The purpose of the present study was three fold. First was the development of highly specific monoclonal antibodies against OFP. These antibodies, which exhibit no cross reactivity with other proteins in the rat system, allowed for the preliminary development of an assay (ELISA) which could screen for the presence of OFP in unprocessed blood plasma. This assay has the potential of screening of human blood plasma for OFP now that anti-human OFP antibodies have become available.

These antibodies have proven to be a powerful tool for defining the expression patterns of OFP. In the fetal system OFP had been previously detected, and through the use of a novel immuno-assay, a highly accurate picture of the level of its expression during prenatal development has been obtained. This is important for the determination of the full scenario of the role of OFP in physiological terms.

The cloning of an OFP cDNA was also made possible through the use of the monoclonal antibodies by screening a cDNA expression library. The determination of the full cDNA sequence, and subsequent determination of the active site of OFP, will allow for an
Interpretation of the significance of the post-translational modifications (tyrosine phosphorylation and glycosylation) detected in this study.
Chapter I Introduction

Potential markers of carcinogenesis include gamma glutamyl transpeptidase (36) and α-fetoprotein (37), both of which have been extensively studied in hepatocarcinogenesis. A number of other proteins have been evaluated as tumor (cancer) markers. Of these, carcinoembryonic antigen and a human chorionic gonadotropin (hCG) have been very useful for specific sites (38). Some oncodevelopmental proteins specified by oncogenes are also currently under investigation as markers (39). These and other phenotypic marker proteins, in combination with morphological markers (i.e. altered foci), are essential for the identification of pre-neoplastic lesions, for establishing cell lineage during carcinogenesis, as well as for diagnosing, monitoring and treating cancer.

This laboratory has previously reported an oncofetal protein (OFP) in the rat which is present during fetal development (28). This antigen is released by pre-neoplastic and neoplastic cells and accumulates in the blood of carcinogen-treated and tumor bearing rats (24, 35). The factor is not induced in rodents by toxins which are not carcinogenic, nor by non-carcinogenic analogs of carcinogens (25). Collectively these studies indicate that OFP production is not restricted to a particular neoplastic tissue. The known biological activity of this protein is to promote the energy-dependent nucleocytoplasmic transport of repetitive DNA transcripts which are normally restricted to the nucleus. These transcripts have been

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shown to be similar to those transported to tumor cell cytoplasm in vivo (31).

A polyclonal antibody raised against OFP was used in several studies (28, 35), but its utility was limited by its cross-reactivity with proteins other than OFP.

The advent of cell hybridization technology for the generation of cell lines which secrete monoclonal antibodies (40, 41), has made it possible to produce very specific antibodies from non-homogeneous antigen preparations. Protocols currently in use involve the fusion of spleen cells from a hyper-immunized animal (mouse or rat) with culture adapted myeloma cells. The culture media from the resultant hybridomas are screened for the presence of antibody of the desired specificity and subsequently clones are established from individual cells. Antibody is then isolated from the cell culture supernatant or from ascitic fluid produced in mice inoculated interperitoneally with the hybridoma cells. The key element of this procedure is the cloning of the individual hybridoma cells, as the original B lymphocyte can produce antibody against only one antigenic epitope (42). Antibodies directed against any of the contaminating proteins will thus be excluded from the final preparation.

Anti-OFP antibodies (MOFP's) prepared as above are described herein and incorporated into the OFP detection assays. The immuno-bioassay is essentially an immunoprecipitation of the OFP prior to analysis in the original bioassay (30). Anti-mouse [IgG(H+L)]-agarose beads are used in place of the standard staphylococcal protein A
Sepharose bead in order to achieve a more quantitative precipitation of the OFP antigen. As has been determined (T.E. Webb, unpublished data), the monoclonal antibodies described in this chapter and used throughout this study are of the IgG1 subclass. This subclass of immunoglobulin is not efficiently adsorbed by protein A (43) except under special buffering conditions which may adversely affect subsequent activity determinations (44).

The development of an enzyme-linked immunosorbant assay (ELISA) is also described. This is the assay of choice for the clinical laboratory processing of biomedical samples due to its ease of handling and its cost effectiveness (45). The first ELISA's were reported in 1971 utilizing both enzyme labelled antibodies (46) and enzyme labelled antigens (47, 48) in place of the traditionally radiolabelled components of the radio-immunoassay (RIA). Many revisions, too numerous to detail here, of these initial reports have been made for the detection of both antigens and antibodies in biological samples (49, 50). Antibody screening in this study was carried out using an indirect ELISA (51) where purified antigen (OFP) was bound to a plastic support followed by incubation with the media from the hybridoma culture. Bound antibodies were detected by a second anti-immunoglobulin antibody which was covalently linked to horse-radish peroxidase (HRP) (52). Presence of bound monoclonal antibody was determined by development with the chromogenic substrate o-phenylenediamine (53).

For the detection of OFP in the blood plasma of rats, a competitive inhibition ELISA was developed as a modification of the
method of Yorde et. al. (54). This assay consisted of purified OFP bound to the plastic support and the purified MOFP pre-incubated with the plasma test sample in separate tubes prior to adding aliquots to the plate. If OFP is present in the sample, it will bind the MOFP and prevent its binding to OFP on the plate. Detection of bound antibody and color development are as per the indirect ELISA. A positive reaction for the presence of OFP in the sample is viewed as the absence of color development in the final step of the assay. The critical element of this assay is the proper dilution of the monoclonal antibody. It must be present in a sufficient abundance to be readily detectable by the second antibody if it binds to the plate-bound antigen, and yet dilute enough that a small amount of antigen in the test sample can quantitatively remove it from solution. This level of optimization has been achieved in this study.

Also shown for the first time using the MOFP's on Western blot analysis is the immunological identity of the tumor cytosolic and blood plasma borne forms of OFP. The protein's temporal expression under a rodent carcinogenic regimen is also presented.
Materials and Methods

In vivo RNA Transport (figure 1.1)

A cell free system designed to measure the release of RNA from isolated nuclei (14) was used extensively in this study. It is described here in detail and hereafter will be referred to as the bioassay.

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) of approximately 250 g were fasted overnight. Following euthanasia by ether anesthesia and exsanguination, the livers were dissected out and homogenized by 6 strokes of a Dounce homogenizer in 0.25 M sucrose-TMK (50 mM Tris-Cl [pH 7.5]; 2.5 mM MgCl₂; 25 mM KCl), 2 ml per gram of wet weight liver. The homogenate was centrifuged for 10 minutes at 12,000 x g (Beckman JA-20 rotor) and the resulting supernatant for 90 minutes at 105,000 x g (Beckman Ti50 rotor). The final supernatant (cytosolic fraction) was removed and dialyzed overnight at 4°C against TMK buffer.

Liver nuclear RNA was pre-labelled in vivo by injecting (i.p.) an ether anesthetized male Sprague-Dawley rat with 100 microCuries (³H) orotic acid. After a 30 minute labeling time, the rat was euthanized by ether anesthesia and exsanguination and the liver removed following perfusion with 10 ml of a solution containing 0.25 M sucrose and 3.3 mM calcium acetate (CaOAc). The liver
Figure 1.1: Flow chart outlining the standard Bioassay for the detection of \textit{in vitro} RNA transport activity.
Bioassay for RNA Transport Factors

Nuclear RNA labelled *in vivo* by I.P. injection of $^3$H-orotic acid

Following 30' incorporation time, liver nuclei prepared and placed in surrogate cytoplasm

Exogenous transport factors added

Incubate at 30°C, 30 min.

Remove nuclei by centrifugation

TCA precipitate supernatant, solubilize and count

Results reported as % nuclear cpm released/mg protein added

1% release of nuclear counts = 1 unit of OFP
tissue was homogenized in 2.3 M sucrose, 3.3 mM CaOAc (15 ml per gram wet weight liver) by 4 strokes of a Dounce homogenizer using a loose fitting pestle with more than usual clearance. The resulting homogenate was centrifuged at 34,000 x g for 60 minutes at 4°C. The pelleted nuclei were rinsed with a solution containing 1 M sucrose, 1 mM CaOAc (1.5 ml/g original liver) and resuspended in the same at 1 ml/g.

The cell free system was constituted to contain 5x10⁶ prelabeled nuclei per ml; 5 mg/ml dialyzed cytosol protein; 50 mM Tris-HCl (pH 7.5); 25 mM KCl; 2.5 mM MgCl₂; 0.5 mM CaCl₂; 0.3 mM MnCl₂; 5.0 mM NaCl; 2.5 mM phosphoenolpyruvate (PEP); 35 units/ml pyruvate kinase; 2.5 mM Na₂HPO₄; 5.0 mM spermidine; 2.0 mM dithiothreitol (DTT); 2.0 mM ATP; 300 μg/ml low molecular weight yeast RNA; 0.4 mg/ml methionine; 0.3 mM GTP; 170 mM sucrose.

This system is limiting in RNA transport factors and is responsive to the addition of exogenous RNA transport factors. An aliquot of up to 100 μl of blood plasma/tissue cytosol or 200 μl of a column fraction to be tested was added to 1 ml of the cell free medium and incubated at 30°C for 30 minutes. The nuclei were pelleted by centrifugation at 1050 x g for 10 minutes and the resulting supernatant decanted into fresh tubes containing 100 μl of 50% trichloroacetic acid (TCA). The samples were vortexed briefly and the resultant precipitate (protein and RNA) allowed to stand at 0°C for 10 minutes before pelleting at 1050 x g for 10 minutes. The pellets were washed with 1 ml cold 100% ethanol to remove excess
water and TCA, and were solubilized by digestion with 0.4 ml Unisol (Isolab, Akron, OH) for 20 minutes at 40°C. The solubilized samples were mixed with 200 µl methanol and then added to 9 ml Unisol Complement (Isolab, Akron, OH) scintillation fluid and the counts per minute (cpm) $^3$H measured in a scintillation counter. Activity was expressed as percent of the total counts transported per milligram exogenous protein added to the assay mix as calculated in the formula:

$$\frac{\text{cpm transported}}{\text{cpm from equal # nuclei} \times 100} \times \frac{\% \text{ labelled RNA transported}}{\text{mg protein added}}$$

Rat OFP Purification (Figure 1.2)

Blood was collected from Buffalo strain rats carrying the transplantable hepatoma 7777 by cardiac puncture using a heparinized syringe. The blood was transferred to heparin containing centrifuge tubes and the cellular components separated by centrifugation in a serofuge. Ammonium sulfate was added to 30% saturation at 4°C and the precipitated proteins pelleted and discarded. Ammonium sulfate was increased to 60% saturation at 4°C and the precipitated proteins pelleted and saved. These proteins were resuspended in 0.02 volumes of 0.1 M KCl-TMK and dialyzed overnight at 4°C against the same buffer. 3 ml of the dialyzed protein per run was fractionated by chromatography on a 1.5 x 40 cm Sepharose CL-6B column (Pharmacia LKB, Piscataway, NJ) and 3 ml fractions were collected using 0.1 M KCl-TMK as the
Figure 1.2: OFP purification scheme using blood plasma of tumor bearing rats.
OFP Purification (From Rat Tumor Plasma)

Draw blood from tumor bearing rat by cardiac puncture using heparinized syringe. Spin to make plasma.

Ammonium Sulfate precipitate plasma between 30 and 60% sat.

Dialyze

Fractionate through a Sepharose CL-6B gel exclusion column

Pool active fractions

Chromatograph on CM-Affi-gel Blue affinity column

Pool active fractions

Dialyze

Chromatograph on single-stranded DNA-Cellulose affinity column

Figure 1.2
column buffer. Fractions which were active for RNA transport activity, as determined by the bioassay in the molecular weight range of 45-60 kD were pooled and applied to a 1.5 x 30 cm CM-Affi-gel Blue (BioRad Laboratories, Richmond, CA) affinity column. Using a continuous flow A280 monitor, non-binding proteins were washed from the column with 0.1 M KCl-TMK. OFP was eluted in 3 ml fractions with TMK containing 0.4 M KCl. The CM-Affi-gel Blue was regenerated with 6 M guanidine-HCl; 0.025 M Tris-Cl, pH 8.0; 0.05 M EDTA and equilibrated with 10 bed volumes 0.1 M KCl-TMK.

The protein was isolated in high purity by affinity chromatography on single-stranded DNA cellulose (Sigma Chemical Co., St. Louis, MO). The column (3.5 x 9.0 cm) was equilibrated with 50 mM NaCl-TMK and the OFP fractions from the CM-Affi-gel Blue column were applied after dialysis against column buffer. The column was washed with 50 mM NaCl-TMK buffer and then the OFP was eluted with 2.0 M NaCl-TMK. The eluted factor was dialyzed against TMK buffer and concentrated in Centriprep-10™ centrifuge concentrators (Amicon Corp, Danvers, MA).

Monoclonal Antibody Production (Figure 1.3)

Hybridomas were obtained by established procedures (55) in the laboratory of Dr. Bruce Zwilling at the OSU Monoclonal Antibody Facility. Balb/c mice were immunized by subcutaneous injection with 1 mg/ml of purified OFP in saline which was emulsified with an equal volume of Freund's complete adjuvant. This subcutaneous
Figure 1.3: Monoclonal antibody production (protocol used in laboratory of Dr. Bruce Zwilling, Ohio State University Monoclonal Antibody Facility).
Monoclonal Antibody Production

Balb/c mice immunized by subcutaneous injection of 1 mg/ml OFP emulsified in Freund's complete adjuvant

Repeat immunization at 3 to 5 weeks

Inject 0.2 ml OFP I.V. after 4 weeks

Select spleen donors by ELISA assay of serum

Spleen cells fused with P3 X63/Ag 8.653 cells (Balb/c myeloma)

Antibodies purified from hybridoma supernatant or ascitic fluid
ascitic fluid by Protein A Chromatography

Figure 1.3
treatment was repeated at 3 to 5 weeks and then the mice were injected intra-venously with 0.2 ml of the antigen after a rest period of one month. Mice were selected as spleen donors based on ELISA assay of the serum. Following fusion of the spleen cells with cell line P3 X63/Ag 8.653 (Balb/c myeloma), supernatants from the hybridoma cell cultures were screened for OFP specific antibody by indirect ELISA and the immuno-bioassay. Clones positive by both assays and which were particularly active in the immuno-bioassay were suspended in medium containing 9 parts serum and 1 part dimethylsulfoxide and cryogenically preserved under liquid nitrogen. Scaled up production of the reactive monoclonal antibodies was done by growing the hybridoma cells as ascitic tumors in pristane-primed Balb/c mice. Ascitic fluid was collected by interperitoneal puncture on a daily basis until the mice became unhealthy in appearance at which time they were euthanized by ether anesthesia. The antibodies were purified from the ascitic fluid using the Protein A MAPS II system® (BioRad Laboratories, Richmond, CA). The system included packed Protein A-Sepharose columns, binding buffer, elution buffer and regeneration buffer. The compositions of the buffers were patented and not revealed in the supplied literature.

The Protein A-Sepharose column was washed with the binding buffer (±10 ml) until a pH of 9.0 of the flow through was obtained. 1 ml of the ascites fluid was loaded (diluted 3 times in binding buffer) and subsequently washed the unbound materials from the column with at least 20 ml of the binding buffer. Elution of the MOFP was with the elution buffer (30 ml total). The column was
regenerated with 10 ml of the regeneration buffer. At the end of the day, the column was washed and stored in phosphate buffered saline (137 mM NaCl; 2.7 mM KCl; 20 mM Na$_2$HPO$_4$; 1.4 mM KH$_2$PO$_4$; pH 7.4) having 0.02% azide. The eluate was neutralized immediately with saturated NaOH (to pH 7.0) followed by dialysis against PBS. Aliquots were frozen at -80°C.

Indirect ELISA (Figure 1.4)

This was carried out essentially according to Kulaga and Sogen (57). 10 ng of purified OFP was bound to 96 well polystyrene microtiter plates (Corning Glass Works, Corning, NY) in 0.10 M Na$_2$HCO$_3$ buffer pH 9.6, 4°C, in a humid chamber overnight. After 3 washes with PBS, open binding sites were blocked by incubation with 350 µl per well of blocking buffer (4% bovine serum albumin in PBS) at 25°C for 4 hours. The plates were washed 3 times with wash buffer (WB: 0.05% bovine serum albumin; 0.05% Tween-20 in PBS) followed by addition of 100 µl of hybridoma supernatant (1:10) containing the antibodies. After incubating for 2 hours at 25°C and washing 3 times with WB, 100 µl of goat anti-mouse (IgG-HRP) conjugate (1:600) (BioRad Laboratories, Richmond, CA) was added and incubated for 2 hours at 25°C. 200 µl per well of the substrate mix (0.015% H$_2$O$_2$, 0.02% o-phenylenediamine in 25 mM citrate/50 mM Na$_2$HPO$_4$; pH 6.0) was then added and color development allowed to proceed in the dark at 25°C for 30 minutes. The extent of color development was determined using an ELISA reader (492 nm
Figure 1.4: Indirect enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of antibody. Representation of a series of reactions; Column A: a solution containing the specific antibody is analyzed, Column B: when non-specific antibody is analyzed. Adapted from Voller et. al. (56).
1) Plate coated with purified OFP

2) Hybridoma supernatant thought to contain anti-OFP antibodies added

3) Enzyme-Second antibody conjugate added

4) Enzyme substrate added

Substrate degradation indicates sample contains antibody

No substrate degradation indicates No antibody

Figure 1.4
filter) after terminating the reaction with 50 μl/well of 4.5M sulfuric acid.

Immunobioassay

This assay relies on the ability of the monoclonal antibodies to bind OFP and remove the detectable activity from solution (immunoprecipitation). The samples to be tested were assayed directly as described in the bioassay (control) as well as after incubation of an aliquot of the sample for 2 hours at 4°C with anti-rat OFP monoclonal antibodies (MOFP) insolubilized on anti-mouse [IgG] agarose beads (Sigma Chemical Co., St. Louis, MO). The agarose bead anti-mouse [IgG(H+L)]-MOFP complex was prepared by first washing the beads with binding buffer (0.01 M phosphate buffer, pH 7.2; 0.25 M NaCl) and then incubating with MOFP for 18 hours at 4°C in the same buffer. The beads were sedimented by centrifugation for 30 seconds at 16,000 x g in a microfuge and non-specific sites were blocked by incubation with 2% non-fat dry milk in 0.5 M NaCl-TMK for 30 minutes at 4°C. After blocking, the beads were washed 3 times in 0.5 M NaCl-TMK and resuspended in an equal volume of the same buffer. 20 μl of the prepared bead-MOFP complex was incubated with each test sample for 2 hours at 4°C. The bead complex, now with OFP bound, was removed by centrifugation as above and the supernatant was tested in the bioassay.

For antibody screening, 100 μl of each hybridoma supernatant or control media was incubated with the agarose-anti-mouse [IgG(H+L)] beads for conjugation. The source of OFP for this set of
experiments was 25 μl of the proteins precipitating between 30% and 60% saturation ammonium sulfate from plasma from a rat carrying a transplantable hepatoma suspended in a minimum volume of 0.1 M KCl and dialyzed against the same buffer. Antibody specificity was confirmed by the inclusion of samples treated with milk blocked agarose bead anti-mouse [IgG(H+L)] without prebinding MOFP.

Competitive Inhibition ELISA (Figure 1.5)

This assay was developed as a modification of the method of Yorde et. al. (55) for the quantification of OFP. 96 well polystyrene microtiter plates were coated with 10 ng/well of purified OFP in 0.10 M Na₂HCO₃ buffer, pH 9.6 at 4°C overnight. Following three washes with PBS, residual binding sites were blocked by incubation with 350 μl/well of blocking buffer at 25°C for 4 hours. The plates were then washed three times with wash buffer (WB). Aliquots of known samples (purified or partially purified OFP) or unknown samples, pre-incubated for 60 minutes on ice with a equal volume of a 1:10 dilution of MOFP, were then added to the wells. After a further incubation (1 hour at 25°C) and washing three times with WB, goat-anti-mouse [IgG]-HRP conjugate (BioRad Laboratories, Richmond, CA) (1:800) was added and incubated for 2 hours at 25°C. The substrate mix (200 μl/well) was added and the reaction allowed to proceed for 30 minutes at 25°C in the dark. Color intensities in the wells were measured using an ELISA reader (492 nm filter) after terminating the reaction with 50 μl/well of 4.5 M H₂SO₄. All ELISA's
Figure 1.5: Competitive Inhibition ELISA for the detection and quantification of antigen. Column A: negative reaction with sample devoid of OFP, Column B: positive reaction with sample containing OFP. Adapted from Voller et. al. (56).
Figure 1.5
were optimized by performing box titrations on each of the components in the assay.

Western Blot Analysis

Rat blood plasma proteins prepared as described were analyzed on Western blots. Cytosolic proteins (normal rat liver and hepatoma 7777) were prepared as for the bioassay from freshly harvested tissue with the inclusion of 1.0 mM phenylmethylsulphonylfluoride (PMSF) in the homogenization buffer. The cytosolic fractions from the 105,000 x g spin were enriched for proteins in the 40-70 kD range by fractionation through a Sepharose CL-6B gel exclusion column and collecting only the fractions within that range.

Proteins were separated according to molecular weight by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (58); 4% stacking gel (4% acrylamide/bisacrylamide (37.5:1) (AmResCo, Solon, OH); 125 mM Tris, pH 6.8; 0.2% SDS), 10% running gel (10% acrylamide/bisacrylamide (37.5:1); 0.375 M Tris, pH 8.8; 0.2% SDS); in a Tall Mighty Small vertical slab cell (Hoefer Scientific Instruments, San Francisco, CA). Proteins were transferred to nitrocellulose in 2.5 mM Tris, 192 mM glycine, 20% (v/v) methanol (59) for 2 hours at 450 mA using a Transblot apparatus (BioRad Laboratories, Richmond, CA). Following transfer, the blots were air dried and unoccupied binding sites were filled by incubation in blocking buffer (TBS [20 mM Tris; 500 mM NaCl; pH 7.5]; 1% (w/v)
non-fat dry milk) for 1 hour at room temperature. After washing three times with TTBS (TBS; 0.05% Tween-20), the blots were incubated with a 1:2 dilution of MOFP in dilution buffer (DB: TBS; 0.05% Tween-20; 0.05% non-fat dry milk) for 2 hours at room temperature with agitation. Following three washes with TTBS, the blots were incubated with goat-anti-mouse [IgG(H+L)]-HRP conjugate (BioRad Laboratories, Richmond, CA) diluted 1:400 in DB for 2 hours at room temperature. Avidin-HRP (BioRad Laboratories, Richmond, CA) was added at a 1:1000 dilution to the second antibody mix for the detection of biotinylated molecular weight markers. The blots were then washed twice with TTBS and twice with TBS and immediately immersed in freshly prepared color development solution (20 ml room temperature TBS with 12 µl cold 30% H₂O₂ added to 5 ml cold methanol containing 15 mg 4-chloro-1-naphthol). Development was allowed to proceed for 5 to 45 minutes at 25°C in the dark. The reaction was stopped by immersion of the membrane in distilled water for 10 to 15 minutes.
Results

Rat Oncofetal Protein (OFP) Purification

As has been shown previously (25), blood plasma proteins from a rat bearing a tumor or treated with a carcinogen exhibited a high level of *in vitro* nucleocytoplasmic RNA transport activity. The activity peaks in fractions eluting in the 50-60 kilodalton range from a Sepharose CL-6B gel exclusion column. In the profile shown in Figure 1.6, 200 µl aliquots of 3 ml fractions eluting from the Sepharose CL-6B column were tested for RNA transport activity in the bioassay. Fractions 36 through 42, which were clearly resolved from the 35 kD normal RNA transport factor and its 700 kD aggregate (30), were pooled and applied to a CM-Affi-gel Blue column. This "pseudo-affinity adsorbent" behaves as an analogue of ADP-ribose and binds to purine-ribose binding sites (60). Figure 1.7 shows the continuous flow absorbance at 280 nm during the course of an Affi-gel Blue column run. Proteins not binding to the matrix (Figure 1.7, peak A) were washed from the column in 0.1 M KCl-TMK. OFP and other proteins having a moderately strong affinity for the blue dye were eluted from the column in 0.4 M KCl-TMK after the absorbance at 280 nm had returned to baseline (Figure 1.7, peak B). A significant purification was achieved at this step as only the proteins eluting in the 0.4 M KCl-TMK were carried on to the next column. The wash proteins (Figure 1.7, peak A) and the column regeneration proteins (Figure 1.7, peak C) were discarded. Peak B
Figure 1.6: Rat OFP Purification (Step 1). Activity profile of fractions eluting from a Sepharose CL-6B column, loaded with 30-60% ammonium sulfate cut rat tumor plasma. Fractions 32 through 40 were collected for further purification.
Figure 1.6

Transport Units vs. Fraction Number
Figure 1.7: Rat OFP Purification (Step 2). Ultra-violet absorbance (280 nm) profile of CM-Affi gel Blue column run. Pooled fractions from the Sepharose CL-6B were loaded and washed (peak A) and eluted (peak B and peak C) as further detailed in the figure. Peak B proteins were collected for further purification.
Figure 1.7
proteins were pooled, dialyzed overnight against 50 mM NaCl-TMK and then applied to a single-stranded DNA cellulose column. OFP has as its known biological function the transport of RNA molecules from isolated nuclei into a surrogate cytoplasm. Our working hypothesis is that the single stranded calf thymus DNA attached to this matrix is mimicking RNA molecules and OFP is associating with it. As shown in Figure 1.8, a small amount of protein is tightly bound and did not elute from this column until a very high salt wash (2 M NaCl-TMK) was applied. It has been shown in the bioassay that the vast majority of the OFP activity applied to this column was recovered in this small high salt elution peak. When analyzed by 10% SDS-PAGE and silver staining major bands at 50 and 55 kD were observed (Figure 1.9). Lane 1 was intentionally overloaded to ensure that any minor contaminating bands would be detected. This protein preparation was used as an immunogen for the production of anti-OFP mouse monoclonal antibodies by standard protocols in the laboratory of Dr. Bruce Zwilling of the Ohio State Monoclonal Antibody Facility.

After fusion of the spleen cells and the Balb/c myeloma cells, 53 hybridoma cell lines screened positive by an initial indirect ELISA using the single-stranded DNA-cellulose purified protein as the plate bound antigen. This indirect ELISA did not distinguish between anti-OFP antibodies and antibodies against any of the minor contaminating proteins in the purified OFP preparation. However, the immuno-bioassay used as a second screen, selected only those antibodies which were able to remove the biological activity
Figure 1.8: Rat OFP Purification (Step 3). Ultra-violet absorbance (280 nm) profile of single-stranded DNA-cellulose column run. Proteins were loaded, washed and eluted (elution in 2.0 M NaCl-TMK) as indicated in the figure.
Single Stranded DNA-Cellulose Column Profile (Continuous Flow A280)

Sample Loading and Washing
50 mM NaCl-TMK

OFP Elution
2 M NaCl-TMK

Figure 1.8
Figure 1.9: Separation of the purified OFP (figures 1.6 - 1.8), on the basis of molecular weight, by SDS-PAGE (10%) followed by silver staining. Lane 1: 15 μg protein, Lane 2: 2 μg protein.
Figure 1.9
(stimulation of RNA transport) from the tumor bearing rat blood plasma. Six monoclonal antibodies were identified which were effective in the immuno-bioassay. These antibodies have been designated MOFP-A through MOFP-F. Subsequent isotyping experiments with rabbit anti-mouse IgG specific for the various subclasses showed that all of the monoclonal antibodies were of the IgG\textsubscript{1} subclass (T.E. Webb, unpublished data).

Shown in Figure 1.10 is the result of the indirect ELISA using MOFP-C isolated from the hybridoma medium by the Protein A MAPS II system\textsuperscript{®} as described. The initial protein concentration of the purified monoclonal antibody was 10 \(\mu\)g/ml. The titer, dilution giving a cut-off value (52), was approximately \(10^{-2}\) for all of the MOFP. Anti-OFP affinity was not detected at any dilution of the negative medium (supplied by Dr. Zwilling).

Figure 1.11 is a histogram showing the relative activities of MOFP-A through MOFP-F and the negative supernatant at a protein concentration of 10 \(\mu\)g/ml using the indirect ELISA assay. MOFP-A, -B and -C show approximately the same activity in the ELISA assay, while MOFP-D, -E and -F show approximately 30% lower activity.

An initial confirmation that the monoclonal antibodies were not immuno-precipitating the normal 35 kD transport factor activity was performed. Each MOFP was evaluated on the capability to discriminate between the two factors in normal and tumor plasma and cytosol in the immuno-bioassay (Table 1.1). There was sufficient insolubilized MOFP added to each test sample to bind approximately 2 \(\mu\)g of OFP, much more than occurred in any of the samples tested.
Figure 1.10: Titration of purified MOFP-C from the medium of hybridoma producing the anti-OFP antibody (•) and of identically treated medium from an unrelated, hybridoma cell line (o).
Figure 1.10

ELISA VALUE (A_{492})

ANTIBODY DILUTION

(+)

(−)
Figure 1.11: Relative activity of the purified MOFP's A through F in the indirect ELISA. The result with a purified negative supernatant is also shown. All the antibodies in this experiment were tested at a dilution of 6 x 10^{-1}. 
Figure 1.11
Table 1.1  Removal of RNA Transport Activity by Immobilized Monoclonal Antibodies in the Immunobioassay.

Activity (Units/mg protein) Removed From:

<table>
<thead>
<tr>
<th>MOFP</th>
<th>Plasma</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor</td>
<td>Normal</td>
</tr>
<tr>
<td>A</td>
<td>1.30</td>
<td>nd</td>
</tr>
<tr>
<td>B</td>
<td>1.53</td>
<td>nd</td>
</tr>
<tr>
<td>C</td>
<td>1.50</td>
<td>nd</td>
</tr>
<tr>
<td>D</td>
<td>1.40</td>
<td>nd</td>
</tr>
<tr>
<td>E</td>
<td>1.40</td>
<td>0.05</td>
</tr>
<tr>
<td>F</td>
<td>1.40</td>
<td>0.07</td>
</tr>
</tbody>
</table>

nd = not detectable
Incubation of the insolubilized MOFP with the plasma or cytosol was for 2 hours, although a time course (D.E. Schumm, unpublished data) indicated that the binding of OFP was completed by 1 hour and did not change with an additional 3 hours of incubation. Because the immuno-bioassay utilized MOFP in excess over the antigen, MOFP-D, -E and -F which showed less activity in the indirect ELISA assay, showed activity very similar to MOFP-A, -B and -C in this assay. None of the monoclonals showed any significant activity against at least 15 separate preparations of normal rat plasma or normal rat liver cytosol when the non-specific adsorption to sites on the agarose were blocked with non-fat dry milk. Since the normal 35 kD RNA transport factor was present in appreciable concentrations in these preparations, the data confirm that antibodies against OFP do not cross-react with the normal factor.

To further confirm the specificity of the monoclonals to OFP, plasma from hepatoma 7777-bearing rats was fractionated on a Sepharose CL-6B column and the fractions were assayed for OFP using MOFP-B or MOFP-F in the immuno-bioassay. The results using 'B', which is identical for 'F' and typical of the other MOFP's, are presented in Figure 1.12. The results clearly show a marked reduction on the RNA transport activity in the region of the profile corresponding to OFP (fractions 44-52). Again, the activity of the normal 35 kD factor (fractions 62-68) was not affected. Figure 1.13 shows a Sepharose CL-6B column profile of normal rat plasma which was treated in an identical manner as the rat tumor plasma in Figure
Figure 1.12: Sepharose CL-6B profile of RNA transport activity in the plasma of a tumor bearing rat before (o) and after (*) treatment with MOFP immobilized on anti-mouse [IgG]-agarose beads in the immuno-bioassay. Mw 55 and 35 kD molecules are localized near fractions 48 and 64 respectively.
Before Treatment

After Treatment

Figure 1.12
Figure 1.13: Sepharose CL-6B profile of RNA transport in the plasma of a normal rat before (*) and after (o) treatment with MOFP A+B+C) agarose beads in the immuno-bioassay. MW 35 kD is localized around fraction 54.
Figure 1.13
1.12. Again, there was no reduction of the normal 35 kD RNA transport activity.

The specificity of MOFP-A, -B, -C and -E was assessed by Western blot analysis of Sepharose CL-6B fractionated tumor-bearing rat blood plasma. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose. It can be seen clearly in Figure 1.14 that MOFP-A through MOFP-C react strongly with a 50 and 55 kD proteins, while MOFP-E (typical of MOFP-D and MOFP-F) reacts strongly with the same proteins as MOFP-A, -B and -C, but also weakly with several other proteins on the blot. For this reason, MOFP-A, -B and -C were chosen for future experiments involving rat OFP. MOFP-E, as will be detailed later, was shown to cross-react with the human tumor form of OFP.

The competitive inhibition ELISA was developed to measure OFP in the plasma, serum or cytosol of carcinogen-treated and tumor-bearing rats. Figure 1.15 shows a dose-response curve for Sepharose CL-6B fractionated blood plasma of a hepatoma 7777 tumor-bearing and normal (control) rat of the Buffalo strain. The curves shown were obtained with MOFP-B, and were typical of the curves obtained for the other monoclonals. The dose-response curve is relatively steep and is approximately linear up to 20 ng of plasma protein when OFP is present. The linear portion of the curve has been shown to be useful in the comparison of antigen preparations (51). OFP was not detectable at any concentration in normal rat plasma.
Figure 1.14: Western blot of Sepharose CL-6B fractionated plasma from a tumor bearing rat. Proteins were separated by SDS-PAGE, (10%), transferred to nitrocellulose and developed using (a) MOFP-A (b) MOFP-B, (c) MOFP-C or (e) MOFP-E as the primary antibody.
Figure 1.15: Determination of OFP in the plasma of normal rats (•) and of a rat bearing hepatoma 7777 (○) using the competitive inhibition ELISA. A/A₀ represents the ratio of the absorbances of the test sample to a reference sample containing buffer only.
Figure 1.15

Graph showing the relationship between A/A₀ x 100 and OFP (ng plasma protein/100 μl).
The immunological relationship between the blood plasma form and the tumor cytosolic form of OFP was shown by the common reactivity of the MOFP's to the protein isolated from both sources in Western blot analysis (Figure 1.16). OFP-containing plasma from a tumor-bearing rat (lane 2) and cytosol from a rat tumor (lane 3) was subjected to 10% SDS-PAGE, then transblotted to nitrocellulose. MOFP-A reacted with two bands corresponding to proteins of MW approximately 50 and 55 kD. The relationship of these two proteins will be detailed in Chapter 2. No reactivity was observed in the normal liver cytosol (lane 1) and no reactivity against OFP was detected on an identical Western blot developed with a primary antibody of unrelated specificity (data not shown). Identical results were obtained with rat mammary tumor cytosol and blood plasma as well as with plasma from a rat 21 days after a 2/3 partial hepatectomy and administration of a carcinogenic dose of diethylnitrosamine (DENA).

As an initial study to show the utility of these antibodies and the immuno-bioassay in the study of a model carcinogenesis system, dose-response curves were generated for OFP induction after treatment of rats with various doses of DENA. Rats were 2/3 partially hepatectomized and treated 24 hours later with 0, 20, 35 and 50 mg/kg of N,N-diethylnitrosamine. The concentration of OFP was determined in the liver cytosol and plasma at 21 days post-treatment. The data (Figure 1.17) show a clear linear response between the dosage of the carcinogen and the concentration of OFP in the liver cytosol and in the blood plasma. It is also obvious that OFP
Figure 1.16: Western blots of (1) normal rat plasma, (2) plasma from a rat bearing hepatoma 7777 and (3) cytosol from a 7, 12-dimethylbenz(a)anthracene induced primary mammary tumor. The plasma and cytosol were fractionated by Sepharose CL-6B chromatography prior to SDS-PAGE (10%). After transfer to nitrocellulose, the blots were developed using a mixture of MOFP-A, -B and -C.
Figure 1.17: Levels of OFP activity (by the immuno-bioassay) in the liver cytosol (○) and in the plasma (●) of rats treated 24 hours post-partial hepatectomy with the indicated doses of diethylnitrosamine. Samples were collected 21 days post-carcinogen treatment and the results expressed as units/ mg protein. Each point represents the average from 3 rats +/- S.D.
Figure 1.17
accumulated to higher levels in the plasma. The reason for this accumulation is as yet, unclear.
Discussion

The use of specific proteins produced by tumor cells as markers of carcinogenesis has become a critical element in basic and applied cancer research. There are many examples of marker proteins specific for a particular tumor type which are in widespread use by clinicians for follow-up and diagnosis of cancer patients. Carcinoembryonic antigen (CEA), human chorionic gonadotrophin (hCG) and γ-glutamyl transpeptidase are but a few of these tumor specific antigens, and more are being reported in the literature on a regular basis. Previous studies from this laboratory (61) along with unpublished data (D.E. Schumm and T.E. Webb) have shown that OFP is present in all tumor types, both human and rat, that have been tested thus far. Tissues tested have ranged from lung tumors to human retinoblastoma. In so far as has been reported, OFP is the only potential tumor marker protein currently under investigation which is expressed in such a broad range of cancer phenotypes. For this reason, it would be extremely valuable as a research and clinical tool to develop a means of accurately determining the presence or absence of OFP in the blood. Such an assay would involve high affinity and extremely specific antibodies against OFP. As has been shown in this chapter and in previously published material, production of such antibodies has been achieved for the rat form of OFP. Previous attempts to produce a polyclonal antibody to rat OFP
(34, 35) gained only limited success due to the inability to purify a homogeneous preparation of the protein and the concurrent production of antibodies to the contaminating proteins. The advent of monoclonal antibody technology has allowed this obstacle to be circumvented. In the process of making the hybridoma cell lines, single spleen cells are isolated, each of which is capable of producing only one antibody targeted to a single antigenic epitope. Because of this, mice immunized with non-homogeneous antigen preparations can still be the source of highly specific antibodies when their hybridoma supernatants are rigorously screened as they have been in this study. The addition of an immuno-precipitation step to the standard bioassay using the monoclonal antibodies (MOFP's), coupled with the MOFP's specificity for only OFP-50/55, has allowed the discrimination of samples containing OFP from those having a similar RNA transport activity while avoiding preliminary column chromatography to separate the normal 35 kilodalton RNA transport protein.

The demonstration that the immuno-bioassay is dose-responsive to the administration of carcinogens in a model rat carcinogenic protocol is another indication of the sensitivity of this assay. Other studies ongoing in this laboratory using MOFP-E, the only MOFP which has any reactivity with the human tumor OFP in the immuno-bioassay, have shown preliminary promise in the screening of human blood plasma samples for OFP activity. The relatively high proportion of false negative results (but no false positives) which have been obtained may be due to MOFP-E being
directed against a non-peptide epitope. A carbohydrate epitope, for example, may show heterogenicity in its complexity dependent on the tumor type in which it is produced. The actual utility of this assay is limited to the research laboratory as the high level of animal use and the complexity of the transport reaction does not lend itself readily to its use as a clinical diagnostic kit.

ELISA (Enzyme-linked Immunosorbant Assay) kits for detecting proteins are heavily used in areas as varied as AIDS testing and home pregnancy tests. In order for OFP to become a useful tool for physicians and ultimately the general public, its detection must be transferred to an ELISA based format or some similar assay which can be easily performed by a clinical laboratory technician. In pursuit of this goal, we have developed the competitive inhibition ELISA with which we have been able to detect the presence of OFP in as little as 5 ng of total blood plasma protein. This is a very sensitive assay, but its utility is limited for now due to the fact that only anti-rat OFP antibodies are currently available.

The human placental OFP preparation outlined in chapter 2 and submitted as an immunogen for monoclonal antibody production has recently yielded 7 hybridoma cell lines which have tested positive by a preliminary ELISA. These cell lines are currently being expanded and should any of them pass the screening of future ELISA's and the immuno-bioassay, they may provide the means of transferring OFP detection to the clinical laboratory.

Previous studies indicated that the source of the blood plasma form of OFP, ultimately the clinically useful form, was the tumor in
the animal (34). The demonstration in this study that the blood, plasma and tumor cytosolic forms of OFP appear immunologically identical indicates that OFP may be applicable as an avenue for tumor imaging with radio-labelled antibodies. Immunohistochemical studies (A. Koolemans-Beynen, unpublished data) have shown that these antibodies do enter the tumor cells. The observation that the two forms of OFP are immunologically identical by Western blot analysis using these antibodies does not preclude the possibility that variations in other portions of the protein structure, which may be non-immunogenic, may exist. The development of the anti-human placental OFP monoclonal antibodies may also lend further insight into this question. Detectable and consistent differences between the cytosolic and blood borne forms may be the handle necessary for OFP to be useful as a chemotherapeutic target.
Chapter II Introduction

The expression of specific proteins by tissues other than the "normal" sites of such production is termed ectopic, meaning "out of place" (62). Many proteins have been found to be expressed ectopically. This protein expression in tumors has been shown to be so widespread as to be considered a universal phenomena of neoplasia (63). A common tenant among many of the ectopically expressed proteins of tumors is the fetal and placental origin of their normal expression.

The early observation that OFP is present in rat fetal tissue (28) along with the more recent immunohistochemical detection of this protein in rat fetal liver and placenta (32 and A. Koolemans-Beynen, unpublished data), but never in normal adult tissue puts it in the class of ectopically expressed oncofetal proteins. OFP is also classified as a tumor-associated antigen based upon its elicitation of an immune response from the host animal (64). Building upon this information, an attempt is made in this study to better define the in vivo expression patterns of OFP in the rat fetal system.

As OFP activity is also detectable at a high level in human placenta, this tissue was used as a source of OFP for characterization of post-translational modifications for comparison to the tumor form.

Many proteins (enzymes) are known to be affected in their reactivity by their state of phosphorylation. They can be activated
or deactivated by phosphorylation or dephosphorylation. Maturation promoting factor (MPF) is a regulatory protein stored in *Zenopus sp.* oocytes arrested in prophase I. It has been shown to be activated *in vivo* and *in vitro* by phosphorylation (65). In contrast, protein synthesis in eukaryotes can be inhibited by the phosphorylation of the translational initiation factor eIF-2 (66).

The vast majority of proteins affected by phosphorylation/dephosphorylation as a mechanism for rapid activation/deactivation are modified on serine or threonine residues. In normal cells, 0.03% of the phosphoamino acid content is phosphotyrosine (67). The level of phosphotyrosine residues is elevated in malignant cells (68), the significance of this being that many growth factor receptors, including EGF, PDGF, IGF-1, and insulin receptors, exhibit tyrosine kinase activity (69). Interesting homologies between these receptors and several oncogene products have been uncovered. Examples include the EGF receptor and erb-B; PDGF receptor and v-sis; insulin receptor and v-ros (70, 71). Many of the phosphorylation events occurring in the tumor cell may be due to aberrant activity of these or other kinases and thus inconsequential to cellular growth and tumor expansion. A sub-set of the phosphorylation events may be partially responsible for expression of the malignant phenotype (68-71).

Another post-translational modification which is relevant to the action of many proteins, especially those of the serum, is the covalent attachment of complex carbohydrate structures or glycosylation. Almost all plasma proteins in mammalian systems,
except albumin, are glycosylated, as are many membrane bound proteins (72). Glycoproteins are known to be involved in a myriad of cellular functions ranging from structural molecules (e.g. collagen) to immunoglobulins. One of the most important potential functions, from the cancer researcher’s point of view, is the possible involvement of altered cell-surface-protein glycosylation patterns and the process of metastasis (73, 74). The altered carbohydrate structures may allow the circulating cells to circumvent normal cell/cell interactions. An example is the penetration of the basement membrane by the metastatic cell. The type of sugar residue and the complexity of the carbohydrate moiety has also been shown to control intracellular targeting of proteins to organelles (75) and may also regulate the shuttling of secreted proteins (76). One of the most dramatic examples of glycosylation mediated targeting is in patients suffering from I-cell disease. These patients lack all of the normal lysosomal enzymes intracellularly, but the enzymatic activities are found in high levels in the serum (77). Further investigation demonstrated that the origin of the disease was a deficiency in the N-acetylglucosamine phosphotransferase enzyme causing aberrant glycosylation and thus the concomitant mis-targeting of the proteins.

A powerful tool for the characterization of the sugar residues present in the glycoprotein structure is the specificity of lectins for the individual sugars. Lectins in common laboratory use are plant derived proteins that can bind one or more sugars and can discriminate between the anomers (α or β) of the glycosidic linkages (78, 79). Used in combination with the Western blot technique, the
nature of the glycosylation patterns of individual proteins can be investigated without purification of the target protein to homogeneity (80). This is in contrast to analysis of sugar residues or carbohydrates which are released by digestion with endoglycosidases or exoglycosidases (81).

These initial studies are one part of the long term goal of the full elucidation of the in vivo function of OFP. Differences detected in phosphorylation or glycosylation between the tumor (secreted) and fetal (retained) forms of OFP may be linked to its state of biological activity and mode of action or they may simply be a consequence of the site of the ectopic production and the tumor associated metabolic aberrations.
Materials and Methods

Preparation of Rat Fetal Cytosol

Sprague-Dawley timed first-time-pregnant rats were purchased from Harlan Sprague Dawley (Indianapolis, IN) and maintained on a standard chow diet. After euthanasia by anesthesia and exsanguination, rat fetuses were removed, decapitated, and immediately homogenized in 0.25 M sucrose-TMK buffer having 10 mM phenylmethylsulfonylfluoride (PMSF) using a Sorvall Omnimixer blade homogenizer. All of the fetal tissue obtained from a single pregnant rat was pooled prior to homogenization. The homogenate was centrifuged for 90 minutes at 105,000 x g to produce the cytosol fraction.

Antibodies and Immuno-bioassay

Monoclonal antibodies against OFP (MOFP's) and the immuno-bioassay were essentially as described in Chapter I. Of the six monoclonal antibodies produced in Balb/c mice three; MOFP-A, MOFP-B and MOFP-C; were shown to be very specific for rat OFP. MOFP-B, purified by Protein A MAPS II system® (BioRad, Richmond, CA) chromatography was bound to agarose-anti-mouse [IgG(H+L)] and used to immunoprecipitate the OFP from the fetal cytosol test samples prior to their analysis in the bioassay. The amount of OFP present in the sample was estimated from the difference in RNA
transport activity before and after treatment with the monoclonal antibody containing beads. One unit of activity is defined as the percent of nuclear counts released to the media after a 30 minute incubation at 30°C.

Western Blot Analysis (Fetal Proteins)

Proteins from the 16 day fetal rat cytosol were electrophoresed in parallel with equal amounts of adult rat liver cytosol and hepatoma 7777 cytosol on a 10% SDS-PAGE gel with a 4% stacking gel. Proteins were transferred to nitrocellulose for 2 hours at 450 mA using a transblotter. The blots were processed as described in Chapter I using a mixture of MOFP-A, MOFP-B and MOFP-C at a final dilution of 1:2 in dilution buffer as the primary antibody.

Anti-phosphotyrosine Detection

Blood plasma from a Buffalo strain rat carrying a hind leg transplantable hepatoma 7777 was fractionated by precipitating the proteins between 30% and 60% saturation ammonium sulfate followed by chromatography on Sepharose CL-6B as described in Chapter I. 12 µg of this protein preparation per lane was separated by 10% SDS-PAGE and transferred to nitrocellulose. One lane of this blot was developed using a mixture of MOFP-A, MOFP-B and MOFP-C as the primary antibody. Another strip was developed in parallel using an anti-phosphotyrosine antibody (monoclonal antibody PY20; ICN Biochemical, Cleveland, OH) as the primary antibody.
Human Placental OFP Purification (Figure 2.1)

Term placenta was obtained from the Labor and Delivery Department of the Ohio State University Hospitals through the Tissue Procurement Service of the OSU Comprehensive Cancer Center. With the material constantly kept on ice, the umbilical cord and amniotic sac were removed. The fetal membranes were dissected away from the placental lobules (cotyledons) and discarded. 40 gram sections of the remaining tissue were stored frozen at -80°C until processing. While still frozen, 40 grams of placenta was shaved into thin sections using scissors. 1.5 ml of 0.25 M sucrose-TMK per gram weight placenta was added along with PMSF to 10 mM final concentration. The tissue was homogenized by five strokes of a Dounce homogenizer. Cellular debris were removed by centrifugation for 10 minutes at 12,000 x g. The supernatant was centrifuged for 90 minutes at 105,000 x g and the cytosolic fraction decanted. Cytosolic proteins which precipitated between 30%-60% (NH₄)₂SO₄ saturation were fractionated on a Sepharose CL-6B column as described in Chapter I. Active fractions from the Sepharose CL-6B column, as determined by the bioassay, were pooled, lyophilized and resuspended in a minimum volume of 20 mM Tris, pH 8.0 and dialyzed overnight at 4°C against the same buffer. Approximately 70 mg protein was chromatographed on a fast protein liquid chromatogram (FPLC) Mono-Q HR 5/5 anion exchange column (Pharmacia LKB, Piscataway, NJ). Proteins were eluted by a linear 0 M to 1 M NaCl gradient and 1 ml fractions collected on ice. Active fractions were pooled and dialyzed overnight against 50 mM NaCl-
Figure 2.1: Flow chart outlining strategy for OFP purification from human placenta.
OFP Purification (From Human Placenta)

Homogenize fresh term placenta in buffer containing PMSF

Pellet debris at 12,000g, 10', 4 °C

Centrifuge supernatant at 106,000g, 4 °C, 90'

Ammonium Sulfate cut supernatant between 30-60% sat.

Dialyze

Fractionate through a Sepharose CL-6B gel exclusion Column

Pool active fractions

Lyophilyze

Dialyze

Chromatograph on FPLC Mono-Q column

Pool active fractions

Dialyze

Chromatograph on single-stranded DNA-Cellulose affinity column

Figure 2.1
TMK. 35 mg were chromatographed on a single-stranded DNA cellulose column as described in Chapter I.

Two-dimension Gel Electrophoresis

Protein samples were precipitated with two volumes of acetone at -20°C overnight followed by centrifugation at 16,000 x g for 5 minutes. Pellets were resuspended in lysis buffer [55% (w/v) urea; 2% NP-40; 5% β-mercaptoethanol; 1.6% ampholyte pH 5-8; 0.4% ampholyte pH 3-10] and loaded onto prefocused 1.5 x 130 mm isoelectric focusing (IEF) tube gels (Hoefer Scientific Instruments, San Francisco, CA) [55% (w/v) urea; 10% acrylamide/bisacrylamide (37.5:1); 2% NP-40; 5% ampholyte pH 4-6.5; 1% ampholyte pH 3-10]. After capping with overlay buffer [55% (w/v) urea; 2% NP-40; 5% β-mercaptoethanol; 0.8% ampholyte pH 5-8; 0.2% ampholyte pH 3-10], the proteins were focused for 16 hours at 400 V, 1 hour at 800 V, and 1 hour 400 V (14). The anode solution was 10 mM H₃PO₄ and the cathode solution was 20 mM NaOH. The second dimension was carried out by equilibrating the IEF gels in 10% glycerol; 5% β-mercaptoethanol; 2.3% SDS; 0.0625 M Tris-Cl pH 6.8 for 30 minutes at room temperature and loading them on 10% SDS-PAGE gels with a 5% stack (82). The stacking gels were poured without a comb and layered with water saturated butanol to form a flat interface. Approximately 3 mm of 1% agarose in equilibration buffer was layered over the stacking gel and the IEF tube gels placed horizontally on the agarose. A single tooth parafilm comb was placed at one end of the gel almost touching the agarose. Finally the tube
gel and comb were sealed in 1% agarose in equilibration buffer, the comb removed, and the appropriate amount of protein standards loaded (Figure 2.2). Gels were run at 50 V constant voltage until the dye front reached 1 cm from the bottom of the gel. The gels were then either stained or blotted to nitrocellulose.

Polyacrylamide Gel Staining

For qualitative determination of the progress of the protein purification, gels were silver stained using a silver stain kit (BioRad Labs, Richmond, CA). Gels were fixed in 40% methanol, 10% acetic acid (v/v) for 1 hour and then in 10% ethanol, 5% acetic acid (v/v) twice for 30 minutes each. Proteins were oxidized in a potassium dichromate, nitric acid solution for 30 minutes and then rinsed in distilled water for 45 minutes with a minimum of five changes of water to remove excess oxidizer. Gels were then immersed in silver reagent (silver nitrate) for 30 minutes, rinsed with distilled water for 2 minutes and transferred to freshly prepared developer solution (sodium carbonate and paraformaldehyde). Development was allowed to proceed for 10 to 20 minutes at which time it was terminated by submerging the gel in 5% (v/v) acetic acid.

Gels were stained with Coomassie Blue R-250 when the protein spots were to be recovered for future use. Gels were immersed in stain/fix solution [0.124% (w/v) Coomassie Blue R-250; 10% (v/v) acetic acid; 50% (v/v) methanol)] for a minimum of 1 hour. Stain/fix solution was decanted for future use, excess solution removed with a quick rinse in distilled water and Destaining Solution #1 added [7.5%
Figure 2.2: Diagrammatic representation of the set-up for the second dimension (SDS-PAGE) run of the two-dimensional gel electrophoresis.
Figure 2.2
(v/v) acetic acid; 50% (v/v) methanol]. After shaking for 2 hours, the Destaining Solution #1 was discarded and Destaining Solution #2 [7.5% acetic acid; 5% methanol] was added. The gels were then destained overnight on an orbital platform shaker.

Auto-Antibody Detection and Glycosylation Determination

In order to assay for an auto-immune response to OFP produced post-natally by naturally occurring tumors, 2-D gel Western blots of human placental OFP were developed using serum collected from a human breast cancer patient as the primary antibody. Normal human serum was used as the control primary antibody. Goat anti-human [IgG(H+L)]-HRP (BioRad Labs, Richmond, CA) was the second antibody and development was with 4-chloro-1-naphthol as described in Chapter I.

For the detection of carbohydrate moieties on OFP, rat and human Western blots, blocked with 1% BSA in TBS, were developed using a series of biotinylated lectins (Vector Labs, Burlingame, CA) in place of any primary antibody. The bound lectins were detected by incubation with Avidin-HRP conjugate and then developed with 4-chloro-1-naphthol.

N-terminal Protein Sequencing

Purified human placental OFP was separated by two-dimensional gel electrophoresis (500 μg/gel) as described above only using first dimension tube gels of 5 mm diameter. This allowed up to ten times more protein to be loaded as compared to the 1.5 mm
diameter gels. Second dimension 10% SDS-PAGE gels were 4.5 mm thick. After the second dimension run was completed, gels were stained with Coomassie Blue R-250 as described above and destained until the spots were just visible when viewed on a light box. The spots were excised using a fresh razor blade, frozen at -80°C and lyophilized. Gel pieces were de-acidified by three rounds of rehydration with distilled water and lyophilization. The de-acidified gel pieces were finally rehydrated with 1x SDS-PAGE loading buffer (62.5 mM Tris, pH 6.8; 2% SDS; 10% glycerol; 0.0125% bromophenol blue; 5% β-mercaptoethanol) and loaded directly into the wells of a 1.5 mm 10% SDS-PAGE (5% stack) gel and electrophoresed at 50 V constant voltage overnight. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane for 2 hours at 450 mA in CAPS buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid; 10% methanol; pH 11.0) (83). Prior to protein transfer, the PVDF membrane was pre-wetted with 100% methanol for 5 to 10 seconds and then soaked in CAPS buffer. After transfer, the membranes were air dried, washed in deionized water for 5 minutes, stained with 0.1% Coomassie Blue R-250 in methanol for 5 minutes and then destained in 50% methanol, 10% acetic acid for 10 minutes at room temperature. Membranes were then rinsed in deionized water for 10 minutes, air dried, sealed in boilable cooking pouches (Dazey Corp., Industrial Airport, KS) and stored at -20°C. The proteins on the PVDF membrane were sent to the laboratory of Dr. Paul Matsudaira and N-terminal sequencing was performed by Dr. Chuck Burkins by the automated Edman Degradation using an Applied
Biosystems model 470 sequencer equipped with on-line PTH analysis using the regular program 03RPTH.
Results

Earlier studies using the biochemical assay showed the presence of a protein with RNA transport activity in 18 day rat fetal cytosol (28). The ability to stimulate the release of RNA from isolated nuclei is also a characteristic of the 55 kilodalton factor (OFP) produced by tumor cells (14, 25-32). The monoclonal antibodies, raised against rat OFP have now been shown to cross-react with a protein of the same molecular weight isolated from the fetal rat cytosol. Shown in Figure 2.3 is the temporal change in the level of MOFP inhibitable RNA transport activity during rat fetal development. Activity remains at a constant level from day 9 (the earliest time at which a sufficient amount of tissue could be obtained) to day 14 of gestation. It then increases to a peak of 0.4 units of activity at 16 days gestation (5 days prior to birth). The levels then decrease sharply and reach non-detectable levels around day 20, i.e. just before birth. OFP remains at non-detectable levels post-natally except for its reappearance in tumors. Although insufficient material was available for evaluation prior to 9 days gestation, the protein is clearly phase-specific during fetal development.

At days 12 and 14 of gestation, essentially all of the RNA transport activity could be removed by incubation with monoclonal antibody B. However, at 16 days gestation only 50% was removed. At 2 days post-natally, the total transport activity was at 0.5 units
Figure 2.3: Temporal changes in RNA transport activity during rat fetal development. This activity is associated with OFP as determined by the immuno-bioassay. Day 21 represents birth.
Figure 2.3
per mg of protein, none of which could be removed by the monoclonal antibody. This indicates a gradual rise in the level of the normal 35 kD transport factor during development.

As shown in Table 2.1, the monoclonal antibodies did not detect any of the putative oncofetal protein in the maternal plasma of the 16 day pregnant rat although it is present in the blood from tumor-bearing rats. This indicated that OFP does not cross the placental barrier to maternal circulation.

Shown in Figure 2.4 are Western blots of the oncofetal protein in the cytosol of the 16 day rat fetus and in the cytosol of the rat hepatoma 7777. The two species in the cytosol of the tumor and fetus appear identical in molecular weight to one another and identical to the factor in the tumor plasma. From the markers the molecular weights of the 2 species were estimated to be 50 and 55 kD. These factors were not detectable in normal liver cytosol. The normal liver and tumor cytosol were loaded on the polyacrylamide gel at a higher protein concentration (10 μg/lane) than the fetal cytosol (6 μg/lane).

Investigations using the MOFP's for immuno-histochemistry have shown that the rat fetal OFP is produced in the liver and placenta (32, A. Koolemans-Beynen, unpublished data) and does not appear to be shed to the blood. In order to gain some insight into a probable mechanism whereby the tumor form of OFP is excreted in contrast to the fetal form, purification and analysis of the fetal OFP was undertaken. Human placenta was used as the source of OFP in these studies as it was the only fetal tissue available in sufficient
Table 2.1  Occurrence of OFP in the blood plasma of normal, pregnant and tumor bearing rats.

<table>
<thead>
<tr>
<th>Source of Plasma</th>
<th>Units of OFP per mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Normal rat</td>
<td>ND</td>
</tr>
<tr>
<td>(2) 16 d pregnant rat</td>
<td>ND</td>
</tr>
<tr>
<td>(3) Rat bearing Hepatoma 7777</td>
<td>1.45</td>
</tr>
<tr>
<td>(4) Rat bearing primary mammary tumor</td>
<td>1.40</td>
</tr>
</tbody>
</table>

ND = non-detectable
Figure 2.4: Western blots of protein samples prepared from (1) normal liver cytosol, (2) 16 day fetal cytosol and (3) hepatoma 7777 cytosol. Proteins were separated according to molecular weight by SDS-PAGE (10%) prior to transfer to nitrocellulose. All the blots were developed using MOFP-(A+B+C) as the primary antibody.
quantities for our purposes. Figure 2.5 shows a typical Sepharose CL-6B column activity profile of human placental cytosol as determined in the bioassay. The immuno-bioassay was not used in the human placental studies as none of the six anti-rat MOFP's cross-react with human placental OFP. The 25 kD adult (homologous to the 35 kD factor in the rat) RNA transport factor has not been detected in this tissue, eliminating the need for the discrimination between the two forms (D. E. Schumm, unpublished data). The active fractions (42-52) were pooled, dialyzed overnight against 20 mM Tris, pH 8.0, applied to a FPLC Mono-Q column and eluted with a linear 1 M NaCl gradient. The 1 ml fractions exhibiting biological activity (Figure 2.6, fractions 12-14) were pooled for application to a single stranded DNA-cellulose column. The active fractions from the Mono-Q column correspond to the same active fractions eluted when human cancer patient plasma was chromatographed on the same column (D.E. Schumm, unpublished data). Since monoclonal antibodies could not be used, this gave an added measure of confidence in the authenticity of the activity found in these fractions. The final purification was by single-stranded DNA cellulose. Typically, 1 mg of total protein could be obtained from this final step per 160 gm of placental tissue starting material. Two-dimensional gel electrophoresis followed by silver staining (Figure 2.7) revealed a major protein spot at 55 kilodaltons molecular weight and possessing an isoelectric point of 6.2. While no antibodies reacting with the human placental form of OFP are currently available, several pieces of evidence strongly suggest that this is the protein of interest. First,
Figure 2.5: Placental OFP Purification (step 1): Sepharose CL-6B RNA transport profile (bioassay) of human placental cytosol proteins. Activity is expressed as counts per minute (cpm) for the RNA released. Fractions 42 through 52 were collected and pooled for further purification by the FPLC Mono-Q column.
Figure 2.5
Figure 2.6: Placental OFP Purification (step 2): FPLC Mono-Q column elution and activity profiles. 70 mg of protein from the pooled fractions off the Sepharose CL-6B column was loaded for each run of the Mono-Q column. The bioassay was used to determine the RNA transport activity (cpm) of aliquots of eluate collected from the Mono-Q column.
Counts Per Minute (cpm)

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>0 M NaCl</th>
<th>1.0 M NaCl</th>
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<tbody>
<tr>
<td>0</td>
<td>~150</td>
<td></td>
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<tr>
<td>1</td>
<td>~150</td>
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<tr>
<td>30</td>
<td>~150</td>
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</tbody>
</table>

Figure 2.6
Figure 2.7: Two dimension gel (first dimension iso-electric focusing tube gels; second dimension SDS-PAGE (10%)) of human placental OFP. The protein was purified by sequential chromatography on Sepharose CL-6B, FPLC Mono-Q and single-stranded DNA cellulose. The proteins were visualized by silver staining.
Figure 2.8: Two dimension gels of human placental OFP preparations (a) after chromatography on Sepharose CL-6B and FPLC mono-Q column and (b) after chromatography as above plus chromatography on single-stranded DNA cellulose column. The proteins were visualized by silver staining.
Figure 2.8
Figure 2.9: Western blot of human plasma proteins from a patient bearing a tumor; developed with MOFP-E as the primary antibody. Proteins were fractionated by Sepharose CL-6B chromatography and SDS-PAGE (10%) prior to transfer to nitrocellulose.
Figure 2.10: Western blot of a two-dimensional gel loaded with plasma from a H7777 bearing rat. Proteins were purified by Sepharose CL-6B and CM-Affi gel Blue prior to electrophoresis. After transfer to nitrocellulose, the blot was developed using MOFP-B as the primary antibody.
Figure 2.11: Two dimensional electrophoretic separation of human placental OFP purified by sequential chromatography over Sepharose CL-6B, FPLC Mono-Q and single-stranded DNA cellulose. (a) proteins were transferred to nitrocellulose and the blot developed using serum from a human breast cancer patient as the primary antibody. (b) proteins were stained with Coomassie Blue R-250. The arrow points to the spot corresponding the the area indicated in (a), this was excised and processed for N-terminal protein sequencing.
Figure 2.11
Figure 2.12: Two dimensional gel loaded with normal human plasma protein purified by sequential chromatography on Sepharose CL-6B, FPLC Mono-Q and single-stranded DNA cellulose. Protein spots were visualized by silver staining. The arrow indicates the area OFP was identified on a gel run in parallel and loaded with purified human placental protein.
this preparation had very high RNA transport activity in the bioassay prior to electrophoresis. Second, this protein was highly enriched after single-stranded DNA cellulose column fractionation as shown in Figure 2.8. Third, the major spot on the two-dimensional gel (Figure 2.7) showed the same molecular weight as the human cancer patient OFP analyzed on a single dimension Western blot developed with MOFP-E (Figure 2.9). Fourth, this spot corresponds exactly to the spot detected on a Western blot when rat tumor plasma was purified by Sepharose CL-6B, CM-Affi gel Blue, separated by 2-D gel electrophoresis, transferred to nitrocellulose and developed with MOFP-B (Figure 2.10). Fifth, Western blots of 2-D gels loaded with human placental OFP and developed with serum from a breast cancer patient (Figure 2.11) as the primary antibody showed reaction of a 55 kD spot exclusively with the breast cancer patient serum. This spot was not detected when normal human serum was used as the primary antibody (data not shown). This was a test for the presence of an auto-antibody against the OFP. Production of auto-antibodies has been shown previously for rat tumor OFP (32). The serum from the breast cancer patient identified a protein spot which corresponded with the major protein spot on the Coomassie Blue R-250 stained 2-D gel from which the protein for N-terminal sequencing was excised (see below). Finally, normal human blood plasma, run through the same battery of column chromatography steps as the human placental cytosol and analyzed by 2-D gel electrophoresis and silver staining, lacked any spot corresponding to the placental OFP 2-D gel major spot (Figure 2.12).
Based on the above cited evidence, the spot identified in Figure 2.7 was chosen for further investigation. Large format (5 mm I.D.) two dimension gels were run, each loaded with 500 μg of the purified placental OFP preparation, and stained with Coomassie Blue R-250. The OFP spot was identified, excised from the gel and processed for N-terminal sequencing. A 13 amino acid residue sequence was obtained (Figure 2.13) from Dr. Chuck Burkins at the Whitehead Institute which was shown to be unique when checked against the Wisconsin System Data Bank for DNA and protein sequences. Up to this point in time, no other laboratory has reported a protein sequence or a DNA sequence which would be translated into a protein which matches with this obtained sequence.

A series of Western blot experiments were undertaken in an attempt to better define the characteristics of the different forms of OFP. Previous studies have shown that rat tumor OFP can be phosphorylated in vitro using [γ-32P]ATP and the catalytic subunit of cAMP dependent protein kinase from bovine heart (28). Western blots were performed not simply to show phosphorylation or no phosphorylation, but to determine the amino acid (serine, threonine or tyrosine) which was phosphorylated. One dimension Western blots using 12 μg purified rat tumor plasma OFP were developed using an anti-phosphotyrosine monoclonal antibody as the primary antibody. As is seen in Figure 2.14, this antibody clearly reacts with the 55 kD species and not with the 50 kD species. From this data, the preliminary conclusion is that the presence of the two species of OFP (50 and 55 kD) present in most preparations of the protein is due to
Figure 2.13: N-terminal amino acid sequence of Human Placental OPF.
Figure 2.13

\[
\begin{array}{cccccc}
\text{NH}_2 & \text{E} & \text{F} & \text{F} & \text{N} & \text{D} \\
\text{GLU} & \text{PHE} & \text{PHE} & \text{ASN} & \text{ASP} \\
\text{D} & \text{T} & \text{L} & \text{E} & \text{L} \\
\text{ASP} & \text{THR} & \text{LEU} & \text{GLU} & \text{LEU} \\
\text{V} & \text{D} & \text{S} & \text{COOH} \\
\text{VAL} & \text{ASP} & \text{SER} \\
\end{array}
\]
Figure 2.14: Western blot of Sepharose CL-6B fractionated plasma from a rat bearing a hepatoma 7777. Proteins were separated according to molecular weight by SDS-PAGE (10%) prior to transfer to nitrocellulose. (M) biotinylated protein molecular weight markers (BioRad Laboratories, Richmond, CA) detected with avidin-HRP. (1) blot of plasma proteins developed with MOFP-(A+B+C) as the primary antibody. (2) plasma proteins developed with anti-phosphotyrosine as the primary antibody.
Figure 2.14
the differential phosphorylation of a portion of the OFP and its concurrent shift in mobility on the SDS-polyacrylamide gels. Interestingly, human placental OFP does not react with the anti-phosphotyrosine antibody, suggesting its lack of phosphorylation at a tyrosine residue (data not shown).

In addition to differences in phosphorylation, tumor and fetal OFP show a different pattern of secretion. This may be due to a modified glycosylation pattern in the tumor tissues as has been demonstrated for the procathepsins (76).

Identical Western blots were developed with a series of biotinylated lectins followed by avidin-HRP in an attempt to 1) show whether OFP is a glycoprotein and 2) determine whether or not the fetal and tumor forms exhibit differential reactivity to the lectins, an indication of modified glycosylation. Figure 2.15 (1 = Rat tumor plasma OFP; 2 = human tumor plasma OFP; 3 = Human placental OFP; 4 = rat fetal OFP) shows examples of positive reactions to the lectin Concanavalin A which has a binding specificity for 2-o-methyl-α-D-mannopyranoside (84). Similar results were obtained for the other lectins which bound OFP (data not shown). The results of these studies were tabulated, along with the specificities of the individual lectins, and shown in Table 2.2. All forms of OFP are glycosylated as determined by their detection on Western blots by the biotinylated lectins. However, the human placental OFP is not reactive with the same subset of lectins as rat or human tumor plasma OFP nor does it appear to have the same glycosylation pattern as the rat fetal OFP. The only common reactivity shown with the lectins evaluated is that
Figure 2.15: Western blots of (1) Sepharose CL-6B, FPLC Mono-Q, and single-stranded DNA cellulose purified OFP from a rat bearing a hepatoma 7777 (2) plasma from a cancer patient having metastatic colon carcinoma (3) cytosol from a normal human placenta purified on Sepharose CL-6B, FPLC Mono-Q and single-stranded DNA cellulose and (4) 18 day rat fetal cytosol. Each of these blots was developed with the biotinylated lectin Concanavalin A followed by avidin-HRP. (5) rat H7777 plasma proteins developed with MOFP-(A+B+C) as a positive control for the other blots.
Table 2.2 Identification of Oligosaccharide Moiety on OFP using Biotinylated Lectins on Western Blots

<table>
<thead>
<tr>
<th>Type of Lectin</th>
<th>Specificity of Lectin for Oligosaccharide Residue</th>
<th>Reaction with OFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td>D-mannose (α-linked in oligosaccharide core)</td>
<td>- - - -</td>
</tr>
<tr>
<td>WGA</td>
<td>N-acetylgalactosamine (sialic acid)</td>
<td>+ - - -</td>
</tr>
<tr>
<td>SWGA</td>
<td>N-acetylgalactosamine (not sialic acid)</td>
<td>- NT - -</td>
</tr>
<tr>
<td>PSA</td>
<td>D-mannose (α-linked with N-acetyllactosamine linked α-fucose core)</td>
<td>- - - +</td>
</tr>
<tr>
<td>LCA</td>
<td>D-mannose (α-linked in oligosaccharide core)</td>
<td>+ - - -</td>
</tr>
<tr>
<td>SJA</td>
<td>N-acetyl-D-galactosamine, galactose</td>
<td>- NT - -</td>
</tr>
<tr>
<td>UEA-I</td>
<td>α-linked fucose</td>
<td>- NT - -</td>
</tr>
<tr>
<td>SBA</td>
<td>Terminal α or β N-acetylgalactosamine</td>
<td>- - - -</td>
</tr>
<tr>
<td>DBA</td>
<td>N-acetyl-D-galactosamine</td>
<td>- + - -</td>
</tr>
<tr>
<td>BSL-1</td>
<td>α-galactose, α-N-acetylgalactosamine</td>
<td>- - - -</td>
</tr>
</tbody>
</table>

Abbreviation Key


SWGA: succinylated wheat germ agglutinin

NT: not tested
all react with Concanavalin A indicating carbohydrate moieties containing $\alpha$-linked D-mannose. A more rigorous approach using, for example, specific endoglycosidases will be necessary to fully define the extended carbohydrate structures on the tumor plasma and placental OFP's.
**Discussion**

Based on the information presented in this chapter which shows similar biological activities between the rat tumor and fetal forms of OFP in the immuno-bioassay along with the immunological identity of the two proteins on Western blots, it is highly probable that the factors are products of the same gene. The expression of OFP during fetal development and its re-expression under neoplastic conditions suggest that it may be involved in some, as yet unknown developmental sub-program. The present hypothesis is that OFP may be controlling a "window of differentiation" through which cells must pass when undergoing differentiation (in the fetus) or de-differentiation (in the tumor). This assumes that the current view of neoplasia as a reversal of ontogeny is correct. It is of interest that fetal OFP does not appear to cross the placental barrier and enter the maternal circulation. An earlier study (28) using the anti-OFP polyclonal antibody yielded preliminary evidence that the tumor form and the form detected in the amniotic fluid were immunologically similar. The low levels of activity found in the amniotic fluid may originate from dying cells shed from the fetus which then release their cytosol proteins to the medium. Even so, the OFP activity has never been associated with maternal circulation. Perhaps the pregnant female must be protected from proteins which influence developmental programs and ultimately genetic expression.
Other fetal antigens, α-fetoprotein being the most notable example, readily cross the placental barrier and are used as monitoring agents in the maternal blood. The fact the tumors do exert an effect, usually detrimental, on the host and that this protein is also a tumor antigen may help clarify this lack of crossover. OFP also elicits an autoimmune response (32; S.N. Larroya-Runge, unpublished data) in the host. This is presumably responsible for the clearance of OFP from the blood plasma early in rat carcinogenic protocols. A similar response early in pregnancy may remove any small amounts of OFP which have entered the system.

As OFP is found in the placenta, this tissue was used as a readily available and abundant source of human OFP. Although it is immunologically distinct from the human tumor form (using MOFP-E), based on the data available for the rat fetal and tumor forms, antibodies raised against peptide epitopes of the placental OFP should also react with tumor OFP.

With the purified preparation of human placental OFP available, studies were undertaken to investigate the relationship in structural modifications between it and the tumor plasma forms of OFP. The biochemical mechanisms underlying the ectopic release of OFP from tumor cells is at present unclear. However, it is known that derangements in glycosylation, including miscompartmentalization of glycosylating enzymes may lead to enhanced secretion of proteins by tumors (76). One example supporting this hypothesis is the procathepsins, the significant difference from OFP being that the procathepsin proteins are present in the adult cell before
transformation. These proteins are normally targeted to the lysosome by the mannose-6-phosphate lysosomal recognition markers (85). Secretory routing of the precursor proteins has been shown in malignant cells including breast tumors (86), pancreatic carcinomas (87) and transformed mouse fibroblasts (88). The collective data suggest that OFP is glycosylated in both tumor and in the fetal tissue, but that the branching of their complex oligosaccharides is modified in the tumor secreted form. Tumor but not fetal OFP exhibits reactivity with Pisum satium agglutinin. This lectin has a specificity for D-mannose (α-linked with N-acetylchitobiose linked α-fucose core). The phenomena of altered glycosylation is not uncommon to neoplasia (89, 90). This modification may be responsible, at least in part, for the secretion of OFP from the tumor cells.

As is seen in several of the Western blots presented thus far, rat OFP is many times present as two forms of 50 and 55 kD respectively. Previous studies (28) using in vivo and in vitro phosphorylation with 32P and detection on dried SDS-PAGE gels by autoradiography have shown rat OFP to be a phosphoprotein. This aspect of OFP post-translational modification was further investigated and more precisely defined using a monoclonal antibody directed against phosphotyrosine residues. The Western blot of rat OFP developed with this antibody displayed clearly that the 55 kD species is phosphorylated at a tyrosine residue. This does not preclude either species being phosphorylated threonine or serine residues. It is interesting to note that the 55 kD OFP from human
placenta is non-reactive with this same anti-phosphotyrosine antibody. The significance of this is not yet known and the phosphotyrosine in the rat tumor form may simply be a consequence of aberrant activity of a tyrosine kinase in the tumor. The possibility also exists that this phosphorylation event in the tumor may influence the \textit{in vivo} activity of OFP (as yet unknown) in the tumor or other sites in the host animal affected by OFP. It is unlikely that this protein is being shed to the blood by all tumors and yet exhibits no biological function. The observation of an auto-immune response to OFP in both rats and humans suggests that it may be functioning in a manner which is not beneficial to the host.

The determination of the N-terminal protein sequence of OFP was undertaken for several reasons. First and foremost it was important to determine the uniqueness of this protein or its relatedness to other proteins. By screening the Wisconsin systems data bank of protein and DNA sequences, this protein has proven to be unique. Due to the short sequence which has been obtained thus far, no determinations of functional homologies with other proteins has or can be made. Future sequence determinations on enzymatic or chemically cleaved peptides may open avenues for this type of analysis. These future sequence determinations may also reveal potential sites of glycosylation and phosphorylation. Finally, knowledge of the N-terminal amino acid of the protein will simplify the determination of the far 5' end of the cDNA clone and possibly tell which AUG in the cDNA sequence is used for translational initiation.
Chapter III Introduction

For the molecular analysis of eukaryotic proteins and the genes which code for them, the conversion of messenger RNA to double stranded cDNA has become a fundamental tool (91).

The first step is constructing a cDNA library is the preparation of the messenger RNA (mRNA). For obvious reasons, it is critical to isolate high quality, undegraded RNA for the synthesis of full-length cDNA molecules. This is especially true for mRNA's greater than 2.5 kb in length. This can be accomplished through the constant use of gloves (as fingers are the most common source of contaminating ribonucleases) (92) and the inclusion of ribonuclease inhibitors (e.g. vanadyl ribonucleoside complexes) (93) in solutions used for RNA extraction. As most eukaryotic mRNA's are polyadenylated at the 3' end (the histone messages being the most notable exception), this class of RNA can be separated from the bulk of cytoplasmic RNA's (ribosomal and transfer RNA) by affinity chromatography on oligo-dT cellulose. The integrity of the isolated mRNA is most commonly assessed by fractionation of a small amount (~0.5 μg) by agarose gel electrophoresis and staining with ethidium bromide. Intact mRNA will appear as a smear between 500 bases and 8 kilobases (91).

The synthesis of the complementary DNA to the mRNA is accomplished by using an RNA dependent DNA polymerase (reverse transcriptase) isolated from an avian retrovirus or E. coli containing
a cloned copy of the Moloney murine leukemia virus (MMLV) reverse transcriptase gene. Both enzymes require a primer to initiate DNA synthesis. In most cases, an oligo-dT molecule is annealed to the poly A tail to serve this function. Synthesis proceeds in the 5' to 3' direction.

For many years, the synthesis of the second strand was carried out by a self priming technique in which the hairpin loops formed at the 3' termini of the single stranded cDNA molecules served as the primer (94, 95). The explanation for the formation of the hairpin loops has not been fully elucidated. Nonetheless, this phenomenon was exploited for the second strand synthesis by E. coli DNA polymerase I. The hairpin loop was then digested with the single strand specific nuclease S1 (95, 96). A more efficient and better characterized method of second strand synthesis has superseded the self-priming protocol. In this method, the RNA backbone of the mRNA:cDNA hybrid is nicked by RNase H but not completely removed. The residual RNA segments are then used as primers by E. coli DNA polymerase I for DNA synthesis by nick translation (97, 98).

Following the completion of cDNA synthesis and the ligation of appropriate restriction site linkers for vector insertion, the cDNA of interest can be greatly enriched for by size selection of the total cDNA. This is possible if the size of the mRNA of interest is known. The cDNA is fractionated by agarose gel electrophoresis, stained with ethidium bromide and the cDNA excised, discarding the molecules significantly smaller than the sought after cDNA. As most of the
cDNA's synthesized are smaller than 1 kb, greater than a 100 fold enrichment can be attained by this protocol (99). Unfortunately, if the cDNA of interest is not represented as a full length molecule, it may be accidentally discarded.

Vectors for cDNA cloning vary widely and are generally derived from bacterial plasmids or bacteriophage. The choice of vector depends on the strategy to be used for selection of the clone. For immuno-detection methods, engineered versions of the *E. coli* bacteriophage lambda are the vectors of choice. The most commonly used of these is the λgt11 expression vector developed by Young and Davis (100). This vector contains the *E. coli* Lac Z gene which codes for a functional β-galactosidase and is inducible with the lactose analog isopropylthio-β-D-galactoside (IPTG). Near the 3' end of this gene a unique EcoRI restriction enzyme target site has been engineered so as to allow the insertion of foreign DNA of up to 7.2 kb in length. If the DNA is inserted in the proper orientation and correct reading frame, it will be expressed as a β-galactosidase fusion protein. The protein can then be transferred to a membrane and detected with antibodies specific to the protein of interest.

The expression of a fusion protein can be useful for characterization of the protein itself. Presented in this chapter is a novel fusion protein induction strategy using λgt11 and the subsequent analysis of the product. The most commonly recommended methods for analyzing fusion proteins involve either subcloning into a suitable plasmid expression vector or production of lysogens in *E. coli* from which the proteins can be induced (101,
102). As has been noted previously (103), the subcloning of cDNA inserts from λgt11 can prove to be quite a labor intensive job and may require several attempts before it is successful. Lysogen production in *E. coli* Y1089 requires a minimum of three working days to prepare (allowing for multiple overnight incubations) before the fusion protein can be induced. The method described in this chapter allows for the fusion protein to be prepared the same day that a single positive plaque is picked from a λgt11 library screen. Analysis by Western blotting and/or biological activity determination can be performed the following day.
Materials and Methods
cDNA Synthesis and λgt11 Library Construction

DMBA-induced mammary tumor mRNA was isolated according to Zehner et al. (104). 3.0 g of tumor tissue was ground with a mortar and pestle under liquid nitrogen and transferred to a sterile 40 ml centrifuge tube. After the N₂ had evaporated, 12 ml of solution A (25 mM Tris, pH 8.0; 0.5% SDS; 25 mM EDTA; 75 mM NaCl) and 15 ml of buffered phenol (AmResCo Inc., Solon, OH) were added and rapidly mixed. The slurry was immediately transferred to a glass mortar and blended by one down stroke of a tight teflon pestle. Following a 30 minute incubation on ice, 6 ml of chloroform was added and the mixture centrifuged at 12,000 x g, 30 minutes, 4°C. The aqueous layer was carefully withdrawn to avoid taking any of the white interface, transferred to a fresh tube and the nucleic acids precipitated with ethanol (2.5 volumes ethanol, 0.1 volumes 3 M sodium acetate) for 15 minutes at -80°C. The resultant pellet from a 10 minute, 12,000 x g spin was dissolved in 12 ml of 1.0 M CsCl with 1% β-mercaptoethanol and layered over a 5 ml cushion of 5.7 M CsCl containing 1% β-mercaptoethanol. RNA was pelleted while DNA banded at the interface after spinning 24 hours at 15°C, 22,000 rpm in a Beckman SW-28.1 rotor. Poly A⁺ RNA was selected by chromatography over oligo-dT cellulose (Pharmacia LKB, Piscataway, NJ) by the method of Kingston (105). All solutions, columns,
glassware and plasticware were treated with 0.1% diethylpyrocarbonate (DEPC) overnight and autoclaved for 30 minutes at 20 psi to remove contaminating ribonucleases (92). A drawn out, siliconized-glass Pasteur pipet was plugged with siliconized glass wool and washed with 10 ml of 5 M NaOH and then rinsed with DEPC treated water. 0.25 ml of dry oligo-dT cellulose powder was suspended in 700 µl of 0.1 M NaOH and the slurry transferred to the glass column and rinsed with 10 ml of DEPC-water. The column was equilibrated with 10 ml of loading buffer (0.5 M LiCl; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA; 0.1% SDS). Total RNA (3 mg) in 1 ml water was heated for 10 minutes at 70°C in a water bath to denature any possible secondary structure. The solution was then made to 0.5 M LiCl by the addition of 50 µl of 1 M LiCl. The RNA was loaded onto the column, rinsed with 1 ml of loading buffer, the eluate was collected and again passed over the column. Unbound RNA was rinsed from the column with 2 ml of middle wash buffer and the poly A+ RNA eluted with 1 ml of elution buffer (2 mM EDTA; 0.1% SDS) into siliconized 1.5 ml microfuge tubes. RNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol at -80°C overnight. Following centrifugation for 20 minutes at 16,000 x g in a microfuge at 4°C, the poly A+ RNA was resuspended in 200 µl of DEPC-water and stored frozen at -80°C.

The corresponding cDNA was synthesized using the Pharmacia® cDNA synthesis kit from 2 µg of poly A+ RNA (figure 3.1). First strand synthesis was with Moloney Murine Leukemia Virus (MMLV) reverse transcriptase and was oligo-dT primed. Following RNase H
Figure 3.1: Brief summary of the reactions involved in the synthesis of double stranded cDNA. This strategy was employed in this study using the Pharmacia® cDNA synthesis kit.
cDNA Synthesis Protocol

mRNA

Anneal oligo-dT primer

Synthesize first strand cDNA using Reverse Transcriptase

Treat with RNase H

Synthesize second strand cDNA using DNA pol I, Ligase and Klenow

Double Stranded cDNA

Figure 3.1
Figure 3.2: Diagram of the "Spun column" provided with the Pharmacia® cDNA synthesis kit. Solutions to be passed over the column were applied directly to the top of the packed bed of Sephacryl® S-200.
Figure 3.2
digestion to nick the RNA strand, second strand synthesis was performed by nick translation using *E. coli* DNA polymerase I. The resulting double stranded DNA was blunt ended with *E. coli* DNA polymerase I Klenow fragment and the cDNA was extracted once with phenol/chloroform (1:1) and passed through a Sephacryl® S-200 spun column (figure 3.2) (Pharmacia LKB, Piscataway, NJ). The column was equilibrated with two applications of 4 ml ligation buffer [66 mM Tris-Cl, pH 7.6; 1 mM spermidine; 10 mM MgCl₂; 15 mM DTT; 0.2 mg/ml BSA (RNase/DNase free)] and then centrifuged at 400 x g for 2 minutes in a swinging bucket rotor. After preparation of the column, the aqueous cDNA solution from the phenol/chloroform extraction was applied to the top of the Sephacryl® S-200 bed and the column again centrifuged at 400 x g for 2 minutes while collecting the effluent in a 1.5 ml microfuge tube. To the cDNA in ligation buffer, 3 µl of EcoRI adaptor solution (Pharmacia LKB, Piscataway, NJ.), 1 µl ATP and 3 µl T4 DNA ligase were added, mixed gently, and incubated at 14°C overnight. The following morning, the cDNA with adaptors was heated to 65°C for 10 minutes to denature the DNA ligase and 10 µl of ATP solution, 1 µl of T4 polynucleotide kinase were added. After incubating at 37°C for 30 minutes, the aqueous DNA solution was phenol/chloroform extracted to remove the kinase and unligated adaptors were removed by passage of the cDNA solution through a spun column as above. The cDNA was eluted while the free adaptors remained in the Sephacryl® matrix. The volume of the final spun column effluent was adjusted to 150 µl using STE buffer (10 mM Tris-Cl, pH 7.5; 1 mM EDTA; 150 mM NaCl)
and the cDNA inserted into EcoRI digested, Calf Intestinal Alkaline Phosphatase treated λgt11 arms (Stratagene, LaJolla, CA) (figure 3.3). 30 μl of cDNA in STE buffer was combined with 2 μl of λgt11 arms and the DNA co-precipitated with a 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol as above. The resultant DNA pellet was dried in a SpeedVac (Savant Instruments, Farmingdale, NY) for 10 minutes and resuspended in 9 μl of ligation buffer. To this, 1 μl of ATP solution (diluted 1:10 in ligation buffer) and 1 μl of T4 DNA ligase was added and incubated at 14°C for 24 hours. The recombinant λgt11 DNA was packaged into phage heads in vitro using the Packagene® extract system (Promega Biotechnology, Madison, WI). One tube of packaging extract (50 μl) was removed from the -80°C freezer and the entire volume of cDNA added immediately. The still frozen mixture was thawed on ice for 45 minutes and then transferred to a 22°C water bath and incubated for 2 hours. The packaged library was diluted to 500 μl with SM (0.1 M NaCl; 8 mM MgSO₄ · 7H₂O; 50 mM Tris-Cl, pH 7.5; 0.01% gelatin), 50 μl of CHCl₃ was added and the mixture stored at 4°C.

λgt11 Library Screening

Library screening (106) was performed by plating the phage on a lawn of E. Coli Y1090R⁻ at a density of approximately 25,000 plaque forming units (pfu) per 150 mm plate. E. coli Y1090R⁻ (single colony) were inoculated into 50 ml LB (per liter: 10 g bactotryptone; 5 g yeast extract; 10 g NaCl; pH 7.0) + 50 μg/ml ampicillin and grown to saturation at 37°C with shaking. Cells were
Figure 3.3: Important features of the cloning vector λgt11. Lac Z = *E. coli* β-galactosidase gene; EcoRI = Engineered Cloning site; CI857 = temperature sensitive λ repressor; nin5 = mutation allowing expression of delayed early genes independent of N protein activity; Sam100 = amber mutation making phage lytic defective in hosts not having SupF amber repressor. The β-galactosidase reading frame is also shown in relation to the EcoRI cloning site. Adapted from Young and Davis (100) and Ausubel *et. al.* (92).
pelleted by centrifugation at 3000 x g, 5 minutes, 4°C and resuspended in 25 ml of 10 mM MgSO₄. Phage from the packaged library were mixed with 400 µl Y1090R⁻ per plate and incubated at 37°C, 15 minutes with shaking to allow phage attachment. 6 ml of melted top agar (LB + 0.7% agarose) warmed to 55°C was added to the phage/Y1090R⁻ and immediately poured onto a 150 mm LB + amp plate (LB + 15 g/l noble agar; 50 µg/ml ampicillin). After the top agar had solidified, the plates were incubated for 2 to 4 hours at 42°C (i.e. until the plaques were just visible) and then overlayed with dry nitrocellulose filters which had been previously soaked in 10 mM isopropylthio-β-D-galactoside (IPTG). The incubation was continued at 37°C for 3 hours. The filter positions were marked using an 18 gauge needle dipped in Indian ink and the filters carefully removed from the plates and air dried. Unbound sites were blocked with 5% non-fat dry milk in TBS for 30 minutes followed by a 1 hour room temperature incubation in 2.5% non-fat dry milk in TBS containing MOFP (A + B + C). Following successive washes with TBS, TBS + 0.1% NP-40, and TBS, each 137 mm filter was incubated with 1.0 µCi of ¹²⁵I-Protein A (New England Nuclear, Boston, MA) in 2.5% milk in TBS for 1 hour at room temperature. Filters were washed as above and exposed to Kodak XAR-5 film with intensifying screens (Dupont Company, Inc., Wilmington, DE) for 72 hours at -80°C. Positive plaques were identified and picked as agar plugs using the wide end of a sterile Pasteur pipet and each soaked in 1 ml of SM. Phage from each plaque picked were re-plated at low density (less than 50 plaques per 90 mm LB + Amp plate) and re-screened as
Figure 3.3

\[ \lambda gt11 \]

\[ 3'...GAC CTT AAG GCG...5'\]

\[ gin\ phe\ glu\ ala\ ]
above. This repeat screening was carried through at least two rounds until a pure culture (single positive plaque) was obtained. The entire cloning strategy is outlined in Figure 3.4.

β-Galactosidase Fusion Protein Analysis

In order to confirm the identity of the plaques which were picked from the library screens, cultures were set-up for the purpose of inducing the expression of the fusion proteins from these λgt11 clones as well as from several negative control plaques. Single plaques were soaked in 1 ml of SM for greater than 2 hours at room temperature to elute the phage particles. The cultures consisted of 8 ml LB media, 8 μl ampicillin (50 mg/ml), 400 μl Y1090R- (prepared as above) and 100 μl phage in SM. Cultures were incubated at 37°C with shaking for 2 hours. 80 μl of 1 M IPTG was then added to each culture (10 mM final concentration) to induce the lac Z promoter and the incubation continued for various time periods.

For Western blot analysis of the fusion proteins (101), 1 ml aliquots of the cultures were collected at 0, 1, 2, 3, 4, and 17 hours post induction and the cells pelleted by centrifugation at 12,000 x g for 1 minute at room temperature in a microfuge. The supernatant was removed by aspiration and each pellet rapidly resuspended in 100 μl of 1x SDS loading buffer (50 mM Tris-Cl, pH 6.8; 100 mM DTT; 2% SDS; 0.1% bromophenol blue; 10% glycerol) by pipetting up and down. Samples were floated in a boiling water bath for 3 minutes to lyse the cells, and 1 μl of PMSF was added, the contents vortexed rapidly and then frozen at -80°C. Subsequently, samples were
Figure 3.4: Flow chart outlining the procedure used for the isolation of the cDNA clone from λgt11.
OFP cDNA Cloning

- Total RNA prepared from rat Hep. 7777 or DMBA induced mammary carc.
- Poly A+ RNA selected on oligo-dT column
- cDNA synthesized using BRL or Pharmacia cDNA synthesis kit
- cDNA inserted into λgt11 following linker addition
- Phage plated on Y1090R- (25,000 pfu/150mm plate)
- Proteins lifted onto nitrocellulose filters saturated with 10mM IPTG, 4h.
- Fusion proteins detected with a mixture of monoclonal antibodies followed by 125-I Protein A.
- Autoradiography

Figure 3.4
thawed, centrifuged at 12,000 x g for 1 minute in a microfuge and a 25 µl aliquot of each was separated by discontinuous SDS-PAGE (4% stacking gel, 8% running gel). Proteins were electroblotted to nitrocellulose for 2.5 hours at 450 mA. Unstained protein molecular weight marker lanes were cut from the nitrocellulose membrane and stained with Coomassie Blue R-250 (83). Identical Western blots were developed first using either a mixture of MOFP A + B + C or anti-β-galactosidase polyclonal antibody (Sigma Chemical Co., St. Louis, MO) followed by goat anti-mouse [IgG(H+L)]-HRP conjugate and 4-chloro-1-naphthol as the substrate. For a clone expressing a fusion protein which was positive by Western analysis using MOFP (A + B + C), 25 µl per lane of the 4 hour and 17 hour post IPTG induction protein preparations was separated by SDS-PAGE and transferred to nitrocellulose as above. Strips having one 4 hour and one 17 hour lane were developed individually using MOFP-A, MOFP-B, and MOFP-C alone in order to define which antibody recognized the epitope being expressed by the positive clone.

Fusion protein cultures to be tested for the presence of OFP biological activity (in vitro nucleocytoplasmic RNA transport) were prepared by a method described for use with induced bacteriophage λgt11 lysogen cultures (101). At the appropriate times post IPTG induction, cells were harvested from 8 ml of culture by centrifugation at 12,000 x g for 30 seconds at 4°C. The medium was removed by aspiration and the bacterial pellets rapidly resuspended by pipetting up and down in 400 µl extraction buffer (50 mM Tris-Cl, pH 7.5; 1 mM EDTA, pH 8.0; 5 mM DTT; 50 µg/ml PMSF). The cells
were transferred to microfuge tubes and immediately submerged in liquid nitrogen for 2 minutes. The tubes were recovered from the liquid nitrogen and held between the fingers until the contents just thawed. 40 μl per tube of freshly prepared lysozyme (10 μg/ml in 10 mM Tris-Cl, pH 8.0) was added and the tubes placed on ice. After 15 minutes, 500 μl of 5 M NaCl was added to each tube, the contents mixed by holding the tube sideways and flicking with a finger and the mixtures incubated at 4°C for 30 minutes on a rotating wheel. Cellular debris were pelleted by centrifugation at 12,000 x g for 30 minutes at 4°C in a microfuge. The supernatants of this final spin were transferred to dialysis bags and dialyzed overnight at 4°C against 0.1 M KCl-TMK containing 50 μg/ml PMSF. Aliquots were tested for the presence of MOFP inhibitable activity in the immuno-bioassay.

Subcloning and DNA Sequencing

The cDNA insert from the λgt11 phage which tested positive by the Western blot and the immuno-bioassay was excised with the restriction endonuclease EcoRI and subcloned into the EcoRI site of the plasmid pUC19 (Figure 3.5).

Liquid lysates were set-up by combining in sterile 20 x150 mm tubes, 200 μl phage plaque elution in SM plus 400 μl E. coli Y1090R- in 10 mM MgSO₄ (as described above) and shaking at 37°C for 15 minutes to allow phage adsorption. LB media (8 ml) + 8 μl ampicillin (50 mg/ml) was added to each tube and the incubation continued for 7-9 hours at which time lysis could be observed by the clearing of
Figure 3.5: Map of plasmid pUC19. Adapted from Ausubel *et. al.* (92). Map constructed on MacPlasmap v.1.6 software (107).
Figure 3.5
the media and the presence of large amounts of cell debris. At this point, 100 μl of CHCl₃ was added to each tube and the incubation continued for an additional 20 minutes. This insured a complete lysis. The chloroform was removed with a Pasteur pipet and 80 μl per tube of lambda prep nuclease (5 mg/ml DNase I; 5 mg/ml RNase A; 30 mM NaOAc; 50% glycerol) was added, vortexed, and incubated at 37°C for 45 minutes to digest the bacterial nucleic acids. The solutions were transferred to fresh polypropylene centrifuge tubes each containing 0.46 g of NaCl. The NaCl, which releases the phage from the bacterial debris, was dissolved by gently inverting the capped tubes. The bacterial cell debris were then pelleted at 14,400 x g in a Beckman JS-13 swinging bucket rotor. Supernatants were transferred to fresh centrifuge tubes containing 0.8 g of polyethylene glycol (PEG) 8000 and placed on a rotating Lab Quake Shaker™ (Lab Industries, Inc., Berkeley, CA) overnight at 4°C to dissolve the PEG and precipitate the phage particles. The next morning, the phage particles were pelleted by centrifugation at 14,000 x g in a JS-13 rotor and gently resuspended in 500 μl of SM per pellet, then transferred to fresh 1.5 ml microfuge tubes. Residual PEG was removed by extraction with 500 μl of CHCl₃, 20 μl of 0.5 M EDTA, 10 μl of 10% SDS, and 10 μl of proteinase K (2.5 ml/ml) was added to the aqueous supernatant. After a 30 minute incubation at 65°C to disrupt the phage heads, proteins were removed by extracting once with an equal volume of phenol/chloroform (1:1), then once with an equal volume of chloroform. DNA was precipitated from the aqueous phase by the addition of 170 μl of 5 M NH₄OAc and
700 μl of isopropanol, mixing, chilling at -80°C for 15 minutes, and centrifuging at 16,000 x g for 20 minutes at room temperature in a microfuge. Pellets were rinsed twice with 80% ethanol, dried in a SpeedVac concentrator and dissolved in 20 μl each of TE pH 7.6 (10 mM Tris-Cl, pH 7.6; 1 mM EDTA, pH 8.0). The DNA was quantitated by measuring the absorbance at 260 nm and using the formula:

\[(\text{O.D.} \ 260) \times (50 \ \mu\text{g/ml}) \times \text{(dilution factor)} = [\text{DNA}] \ (\mu\text{g/ml})\]

20 μg of the λ DNA was further purified by passage over an Elutip-d® syringe column. The column was primed by forcing 2 ml of high salt solution (1.0 M NaCl; 20 mM Tris-Cl, pH 7.4; 1.0 mM EDTA) through and then flushing with 5 ml of low salt solution (0.2 M NaCl; 20 mM Tris-Cl, pH 7.4; 1.0 mM EDTA). The DNA sample was diluted with 500 μl of low salt solution and drawn into a 3 ml syringe using an 18 gauge needle. The DNA solution was then slowly forced through the column. The column, with the DNA bound, was rinsed with 3 ml of low salt buffer and subsequently the DNA was eluted by the slow passage of 400 μl of high salt buffer. The purified DNA was precipitated with 1 ml of ethanol at -80°C for 15 minutes and pelleted at 16,000 x g, 20 minutes in a microfuge. The pellet was washed two times with 80% ethanol, dried in a SpeedVac™ and dissolved in 34 μl sterile distilled water. All of the DNA was digested with EcoRI by the addition of 4 μl REact® 3 10x reaction buffer (final concentration : 50 mM Tris-Cl, pH 8.0; 10 mM MgCl₂; 100 mM NaCl)
and 2 μl EcoRI (Bethesda Research Laboratories, Bethesda, MD) (20 units) and incubating at 37°C for 2 hours. 10 μl of the digested DNA was mixed with 1 μl of 10x Ficoll loading buffer (0.4% bromophenol blue; 0.4% xylene cyanol FF; 25% Ficoll) and separated by size on a 1.5% agarose gel in 0.5x TBE buffer (0.045 M Tris-borate; 0.001 M EDTA) containing 0.5 μg/ml ethidium bromide. pUC19 plasmid DNA (1μg) was also digested with EcoRI and electrophoresed on the same gel with the digested λDNA. The cDNA insert band (approximately 200 base pairs long) and the digested pUC19 band were both excised from the gel using a fresh razor blade while viewing the gel on a ultra-violet (UV) transilluminator. The DNA's were co-eluted from the agarose gel pieces by spin elution.

An elution device (figure 3.6) was assembled by de-capping a 1.5 ml microfuge tube and inserting a 0.5 ml microfuge tube which had been punctured near the base with a 24 gauge needle and then packed 1/3 full with siliconized glass wool. The gel pieces were placed inside the 0.5 ml tube and the device spun at 12,000 x g, 5 minutes at room temperature. The elute containing the DNA was collected from the bottom tube and transferred to a fresh 1.5 ml microfuge tube. The device was re-centrifuged and the eluate recovered and pooled with the first batch. The device having the retained agarose was discarded. The volume of the eluate was brought to 200 μl with TE pH 7.6 and extracted twice with 1 ml of water saturated butanol to remove the ethidium bromide. Other contaminants were removed by one phenol/chloroform (1:1) extraction and one chloroform extraction followed by ethanol
Figure 3.6: Assembly of a spin elution device for recovery of DNA from agarose gel slices.
De-capped 1.5 ml Microcentrifuge Tube

0.5 ml microfuge tube

Silicanized Glass Wool

Agarose Gel Pieces

Needle Puncture

Assembled Elution Device

Figure 3.6
precipitation. This DNA pellet was washed twice with 80% ethanol, dried and resuspended in 23 μl of sterile distilled water. Ligation was carried out by the addition of 6 μl of 5x ligation buffer (250 mM Tris-Cl, pH 7.6; 50 mM MgCl₂; 5 mM ATP; 5 mM DTT; 25% (w/v) PEG-8000) and 1 μl of T4 DNA ligase (6.5 units/ml; Pharmacia LKB, Piscataway, NJ) followed by incubation at 12°C for 16 hours.

Competent cells (E. coli DH5α F') were prepared for transformation. 0.5 ml of LB media was inoculated with a single colony picked from a DH5α F' streak plate and incubated for 30 minutes at 37°C with vigorous shaking. The entire 0.5 ml was then added to 50 ml of LB and incubated at 37°C with shaking until the O.D. 600 reached 0.4-0.6. Cells were pelleted at 10,000 x g, 4°C for 10 minutes, resuspended in 25 ml of ice-cold 50 mM CaCl₂ and incubated on ice for 20 minutes. Cells were again pelleted as above, resuspended in 2.5 ml of 50 mM CaCl₂, and incubated on ice for 40 minutes. The cells were made 15% in sterile glycerol and stored as 200 μl aliquots at -80°C.

Transformation was carried out by adding the entire volume of the ligation mix to 200 μl of frozen DH5α F' competent cells and incubating on ice for 40 minutes. Cells were heat shocked at 42°C for 45 seconds, 1 ml of LB media was added and recovery carried out at 37°C for 1 hour. The tubes were then mixed by inverting. 100 μl aliquots were plated on LB/LacZ inducing plates (LB agar; 50 μg/ml ampicillin; 0.1 mM IPTG; 0.004% 5-bromo-4-chloro-3-indolyl-β-D-galactoside [X-gal]) and incubated at 37°C overnight. White colonies were picked with sterile toothpicks and dropped directly into 3 ml
LB + ampicillin and incubated with shaking at 37°C overnight. Blue colonies represent bacteria which have been transformed with self-closed pUC19 and thus express a functional β-galactosidase α fragment which, in DH5αF, can α complement (108) and cleave the X-gal, resulting in the blue color.

Plasmid DNA was prepared from the overnight cultures by a mini-prep protocol (109). Bacterial cells (2 ml) were harvested by 16,000 x g centrifugation (1 ml each time) in a microfuge for 1 minute. Pellets were resuspended in 100 μl of GET buffer (50 mM glucose; 10 mM EDTA, pH 8.0; 25 mM Tris-Cl, pH 8.0). The cells were lysed by the addition of 20 μl of freshly prepared 0.2 N NaOH; 1% SDS and mixing by inversion 5-6 times. After incubating on ice for 5 minutes, 150 ml of 3 M K, 5 M acetate, pH 4.8 was added, the tubes inverted 5-6 times and incubated on ice for 5 minutes (neutralization). Following a 5 minute spin, 16,000 x g, 4°C in a microfuge, the supernatant was collected and 5 μl of (10mg/ml) DNase free RNase A was added and incubated at 37°C for 30 minutes to digest the bacterial RNA. RNase A and other contaminants were removed by one phenol/chloroform extraction followed by one chloroform extraction. The DNA was ethanol precipitated, washed twice with 80% ethanol, dried and dissolved in 20 μl of TE pH 7.6. 4 μl of each mini-prep was digested with EcoRI and run on a 1.5% agarose gel with 0.5 μg/ml ethidium bromide to confirm the presence of an insert of the proper size. 1 ml of each culture having a plasmid with the proper insert size was made 15% in sterile glycerol and frozen at -80°C.
DNA Sequencing

The cDNA insert in pUC19 (pOFP) was sequenced in the forward and reverse directions by the dideoxy chain termination method of Sanger (110) using Sequenase® Version 2.0 (United States Biochemical Corp, Cleveland, OH). Sequencing primers (0.5 pmole) (Forward reaction: 17-mer universal M13 primer (-40) 5'-GTTTTCCCAGTCACGAC-3'. Reverse reaction: 16-mer reverse M13 sequencing primer, 5'-TTCACACAGGAAACAG-3') (United States Biochemical Corp., Cleveland, OH) were combined in microfuge tubes with 2 μg each of pOFP DNA (10 μl total volume) from the mini-prep described above. The plasmid DNA was denatured by heating the capped tube to 65°C for 2 minutes and the primers annealed to the pOFP template by transferring the warmed tubes to a 37°C water bath for 30 minutes. The annealed primers were labeled by limited extension for 3 minutes at room temperature with 1.0 μl of DTT (0.1 M), 2.0 μl of dGTP labeling mix (1.5 μM dGTP; 1.5 μM dCTP; 1.5 μM dTTP), 0.5 μl [α-35S]dATP (1000-1500 Ci/mmole; 5 μCi) (New England Nuclear, Boston, MA) and 2.0 μl of Sequenase® Version 2.0 enzyme (diluted 1:8 in enzyme dilution buffer [10 mM Tris-Cl, pH 7.5; 5 mM DTT; 0.5 mg/ml BSA]). 3.5 μl of the labelled mixture was transferred to tubes labelled G, A, T, and C which contained 2.5 μl of pre-warmed (37°C) ddGTP, ddATP, ddTTP, and ddCTP termination mix respectively. Each termination mix contained 80 μM dGTP, 80 μM dATP, 80 μM dCTP, 80 μM dTTP, 8 μM ddNTP (G, A, T or C respectively for each tube) and 50 mM NaCl. All tubes were
incubated for 8 minutes at 37°C for the extension/termination reactions to be completed and then quenched with 4 μl per tube of stop solution (95% formamide; 20 mM EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol FF).

Sequencing reactions were run on an International Biotechnologies Inc. STS-40 vertical gel apparatus using a 0.2 mm denaturing 6% polyacrylamide gel made in 1x TBE. The short glass plate (which contacts the gel box directly) was siliconized by thoroughly rubbing on Prosil® (Baxter Scientific, McGaw Park, IL) solution (25 ml distilled water; 250 μl Prosil®) and baking at 70°C for 1 hour. The long plate was treated with a solution of 25 ml 95% ethanol, 125 μl methacrylopropyltrimethoxysilane (Aldrich Chemical Co., Milwaukee, WI) and 840 μl of 10% acetic acid. This allows the gel to stick to the long plate when the plates are peeled apart after the gel has been run. The plates were assembled by taping with the 0.2 mm spacers in place. The gel mix [22.3 ml dH2O; 14 ml 5x TBE; 35 g urea; 70 mg ammonium persulfate; 10.5 ml acrylamide/bisacrylamide, (19:1)] was carefully poured in, the plates positioned horizontally on the lab bench, the conventional comb inserted, clamped and the gel allowed to polymerize overnight. The following day, the comb was removed, the gel assembled on the gel box, buffer added, the wells rinsed thoroughly using a 20 ml syringe and an 18 gauge needle and the system pre-run at 2000 volts for 30 minutes. After again rinsing the wells, the termination reactions (G,A,T,C) were heated to 80°C for 2 minutes and 3 μl of each was loaded onto the gel. Electrophoresis was carried out at 2000 volts
until the bromophenol blue marker had just reached the bottom of the gel. At this time fresh lanes were rinsed and the samples re-loaded as above and the electrophoresis continued. Three rounds of loading were carried out in order to maximize the amount of sequence data which could be obtained from a single gel. When the bromophenol blue tracking dye from the third loading had almost reached the bottom of the gel, the run was stopped, the glass plates pried apart and the gel soaked in 10% acetic acid for 15 minutes to remove the urea. Following a thorough rinse with tap water, the gel was dried on the glass plate overnight at 60°C. The sequencing ladders were visualized by exposing the dried gel directly to Kodak™ XAR-5 film at room temperature for 48 hours.

For the determination of the orientation of the OFP cDNA clone and its proper reading frame, DNA sequencing was performed directly on the λgt11-OFP clone. This procedure requires the template DNA to be of very high purity. This was achieved by growing a large scale liquid lysate of the phage and purifying the particles by CsCl gradient centrifugation. 500 ml of LB media containing 50 μg/ml ampicillin; 0.2% maltose (to induce high level expression of the maltose receptor, the site of attachment for the λ phage); 8mM MgSO₄ was inoculated with 500 μl of E. coli Y1090R⁻ in 10 mM MgSO₄ and incubated at 37°C with vigorous shaking until the OD₆₀₀ reached 0.5. At this time, 1 x 10¹⁰ phage were introduced and the incubation continued until the culture lysed (approximately 6 hours). Upon lysis, 10 ml of CHCl₃ was added, the incubation continued for an additional 20 minutes, then the cultures were
stored at 4°C overnight. The next morning, the CHCl₃ was removed and the culture was warmed to room temperature. Lambda prep nuclease (100 µl/500 ml culture) was added, the solution was mixed and then incubated at 37°C for 45 minutes. NaCl was added to a final concentration of 1 M to dissociate the phage particles from the cellular debris, and the debris was pelleted at 11,000 x g for 10 minutes at 4°C. 50 g of PEG-8000 was added and dissolved at room temperature followed by a 1 hour incubation in ice water to precipitate the phage. Phage were recovered by centrifugation at 11,000 x g and the pellet was resuspended in 6 ml of SM. PEG-8000 and residual cellular debris were removed by 1 extraction with an equal volume of CHCl₃. The volume of the collected aqueous phase was measured and 0.5 g of CsCl per ml was added and dissolved (ρ = 1.15 g/ml). This solution was carefully layered over a CsCl step gradient (101)(ρ = 1.7 g/ml; ρ = 1.5 g/ml; ρ = 1.45 g/ml) which had been premade in a SW-28 ultra-centrifugation tube (figure 3.7). The gradient was centrifuged at 22,000 rpm for 2 hours at 4°C in a Beckman SW-28 rotor. The blue band of phage particles which formed at the interface between the 1.45 g/ml layer and the 1.5 g/ml layer was collected by puncturing the side of the ultracentrifugation tube with an 18 gauge needle attached to a 10 ml syringe just below the phage band and carefully pulling the band into the syringe. This solution was placed in a 15 x 87 mm ultracentrifugation tube, filled with CsCl solution (1.5 g/ml in SM), the tube was capped with a titanium cap and centrifuged at 38,000 rpm for 24 hours at 4°C in a Ti50 rotor. The phage band was collected as described above
Figure 3.7: CsCl step gradient for the purification of phage particles. $\rho =$ solution density in g/ml. Figure adapted from Sambrook et. al. (101).
(0.5 ml collected), 50 µl of 2 M Tris-Cl (pH 8.5)/0.2 M EDTA was added and the solution mixed by inverting. The EDTA chelated all of the available Mg\(^{2+}\) ions causing the phage heads to rupture. 0.5 ml of formamide was added, mixed and incubated at room temperature for 30 minutes. 1 ml of 100% ethanol was added, mixed gently, the solution divided between two 1.5 ml microfuge tubes and centrifuged at 16,000 x g, room temperature in a microfuge for 2 minutes. The resulting pelleted DNA was washed once with 80% ethanol and dissolved in 100 µl of TE pH 7.6.

**Direct Lambda DNA Sequencing**

Direct \(\lambda\) DNA sequencing was performed using the TAQuence\({\textsuperscript{TM}}\) DNA sequencing kit (U.S. Biochemical Corp., Cleveland, OH). The elevated chain extension temperature (72°C) used in this protocol aids in eliminating compressions in the sequence caused by reannealing of the \(\lambda\) DNA template. \(\lambda\)gt11 forward sequencing primer (24-mer: 5'-GGTGGCGACGAC- TCCTGGAGCCCG-3'; 10 pmole/µl) (New England Biolabs, Beverly, MA) was \(\textsuperscript{32}P\) end labelled by combining 0.5 µl of primer (5 pmole), 1.5 µl of [\(\gamma\)-\(\textsuperscript{32}P\)]ATP (3000 Ci/mmmole; 10 µCi/µl) (New England Nuclear, Boston, MA), 0.5 µl of 10x T4 polynucleotide kinase buffer (0.5 M Tris-Cl, pH 7.6; 0.1 M MgCl\(_2\); 50 mM DTT; 1 mM spermidine HCl; 1 mM EDTA, pH 8.0), 1.5 µl of sterile distilled H\(_2\)O, 1 µl of T4 polynucleotide kinase (5 u/µl) (Promega Biotechnology, Madison, WI) and incubating at 37°C for 10 minutes. After this incubation, the kinase was denatured by heating at 90°C for 2 minutes. 10 µg of the CsCl purified \(\lambda\) DNA was alkali
denatured in preparation for DNA sequencing. To the DNA (in 100 μl sterile distilled H₂O) 10 μl of a 2 M NaOH, 2 mM EDTA solution was added and incubated at room temperature for 5 minutes. The reaction was neutralized by the addition of 10 μl of 2 M ammonium acetate (pH 4.6) and mixed by vortexing. The denatured DNA was immediately precipitated with 375 μl of ethanol at -80°C and pelleted at 16,000 x g in a microfuge for 10 minutes. The pellet was washed once with 80% ethanol, dried and dissolved in 11.5 μl of sterile distilled H₂O. The λgt11 forward primers, which were previously end labelled, were immediately annealed to the denatured template DNA. To the template, 2 μl 5x TAQence™ reaction buffer and 1.5 μl (1.5 pmole) primer were added and the mixture incubated at 37°C for 10 minutes. Diluted (1:8) Taq DNA polymerase (2 μl) was added to the annealed template/primer mix and 4 μl of the mix transferred to each dideoxy termination tube (G,A,T,C) and the reactions performed as for the Sequenase® Version 2.0 protocol except that the incubation was carried out at 72°C. Samples were run on a denaturing 6% polyacrylamide sequencing gel and exposed to Kodak XAR-5 film as before.

Northern Blot Analysis

Poly A⁺ RNA was prepared from normal adult rat liver, DMBA induced rat mammary carcinoma, hepatoma 7777 and full term human placenta as described for the cDNA library construction. 0.67 μg of each poly A⁺ RNA was prepared for gel loading as described by Sambrook et. al. (91). RNA samples in 4.5 μl of DEPC-
H₂O, 2.0 µl of 5x MOPS buffer, 3.5 µl of formaldehyde, and 10.0 µl of formamide (AmResCo, Solon, OH) were heated to 65°C for 15 minutes and then chilled on ice to remove any secondary structure. 3 µl of BRL 0.24-9.5 kb RNA ladder (Bethesda Research Laboratories, Bethesda, MD) was similarly treated. 2 µl of sterile, DEPC treated formaldehyde gel-loading buffer (50% glycerol; 1 mM EDTA, pH 8.0; 0.23% bromophenol blue; 0.25% xylene cyanol FF) was added to each sample and the samples loaded into the wells of a denaturing formaldehyde 1% agarose gel. A 12 x 25.5 gel was poured by mixing 3 g agarose in 189 ml DEPC treated water (DEPC-H₂O), melting the agarose in a microwave oven and adding 57 ml of 5x MOPS buffer (0.1 M 3-(N-morpholino)propanesulfonic acid, pH 7.0; 40 mM sodium acetate; 5 mM EDTA, pH 8.0) plus 54 ml of formaldehyde (37% solution; Sigma Chemical Co., St. Louis, MO) mixing and pouring into a pre-taped plastic gel tray with a 15 well comb inserted. After sample loading, the gel was run submerged in 1x MOPS buffer until the bromophenol blue tracking dye had travelled approximately 2/3 of the length of the gel. The BRL RNA ladder lane was cut from the gel and stained in 0.5 µg/ml ethidium bromine in DEPC-H₂O and photographed while viewed on a UV transilluminator. The nucleic acids were transferred to a Nytran® (Schleicher and Schuell, Keene, NH) nylon membrane by capillary action overnight in 20x SSC (3 M NaCl; 0.3 M sodium citrate; pH 7.0) by standard procedures (91). After approximately 18 hours of transfer, the membrane was removed from the gel, rinsed briefly in 2x SSC to remove any
adhering gel particles, air dried and baked for 2 hours at 80°C in a vacuum oven to permanently bind the RNA.

pOFP insert DNA was prepared from a large scale plasmid prep and used as a probe on the Northern blot. 250 ml LB + amp media was inoculated from the stock of pOFP transformed DH5αF' cells stored at -80°C and incubated with shaking overnight at 37°C. The cells were pelleted at 5000 x g, 15 minutes at 4°C and resuspended in 6 ml of lysis buffer (25 mM Tris-Cl, pH 7.5; 10 mM EDTA; 15% (w/v) sucrose) by gentle pipetting and set on ice for 20 minutes. 12 ml of freshly prepared alkaline lysis solution (0.2 M NaOH; 1% SDS) was added, mixed by inverting 5-6 times and set on ice. After a 10 minute incubation, the cellular debris and bacterial genomic DNA was pelleted at 10,000 x g for 15 minutes at 4°C. The supernatant containing the plasmid DNA was carefully transferred to a fresh tube (avoiding any of the pelleted material) and 50 μl boiled RNase A (1 mg/ml) was added and incubated at 37°C for 30 minutes to digest the bacterial RNA. Following 2 extractions with phenol/CHCl₃ (1:1) and 1 extraction with CHCl₃ to remove the RNase A and other contaminating proteins, the DNA was ethanol precipitated and pelleted at 10,000 x g for 20 minutes at 4°C. This pellet was dissolved in 1.6 ml sterile H₂O. 0.4 ml of 4 M NaCl and 2 ml of 13% PEG-8000 were added, mixed and incubated on ice for 1 hour to precipitate the plasmid DNA at high purity. Plasmid DNA was pelleted at 10,000 x g for 10 minutes at 4°C, washed twice with 80% ethanol and dried. The insert DNA was excised from 200 μg of plasmid DNA by digestion with EcoRI, as described earlier except that
100 units of enzyme were added after the initial 2 hours of digestion and the incubation continued for an additional 2 hours. The entire reaction was loaded onto a preparative 1% agarose gel with 0.5 \( \mu \text{g/ml} \) ethidium bromide and electrophoresed at 80 volts for 1 hour. The large insert band was excised from the gel and the DNA eluted by the spin elution technique described earlier.

For use as a probe on the Northern blot, 100 ng of the insert DNA was labelled to high specific activity \((10^9 \text{ cpm/\mu g})\) using \([\alpha-^32\text{P}]d\text{ATP}\) and a random primer labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). The DNA was diluted to 9 \( \mu \text{l} \) in sterile \( \text{H}_2\text{O} \), denatured in a boiling water bath for 5 minutes, and snap cooled on ice. To this was added from the kit 1 \( \mu \text{l} \) each of dCTP, dGTP, dTTP, 2 \( \mu \text{l} \) of reaction mix (buffer and primers), 1 \( \mu \text{l} \) of \( E. \text{coli} \) DNA polymerase I Klenow fragment (on the side of the tube) along with 5 \( \mu \text{l} \) of \([\alpha-^32\text{P}]d\text{ATP}\) (3000 Ci/m mole; 10\( \mu \text{Ci/\mu l}; \) New England Nuclear, Boston, MA). The tube was spun briefly to introduce the Klenow fragment into the mix and then incubated at 37\(^\circ\)C for 45 minutes. The labelled DNA was Elutip-d\(^\text{TM}\) purified to remove unincorporated nucleotides and the Klenow protein as described earlier, except that the low salt rinse after DNA binding was omitted and the DNA was eluted with 700 \( \mu \text{l} \) of high salt buffer. A 7 \( \mu \text{l} \) aliquot was counted in 10 ml of Scintiverse\(^\text{©}\) (Fisher Scientific, Fair Lawn, NJ) liquid scintillation cocktail to insure that the DNA was labelled properly.

The baked Northern blot was soaked in prehybridization solution (50% (v/v) formamide; 5x Denhardt's solution [0.1% (w/v)
Ficoll; 0.1% (w/v) polyvinylpyrrolidone; 0.1% (w/v) bovine serum albumin; 1% SDS; 0.1 mg/ml salmon sperm DNA) to block non-specific binding sites for a minimum for 30 minutes at 42°C. The prehybridization solution was discarded and the probe (in 3 ml prehybridization solution, boiled 5 minutes and chilled on ice) added to the blot in a minimum volume (20 ml) of fresh prehybridization solution. Hybridization was carried out at 42°C for 16 hours. The blot was washed for 15 minutes at room temperature in 2x SSPE (3 M NaCl; 0.2 M NaH₂PO₄; 25 mM EDTA; pH 7.4), 0.1% SDS, then for 30 minutes at room temperature in the same buffer. This was followed by a 30 minute wash at 42°C in 1x SSPE, 0.1% SDS and then 30 minutes at 65°C with 0.1x SSPE, 0.1% SDS. The damp membrane was sealed between layers of Saran Wrap® and exposed to Kodak™ XAR-5 film at -80°C with intensifying screens for 10 days.

Cell Culture

Hepatoma 7777 cells were thawed from under liquid nitrogen and resuspended in culture medium [D-MEM (Dulbecco's Modified Eagle Medium, Bethesda Research Laboratories, Bethesda, MD), high glucose, enriched with 15% FBS (fetal bovine serum)]. 1.5 x 10⁶ cells were inoculated into each T-75 culture flask (Falcon™ Division Becton, Dickinson and Co. Oxnard, CA) in 15 ml of culture medium. The cells were allowed to attach to the flasks by incubation at 37°C with 5% CO₂. Cells were fed every 3-4 days by total medium replacement until a confluent monolayer of cells had grown. The cells were then harvested by trypsinization. The spent medium was
removed by suction and 5 ml of Versene (Sigma Chemical Co. St. Louis, MO) was pipetted into each flask to be harvested. The flasks were rotated gently to cover the entire cell sheet, and then the Versene was removed by suction. This step removes residual serum from the cells insuring that the trypsin will not be inhibited. To each flask, 1.5 ml of trypsin (1% in minimal essential medium (MEM)-Hepes, Bethesda Research Laboratories) was added, spread over the cell sheet and allowed to digest at room temperature for 2-3 minutes. After the cells had "rounded up", as checked under a microscope, the cells were loosened by tapping the flask sharply 2-3 times on each side. 10 ml of culture medium, pre-incubated at 37°C, 4% CO₂, was added to each flask. The cells were resuspended by pipetting up and down 2-3 times and then were transferred to 15 ml disposable centrifuge tubes. Cells were pelleted by centrifugation at 1040 x g for 10 minutes at 4°C, resuspended in 10 ml of PBS and repelleted. This final wash removed the FBS used for the neutralization of the trypsin.

Liver Cell Preparation

Liver cells were prepared by ether anesthetizing a male Sprague-Dawley rat and perfusing the liver in situ with 10 ml of PBS (pH 6.8) and then filling the liver (in situ) with a solution of 0.1% collagenase and digesting for ten minutes. The liver was removed to a petri dish and the lightest colored lobe (i.e. the one containing the least blood) cut off and placed in Cellector™ (Belleco, Vineland, NJ). The Cellector™ was placed on top of a 35 mm petri dish and the liver
minced using two scalpels while rinsing copiously with PBS. The minced liver was transferred to a 15 ml disposable centrifuge tube and 10 ml 0.1% collagenase solution was added. After a 20 minute incubation at 37°C, the digestate was transferred back to the Celлектor™ (which had been cleaned with PBS and kept moist). Using the plunger from a 10 ml syringe, the tissue fragments were rubbed through the sieve with a minimal amount of force, rinsing with PBS as before. The effluent was transferred to a 15 ml centrifuge tube and centrifuged at 1040 x g for 10 minutes to pellet the cells. The supernatant was decanted and the cells resuspended in D-MEM plus 200mM L-Glutamine without FBS, vortexed gently and centrifuged as above. This final wash removes free lipids from the cell suspension.

Cytoplasmic RNA Isolation (111)

Each cell pellet (H7777 or liver cells) was resuspended in 375 µl of ice-cold lysis buffer (50 mM Tris-Cl, pH 8.0; 100 mM NaCl; 5 mM MgCl₂; 0.5% (v/v) NP-40; in DEPC-H₂O) and incubated on ice for 5 minutes. Following a 16,000 x g, 4°C, 2 minute spin in a microfuge, to pellet the nuclei, the supernatant was transferred to a fresh microfuge tube containing 8 µl of 10% SDS and mixed. 2.5 µl of 20 mg/ml proteinase K was added and the cellular proteins digested at 37°C for 15 minutes. Contaminants were removed by sequential extractions with phenol/chloroform (1:1) twice and then chloroform once. The RNA was ethanol precipitated on ice for 45 minutes and collected by centrifugation for 15 minutes at 16,000 x g, 4°C in a
microfuge. The pellets were washed once with 80% ethanol and dissolved in 100 μl each of TE pH 7.8 in DEPC-H₂O.

One-half of each preparation was treated with RNase free DNase I to eliminate the possibility that any of the bands visualized on the autoradiogram could be due to contaminating DNA. To 50 μl of each RNA preparation, 50 μl of a cocktail containing 10 μl of 100 mM MgCl₂/10 mM DTT, 0.35 μl of RNase free DNase I (27 units per μl) (Boehringer Mannheim Biochemicals, Indianapolis, IN), 5 μl vanadyl ribonucleoside complexes (200 mM) and 34.7 μl TE pH 7.8 was added and incubated for 15 minutes at 37°C. The DNase I reaction was stopped by the addition of 25 μl per tube of DNase stop solution (50 mM EDTA; 1.5 M sodium acetate; 1% SDS) and mixing. The preparations were extracted once with phenol/chloroform (1:1), once with chloroform, ethanol precipitated and resuspended in 25 μl of TE pH 7.8. 11.7 μg of each cytosolic RNA preparation (RNA and DNase I treated RNA) was loaded and electrophoresed on a denaturing formaldehyde 1% agarose gel as described for the poly A⁺ RNA's.

Cytoplasmic RNA Hybridization

Due to a high background problem encountered when attempting to probe these RNA's when they were transferred to a Nytran™ membrane, the hybridization with the ³²P labelled probe was done in situ on the dried agarose gel (112). Following electrophoresis, the gel was rinsed twice with DEPC-H₂O, placed between two pieces of gel blot paper (Schleicher and Schuell, Keene,
NH) and dried in a slab gel drier (BioRad Laboratories, Richmond, CA) for 30 minutes under vacuum at room temperature and then for 1 hour at 65°C. The gel blot papers were removed by soaking the sandwich in DEPC-H2O and gently peeling them off. The gel was hybridized with 100 ng of 32P labelled OFP cDNA overnight at 42°C in prehybridization solution without prehybridization. Unhybridized probe was washed from the gel with 2 x SSC, 0.01% SDS at room temperature, 3 times for 10 minutes each and then with 1 x SSC, 0.01% SDS at 60°C, 4 times for 15 minutes each. The still moist gel was then exposed to Kodak™ XAR-5 film with intensifying screens at -80°C.
**Results**

A partial cDNA clone was isolated from a DMBA-induced rat mammary tumor cDNA library constructed in the bacteriophage expression vector λgt11 (100). This clone was isolated by repeated rounds of plaque screening (Figure 3.8) of expressed proteins using a mixture of MOFP-A, MOFP-B and MOFP-C. *E. coli* Y1090R− infected with a pure culture of the recombinant λgt11 and treated with IPTG (a gratuitous inducer of the *E. coli* lactose operon promoter) using a novel induction strategy produce a 120 kD β-galactosidase/OFP fusion protein when analyzed on Western blots reacted with a mixture of MOFP's (A + B + C) (Figure 3.9). *E. coli* Y1090R− infected with a culture of λgt11 phage prepared from a negative control plaque and processed in a manner identical to the positive clone exhibited reactivity only with the anti-β-galactosidase antibodies. The fact that the two clones produce an IPTG inducible protein of approximately the same molecular weight was simply a fortuitous event in that both clones must possess inserts of approximately the same size or having similar length open reading frames. Proteins from uninfected Y1090R− cultures yielded no bands by Western blot analysis (data not shown).

Identical Western blots having the 4 and 17 hour induced culture proteins (positive plaque) were developed using MOFP-A, -B and -C alone in an attempt to define which monoclonal antibody is reactive with the insert specific epitope of fusion protein. As seen in
Figure 3.8: Autoradiogram of a positive antibody screen. The arrowed spot corresponded to a positive plaque which was picked for further analysis.
Figure 3.9: Western Blot of fusion proteins produced by recombinant λgt11 phage. Proteins were separated according to molecular weight by SDS-PAGE (8%) prior to transfer to nitrocellulose. The numbers denoting gel lanes represent hours post IPTG induction when samples were collected. 'M' is the molecular weight marker lane. Marker proteins were stained with Coomassie Blue R-250 subsequent to transfer. Identical blots were developed with MOFP-(A+B+C) or Anti-β-galactosidase antibodies.
Figure 3.9
Figure 3.10, both MOFP-A and -B react with the 120 kD fusion protein, albeit the MOFP-B reaction is somewhat stronger. MOFP-C exhibited no reactivity to the fusion protein preparation. It was somewhat surprising to observe that two of the monoclonal antibodies exhibited reactivity considering that, as shown below, the cDNA codes for a very small segment of the OFP. Upon further investigation (T.E. Webb, personal communication), it was found that MOFP-A and MOFP-B were derived from the same original hybridomas and separated during clonal expansion.

Induction cultures were also set-up and the proteins prepared for analysis of RNA transport activity in the immuno-bioassay. Pelleted induced culture cell proteins were processed according to the protocol of Sambrook *et al.* (101) for the preparation of crude lysates from λgt11 lysogens in preparation for DNA-binding protein analysis. As shown in Table 3.1, the λgt11 clone which proved positive by screening and Western blot analysis also exhibited biological activity which was inhabitable with MOFP-B. Y1090R− cultures infected with a negative control clone, or not infected with phage, did not produce a fusion protein which could induce the release of RNA from the isolated nuclei in the bioassay.

The insert from the λgt11 clone which successfully passed the screening Western blot and activity tests was subcloned into the plasmid vector pUC19 and sequenced in both directions by the dideoxy chain termination method of Sanger (110). The proper orientation of the insert in λgt11 and also the correct reading frame for translation in frame with the β-galactoside gene was obtained by
Figure 3.10: Western blot of fusion proteins, produced by the recombinant λgt11 phage which was positive by antibody screening. Identical blots were developed using the individual MOFP antibodies as indicated in the figure. The number above the lanes represents the hours post-IPTG induction at which the samples were collected. M1 marker proteins were stained with Coomassie Blue R-250 after transfer to nitrocellulose. M2 marker proteins were biotinylated. The nitrocellulose membrane was developed with avidin-HRP followed by the substrate, 4-chloro-1-naphthol.
Figure 3.10
Table 3.1: Properties of Fusion Protein Coded by OFP cDNA

<table>
<thead>
<tr>
<th>Results of Screen</th>
<th>Activity in Bioassay (Units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOFP + MOFP(A+B+C)</td>
<td>- MOFP</td>
</tr>
<tr>
<td>1. Positive Clone</td>
<td>145 ± 2 30 ± 27</td>
</tr>
<tr>
<td>2. Negative Clone</td>
<td>-18 ± 23 38 ± 7</td>
</tr>
<tr>
<td>3. Uninfected Y1090R-</td>
<td>0 ± 15 0 ± 13</td>
</tr>
</tbody>
</table>

Activity is expressed as cpm in RNA released from $10^6$ isolated nuclei minus buffer control to medium containing 1.0 mg protein isolated from the bacterial culture.
sequencing the λgt11 clone directly. This was accomplished using a λgt11 specific forward primer, again with the dideoxy chain termination method. The base sequence (5'→3'), together with the protein translation is shown in Figure 3.11. Note that with the in-frame stop codons present in the cDNA sequence, this codes for a highly basic 35 amino acid peptide. The high ratio of basic amino acids in this peptide may offer some insight into its interactions with and nuclear release of RNA in the immuno-bioassay. This has been shown previously for the class of DNA binding proteins possessing zinc finger motifs (113).

The cDNA insert was 32P labelled and used as a probe on a Northern blot of several poly A+ RNA's. Shown in Figure 3.12 are the results of separating 0.67 μg each of rat hepatoma 7777, DMBA induced rat mammary carcinoma, human placental and normal rat liver poly A+ RNA's on a 1% agarose formaldehyde gel, transferring them to a Nytran® nylon membrane and hybridizing with the cDNA probe. The blot was washed at fairly high stringency to ensure that only the OFP messenger RNA would be identified. Due to the low abundance of the message in the tissues, it was necessary to expose the blot to x-ray film for a minimum of 10 days before an interpretable autoradiogram could be obtained. Surprisingly, the poly A+ RNA isolated from normal rat liver contains a message which hybridizes with the 32P labelled probe and is of the same size (2.8 kb) as found in the mRNA's from the other three tissues (Figure 3.12). It is unclear whether the 1.2 kb band also observed in all four tissues is another form of the messenger RNA or a degradation
Figure 3.11: Base sequence (5' -> 3') of the OFP cDNA isolated by antibody screening of a λgt11 cDNA expression library. The proper reading frame was determined by direct sequencing of the λgt11 clone, allowing the determination of the accompanying translation product (i.e. amino acid sequence).
Figure 3.11

5'

GGC ACG AGG GCG ACA GTT GGA GAG
  gly  thr  arg  ala  thr  val  gly  glu

GGA CGT TCC CGG AAA GTT ACC GGT
  gly  arg  ser  arg  lys  val  thr  gly

CAG AAG AAA CTA TCT CCA CAT CAC
  gln  lys  lys  leu  ser  pro  his  his

CAC CTG ACA GGT CAC AAC CAT GTC
  his  leu  thr  gly  his  asn  his  val

AGA CAA AAG TGA ATT AAA AGC TGA
  arg  gln  lys  OPA

GTT GGA ACG TAA GAA GCA ACG GTT
  OCH

GGC CCA AAT CAG AGA ACT CGT GCC

3'
Figure 3.12: Autoradiogram of a Northern blot of poly A+ RNA isolated from: hepatoma 7777 (H7777), DMBA-induced primary rat mammary carcinoma, human placenta and normal rat liver as indicated. RNA's were separated by denaturing 1% agarose formaldehyde gel electrophoresis followed by transfer to a Nytran® nylon membrane. The blot was probed with $^{32}$P-labelled OFP cDNA.
product. The presence of the 2.8 kb band in the human placental poly A⁺ RNA lane leads to the conclusion that there is at least limited homology between the rat tumor and human placental RNA's which code for OFP. The diminished intensity of the band in the human placental lane could be due to incomplete homology with the probe or to a lesser abundance than is found in the rat tissues.

Presence of the OFP message in the normal rat liver in the absence of detectable OFP protein by any of the available assay methods (bioassay, immuno-histochemistry, Western blot analysis) could be explained in at least two manners. The OFP gene could be constitutively expressed and its messenger RNA either 1) retained within the nucleus until the proper signals for its release are received or 2) the message is sequestered in the cytoplasm as a ribonucleoprotein particle until the proper conditions for translation are achieved.

To investigate which mode of control was being exerted in this case, total cytoplasmic RNA was prepared from cultured hepatoma 7777 cells and from normal rat liver cells and analyzed by in situ gel hybridization. As revealed in Figure 3.13 only the cytoplasmic RNA from the hepatoma cells contained a molecule which hybridized with the OFP cDNA probe. Also note that the 1.2 kb hybridizing band observed in the poly A⁺ Northern blot is not present. These results indicate that the expression of OFP is regulated post-transcriptionally by restricting the mRNA to the nucleus in the normal liver cells. The presence of the 1.2 kb band observed in the poly A⁺ RNA Northern blot is not accounted for in this experiment. This molecule may be
Figure 3.13: Autoradiogram of a 1% agarose formaldehyde gel which was dried and the RNA's contained therein hybridized in situ with $^{32}$P-labelled OFP cDNA. Cytoplasmic RNA was isolated from rat hepatoma 7777 and normal rat liver. Each RNA was loaded on the gel directly or after pre-treatment with DNase I. Lane orders are as indicated in the figure.
restricted to the nucleus in each of the tissues tested, or may be a degradation product of the 2.8 kb RNA.
Discussion

The isolation of a complementary DNA (cDNA) to a messenger RNA (mRNA) coding for a protein of interest has become the method of choice for many, if not most, laboratories investigating protein structure and function. Once a cDNA has been cloned, the possibilities for future investigations and approaches to their pursuit are broadened tremendously. A few examples of available techniques include site-directed mutagenesis to define and modify active sites in the protein or subcloning of the cDNA into an appropriate expression vector which will express a sense strand RNA. This RNA can be translated in vivo and the physiological effects of the addition of the protein to the cell studied. Conversely, vectors can be constructed to produce an anti-sense RNA which will hybridize to and effectively sequester the mRNA for the protein of interest and hence disallow its translation into a functional product. Subsequently, the consequences of the loss of the protein on the cellular function can be determined. The cDNA can also be used as a probe to screen a library for isolation of the genomic clone for this protein, followed by analysis of the mechanisms regulating its expression in the cell.

In the pursuit of a more detailed understanding of how OFP is functioning in vivo, a cDNA clone has been isolated using the MOFP-A, -B and -C to screen a λgt11 bacteriophage expression library. In
the course of confirming that the primary clone which was isolated was indeed a copy of the OFP message, a novel strategy for the induction of λgt11 fusion proteins was developed. The protocol is so rapid and technically simple that it is very surprising that it has not been reported previously. Standard manuals of molecular biology techniques (92, 101) along with the suggestions of the original designers of the λgt11 cloning vector (106) recommend the production of integrated lysogens of positive λgt11 clones in E. coli Y1089 prior to induction of the β-galactosidase fusion protein. This process can prove to be a very time consuming and labor intensive task, and as in the experience of this laboratory, it does not always work. It is well known by workers in the field that when trying to amplify a bacteriophage lambda clone by a liquid lysate procedure, failure to optimize the ratio of infecting phage to the bacterial host cell will yield a culture which will 1) lyse prematurely (too many phage) or 2) fail to lyse at all (too few phage). Having this knowledge in hand, liquid "lysate" cultures were prepared in which the phage titer was reduced 50% from the optimum and eventually induced to express the fusion protein. This strategy has worked very well for the purpose of preparing crude lysates for Western blot analysis and biological activity determination. The savings in time and effort for these types of confirmatory experiments has been tremendous. Fusion proteins can potentially be induced on the same day that a positive plaque is isolated from a screening plate and analysis can be performed the following day.
The cDNA clone in hand is believed to be coding for a segment of the OFP protein. Quite fortuitously, this short clone appears to code for the active site of the protein. The presence of an active fusion protein is surprising when looking at the size of the insert DNA, but when considered along with the sequence data bank search information, it does not appear to be totally unreasonable. The OFP cDNA shares limited stretches of homology with at least two nucleotide or nucleic acid binding proteins, human hypoxanthine phosphoribosyltransferase (HPRT) and rat testis specific histone (H1t). As OFP has as its known biological function the transport of RNA out of isolated nuclei, and the protein itself is purified on absorbent matrixes of Affi-gel Blue, which mimics ADP-ribose, and DNA cellulose which mimics RNA. It would seem unlikely that the active site or some other portion of the protein would not contain a nucleotide or a nucleic acid binding entity.

Over the course of more than two years, multiple attempts have been made to isolate a longer and potentially full length cDNA clone for OFP. cDNA has been synthesized on three separate occasions from poly A+ RNA isolated from two separate tissues (transplantable hepatoma 7777 and primary rat mammary tumor) and 7 λgt11 libraries and 1 pUC19 plasmid library have been constructed. Screening attempts using 32P labelled OFP cDNA as the hybridization probe have exhausted the λgt11 libraries unsuccessfully. Thus far, a polymerase chain reaction (PCR) strategy utilizing an OFP cDNA specific primer and various combinations of vector specific primers (114) has proved unsuccessful in generating a
product longer than the original clone. This inability to generate a cDNA of any reasonable length may be due to the presence of significant secondary structure in the messenger RNA which, during first strand cDNA synthesis, the reverse transcriptase is unable to melt and maintain its processivity. Future attempts to isolate the full length clone may have to involve first strand synthesis using an elevated temperature and Taq DNA polymerase. Reverse transcriptase activity of this enzyme has been shown previously (115).

In an effort to determine the length of the OFP messenger RNA, Northern blot analysis of several poly A+ RNA preparations was performed. A 2.8 kilobase band detected on the autoradiogram of this blot is of a reasonable size to code for a 55 kD protein allowing for stretches of untranslated 3' and 5' flanking sequences. The presence of the 1.2 kb species may be the result of a degradation of the full length 2.8 kb message or may be a distinct messenger RNA coding for another RNA transport protein, possibly the normal 35 kD factor. The fact that the MOFP's do not cross-react with this 35 kD protein does not rule out the possibility that these two proteins could share significant homology in their DNA sequences, especially in their active site region. The active site of the 35 kD RNA transport protein may not be accessible as an epitope for antibody binding.

The observation that the OFP message was present in the human placental poly A+ RNA was very encouraging as this tissue produces a similar, if not the same protein, as the rat tumor from which the cDNA clone was isolated. The presence of the same size
message hybridizing in the normal rat liver poly A+ RNA was very surprising. There exists no evidence from this or any other laboratory for the presence of OFP-related activity in any normal tissue. Analysis has included bioassay of column fractionated cytosol from normal tissues and immuno-histochemical studies on cultured and freshly sectioned normal liver cells, along with Western blot analysis of the same proteins. The possibility exists that the OFP gene is being constitutively transcribed, but that control is exerted at the level of nuclear restriction of the mRNA. Many examples of control at this level have been reported (5). Analysis of total cytoplasmic RNA from liver cells and cultured hepatoma 7777 cells indicated that the message is being released from the nucleus only in the tumor cells indicating that nuclear restriction is regulating the expression of OFP. The identity of the 1.2 kb message may be resolved by analyzing nuclear RNA preparations on Northern blots. The identification of a molecule of 1.2 kb hybridizing on these blots will be a strong indication that the message is not being transported. Should the 1.2 kb band not appear on these autoradiograms, it would strongly suggest that significant degradation of the RNA is occurring during poly A+ selection.
General Discussion

OFP, by the simple observation of its ubiquitous presence in carcinogenesis and tumorigenesis, is potentially the most promising tumor marker protein currently under investigation. The availability of a single protein which could be used in a follow-up diagnostic test in a broad spectrum of cancers would greatly simplify the monitoring of disease treatment. At the present time, the most useful tumor markers are only specific for a subset of sites of neoplasia. Carcinoembryonic antigen (CEA) was originally thought to be a specific marker for colorectal cancer (116). It was subsequently shown that the antigen is found in normal liver tissue, and the columnar epithelium of the digestive tract and bronchus (38). Alpha-fetoprotein (AFP) is clinically useful for patients having hepatocellular carcinoma and germ cell tumors of the testis, ovaries or extragonadal sites (117), and is one of the most widely used tumor marker proteins. AFP is also present normally at low levels in the adult and its serum levels elevated under conditions of pregnancy and rapid liver regeneration (38, 117). Human chorionic gonadotropin (hCG) is also useful, in combination with AFP, in the management of gestational trophoblastic tumors and germ cell tumors (117).

What all of these markers have in common are the low levels of expression in the normal adult, re-expression under non-
malignant conditions and a fairly narrow range of tumor types in which they are expressed. In as far as has been tested, OFP exhibits none of these potential drawbacks.

As far as basic research is concerned, the full elucidation of the \textit{in vivo} activity of this protein must be determined. This is critical to the long term potential of exploiting the biological function of OFP as a means of intervening in the carcinogenesis process. For a significant period of time, it has been the working hypothesis in this laboratory that OFP is the key to opening a "window of differentiation" through which cells must pass during fetal development and carcinogenesis. When assessing the data as a whole, both presented here and elsewhere (32), it appears now that this hypothesis must be modified. First it has to be noted, as is obvious, that the cellular processes of growth and differentiation can be viewed (or are) wholly independent of each other. The demonstration that the addition of MOFP-A to cultured hepatoma cells causes an inhibition of the rate of tumor cell growth (T.E. Webb, P.C. Stromberg, unpublished data) is significant yet independent of the process of cellular differentiation. The cells being treated have already undergone de-differentiation. Likewise, the use of anti-sense oligonucleotides based on the cloned cDNA sequence to inhibit the growth of human leukemia HL-60 cells (T. E. Webb, A. Koolemans-Beynen, unpublished data) is a demonstration of OFP regulation of cell growth, not cell differentiation. This information is supported with the time course expression of OFP in the rat fetal development. OFP activity peaks during the final one-third of rat
fetal development period. The bulk of differentiation occurs early in development and the final trimester is used primarily for the maturation of internal organs and the rapid growth of the developing fetus (118). This implies that growth is being regulated by OFP as opposed to differentiation. Immuno-histochemical observations that OFP is present in pre-neoplastic lesions was initially interpreted as supporting the "window of differentiation" model. The OFP present in these cells may be acting as the manager molecule which allows the transformed cells to clonally expand. As yet unresolved is the absence of OFP expression under non-tumorigenic liver regeneration (25, 30). The regenerating liver was tested for OFP activity at only one time point, that being 24 hours post - 2/3 partial hepatectomy. OFP may be transiently produced early in liver regeneration and subsequently down-regulated prior to the point at which the tissue samples were harvested. Further investigation should include time points prior to 24 hours and later than 24 hours post-partial hepatectomy. The preparation of antibodies specific for OFP will allow the exploration of this new hypothesis. If true, expression of OFP should be minimal during embryogenesis, a time point not yet evaluated. OFP should also have a pronounced effect on metastases as the secondary tumor colonies expand. Should OFP be controlling differentiation, one would expect little or no effect on secondary tumors. This question may be resolved by evaluating the relative levels of OFP in primary and metastatic tumors and comparing the levels with those in tumors which do not metastasize.
One series of experiments which may build a foundation supporting this hypothesis involves determining any correlation between OFP levels and the growth rate of tumors. This study is ongoing utilizing a series of rat mammary tumors having known and widely different rates of growth.

Future investigations will need to involve further characterization of the in vivo activity of OFP and the regulation of its expression pattern. Analysis of in vivo function should include not only the gross analysis of cell growth and morphology incident to the introduction or removal of OFP from the cell, but also effects on second messenger molecules such as cyclic AMP. Correlating the OFP levels to the number and type of hormone receptors on the cells producing or affected by OFP may have a bearing on OFP's mechanism of action. Protein coupling experiments followed by immunoprecipitation may yield insight into other proteins which may be acting in concert with OFP. The introduction of a fluorescent or radio-labelled preparation of OFP into the circulation of an animal having metastatic disease and subsequent localization in the animal may yield preliminary evidence as to its potential role in secondary tumor colonization. This may also show whether or not a subset of the cells in a solid tumor express a receptor for OFP.

It is interesting that OFP expression appears to be regulated post-transcriptionally at the level of nucleocytoplasmic transport. This finding suggests that the OFP messenger RNA must be mobilized fairly rapidly when the signals for its expression are received. This
data supports the hypothesis that the presence of OFP is necessary during the very early stages of carcinogenesis.
REFERENCES


44) Proteins A MAPS® kit. Literature provided by BioRad Laboratories, Richmond, CA.


