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CONSTRUCTION AND CHARACTERIZATION OF STABLE CELL LINES THAT GENERATE RECOMBINANT ADENO-ASSOCIATED VIRUS (rAAV)

DISSEYATION

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

By

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ABSTRACT

Adeno-associated virus (AAV) is a replication defective parvovirus that is being developed as a vector for human gene transfer. However, production of AAV vectors in the laboratory has been cumbersome because of the lack of efficient systems to package recombinant virions (rAAV). Our laboratory has developed a novel strategy for rAAV production that involves the construction of stable cell lines that generate rAAV upon infection with adenovirus. We describe here the construction and characterization of the stable cell lines H44 and D6 that package the lacZ gene of E. coli into recombinant virions (rAAV/β-gal). The lacZ gene is a reporter gene that can be conveniently detected using X-gal histochemical staining. We analyzed the cell lines for wild-type AAV generation and demonstrated that rAAV/β-gal generated from the D6 cell line was apparently free of wild-type AAV. Protein expression and RNA in situ hybridization analyses established that rAAV/β-gal was generated from approximately 50% of the D6 cells. A situation similar to our producer cell lines is seen during infection of tissue culture cells with wild-type AAV and adenovirus. At multiplicities of infection where most of the cells are expected to express the AAV capsid proteins, only a small percentage of the cells express these proteins. This phenomenon has been attributed to AAV autointerference. Our studies with several genotoxic agents suggested that it might be possible in the future to modulate rAAV production in producer cell lines. The ability to
produce rAAV easily and efficiently in the laboratory will now enable us to expand investigation on the biology of gene transfer mediated by rAAV in animal models.
To my brother, Niko
ACKNOWLEDGMENTS

The author wishes to thank her adviser, Philip R. Johnson, M. D., for constant intellectual support and encouragement during the completion of this work.

The author also wishes to thank, K. Reed Clark, Ph. D., for scientific discussions and valuable input, Tomas Sferra, M. D., for computer help, David M. Fraley for screening the neomycin resistant cell lines, Mary Connel for taking the photographs and Brenda Cain for her unlimited help and kind nature.

Finally, the author wishes to thank George W. Chacko, Ph. D., for being supportive and very patient during the entire painstaking process.
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CHAPTER 1

INTRODUCTION

1.1 Overview of gene transfer.

The treatment of human disease by gene transfer has been termed gene therapy. Somatic cell gene transfer affects the genetic composition of somatic cells without transmitting the genetic modification(s) to future generations. Germ line gene transfer involves the alteration of genes in the germ cells with the consequence of inheriting the genetic alteration(s) to future generations (Dube' and Cournoyer, 1995; Miller, 1992).

Transfer of a gene into a human host for therapeutic purposes needs to fulfill the following requirements in order to achieve the desired affect. First, the gene of interest and its regulatory sequences need to be isolated and their function established. Second, the gene of interest should be introduced into the recipient host in a stable fashion so that expression is maintained over long periods of time. Third, the method of introduction of the foreign gene into the host must be safe and efficient, and the gene should be expressed in the appropriate target organ(s) in a manner that can be regulated. Finally, for at least some applications (i.e., cancer treatment), the vector should be capable of transducing up to 100% of the cells.
1.2 Non-viral gene transfer vectors.

Non-viral vectors for gene transfer by and large introduce naked DNA into the host. To augment gene transfer, some non-viral vectors utilize viral products to introduce DNA into the host. The main advantages of the non-viral vectors is their safety and the lack of immunogenicity upon entry into the host. The non-viral vectors that utilize viral products to augment gene transfer face the safety issues of viral vectors and they can be immunogenic upon entry into the host cell. The disadvantages of the non-viral vectors is their low transduction efficiency and the transient nature of gene expression after introduction of the foreign gene into the cell. The most important non-viral vectors for gene transfer are described briefly below.

1.2.1 Particle bombardment.

This method involves the coating of gold beads with plasmid DNA and then introducing the coated particles into cells by bombardment (Yang et al., 1990). In reports to date, once inside the cell, the DNA remains extrachromosomal (Wolff et al., 1990) and gene expression is low and does not persist (Williams et al., 1991). Expression of the transgene varies dramatically among tissue types and promoters and makes the use of gene delivery by particle bombardment inconsistent (Cheng et al., 1993). However, the method may be useful in determining the activity of various promoters in vivo (Cheng et al., 1993). In summary, the transient and low expression of the transgene makes the particle bombardment method of gene transfer at present unsuitable for many types of in vivo gene transfer.
1.2.2 Direct nucleic acid injection.

This method involves direct injection of nucleic acid into the target tissue where the DNA remains extra-chromosomal. The earliest reports of this approach involved direct injection of a luciferase construct into mouse skeletal muscle and resulted in luciferase expression for at least two months (Wolff et al., 1990). The method is safe and easy and leads to prolonged expression of the transgene (at least in muscle). However, gene expression depends on the differentiated state of the cell since regenerated muscle becomes transduced more efficiently than mature muscle in mice (Davis et al., 1993). Furthermore, the promising results of direct DNA injection in mice may not apply to primate animal models, suggesting limitations in the application of this method in humans (Jiao et al., 1992).

1.2.3 Lipofection.

In this approach, liposomes are prepared by any number of different methods, and when mixed with DNA under appropriate conditions, liposomes encapsulate the DNA. Liposomes prepared with various phospholipids enter the cell by endocytosis and traffic through the lysosomal pathway where DNA might be extensively degraded (Farhood et al., 1994; Hug and Sleight, 1991).

Cationic lipids form liposomes which interact with DNA spontaneously to form lipid/DNA complexes. The complexes enter the cell by fusion of the liposomal vesicle with the cell membrane and avoid trafficking through the lysosomal pathway (Felgner et al., 1987). Although transfection efficiencies using cationic liposomes are high, the method is variable and depends on the proliferative state of the cell (Takeshita et al., 1994). Gene delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene into the airway of
the Edinburgh insertional mutant mouse (CF transgenic mouse) using cationic liposomes was efficient, easy, and safe indicating that the method may have utility in *in vivo* applications (Alton *et al.*, 1993).

The main advantage of liposome-based vectors is their ability to transfer large DNA fragments into the cell. In addition, cationic liposomes, in contrast to other liposomes, do not enter the cell by endocytosis and therefore they avoid the lysosomal pathway where DNA degradation is extensive.

1.2.4 Receptor-mediated gene transfer.

Receptor-mediated gene transfer takes advantage of binding of ligands to specific receptors on the cell surface. DNA can be conjugated to a ligand of a cell surface receptor by a linking poly-cation (i.e. poly-L-lysine) and the complex is targeted to the appropriate cell type(s) due to the specificity of the ligand/receptor interaction. The DNA/ligand complex is internalized by the cell and transferred into the nucleus where DNA is expressed (Wu *et al.*, 1989; Wu and Wu, 1988). The internalization pathway of the DNA/ligand complex depends on the type of receptor the ligand targets. Many receptors, following binding to their cognate ligand, traffic to lysosomes where the DNA is degraded. In contrast, other receptors do not traffic through the lysosomes but instead they recycle back to the membrane or move to the apical surface of the cell (Guy *et al.*, 1995; Phillips, 1995).

Curiel and co-workers took advantage of the ability of adenovirus to disrupt endosomes and introduced transferrin/poly-L-lysine/DNA conjugates into adenovirus infected cells (Curiel *et al.*, 1991). More than 2000-fold increase in transfection efficiency was observed when transferrin/poly-L-lysine/DNA conjugates were introduced into adenovirus infected cells.
Adenovirus enters the cell by receptor mediated endocytosis. The low pH of the endosome causes a conformational change in the capsid protein of adenovirus that results in the escape of the adenoviral particle from the endosome (Greber et al., 1993). It is believed that the presence of adenovirus allows the transferrin/poly-L-lysine/DNA conjugates to escape the endosome more efficiently (Curiel et al., 1991).

Cotten and co-workers used adenovirus inactivated by treatments that damage the viral DNA but leave the protein intact (such as UV irradiation or psoralen). The authors reported that the inactivated adenovirus augmented transfection of cells by the transferrin/poly-L-lysine/DNA conjugate more than 1000-fold (Cotten et al., 1992).

Wagner and co-workers avoided using whole viral particles by conjugating synthetic fusogenic peptides of the influenza virus hemagglutinin to transferrin/poly-L-lysine/DNA complexes. The hemagglutinin protein of influenza virus mediates the fusion of the viral membrane with the endosomal membrane at acidic pH. The authors reported increased transfection efficiency of transferrin/poly-L-lysine/hemagglutinin peptide/DNA complexes (Wagner et al., 1992).

The major advantage of gene transfer by receptor mediated endocytosis is the ability to target the transgene to a specific tissue or cell type. However, the transgene expression is low and transient.

1.3 Viral gene transfer vectors.

Many viruses have evolved mechanisms to attach, penetrate, and evade the host cell efficiently. Viral vectors for gene transfer are generated through genetic manipulation to introduce foreign genes into the host efficiently, safely,
and stably. The main advantages of the viral vectors is the high level of transduction and expression of the transgene for extended periods of time. The disadvantages of the viral vectors include safety concerns and immunogenicity. The most important viral vectors studied today are outlined below.

1.3.1 Herpes simplex virus (HSV) vectors.

HSV is a large DNA virus that can undergo both a lytic and latent life cycle. HSV has a broad cell tropism and can infect both dividing and non-dividing cells, but it can establish a latent infection only in non-dividing cells (Glorioso et al., 1992; Glorioso et al., 1994). During latency the HSV genome exists in the cell nucleus as a mini-chromosome (Deshmane and Fraser, 1989). The ability to establish a latent infection in non-dividing cells and carry large DNA fragments makes HSV an attractive candidate vector for gene therapy. Because of the biology of herpes simplex viruses, HSV vectors are ideal candidates for gene transfer into the postmitotic cells of the brain (Glorioso et al., 1994; Kennedy and Steiner, 1993).

HSV vectors are genetically manipulated to eliminate the lytic functions of the virus so that foreign genes can be expressed in cells latently infected with the recombinant virus (Dobson et al., 1990; Glorioso et al., 1995). However, the attenuated HSV vectors, although unable to cause a lytic infection, remain cytotoxic (Boviatsis et al., 1994; Johnson et al., 1992). Even when a large reduction in cytotoxicity was achieved by additional manipulations of the HSV genome (Glorioso et al., 1995), gene expression was transient in HSV vectors (Andersen et al., 1992; Chiocca et al., 1990). Ramakrishnan and co-workers showed that the transient gene expression is not the result of loss of viral genomes over time but rather the result of silencing of the transgene over time.
Ramakrishnan et al., 1994). Elimination of the cytotoxic functions of the HSV vectors and establishment of conditions that allow transgene expression for prolonged periods of time need to be developed in order for HSV vectors to be used for gene transfer applications.

1.3.2 Retroviral vectors.  

Retroviruses are diploid single-stranded RNA viruses that convert their genome into double stranded DNA which becomes integrated into the chromosome of the host (Brown et al., 1987). The high level of transduction and the potential of stable expression of a foreign gene from an integrated provirus makes retroviruses good candidate vectors for gene therapy.

Most retroviral vectors have been generated from murine C-type retroviruses. C-type retroviruses integrate their genome into the host chromosome during the S-phase of the cell cycle but require cell division to activate viral expression from the integrated provirus (Humphries et al., 1981). The requirement of cell division for retroviral integration and gene expression makes retroviral vectors most suitable for ex vivo gene delivery. However, ex vivo gene delivery is expensive and complex and will limit the broad application of this treatment.

The alternative to ex vivo treatment is the direct administration of retroviral vectors into the host. However, generation of replication competent retroviruses (RCR) in producing cell lines and subsequent germ line transduction are concerns in the direct retroviral vector administration protocols (Sajjadi et al., 1994). Third generation packaging cell lines have been developed which generate retroviral vectors virtually free of RCR (Miller, 1990).
Another drawback for the use of retroviral vectors in gene therapy is their random chromosomal integration and the resulting potential to either transactivate neighboring genes through the strong promoter and enhancer elements present in the long terminal repeats (LTR) or to inactivate cellular genes by insertional mutagenesis (Cometta, 1992). Also, due to the high error rate of reverse transcriptase, genes cloned into retroviral vectors could become mutated during reverse transcription (Varela-Echavarria et al., 1993). Finally, the efficiency of existing packaging cell lines that can only generate recombinant retroviruses of low titers such as $10^6$-$10^7$ infectious particles per milliliter may limit widespread use (Russell, 1991).

1.3.3 Adenoviral vectors.

Adenoviruses are double-stranded DNA viruses that enter the cell by receptor mediated endocytosis. The low pH of the endosome triggers the release of the viral capsid into the cytosol. After release of the viral capsid from the endosome into the cytosol, the capsids reach the nuclear pores and DNA is released into the nucleus where transcription of the viral genome begins (Greber et al., 1993). Once inside the nucleus, adenoviral DNA remains extrachromosomal (Akii et al., 1993; Bajocchi et al., 1993; Schaack et al., 1990; Stratford-Perricaudet et al., 1990). However, since adenoviruses do not integrate into the chromosome, they only provide transient expression of the foreign gene.

Human adenoviruses will infect almost all cell types, including dividing and non-dividing cells, making these vectors likely candidates for in vivo gene
delivery (Kozarsky and Wilson, 1993). Adenoviruses are considered to be safe because even though they cause respiratory disease in humans they are not associated with human malignancies (Horwitz, 1991).

First generation adenoviruses have been constructed by deleting the E1 region of the virus (Kozarsky and Wilson, 1993). In vivo use of first generation adenoviral vectors resulted in transient transgene expression associated with pathology. Infection of immunocompetent mice with first generation adenoviral vectors elicits a CD8+ cytotoxic T cell (CTL) response that results in the elimination of the virus infected cells (Akli et al., 1993; Yang et al., 1994b; Yang et al., 1995; Yang et al., 1994c).

Second generation adenoviral vectors have been constructed that further cripple the virus by deleting or mutating the E2A or E4 regions of adenovirus in addition to the E1 region. Second generation vectors, although able to express the transgene for an extended period of time, are still associated with cytopathology although much reduced compared to the cytopathology observed with the first generation adenoviral vectors (Armentano et al., 1995; Engelhardt et al., 1994; Yang et al., 1994d; Yeh et al., 1996).

Finally, recombinant adenoviral vectors have been constructed with deletions in all viral open reading frames. These "gutless" adenoviral vectors are replicated in 293 cells in the presence of E1 deleted helper viruses and can potentially overcome the present limitations of adenoviral vectors (Fisher et al., 1996a; Mitani et al., 1995).

Tripathy and co-workers showed that the major determinants of the stability of transgene expression are the cellular and humoral immune responses directed against the foreign transgene product and not the adenovirus itself (Tripathy et al., 1996). These findings have significant
implications towards gene transfer applications suggesting that long term transgene expression can be achieved if recombinant adenoviruses encoding self proteins are used (Tripathy et al., 1996).

An additional problem associated with adenoviral vectors is the antibody response elicited by the host after readministration of the recombinant virus. Manipulation of the host's immune response may be required if readministration of recombinant adenoviruses is desired (Tripathy et al., 1996; Yang et al., 1995).

1.3.4 Adeno-associated virus (AAV) vectors.

Adeno-associated virus (AAV) is a dependovirus that requires a helper virus, (usually an adenovirus or herpes virus), for a productive infection (Siegl et al., 1985). In the absence of helper virus AAV can integrate into chromosome 19 (Kotin et al., 1992; Kotin et al., 1991; Kotin et al., 1990; Samulski et al., 1991). AAV is not associated with any disease, has a broad cell tropism, and can infect both dividing and non-dividing cells efficiently (Blacklow et al., 1968; Khleif et al., 1991). The ability of AAV to integrate in a site specific fashion combined with its ability to infect dividing and non-dividing cells makes this virus a good candidate vector for gene transfer. The main disadvantage of AAV is its small genome; AAV can package foreign sequences no longer than 110% of wild-type size (Muzychka, 1992).

1.4 Natural infection by AAV.

Adeno-associated viruses (AAV) belong to the family of Paroviridae, genus dependovirus. Paroviruses are small single stranded DNA viruses that replicate in the nuclei of infected cells. AAV requires a helper virus, (adenovirus
or herpes virus), for a productive infection (Siegl et al., 1985). There are five serotypes of AAV, (AAV-1 to AAV-5), of which AAV-2 is the best characterized. Seroconversion occurs early in life and about 90% of the adult population is positive for AAV antibodies (Blacklow et al., 1968; Blacklow et al., 1971). Although AAV is widely distributed in the population, it is not associated with any disease (Blacklow et al., 1968; Grossman et al., 1992; Khleif et al., 1991). In tissue culture, AAV has been shown to suppress oncogenic transformation of other DNA viruses and to down regulate the expression of cellular oncogenes (de la Maza and Carter, 1981; Hermonat, 1989; Hermonat, 1994; Yang et al., 1992).

1.5 The AAV genome.

The AAV particle is an icosahedral, non-enveloped structure, 20-24 nm in diameter (Hoggan, 1970; Melnick et al., 1965) which contains a single stranded DNA genome of approximately 4.7 Kb in length (Ruffing et al., 1994; Srivastava et al., 1983). Strands of both polarities are packaged into virions with equal efficiency and they are both infectious, (Bems and Rose, 1970; Laughlin et al., 1979a; Samulski et al., 1987), but only the negative strand is transcribed (Carter et al., 1976; Rose and Koczot, 1971).

The AAV genome is composed of two open reading frames (Figure 1). The left open reading frame, called rep, encodes four proteins required for viral DNA replication (Hermonat et al., 1984; Yang et al., 1992), rescue of the viral genome from the integrated state (Hermonat et al., 1984), and single strand DNA accumulation (Chejanovsky and Carter, 1989). The Rep proteins are transcribed from the p5 and p19 promoters located at the corresponding map units 5 and 19 (Mendelson et al., 1986). The right open reading frame, called
cap, encodes the three capsid proteins of AAV which are transcribed from the p40 promoter located at map unit 40 (Green and Roeder, 1980). All AAV transcripts are 3' coterminally and share the same polyadenylation sequence at map unit 95 (Green and Roeder, 1980; Laughlin et al., 1979b; Srivastava et al., 1983). The AAV genome contains an intron shared by all transcripts and spliced and unspliced forms of all transcripts are present in infected cells (Green and Roeder, 1980; Laughlin et al., 1979b).

The four Rep proteins of AAV are 78, 68, 52, and 40 Kd in size (Mendelson et al., 1986; Srivastava et al., 1983). Rep78 and Rep68 are translated from the same primary transcript generated from the p5 promoter and Rep68 is the spliced form of Rep78. Rep78 and Rep68 regulate AAV gene expression (Beaton et al., 1989; Trempe and Carter, 1988b) and DNA replication (Im and Muzyczka, 1990; Im and Muzyczka, 1992; McCarty et al., 1994). Rep52 and Rep40 are translated from the same primary transcript generated from the p19 promoter and Rep40 is the spliced form of Rep52. Rep52 and Rep40 are required for AAV single strand DNA accumulation in infected cells (Chejanovsky and Carter, 1989).

The three capsid proteins of AAV are named VP1, VP2, and VP3, and are 87, 73, and 62 KD in size, respectively. VP3 is the smallest and most abundant capsid protein, accounting for 90% of all protein in the virion, whereas VP1 and VP2 account for 5% each (Janik et al., 1984; Johnson et al., 1977). VP1 is translated from an alternatively spliced p40 transcript which utilizes a splice acceptor site 27 nt. upstream of the conventional one (Becerra et al., 1988; Cassinotti et al., 1988; Muralidhar et al., 1994; Trempe and Carter, 1988a). This alternative splicing event exposes an ATG codon located within the intron sequence. VP2 is translated from the same transcript as VP3, but VP2 uses the
alternative initiation codon ACG located 195 nt. upstream of the conventional ATG codon utilized by VP3 (Becerra et al., 1988; Muralidhar et al., 1994).

The rep and cap open reading frames are flanked by inverted terminal repeat sequences (TR), 145 nt. long (Figure 1). The first 125 bases of the terminal repeats are composed of three palindromic sequences which can base pair to form a hairpin structure (Lusby et al., 1980). The terminal repeats contain the cis elements required for DNA replication, rescue, encapsidation, and integration of the viral genome (McLaughlin et al., 1988; Samulski et al., 1983; Senapathy et al., 1984). A number of investigators identified a sequence within the AAV terminal repeats where the Rep78 and Rep68 proteins bind (McCarty et al., 1994; Ryan et al., 1996). McCarty and co-workers showed that although Rep binds to the palindromic sequence in its linear form, it binds to the sequence in the hairpin configuration with greater affinity (McCarty et al., 1994).

1.6 The AAV life cycle.

The AAV life cycle is unique in that it requires a helper virus, (usually adenovirus or herpes virus), to initiate a productive infection in tissue culture cells (Atchison, 1970; Siegl et al., 1985). Vaccinia virus can also function as a helper virus for AAV (Schlehofer et al., 1986). The cell receptor for AAV has not been identified, but since AAV can infect every cell type studied so far, it is believed that the virus adsorbs to an apparently ubiquitous cell receptor and becomes internalized through receptor mediated uptake. Inside the cell, AAV is transported to the nucleus and becomes uncoated (Hunter and Samulski, 1992; Rose and Koczot, 1971). In the presence of helper virus, AAV enters the productive cycle of viral infection where viral DNA is transcribed, replicated, and translated into viral proteins. The Rep and Cap proteins accumulate in the
nucleus where capsid assembly occurs and viral DNA becomes encapsidated (Hunter and Samulski, 1992; Wistuba et al., 1995). In the absence of helper virus, AAV enters the latent cycle of infection by integrating into chromosome 19 (Kotin et al., 1992; Kotin et al., 1991; Kotin et al., 1990; Samulski et al., 1991). The virus remains in the latent state until certain stimuli such as carcinogens, UV irradiation, metabolic inhibitors, or super-infection of the cell with helper virus, stimulate the virus to enter the productive cycle of viral infection (Schlehofer et al., 1986; Yakobson et al., 1989; Yakobson et al., 1987; Yalkinoglu et al., 1988).

1.7 Regulation of AAV gene expression.

As mentioned before, AAV is a dependovirus that requires a helper virus, (usually adenovirus or herpes virus), for a productive infection (Atchison, 1970; Siegl et al., 1985). The helper functions of adenovirus have been studied more extensively and has been shown that AAV gene expression is regulated by both the AAV Rep proteins and adenoviral proteins. Tissue culture cells infected with AAV in the absence of adenovirus do not synthesize AAV mRNA that can be detected by Northern blot analysis (Clark et al., 1995; Laughlin et al., 1982; Rose and Koczot, 1971). However, low levels of AAV mRNA in helper virus uninfected cells can be detected by a sensitive RT-PCR assay (Clark et al., 1996b). Infection of cells with adenovirus and AAV results in transactivation of AAV transcription from all three AAV promoters (Clark et al., 1995; Janik et al., 1989)

Richardson and co-workers identified the adenoviral genes that provide the helper functions for AAV DNA replication. They reported that the early region 4 (E4) of adenovirus provides a helper function for AAV DNA replication.
while, the early regions 1A and 1B (E1A, E1B) and early region 2A (E2A) are required to transactivate E4 transcription (Richardson and Westphal, 1981; Richardson and Westphal, 1984).

The adenoviral proteins E1A and E1B are the first genes expressed during adenovirus infection and they are transcriptional activators of the adenoviral early genes (Richardson and Westphal, 1981). E1A transactivates the p5 (Chang and Shenk, 1989) and p19 (Tratschin et al., 1984) promoters of AAV and results in upregulation of Rep protein expression in the infected cell (Flint and Shenk, 1989). The Rep proteins in turn transactivate the AAV p5, p19, and p40 promoters which leads to high levels of all AAV transcripts (Labow et al., 1986; Yang et al., 1994a; Yang et al., 1992). In the absence of adenovirus infection, the Rep proteins repress expression from all AAV promoters (Kyostio et al., 1994; Labow et al., 1986; Trempe and Carter, 1988b).

The adenoviral protein E2A encodes a DNA binding protein (DBP) required for adenoviral DNA replication, whereas the E4 region of adenovirus is involved in the inhibition of host macromolecular synthesis (Richardson and Westphal, 1981). E2A and E4 facilitate the accumulation of AAV mRNAs in the infected cell by either stabilizing the message or enhancing the transport of mRNA to the cytoplasm (Samulski and Shenk, 1988).

Janik and co-workers showed that the adenoviral virion associated-1 (VA1) RNA also provides help for AAV replication and in conjunction with E2A they regulate AAV expression at the level of translation by increasing the efficiency of translation and the steady state levels of capsid proteins (Janik et al., 1989; Janik et al., 1981).
1.8 AAV DNA replication.

AAV is a single stranded DNA virus that replicates its genome by a single strand displacement mechanism (Hong et al., 1994; Lusby et al., 1980; Straus et al., 1976; Ward and Bems, 1995). Two regions of the AAV genome are required for DNA replication: the inverted terminal repeats (Senapathy et al., 1984) and the rep gene which codes for the four Rep proteins Rep78, Rep68, Rep52, and Rep40 (Hermonat et al., 1984; Yang et al., 1992). The inverted terminal repeats contain two small palindromes within a larger palindrome that can fold into a hairpin and serve as primers for DNA synthesis (Lusby et al., 1980).

DNA replication is initiated at the AAV terminal repeats which when folded into a hairpin configuration function as primers for AAV DNA synthesis (Figure 2). AAV DNA replication starts at the 3' OH end of the terminal repeats and continues in the 5' to 3' direction to synthesize the complementary AAV strand. The synthesis of the complementary AAV strand results in a double stranded replicative intermediate in which one end is covalently joined. The covalently closed end is then resolved into an open end by a process called terminal resolution or hairpin transfer (Snyder et al., 1990; Tattersall and Ward, 1976). The terminal resolution process is carried out by the AAV proteins Rep78 and Rep68 which possess helicase activity and a site and strand specific endonuclease activity (Im and Muzychka, 1990; McCarty et al., 1994; Ni et al., 1994; Snyder et al., 1990). Rep78 and Rep68 create a cut at the terminal resolution site (trs) in the parental strand located opposite the original 3' OH primer. This results in the transfer of the terminal repeat to the progeny strand and also in the generation of a new 3' OH end in the parental strand. This 3' OH end is then used to repair the parental strand by synthesizing a new
terminal repeat in the inverted orientation. Following resolution, the palindromic sequences in the end of the molecule assume the hairpin configuration providing a 3' OH end for displacement synthesis. Primer elongation results in the displacement of a single stranded molecule, which is potentially packaged into virions, and a new replicative intermediate that can undergo a second round of terminal resolution (Hong et al., 1994; Ni et al., 1994; Ward et al., 1994).

Another form of replication intermediate found during AAV DNA replication consists of dimeric molecules (concatamers) joined in a head-to-head or tail-to-tail configuration. These molecules most likely are the result of replication of the monomeric molecules before the terminal resolution process has been completed (Hong et al., 1994; Ni et al., 1994). The dimeric molecules are most likely resolved into monomeric molecules by Rep78 and Rep68 since McCarty and co-workers identified the binding of Rep78/68 to the terminal repeat sequences in the linear conformation (McCarty et al., 1994). In addition, Rep68 cleaves the dimeric replicative intermediates into the monomeric molecules (Ni et al., 1994; Ward et al., 1994). Ni and co-workers reported the inability of Rep52 to support DNA replication in an in vitro replication assay (Ni et al., 1994), whereas Im and Muzyczka reported the lack of binding of Rep52 and Rep40 to AAV terminal repeats (Im and Muzyczka, 1992).

As mentioned earlier, AAV requires a helper virus coinfection to establish a permissive environment for DNA replication. It is not clear whether a helper virus protein(s) is responsible for providing the permissive environment for AAV DNA replication, or whether cellular factors expressed after infection of the cell with helper virus are involved. To identify the viral and perhaps cellular factors involved in AAV DNA replication, in vitro DNA replication assays have been
developed wherein DNA is replicated after the addition of purified Rep proteins to HeLa cell lysate extracts supplemented with deoxyribonucleotides, MgCl₂ and an ATP generating system. Ward and co-workers developed an in vitro DNA replication assay where plasmid pAV2 serves as template (Ward et al., 1994). pAV2 is a molecular clone of AAV (Laughlin et al., 1983) that contains the AAV genome cloned into pBR322 by BglIII linkers. In vitro DNA replication of pAV2 requires adenovirus infected HeLa cell lysate. Uninfected HeLa cell lysate, although unable to replicate pAV2, can excise the AAV sequence from the plasmid. Similar results have been reported when AAV linear DNA with covalently closed ends (no-end DNA) was used as the template for DNA replication (Ni et al., 1994). In vitro DNA replication of no-end DNA requires adenovirus infected HeLa cell lysate. Uninfected HeLa cell lysate, although unable to replicate no-end DNA, can support terminal resolution by the Rep78/68 proteins. Since individual adenoviral proteins cannot support AAV DNA replication, it has been suggested that a cellular enzyme either induced or derepressed after helper virus infection may be responsible for establishing a permissive environment for AAV DNA replication (Ni et al., 1994).

1.9 AAV rescue.

The AAV life cycle involves excision of the viral genome from the integrated state and replication after helper virus infection. Infectious clones of wild-type AAV such as psub201 (Samulski et al., 1987) or pSM620 (Samulski et al., 1982) have been used to study AAV rescue and replication. Samulski and co-workers showed that the AAV terminal repeats are required for rescue and replication of the AAV genome from the plasmid, and large regions of the terminal repeat sequences can be deleted without effecting the ability of the
AAV plasmid to rescue and replicate (Samulski et al., 1983). The AAV genome is capable of repairing the terminal repeats. Specifically, Samulski and co-workers showed that the AAV terminal repeats need to retain the non-palindromic sequences and at least one copy of the palindromic sequences for repair to take place (Samulski et al., 1983). The implications of the existence of self-repairing mechanism during rescue of AAV are profound. During integration of the AAV genome into the chromosome, substantial rearrangements of the cellular and viral sequences take place resulting in disruption of the wild-type sequence of the AAV terminal repeats (Kotin et al., 1992; Samulski et al., 1991). The ability of the virus to repair the rearrangements under conditions permissive for replication is vital for its perpetuation.

AAV DNA replication can take place either during a productive AAV infection, where single stranded AAV DNA is the substrate for DNA replication, or in a cell latently infected with AAV and super-infected with helper virus, where the substrate for DNA replication is a double stranded genome integrated into the chromosome of the host. The result of AAV DNA replication in a latently infected cell after helper virus infection is the excision and rescue of the AAV genome from the chromosome and its subsequent replication. It is not known however, whether excision and rescue of the AAV genome is required before DNA replication can proceed or whether excision and rescue of the AAV genome is the outcome of DNA replication. Ward and co-workers developed an in vitro DNA replication assay where the template is the plasmid pAV2 (Ward and Bems, 1991; Ward et al., 1994). pAV2 is a molecular clone of AAV (Laughlin et al., 1983) which contains the AAV genome cloned into pBR322 by BglIII linkers. Uninfected HeLa cell lysate, although unable to support
replication of pAV2, can excise the AAV sequence from the plasmid. However, the rescued products were not substrates for DNA replication in this in vitro system, indicating that DNA replication takes place before excision. Although the data of Ward and co-workers (Ward and Berms, 1991; Ward et al., 1994) indicate that the rescued products are the outcome of DNA replication, the authors cannot eliminate the possibility that these results are an artifact of their in vitro DNA replication assay. Gottlieb and Muzychka identified a cellular activity in HeLa cell nuclear extracts capable of rescuing AAV DNA in vitro and in the absence of DNA synthesis. They called this activity endo R (Gottlieb and Muzychka, 1988). The existence of a cellular activity that rescues AAV proviruses in vitro and in the absence of DNA replication, argues against the products of rescue being the outcome of AAV DNA replication. In addition, Ni and co-workers showed that when no-end DNA was used as a substrate in an in vitro DNA replication assay, uninfected HeLa cell lysates could support terminal resolution when supplemented with Rep78 or Rep68 in the absence of DNA replication (Ni et al., 1994). Their results indicate that Rep proteins may be involved in the excision of proviruses from the integrated state by Rep mediated cleavage at the terminal resolution sites of the integrated AAV genome.

1.10 AAV integration.

AAV integrates into the human chromosome in a site-specific fashion. The preferred site of integration has been mapped to chromosome 19 q13.3- qter and has been termed AAVSI (Kotin et al., 1992; Kotin et al., 1991; Kotin et al., 1990; Samulski et al., 1991). The G+C content of the AAVSI sequence is 65% with some regions being up to 82% G+C rich. The AAVSI site contains a
mini-satellite sequence repeated 60 times within the genome and all 60 sites are located within the q arm of chromosome 19. In addition, 21 other direct repeats are present within the AAVSI sequence (Kotin et al., 1992).

CpG islands are present within the AAVSI in the region that is 82% G+C rich. CpG islands have been found in the promoter regions of genes that lack TATA boxes (Cross and Bird, 1995). In addition, a binding site for the cAMP response element (CRE) (Montminy et al., 1986) and nine binding sites for the upstream binding factor 1 (UBF-1) (Bell et al., 1988) are located within the CpG island. Although northern blot analysis did not reveal a putative transcript, reverse transcription-polymerase chain reaction (RT-PCR) revealed the presence of a transcript in that region (Kotin et al., 1992).

There is no extensive homology between the AAV terminal repeats and the AAVSI site suggesting that integration is accomplished by non-homologous recombination (Kotin et al., 1992). Several investigators identified the Rep recognition sequence GAGC within the AAV integration locus AAVS1 (Chiorini et al., 1994; Weitzman et al., 1994). Rep78/68 has been shown to bind to the GAGC sequence present in the AAV terminal repeats and the p5 and p19 promoters (McCarty et al., 1994). It is possible that Rep mediates integration of the AAV genome through binding to its cognate sequence present in both the AAVS1 and the AAV terminal repeats. Supporting evidence comes from the work published by Giraud and co-workers who showed recombination between the AAV genome and the AAVS1 sequence cloned in an Epstein-Barr virus (EBV) shuttle vector (Giraud et al., 1994).

The structure of the AAV provirus has been analyzed and it is shown to be a tandem head-to-tail integrant containing three to five copies of AAV of which one copy at least remains intact (Berns et al., 1975; Cheung et al., 1980;
Laughlin et al., 1986). Recombination is observed in both the viral and cellular sequences (Cheung et al., 1980; Kotin et al., 1992; McLaughlin et al., 1988) and AAV integrates into only one locus regardless of multiplicity of infection (Samulski et al., 1991). Since the model of AAV DNA replication predicts the existence of head-to-head or tail-to-tail concatamers, proviral DNA is most likely not a DNA replication intermediate. It is not known whether single stranded input viral DNA needs to be converted to the double stranded form before integration or whether the virus can integrate into the genome in its single stranded form.

1.11 The effect of Rep on AAV integration.

The effect of Rep on integration is not well documented. Work published on the integration of recombinant AAV (rAAV) before 1990 showed that both Rep⁺ and Rep⁻ vectors were capable of integration into the chromosome of the host. In addition, the Rep⁺ and Rep⁻ vectors integrated into the chromosome in a head-to-tail orientation and only at a single integration locus (McLaughlin et al., 1988; Samulski et al., 1989; Tratschin et al., 1985).

In 1990, Kotin and co-workers reported the integration of wild-type AAV into a specific location on chromosome 19 (Kotin et al., 1990). This was the first demonstration of a DNA virus exhibiting targeted integration. A number of studies were undertaken to determine whether Rep⁻ vectors integrated into the same location on chromosome 19 or whether Rep was required for targeted integration. A variety of conditions which might have been required for targeted integration were also investigated and specifically the requirements for cell division and DNA replication.
To determine whether cell division or DNA replication was required for integration of the AAV genome into the chromosome, Russell and co-workers used the Rep- vector rAAV/LAPSN (Russell et al., 1994). rAAV/LAPSN contains the alkaline phosphatase gene under the control of the Molony leukemia virus (MLV) long terminal repeat (LTR) promoter and the neomycin resistance (neo\(^R\)) gene under the control of the SV40 promoter, both genes positioned between the AAV terminal repeats. Dividing and non-dividing primary fibroblasts were infected with the virus and cells were selected for neomycin resistance after day 3 (DNA replication and cell division occurred in both dividing and stationary cultures) or assayed for alkaline phosphatase expression after day 2 (DNA replication and cell division only occurred in dividing cells). Parallel cultures were prepared for Southern blot hybridization experiments to determine the status of the rAAV genomes in the stationary and dividing cultures. The authors reported that in stationary cultures which did not undergo DNA replication and cell division (alkaline phosphatase assay), the AAV input genomes were maintained in the single stranded form and integration into the chromosome could not be detected by Southern blot analysis. However, integration was detected in stationary cultures which underwent DNA replication and cell division for selection and it appeared to be random in nature. The results of Russell and co-workers indicate that cell division or DNA replication may be required for integration and that Rep is required for AAV targeted integration. Their data are supported by Shelling and Smith who used the Rep\(^+\) pAAV/p40/neo vector to either transfect or infect human HeLa and 293 cells (Shelling and Smith, 1994). The authors reported that 75% of the neomycin resistant HeLa or 293 clones transfected with pAAV/p40/neo and 82% of the neomycin resistant HeLa or 293 cells infected with rAAV/p40/neo showed site

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specific integration into chromosome 19 as determined by Southern blot analysis. These results implicate Rep in targeted integration of rAAV vectors.

1.12 The effect of Rep on promoter activity.

The rep open reading frame of AAV encodes four proteins, Rep78, Rep68, Rep52, and Rep40. The Rep proteins are the regulatory proteins of AAV and are collectively referred to as Rep. Several reports in the literature suggest that Rep inhibits the expression of homologous (p5, p19, p40) and heterologous promoters.

The effect of Rep on the p5 and p19 AAV promoters was reported by Beaton and co-workers (Beaton et al., 1989). The chloramphenicol acetyl transferase (CAT) gene under the control of the p5 or p19 promoters was positioned between the AAV terminal repeats (p5/CAT or p19/CAT) and CAT expression was used to assay promoter activity in HeLa cells. p5/CAT or p19/CAT vectors were transfected into HeLa cells with or without a wild-type AAV plasmid and assayed for CAT activity. The authors observed a negative regulatory effect of Rep on the activity of p5 and p19 promoters in HeLa cells in the absence of adenovirus. In addition, the authors observed that p5 and p19 promoters became adenovirus responsive only in the presence of Rep. In the absence of Rep, adenovirus had a minimal effect on p5 and p19 promoters.

Similar results were reported by Kyostio and co-workers on the effect of Rep on the activity of the p5 and p19 promoters (Kyostio et al., 1994). The authors reported that Rep78 and Rep68 down regulate the mRNA levels from the p5 and p19 promoters in uninfected 293 cells.

Tratschin and co-workers determined the effect of Rep on CAT gene expression driven by the AAV p40 promoter (Tratschin et al., 1986). The
authors developed a variety of Rep+ and Rep− constructs which they transfected into 293 and HeLa cells in the presence or absence of adenovirus. The authors suggested that Rep is a negative regulator of p40 promoter in uninfected 293 cells and a transactivator of p40 promoter in both infected and uninfected HeLa cells and adenovirus infected 293 cells. In addition, when Rep was supplied either in cis or in trans, the p40 promoter became adenovirus responsive in both HeLa and 293 cells. Similar results were reported by Trempe and Carter on the effect of Rep on the p40 promoter in 293 cells in the absence of adenovirus (Trempe and Carter, 1988a).

Labow and co-workers reported on the effect of Rep on a variety of heterologous promoters using both stable and transient transfection methods (Labow et al., 1987). In co-transfection experiments of pSV40/neo and various Rep+ and Rep− AAV constructs, the authors analyzed the effect of Rep on an SV40 promoter as measured by isolation of neomycin resistant colonies. The authors observed an inhibitory effect on transformation mediated by the AAV rep gene. A similar inhibitory effect on transformation was observed on the herpes simplex virus tk gene under the control of the TK promoter and the neoR gene under the control of the mouse metallothionein promoter.

1.13 The effect of Rep on the transduction efficiency of rAAV vectors.

McLaughlin and co-workers infected Detroit 6 cells (D6), human KB (oral epidermoid carcinoma) cells, and mouse L thymidine kinase negative cells (Ltk−) with the Rep+ (dl52-91/neo) and Rep− (dl3-94/neo) vectors (Hermonat and Muzyczka, 1984; McLaughlin et al., 1988). Vector dl3-94/neo retains the AAV terminal repeats and AAV polyadenylation signal whereas, vector dl52-91/neo
retains the AAV terminal repeats and the AAV rep genes. Both vectors contain the neo\textsuperscript{f} gene between the AAV terminal repeats. Neomycin resistant colonies were isolated and transduction frequencies were calculated for both the Rep\textsuperscript{+} and Rep\textsuperscript{-} rAAV vectors. The Rep\textsuperscript{+} vector showed a transduction frequency of 0.4\% whereas the Rep\textsuperscript{-} vector showed a transduction frequency of 80\%. The authors concluded that the presence of Rep inhibits transduction. Similar results were reported in HeLa and D6 cells using the Rep\textsuperscript{-} vector rAAV/SV40/neo (Samulski et al., 1989).

The above studies established that Rep inhibits the transduction of immortalized cells by rAAV. The effect of Rep on the transduction of primary cells was also investigated. Halbert and co-workers reported on the transduction of Rep\textsuperscript{-} rAAV vectors in primary and immortalized cells (Halbert et al., 1995). Primary epithelial cells from CF patients and the IB3 cell line (immortalized by an adenovirus-SV40 chimeric virus) were infected with rAAV/LAPSN vector. rAAV/LAPSN contains the alkaline phosphatase gene under the control of the MLV LTR promoter and the neo\textsuperscript{f} gene under the control of the SV40 promoter, both genes positioned between the AAV terminal repeats. The authors concluded that the transduction efficiency of the Rep\textsuperscript{-} rAAV vector, as measured by alkaline phosphatase, was higher in immortalized cells compared to primary cells.

The requirements for DNA replication and cell division on the transduction of cells by rAAV was investigated using dividing and non-dividing cells. The transduction efficiency of dividing and non-dividing primary cells with the Rep\textsuperscript{-} vectors rAAV/SV40/neo and rAAV/LAPSN was reported by Russell and co-workers (Russell et al., 1994). Dividing and non-dividing primary fibroblasts were infected with the Rep\textsuperscript{-} vectors and cells were selected for
neomycin resistance after day 3 or assayed for alkaline phosphatase expression after day 2. The neomycin resistance transduction titer was equivalent between dividing and non-dividing primary cells whereas the alkaline phosphatase transduction titer was >20-fold higher in dividing cells. In addition, ³H thymidine incorporation assays showed that the majority of cells in stationary phase were resistant to transduction by rAAV vectors. The authors showed that in stationary fibroblasts the rAAV genomes remain single stranded and episomal. However, the number of single stranded genomes decreased with time whereas the number of cells expressing alkaline phosphatase increased until transduction levels of the non-dividing cultures reached the levels of the dividing cultures. These data indicated that in non-dividing cells the rAAV genomes remained episomal and were recruited for transduction over time.

Similar results were published by Halbert and co-workers who reported that rAAV vectors transduce primary epithelial cells from cystic fibrosis patients less efficiently than immortalized cells (Halbert et al., 1995). The authors reported that the reduced transduction observed in primary cells was not due to the reduced uptake of rAAV genomes by the cell as determined by quantitative PCR. The input genomes remained episomal as determined by Southern blot analysis whereas Northern blots indicated that the majority of these genomes were transcriptionally inactive showing higher transcriptional levels in immortalized cells compared to primary cells.

It has been established that rAAV vectors transduce dividing cells much more efficiently than non-dividing cells (Halbert et al., 1995; Russell et al., 1994). It was postulated that in dividing cells, DNA replication or DNA repair resulted in the conversion of single stranded genomes to the double stranded
form ensuing enhanced expression of the transgene from those cells. To elucidate the cellular factors responsible for the increased transduction observed in cells in the S-phase of the cell cycle, DNA damaging agents, DNA synthesis inhibitors, and topoisomerase inhibitors were used to treat primary human fibroblasts before transduction with rAAV (Alexander et al., 1994; Russell et al., 1995). DNA damaging agents (UV and γ-irradiation, cis-platinum, and ³H thymidine) increased the transduction of stationary human fibroblasts by two orders of magnitude and these agents had a greater effect on the non-dividing cultures. Agents that arrest cells in mitosis or folic acid antagonists had little or no effect on the transduction efficiency of rAAV indicating that mitosis is not required for transduction of cells by rAAV vectors. DNA synthesis inhibitors (aphidicolin and hydroxyurea) and topoisomerase inhibitors (etoposide) also increased the transduction of stationary fibroblasts indicating that DNA synthesis is not the sole determinant of transduction efficiency. Increased DNA repair may be responsible for increased transduction. Drugs affecting RNA transcription and protein translation had no effect on the transduction efficiency of rAAV vectors on stationary fibroblast cultures (Alexander et al., 1994; Russell et al., 1995).

Transduction of cells by rAAV is greatly increased after adenovirus infection. Several investigators showed that the increased transduction observed after infection of cells with adenovirus is the result of second strand AAV DNA synthesis mediated by the E4 ORF6 gene of adenovirus indicating that viral genomes need to be converted to double stranded DNA in order for gene expression to occur (Ferrari et al., 1996; Fisher et al., 1996b).
1.14 Transduction of stem cells by rAAV vectors.

For rAAV vectors to be used for gene transfer, they should be able to transduce stem cells stably and efficiently. After introduction of a therapeutic gene into the stem cell population, the gene has to be expressed in the appropriate cell type and in high enough levels to have a therapeutic effect. In addition, the transgene expression should be regulated.

To achieve regulated tissue specific expression of the $\alpha\gamma$-globin gene, Miller and co-workers infected human hematopoietic progenitor cells with rAAV containing the DNase I hypersensitive regions HS4, HS3, and HS2 of the human $\beta$-globin locus control region (LCR) driving the expression of the human $\alpha\gamma$-globin gene (Miller et al., 1994). Human peripheral blood mononuclear cells from a patient with sickle cell anemia were enriched in CD34$^+$ cells and infected with rAAV/$\gamma$-globin. Twenty to 30% of the erythroid colonies were positive for $\alpha\gamma$-globin RNA as determined by RT-PCR, whereas non-erythroid colonies were negative for $\alpha\gamma$-globin RNA. In addition, in the erythroid colonies the $\alpha\gamma$-globin gene expression was about 10% that of the endogenous gene indicating that the transduced $\alpha\gamma$-globin gene was introduced successfully into primitive hematopoietic cells and was expressed in mature erythroblasts. To determine whether the expression of $\alpha\gamma$-globin RNA resulted in the production of hemoglobin the authors used HPLC to quantitate the production of fetal hemoglobin (HbF) in the transduced erythroblasts. Fetal hemoglobin was produced in 40% of the total hemoglobin levels in rAAV transduced cells whereas HbF was produced in 26% of the total hemoglobin levels in the mock transduced cells.

Walsh and co-workers reported the transduction of CD34$^+$ hematopoietic progenitor cells by rAAV containing the cDNA for Fanconi anemia (FA) which
corrected the FA phenotype in cells isolated from a FA patient (Walsh et al., 1994). Peripheral blood mononuclear cells from a FA patient were enriched in CD34+ hematopoietic progenitor cells and infected with rAAV/FA. Hematopoietic colonies were isolated 15 days later and incubated with mitomycin C (MMC), a DNA crosslinking agent. CD34+ cells from FA patients are hypersensitive to MMC and their growth is retarded. CD34+ cells isolated from patients with FA, when transduced with rAAV/FA, resulted in four times the number of colonies, compared to the non-transduced cells. In addition, when CD34+ cells isolated from patients with FA and transduced with rAAV/FA were treated with MMC, 8 to 10-fold increase in the number of colonies was observed compared to the non-transduced controls. RT-PCR analysis of the colonies with increased growth rate showed that these colonies express the FA gene.

1.15 In vivo transduction with rAAV vectors.

To determine the efficiency of rAAV vectors to transfer and express foreign genes in vivo, Flotte and co-workers introduced the CFTR gene into primary CF nasal polyp cells and into the airway epithelium of rabbits (Flotte et al., 1993). rAAV/CFTR vector sequences and CFTR protein expression were detected in primary CF nasal polyp cells infected with rAAV/CFTR as determined by in situ PCR and immunofluorescence. Over 75% of the CF nasal polyp cells contained vector DNA and expressed CFTR protein. When the same rAAV/CFTR vector was introduced into the airway epithelium of rabbits recombinant genomes and CFTR protein were detected as early as 3 days after
infection and for at least 6 months. The authors reported lack of inflammation or pathogenicity associated with the introduction of the rAAV/CFTR vector into the airway epithelium of rabbits.

Zeitlin and co-workers used rAAV/β-gal vector to introduce the lacZ gene into the lung of newborn rabbits (Zeitlin et al., 1995). X-gal histochemical staining was used to assay transduction whereas, 5-bromo-deoxyuridine (BRDU) incorporation was used to assay cell proliferation. The authors showed that cells undergoing proliferation were preferentially but not exclusively transduced by the rAAV/β-gal vector. Specifically, transduction in the newborn rabbit lung occurs preferentially in alveolar epithelial type II cells and in the tracheobronchial basal stem cells and non-dividing ciliated cells.

Kaplitt and co-workers introduced the rAAV/β-gal and rAAV/th (tyrosine hydroxylase) vectors into the brain of male Sprague-Dawley rats (Kaplitt et al., 1994). The authors showed that expression of the transgenes persisted for up to 3 months. *In situ* PCR was used to detect the presence of rAAV/β-gal input viral genomes two months after infection. The authors reported a 10% transduction of brain cells three days after infection as detected by β-gal staining. In addition they reported a significant behavioral recovery of lesioned rats injected with rAAV/th vector with implications towards treatment of Parkinson's disease.

Alexander and co-workers introduced the AAV-LAPSN vector into the adult rat brain and overlying scalp muscle (Alexander et al., 1996). They showed that γ-irradiation enhanced the transduction of dividing cell populations within the central nervous system (in the pia-arachnoid and choroid epithelium), and of non-dividing muscle cells in the scalp (striated muscle cells). The
authors reported that γ-irradiation mediated a 12 to 50-fold increase in the transduction of muscle cells whereas, in the pia-arachnoid and choroid epithelium a 50 to 250-fold increase in transduction was observed.

1.16 rAAV as a vector for gene transfer.

The features of AAV described in this section support the application of AAV as a gene transfer vector. Specifically, the use of AAV as a gene transfer vector has certain advantages. First, AAV infection is not associated with any known human or animal disease. Second, in the absence of helper virus infection, AAV vectors containing the Rep gene have the potential for targeted integration into human chromosome 19. Third, AAV can infect every cell type examined to date. Finally, AAV can infect both dividing and non-dividing cells. However, AAV also faces obstacles. First, AAV has a small genome and thus there are packaging constrains. Second, the AAV genome is single stranded and needs to be converted to a double stranded form for transgene expression. Third, the in vivo biology of rAAV is largely unknown partly because of the difficulties in isolating rAAV in the laboratory. Finally, the technology for the production of rAAV in the laboratory is not fully optimized.

To study the biology of rAAV in vitro and in vivo with the prospect of developing rAAV as gene transfer vector rAAV must be efficiently produced in the laboratory. Towards that goal, we developed a novel strategy for rAAV production which involves the generation of stable cell lines that package rAAV upon infection with adenovirus (Clark et al., 1995). In the chapters that follows, we report the construction and characterization of stable cell lines that upon infection with adenovirus package the lacZ gene of E. coli into recombinant
virions (rAAV/β-gal). β-galactosidase is a reporter gene that can be conveniently detected in vitro using X-gal histochemical staining. rAAV/β-gal is produced efficiently, reproducibly, and is free of contaminating wild-type AAV.
CHAPTER 2

MATERIALS AND METHODS

2.1 Cells.

HeLa (human cervical carcinoma cells) and 293 (human embryonic kidney cells) cells were maintained in DMEM and EMEM (Gibco, BRL), respectively, supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin (100 μg/ml). HeLa and 293 cells were obtained from the American Type Culture Collection (Rockville, MD). Neomycin resistant cell lines such as H44, D6, A64, A80, and C12 were grown in DMEM medium supplemented with 500 μg/ml (active) G418 (Clark et al, 1995).

2.2 Isolation of wild-type AAV.

Wild-type AAV (serotype 2) was purified according to established protocols (Berns and Rose, 1970). Briefly, HeLa cells (60-70% confluent) were infected with Ad5 at a multiplicity of infection (moi) of 5 in 2% DMEM. After 45 min of adenovirus infection, the cells were infected with wild-type AAV (moi of 10) and incubated to maximum cytopathic effect (CPE) (all cells rounded, 70% floating). Cells were harvested by centrifugation at 800 x g for 10 min, resuspended in 10 ml of 10 mM Tris pH 8.0 to the final concentration of 3.5x10^6
cells/ml and lysed by three freeze/thaw cycles. Deoxycholic acid (DOC) was added to the final concentration of 0.5%, the cell lysate was incubated at 37°C for 10 min, and then sonicated at maximum setting three times. Cellular debris was removed by centrifugation at 3,000 x g, 10 min, at 4°C. The density of the solution was adjusted to 1.4 g/ml with solid CsCl and centrifuged in an SW28 rotor (Beckman), 25,000 rpm, 10°C for 48 hr. The isopycnic gradient was fractionated in 3.5 ml fractions and the optical density of each fraction was measured using a refractometer. The fractions were analyzed for the presence of wild-type AAV by dot blot hybridization. The wild-type AAV containing fractions were combined and the CsCl was diluted down to 1.1 g/ml with 10 mM Tris pH 8.0. The virus was pelleted in an SW28 rotor (Beckman) by centrifugation at 28,000 rpm, 10°C for 5 hr. The viral pellet was resuspended in 1.5 ml buffer (20 mM Tris pH 8.0, 1 mM MgCl₂, 150 mM NaCl) and purified once more through CsCl isopycnic gradient by bringing the density of the solution to 1.41 g/ml with solid CsCl and centrifugation in an SW41 rotor (Beckman), 30,000 rpm, 10°C for 48 hr. The isopycnic gradient was fractionated in 1 ml fractions and the optical density of each fraction was measured using a refractometer. The fractions were analyzed for the presence of wild-type AAV by dot blot hybridization. The wild-type AAV containing fractions were pooled and dialyzed against 4L of 20 mM Tris pH 8.0, 1 mM MgCl₂, 150 mM NaCl buffer for 48 hr, changing the buffer 4 times. The virus was titered by IFA and stored under liquid nitrogen.
2.2.1 Dot blot analysis of isopycnic fractions.

Twenty µl from each isopycnic fraction were incubated at 37° C for 1 hr in the presence and absence of 350 units of DNase I (Gibco, BRL). The fractions were extracted once with an equal volume of phenol/chloroform and boiled for 10 min. To the samples 20XSSC was added to the final concentration of 6X and loaded on a dot blot apparatus (Gibco, BRL). The wells were rinsed once with 6XSSC and the nitrocellulose filter was air dried and baked 80° C for 2 hr. The filter was hybridized with a 360 bp PCR amplified probe generated using the rep primers 214/215 and analyzed by autoradiography.

2.2.2 Titration of wild-type AAV by a modified fluorescent focus assay.

HeLa cells (2x10^4) were seeded on Titer-tek slides (Nunc Inc.) and infected with serial 1:10 dilutions of wild-type AAV in quadruplicate, in the presence and absence of adenovirus (moi of 5), in 2% DMEM for 2 hr. After infection, the cells were fed with fresh 2% DMEM and incubated for 48 hr. After 48 hr the cells were fixed in 50% methanol/50% acetone and stained with a mouse IgM monoclonal antibody reactive to AAV capsid proteins (kindly provided by Dr. Long-Sheng Chang). The primary antibody was diluted 1:10 in 1XPBS/3% BSA buffer (1XPBS: 15 mM sodium phosphate pH 7.3, 150 mM NaCl), applied to the slides such that it covered the cells completely, and incubated at room temperature for 1 hr in a humidified chamber. After 1 hr, the antibody was removed by washing the slides in 1XPBS/0.1% Tween, 5 min and briefly in 1XPBS. Excess buffer was removed by blotting and the secondary antibody, (anti-mouse IgM FITC conjugate [Sigma]) was diluted 1:50 in
1XPBS/3% BSA buffer and applied to the slides as above. The slides were incubated at room temperature for 30 min in the dark in a humidified chamber. After incubation, the antibody was removed by washing the slides in 1XPBS/0.1% Tween buffer for 5 min and then briefly in 1XPBS. Coverslips were mounted and the slides were observed under the fluorescent microscope (Olympus, BH-2). Wells were scored positive when three or more cells showed positive staining. The TCID$_{50}$ titer of the stock was calculated using the Reed-Muench formula.

2.3 Isolation of adenovirus.

Adenovirus type 5 (Ad5) was purified according to established protocols (Graham and Prevec, 1991) with minor modifications. Briefly, HeLa cells were allowed to reach 70-80% confluency and infected with adenovirus containing inoculum (moi 5-10) in 2% DMEM. Cells were harvested at maximum CPE by centrifugation at 1,000 x g, 15 min at 4° C and resuspended in 100 mM Tris pH 8.0 to the final concentration of 1x10$^7$ cells/ml. Cell lysate was prepared by three rounds of freeze-thaw and three rounds of sonication and cell debris was removed by centrifugation at 3,000 x g, 10 min, at 4° C. Adenovirus was purified through a step gradient using 1.41 g/ml bottom pad and 1.25 g/ml top pad in SW28 rotor (Beckman), 26,000 rpm, 2 hr, at 10° C. The adenovirus band was collected by side puncture using an 18 gauge needle and stored in buffer at a 1:2 dilution (10 mM Tris pH 8.0, 150 mM NaCl, 40% glycerol) under liquid nitrogen. The virus was subsequently titered by plaque assay on 293 cells.
2.3.1 Titration of adenovirus by plaque assay.

Infectivity titrations of Ad5 were performed by plaque assay on 293 cells according to established protocols (Graham and Prevec, 1991). Briefly, 293 cells were seeded on 12-well plates and infected with serial 1:10 dilutions of the adenovirus stock in quadruplicate for 2 hr in 2% EMEM. After infection, the cells were fed with fresh 10% EMEM and incubated until plaques formed on the endpoint dilution wells (usually about one week). The TCID$_{50}$ titer was calculated by the Reed-Muench formula.

2.4 Isolation of rAAV/$\beta$-gal.

rAAV/$\beta$-gal was purified using a method similar to wild-type AAV with minor modifications. Neomycin resistant cell lines (H44 or D6) at 50% confluency were infected with adenovirus (moi of 5) and CPE was allowed to progress for 72 hr. Cells were then collected by centrifugation at 1,000 x g, 15 min, $4^0\ C$ and resuspended at a density of 1x10$^7$ cells/ml in 20 mM Tris pH 8.0 and 1 mM MgCl$_2$. After lysis by three freeze-thaw cycles, the lysate was clarified by centrifugation at 1,000 x g, 15 min, $4^0\ C$. rAAV/$\beta$-gal was concentrated from the clarified lysate by centrifugation through a CsCl cushion (1.31 g/ml) in an SW-28 rotor (Beckman), 16,000 rpm, 15 hr, at $10^0\ C$. The viral pellet was resuspended overnight at $4^0\ C$ and purified further by isopycnic gradient centrifugation by adjusting the CsCl density to 1.41 g/ml and spinning at 30,000 rpm for 48 hr in an SW-41 rotor (Beckman). rAAV/$\beta$-gal containing fractions were desalted by pelleting or dialysis.
2.4.1 Titration of rAAV/β-gal by the C12+Ad assay.

C12 cells were seeded on a 24-well plate and infected with serial 1:10 dilutions of rAAV/β-gal in duplicate, in the presence of adenovirus (moi of 5) in 2% DMEM for 24 hr. After infection, the cells were fed with fresh 2% DMEM and incubated for another day. Forty hours after infection, the cells were stained for β-galactosidase by the X-gal histochemical staining. The titer was calculated by counting the number of blue cells in both wells in the end-point dilutions, taking the average and multiplying that number by the dilution factor and the infection volume. The titer was expressed as blue forming units per ml (bfu/ml).

2.5 Plasmid DNA constructions.

The construction of the tripartite plasmid (pTP) pAAV/CMV-β/rep-cap/neo is shown in Figure 3. Plasmid psub201 (Samulski et al., 1987) was digested with Xbal and the 4.1 Kb fragment containing the pEMBL8+ sequences and the AAV terminal repeats was blunt ended and band isolated to result vector psub201/Xbal. Plasmid pCMV-β (Clonetech) was digested with PstI and the 4.5 Kb fragment was band isolated and blunt ended. The 4.5 Kb PstI fragment contains the CMV immediate/early promoter and enhancer, the SV40 splice donor and acceptor signals, the β-galactosidase sequence and the SV40 small t-antigen polyA signal. The 4.5 Kb PstI fragment was ligated into psub201/Xbal to give plasmid pAAV/CMV-β. Plasmid pAAV/CMV-β was digested with NgoMI, blunt ended using the Klenow fragment of DNA polymerase I, dephosphorylated using alkaline phosphatase from calf intestine, and the 8.6 Kb fragment was band isolated. Plasmid pBluescript KS(-) (Stratagene) was digested with EcoRI and BamHI and the neoR transcription unit (neoR gene
under the control of the SV40 promoter and polyA signal) was ligated into the EcoRI-BamHI sites to generate plasmid pBS/SVneo. The 4.3 Kb rep-cap Xbal fragment was cloned into the XbaI site of pBS/SVneo to give plasmid pBS/rep-cap/neo. pBS/rep-cap/neo contains the neoR gene under the control of the SV40 promoter and SV40 polyA signal and the rep-cap open reading frames of AAV under the control of their native promoters. Plasmid pBS/rep-cap/neo was digested with NotI and EcoRV and the 7.0 Kb fragment was blunt ended, band isolated and ligated into the NgoMI site of pAAV/CMV-β to give plasmid pAAV/CMV-β/rep-cap/neo.

The construction of the tripartite plasmid (pTP) pAAV/CMV-β/rep-cap/neo-tk is shown in Figure 4. Plasmid pAAV/CMV-β was digested with NgoMI, blunt ended and dephosphorylated as described above and the 8.6 Kb fragment was band isolated. pBK-RSV (Stratagene) was digested with Bsu36I and the 1.6 Kb neoR transcription unit (SV40 promoter, neoR gene and the tk polyA signal) was ligated into the EcoRV site of pBluescript KS(-) (Stratagene) to generate pBS/neo-tk. psub201 was digested with XbaI and the 4.3 Kb rep-cap fragment was band isolated and ligated into the XbaI site of plasmid pBS/neo-tk to generate plasmid pBS/rep-cap/neo-tk. Plasmid pBS/rep-cap/neo-tk was digested with Clal and NotI, blunt ended and the 5.9 Kb rep-cap/neo-tk fragment was ligated into the NgoMI site of pAAV/CMV-β to generate pAAV/CMV-β/rep-cap/neo-tk.

The plasmid maps of pGEM/β-gal-1 and pGEM/β-gal-1A are shown in Figure 5. The β-galactosidase sequence was removed from plasmid pAD-β (Clonetech) by NotI digestion and the 3474 bp NotI fragment was ligated into the HindIII/EcoRI sites of pGEM 7zf(+) by blunt end ligation to give plasmid
pGEM/β-gal-1. pGEM/β-gal-1 was digested with Sacl to remove the last 1400 bp of the β-galactosidase sequence. The final plasmid construct (pGEM/β-gal-1A) contained the first 2074 bp of the lacZ gene cloned between the bacteriophage T7 and SP6 promoters in pGEM 7zf(+).

2.6 Transfection of cells by the Ca$_3$(PO$_4$)$_2$ co-precipitation method.

Transfections were performed according to established protocols (Becker et al., 1994). Briefly, cells were allowed to reach 70-80% confluency and when required they were infected with adenovirus (moi of 5). Approximately 3x10$^6$ cells were transfected per 1 ml of transfection medium. The transfection mixture contained 2XHEPES-buffered saline (2XHBS: 280 mM NaCl, 42 mM HEPES, 10 mM KCl, 1.4 mM Na$_2$HPO$_4$·7H$_2$O, 0.2% dextrose pH 7.05-7.15) to the final concentration of 1X, 10-20 μg of plasmid DNA, and water to bring the volume up to 950 μl. Fifty μl of 2.5 M CaCl$_2$ was added to the transfection mixture, resuspended, and incubated at room temperature for 45-60 min. The transfection mixture was kept on the cells over night and the next day the cells were fed with fresh media and analyzed at appropriate times.

2.7 X-gal histochemical staining.

Cells were fixed for 5 min at 4°C in cold 2% paraformaldehyde/0.2% gluteraldehyde in 0.1 M sodium phosphate buffer, pH 7.3. The fixative was aspirated and the cells were washed in 1XPBS and stained with X-gal stain (100 mM sodium phosphate pH 7.3, 1.3 mM MgCl$_2$, 3 mM K$_3$Fe(CN)$_6$, 3 mM K$_4$Fe(CN)$_6$ and 1 mg/ml X-gal in N,N-dimethyl formamide) at 37°C overnight.
2.8 Biological activity of pTP plasmids.

The pTP plasmids were tested for their ability to produce infectious virions capable of expressing β-galactosidase. HeLa cells (5x10^5) were transfected with 5 μg of pTP plasmid DNA for 18 hr using the CaPO_4 coprecipitation method. After transfection, the cells were infected with adenovirus (moi of 5) in 2% DMEM for 2 hr. The cells were fed with fresh 2% DMEM and incubated to maximum CPE. At maximum CPE, cell lysate was prepared by three rounds of freeze/thaw and three rounds of sonication. The cell lysate was clarified by centrifugation at 4000 rpm, 4°C, 15 min and adenovirus was heat inactivated at 56°C for 30 min. HeLa cells (5x10^5) were infected with the cell lysate in the presence and absence of adenovirus (moi of 5) for 1-2 hr and 48 hr later the cells were stained for β-galactosidase by X-gal histochemical staining.

2.9 Indirect immunofluorescence analysis (IFA).

Cells (2x10^4) were seeded on Titer-tek slides (Nunc Inc.) and fixed for 10 min in cold acetone. The primary antibody used was the anti-AAV-VP1-VP2-VP3 monoclonal antibody (American Research Products, Inc.) diluted 1:2 in 1XPBS/3% BSA/0.2% Tween buffer. The primary antibody was applied on the slides such that it covered the cells completely and was incubated at 37°C for 1 hr. After 1 hr, the antibody was removed by washing the slides in 1XPBS/3% BSA/0.2% Tween buffer for 10 min by gentle shaking. Excess buffer was removed and the secondary antibody, anti-mouse IgG whole molecule FITC conjugate (Sigma), was diluted 1:16 in 1XPBS/3% BSA/0.2% Tween buffer and added on the slides such that it covers the cells completely. Occasionally,
Evans blue (0.001%) was added to the 1XPBS/3% BSA/0.2% Tween buffer before making the antibody dilution. The slides were incubated for 30 min at 37°C in the dark. After incubation, the antibody was removed by washing the slides in 1XPBS/3% BSA/0.2% Tween buffer for 10 min with gentle shaking and in water briefly. Coverslips were mounted on the slides and the slides were observed under a fluorescent microscope (Olympus, BH-2).

2.10 Titration of wild-type AAV in rAAV/β-gal preparation.

HeLa cells (2x10^4) were seeded on Titer-tek slides (Nunc Inc.) and infected in quadruplicate with serial 1:10 dilutions of rAAV/β-gal in the presence and absence of adenovirus. The adenovirus infections were done in 2% DMEM (moi of 5) and were kept on the cells for 2 hr. After infection the cells were fed with fresh 2% DMEM and 48 hr later were stained with AAV capsid antibodies by IFA. A TCID_{50} titer was calculated using the Reed-Muench formula.

The sensitivity of the assay was determined by infecting similar number of HeLa cells with serial 1:10 dilutions of wild-type AAV in the presence and absence of adenovirus as above. After infection, the cells were fed with fresh 2% DMEM and 48 hr later were stained with antibodies that were specific to AAV capsid proteins.

2.11 Crude lysate preparation.

Cells were grown to 50% confluency and after all the necessary manipulations were finished, the cells were infected with adenovirus (moi of 5) in 2% DMEM for 24 hr. After infection, the cells were fed with fresh 2% DMEM and incubated to maximum CPE. At maximum CPE, cell lysate was prepared
by three rounds of freeze-thaw and three rounds of sonication. The cell lysate was clarified by centrifugation at 4000 rpm, 4°C, 15 min and adenovirus was heat inactivated at 56°C for 30 min.

2.12 Particle-to-infectivity ratio of rAAV/β-gal and wild-type AAV.

The particle-to-infectivity ratios of rAAV/β-gal and wild-type AAV were determined by comparing the intensity of hybridization of rAAV/β-gal and wild-type AAV to the intensity of hybridization of pCMV-β and psub201 genome equivalents, respectively.

2.12.1 Particle-to-infectivity ratio of wild-type AAV.

Serial 1:10 dilutions of 5x10^7 TCID_{50} of wild-type AAV and 2x10^9 genome equivalents of psub201 were incubated at 37°C for 30 min in the presence and absence of 350 units of DNase I (Gibco, BRL). The DNA was purified by phenol/chloroform extractions, boiled for 10 min, and chilled on ice. To the samples, 20XSSC was added to the final concentration of 6X and the DNA was loaded on a dot blot apparatus (Gibco, BRL). The probe used for hybridization was a 641 bp PCR generated fragment (primers 210/211) located within the rep open reading frame of plasmid psub201.

2.12.2 Particle-to-infectivity ratio of rAAV/β-gal.

Serial 1:10 dilutions of 5x10^7 bfu of rAAV/β-gal and 2x10^9 genome equivalents of pCMV-β were incubated at 37°C for 30 min in the presence and absence of 350 units of DNase I (Gibco, BRL). The DNA was purified by phenol/chloroform extractions, boiled for 10 min, and chilled on ice. To the samples 20XSSC was added to the final concentration of 6X and the DNA was
loaded on a dot blot apparatus (Gibco, BRL). The probe used for hybridization was a 1824 bp Clal/Pvull restriction enzyme fragment located within the β-galactosidase open reading frame of plasmid pAD-β (Clonetech).

2.13 Genomic DNA isolation.

Genomic DNA was isolated according to established protocols with minor modifications (Sambrook et al., 1989). Cells were harvested by trypsinization or shaking, collected by centrifugation at 2,000 x g, 5 min at 4° C, and washed twice in serum free media. The cell pellet was resuspended in proteinase K digestion buffer (0.1 M NaCl, 10 mM Tris pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS and 0.1 mg/ml proteinase K) to 2x10^7 cells/ml and digested at 56° C over night. The next day the digestion was purified by repeated phenol/chloroform extractions, the DNA was collected by precipitation, resuspended in TE (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) buffer, and its concentration was determined by optical density. The integrity of the DNA preparations was examined by agarose gel electrophoresis.

2.14 High molecular weight DNA isolation.

High molecular weight DNA was isolated according to established protocols (Sambrook et al., 1989). Cells were harvested by trypsinization, collected by centrifugation at 1,500 x g, 10 min, 4° C, and washed once with serum free media. The cells were resuspended in TE buffer to a concentration of 5x10^7 cells/ml and 10 ml extraction buffer (10 mM Tris pH 8.0, 100 mM EDTA, 20 µg/ml RNase A and 0.5% SDS) was added per 1 ml of cell suspension. The cells were incubated at 37° C for 1 hr and proteinase K to the final concentration of 100 µg/ml was added to the suspension and incubated at
50° C over night. The suspension was purified by phenol/chloroform extractions three times by gentle inversion to avoid shearing of the DNA. The DNA was dialyzed against 4L of 50 mM Tris, 10 mM EDTA buffer, five times and the concentration was determined by optical density.

2.15 Low molecular weight (Hirt) DNA isolation.

Low molecular weight DNA was isolated by standard methods (Hirt, 1967) with minor modifications. Cells were grown on 6-well or 12-well plates and after all necessary manipulations were finished the cells were washed twice with serum free media and resuspended in 50 µl HBSS (Gibco, BRL). DNA extraction buffer, 400 µl, (0.6% SDS, 10 mM Tris pH 7.6, 10 mM EDTA) was added to the cells and incubated at room temperature for 10 min. NaCl to the final concentration of 1 M was added to the cell lysate and incubated at 4° C over night. The cell lysate was centrifuged at 12,000 x g for 30 min and the supernatant which contains the low molecular weight DNA was saved and purified by phenol/chloroform extractions. Hirt DNA was precipitated, ethanol washed, and resuspended in 30 µl of TE buffer.

2.16 Polymerase chain reaction (PCR).

One µg of total or Hirt DNA and 20 pg of plasmid DNA were used in PCR reactions. The PCR primers annealed on two areas of the AAV genome, the terminal repeats (TR) and the rep sequence (rep). The nucleotide positions of the rep primers are based on the AAV-2 sequence published by (Ruffing et al., 1994) and are the following: 208 (nt. 779-nt. 756), 210 (nt. 1000-nt. 1023), 211 (nt. 1640-nt. 1617), 214 (nt. 381-nt. 406), 215 (nt. 740-nt. 716), 482 (nt. 927-nt. 907), 483 (nt. 1190-nt. 1170), and 484 (nt. 1297-nt. 1277). The positions of the
TR primers, 446, 485, and 480, cannot be defined on the AAV sequence because they have been designed based on the psub201 sequence. The terminal repeat sequences are different between the AAV and psub201 (Samulski et al., 1987), therefore the sequence of the TR primers is given instead: 446 (ccgccatgctaatattctacgtag), 485 (tccatcactaggggttcctt), 480 (gtagttaatgattaacccgc).

All PCR reactions were performed using a PCR kit (Gibco, BRL) according to the manufacturer's instructions. Briefly, 100 μl reactions were set up by adding 10XPCR buffer (200 mM Tris-HCl pH 8.0, 500 mM KCl) to the final concentration of 1X, dNTP's to the final concentration of 0.2 mM, MgCl2 to the final concentration of 1.5 mM, each of the primers to the final concentration of 0.3 μM, and 5 units of Taq DNA polymerase. The reactions were incubated for 30 cycles at 94° C, 2 min; 55° C, 1 min; 72° C, 1 min. Twenty μl from each reaction were fractionated by agarose gel electrophoresis and visualized by ethidium bromide staining.

2.17 Field Inversion Gel Electrophoresis (FIGE).

High molecular weight DNA was fractionated on a 0.8% agarose gel in 0.5XTBE buffer (0.045 M Tris-borate, 0.001 M EDTA). The field inversion was accomplished by the use of the PPI-200 programmable power inverter (MJ Research, Inc.) using the built in program 4. Briefly, high molecular weight DNA was digested to completion with restriction enzymes according to the manufacturer's instructions overnight. The DNA was precipitated with 1/10 volume 3 M NaOAc and 2 volumes of 100% ethanol for several hours. The DNA was washed with 70% ethanol, air dried, and resuspended in 40 μl TE buffer over night, at room temperature. The next day the DNA was loaded on an
0.8% agarose gel and fractionated by field inversion gel electrophoresis. After completion of electrophoresis the gel was processed for Southern hybridization analysis according to the protocol described below.

2.18 Southern hybridization analysis.

DNA was fractionated on an agarose gel and then the DNA was denatured in 1.5 M NaCl, 0.5 N NaOH for 30 min by gentle shaking. The gel was rinsed briefly in water and neutralized in 1.0 M Tris pH 8.0, 1.5 M NaCl for 15 min, twice, by gentle shaking. The gel was rinsed briefly in water and the DNA was either transferred onto nylon membrane (Amersham) in 20XSSC by capillary action, or dried on a gel drier (unblot). The DNA was covalently bound to the nylon membrane by UV crosslinking and then it was incubated in prehybridization buffer [0.5% SDS, 5XSSC, 50 mM PB (0.05 M Na₂HPO₄ and 0.05 M NaH₂PO₄), 2X Denhardt's and 0.2 mg/ml hsDNA] at 60° C, for 2 hr. The filter or the unblot were hybridized in hybridization buffer (0.5% SDS, 5XSSC, 50 mM PB and 1X Denhardt's and 0.1 mg/ml hsDNA) 60° C, over night. The probe used in hybridization was labeled to high specific activity (>5x10⁸ cpm/µg) using the RadPrime labeling kit (Gibco, BRL) according to manufacturer's instructions. Low stringency washes were performed in 2XSSC, 0.1% SDS, 10 min, twice, at room temperature whereas, high stringency washes were performed in 0.2XSSC, 0.1% SDS, 65° C, twice, 30 min. The filters were analyzed by autoradiography.

2.19 Northern hybridization analysis.

Total RNA was isolated using the TRIzol Reagent (Gibco, BRL) according to the manufacturer's instructions. The RNA was fractionated on 1%
agarose/37% formaldehyde gel in HE buffer (50 mM HEPES, 10 mM EDTA pH 8.0) and transferred to a nylon membrane (Amersham) in 20XSSC by capillary action. The RNA was covalently bound to the membrane by UV crosslinking and incubated in prehybridization buffer [50% formamide, 5XSSC, 0.05 M PB, 1% SDS, 4X Denhardt's and 0.2 mg/ml hsDNA] for 2 hr. The filter was hybridized in hybridization buffer [50% formamide, 5XSSC, 0.05 M PB, 1% SDS, 1X Denhardt's and 0.1 mg/ml hsDNA] over night. The prehybridization and hybridization temperatures were 42°C for DNA probes and 60°C for RNA probes. The DNA probes used in hybridization were labeled to high specific activity (>5x10^8 cpm/µg) using the RadPrime labeling kit (Gibco, BRL) according to manufacturer's instructions. The RNA probes were prepared according to the protocol described below. Low stringency washes were performed in 2XSSC, 0.1% SDS, 10 min, twice, at room temperature whereas, high stringency washes were performed in 0.2XSSC, 0.1% SDS, 68°C, twice, 30 min. The filters were analyzed by autoradiography.

### 2.20 Template preparation for *in vitro* transcription.

For the preparation of antisense probe, plasmid pGEM/β-gal-1A (Figure 5) was digested with XbaI to linearize the plasmid at the 5' end of the β-galactosidase sequence. For the preparation of the sense probe, plasmid pGEM/β-gal-1A was digested with SaeI to linearize the plasmid at the 3' end of the β-galactosidase sequence. Complete enzyme digestion was verified by agarose gel electrophoresis and template DNA was purified by phenol/chloroform extractions, collected by ethanol precipitation and resuspended in RNase free water at a concentration of 1 mg/ml.
2.21 Riboprobe preparation.

For both sense and antisense probes, 20 µl reactions were set up using the Riboprobe Gemini system (Promega). Briefly, 5µM of [33P]-UTP were dried down and resuspended in RNase free water. Cold UTP, 20 µM, was added to make the final concentration of UTP in the reaction 25 µM. The following reagents were added to the reaction in the order specified: Transcription buffer to the final concentration of 1X (40 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 2 mM spermidine and 10 mM NaCl), 10 mM DTT, 20 units of Ribonuclease inhibitor, 500 µM of rATP, rGTP and rCTP, 1 µg of linearized template DNA and 20 units of the appropriate RNA polymerase (T7 for the sense probe and SP6 for the antisense probe). The T7 and SP6 reactions were incubated at 37° and 40° C respectively for 1.5 hr. The DNA template was digested with 1 unit of RNase free DNase I at 37° C for 15 min. The probe was hydrolyzed to approximately 300 bases by incubation at 60° C in 4 mM NaHCO3 and 6 mM Na2CO3 for 15 min in 200 µl total volume. The hydrolysis of RNA was terminated by adding 1.3 µl glacial acetic acid and 20 µl of 3 M sodium acetate. The RNA was precipitated with 2.5 volumes of 100% ethanol and 50 µg of yeast t-RNA as carrier. The RNA was collected by centrifugation and the pellet was washed with 70% ethanol, air dried and resuspended in 50 µl RNase free water. Ten µl of 1:100 dilution of the probe were counted on a scintillation counter and the specific activity of the probe was calculated.

2.22 Fixation of cells for in situ hybridization.

Paraformaldehyde was prepared fresh in RNase free sodium phosphate buffer (80 mM Na2HPO4 and 20 mM NaH2PO4), pH 7.3. Cells were fixed in 4% paraformaldehyde, at 4° C, for 15 min. After fixation the slides were washed in
3XPBS, 2 min, once, and in 1XPBS, 2 min, twice. The cells were dehydrated in increased concentrations of ethanol (30, 50, 70 and 95%) and stored in 95% ethanol at 4°C or air dried and processed accordingly.

2.23 Treatment of cells with proteinase K.

After fixation and before in situ hybridization, the cells were permeabilized by proteinase K digestion (Gibco, BRL). Proteinase K, 0.1 μg/ml, was prepared in 100 mM Tris-HCl pH 8.0, 50 mM EDTA buffer, added to the slides and incubated at 37°C, for 30 min. To remove the proteinase K, the slides were washed briefly with water and 0.1 M triethanolamine buffer pH 8.0 (TEA) (Sigma). Acetylation was performed in TEA buffer and 0.25% v/v acetic anhydride (Sigma) at room temperature, for 10 min. After acetylation the slides were washed briefly in 2XSSC and dehydrated in 30, 50, 70 and 95% ethanol and stored in 95% ethanol at 4°C or air dried and processed accordingly.

2.24 Treatment of cells with DNase.

After proteinase K digestion and before in situ hybridization the cells were treated with RNase free DNase I (Sigma). DNase I (100 μg/ml) was prepared in buffer (0.5 M NaOAc pH 6.5, 100 mM MgCl₂, 50 mM CaCl₂), added to the slides and incubated at 37°C for several hours. To remove the DNase I the cells were washed briefly with 2XSSC, dehydrated in 30, 50, 70 and 95% ethanol and stored in 95% ethanol at 4°C or air dried and processed accordingly.
2.25 **In situ** hybridizations.

*In situ* hybridizations were performed in 20 μl hybridization volume (4.84 cm² area) according to established protocols with minor modifications (Angerer and Angerer, 1994). The hybridization volume, 20 μl, was composed of the hybridization cocktail (0.3 M NaCl, 20 mM Tris pH 8.0, 5 mM EDTA, 50% formamide, 10% Dextran Sulfate and 0.5X Denhardt's) and the probe mix (20 μg of yeast t-RNA and 0.63 ng/μl of probe). The hybridization cocktail constituted 81% of the hybridization volume whereas the probe mix constituted 19% of the hybridization volume. The probe mix was prepared as described above, heated at 80°C for 2 min and kept on ice. The hybridization cocktail was added to the probe mix, vortexed and 20 μl were added to the cells. The cells were covered with a coverslip (4.84 cm² area), sealed and hybridized at 50°C over night.

2.26 **In situ** hybridization washes.

After over night hybridization, the cover slips were removed and the slides washed in 4XSSC, 5 min, three times. The cells were then dehydrated in 30, 50, 70 and 95% ethanol (sequentially) containing 300 mM NH₄OAc. A high stringency wash was performed in hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris pH 8.0 and 1 mM EDTA) at 50°C for 15 min. The slides were washed briefly in 2XSSC to remove the formamide and incubated with 20 μg/ml of RNase A in RNase A buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA pH 8.0) at 37°C for 30 min. To remove the RNase A the slides were washed with RNase A buffer for 30 min, changing the wash every 5 min. The slides were washed further with 2XSSC, 15 min, four times, to remove all residual RNase A before the low salt high stringency wash. The low salt wash was performed at
55°C for 15 min. Finally, the cells were dehydrated in increasing concentrations of ethanol (30, 50, 70 and 95%) containing 300 mM NH₄OAc and air dried. After the slides were completely dry, nuclear emulsion (Amersham) was applied on the slides. The nuclear emulsion was exposed for 4-5 days and developed manually.

2.27 In vitro RNA synthesis.

Template preparation for the in vitro synthesis of β-galactosidase antisense and sense transcripts was accomplished by digesting plasmid pGEM/β-gal-1 with XbaI and BamHI respectively (Figure 5). In vitro transcription reactions were set up in 100 μl volume by adding the following components in the order specified: Transcription buffer to the final concentration of 1X (40 mM Tris-HCl, pH 7.5, 6 mM MgCl₂, 2 mM spermidine and 10 mM NaCl), 100 units of Ribonuclease inhibitor, 2.5 mM of rATP, rGTP, rUTP and rCTP, 3 μg of linearized template DNA and 100 units of the appropriate RNA polymerase (T7 for the sense transcripts and SP6 for the antisense transcripts). The T7 and SP6 reactions were incubated at 37°C and 40°C respectively for 1.5 hr. The DNA template was digested with 3 units of RNase free DNase I at 37°C for 15 min and precipitated. The RNA was collected by centrifugation and resuspended in 20 μl RNase free water. The RNA concentration was estimated by optical density. The expected size of the in vitro transcripts (3.5 Kb) was verified by Northern blot analysis.

2.28 Treatment of cells with chemical agents.

D6 cells (10⁵) were treated for 16 hr with 5-aza-cytidine (1, 10, 100, 1000, 10,000 μM), cis-platinum (0.1, 1, 10, 100, 1000 nM), colchicine (0.01,
0.03, 0.06, 0.1, 1, 10, 100 μg/ml), nocodazole (0.025, 0.050, 0.1, 1, 10 μg/ml), hydroxyurea (40, 80, 160, 320 mM), aphidicolin (20, 25, 30, 35, 40 μg/ml), etoposide (3, 30, 60, 120, 240, 300, 600 μM), and cycloheximide (50, 100, 200, 400, 800 μg/ml) in DMEM supplemented with 10% FBS and 500 μg/ml G418. 5-aza-cytidine was prepared as 10 mM stock in 1XPBS, cis-platinum as 0.5 mM stock in DMF, colchicine as 50 mg/ml stock in 100% ethanol, nocodazole as 1 mg/ml stock in DMSO, hydroxyurea as 1.0 M stock in 1XPBS, aphidicolin as 1 mg/ml stock in DMSO, etoposide as 10 mM stock in DMSO, and cycloheximide as 50 mg/ml stock in DMSO. All agents were kept on the cells for approximately 16 hr except for colchicine and nocodazole that were kept on the cells for 5 hr. Toxicity was observed at the following concentrations for each drug: cis-platinum 1,000 nM, colchicine 0.1 μg/ml and above, nocodazole 10 μg/ml, hydroxyurea 320 mM, aphidicolin 35 μg/ml and above, etoposide 300 μM and above, and cycloheximide 400 μg/ml and above. 5-aza-cytidine was not toxic even at high concentrations of the drug. After incubation the cells were washed twice with drug free media and mock or adenovirus infected (moi of 5) for 24 hr. Two days after infection, the cells were stained for β-galactosidase by X-gal histochemical staining or AAV capsid proteins by IFA.
CHAPTER 3

PRODUCTION AND CHARACTERIZATION OF
rAAV PRODUCING CELL LINES

3.1 Introduction.

Production of rAAV in vitro requires the participation of three elements: (i) the recombinant vector (the AAV terminal repeats flanking a gene expression cassette); (ii) the rep-cap open reading frames of AAV; and, (iii) a helper virus, such as adenovirus. The AAV terminal repeats contain the cis elements required for the replication and packaging of the recombinant single stranded DNA genome into virions (McLaughlin et al., 1988; Samulski et al., 1983; Senapathy and Carter, 1984). The rep-cap open reading frames mediate the replication and encapsidation of the recombinant genome in the presence of helper virus (Beaton et al., 1989; Im and Muzychka, 1990; Im and Muzychka, 1992; McCarty et al., 1994; Trempe and Carter, 1988b).

Recombinant vectors are constructed using molecular clones of wild-type AAV. One commonly used molecular clone of wild-type AAV is plasmid psub201 derived by Samulski and co-workers (Samulski et al., 1987) (Figure 3, top). Two XbaI sites were engineered in psub201 which flank the rep-cap open reading frames to facilitate their removal and insertion of foreign sequences between the AAV terminal repeats.
At present, several methods exist for the packaging and production of rAAV in the laboratory. The most prevalent method for rAAV production involves the co-transfection of tissue culture cells with two plasmids: the recombinant vector (the AAV terminal repeats flanking a gene expression cassette) and a helper plasmid that supplies the replication and encapsidation functions of AAV in trans. Adenovirus infection of the transfected cells initiates a cascade of events that leads to the replication and packaging of the recombinant genome into virions (Lebkowski et al., 1988; Samulski et al., 1989) (Figure 6A). The major disadvantage of this method is the need for the introduction of three elements into one cell (recombinant vector, rep-cap expressing plasmid and adenovirus) for rAAV to be produced. This method is, therefore, inherently inefficient and variable. In addition, it generates variable levels of wild-type AAV depending on the helper plasmid used (K. R. Clark, personal communication).

An alternative method for rAAV production involves the generation of stable cell lines that carry functional rep and cap genes. Upon transfection of such cell lines with the recombinant vector and subsequent infection with adenovirus, rAAV is generated (Clark et al., 1995; Vincent et al., 1990) (Figure 6B). This method requires the introduction of two elements into one cell (recombinant vector and adenovirus) for rAAV to be produced. The major drawback of this method remains the need to transfect the cells in culture. However, stable cell lines expressing the rep and cap genes of AAV have been described as a tool to titr rAAV (Clark et al., 1996b).

The need to generate packaging systems that efficiently produce rAAV in high titers is pressing. Towards that goal, our laboratory developed a novel strategy for rAAV production that involves the construction of stable cell lines
that package rAAV by simply infecting the cell lines with adenovirus (Clark et al.,
1995) (Figure 6C). The stable cell lines are generated by transfection of tissue
culture cells with a plasmid that contains three elements: the recombinant vector
(the AAV terminal repeats flanking a gene expression cassette); the AAV rep
and cap genes; and, the neo\(^f\) gene. The recombinant vector is the element that
will be packaged into virions upon infection of the cell line with adenovirus. The
rep and cap genes of AAV provide the replication and encapsidation functions
of AAV in \textit{trans}. Finally, the neo\(^f\) gene is required for selecting stable cell lines.

We were interested in generating rAAV that packages the lac\textit{Z} gene of \textit{E. coli}
into recombinant virions (rAAV/\(\beta\)-gal) so that we could efficiently study the
production and biology of rAAV \textit{in vitro} and \textit{in vivo}. \(\beta\)-galactosidase is a
reporter gene that can be conveniently detected \textit{in vitro} using X-gal
histochemical staining. We report here the construction and characterization of
stable cell lines that upon infection with adenovirus generate rAAV/\(\beta\)-gal.

### 3.2 Construction of the tripartite plasmid pAAV/CMV-\(\beta\)/rep-cap/neo.

Our aim was to generate a stable cell line that would produce rAAV/\(\beta\)-gal
upon infection with adenovirus. To that end, we constructed a tripartite plasmid
which was subsequently used to generate stable cell lines. The tripartite
plasmid consisted of three elements: (i) the lac\textit{Z} gene positioned between the
AAV terminal repeats under the control of the CMV promoter and SV40 polyA
signal; (ii) the rep and cap genes of AAV under the control of the AAV p5, p19,
and p40 endogenous promoters and the AAV polyA signal; (iii) the neo\(^f\) gene
under the control of the SV40 promoter and the SV40 polyA signal (Figure 3).
As our cloning vector, we used plasmid psub201, a molecular clone of wild-type
AAV (Samulski \textit{et al.}, 1987). Briefly, psub201 was digested with Xbal to remove
the rep and cap genes; in their place, the β-galactosidase expression cassette was introduced to generate plasmid pAAV/CMV-β. The rep and cap genes of AAV were introduced into the XbaI site of plasmid pBS/SVneo to generate plasmid pBS/rep-cap/neo. Plasmid pBS/rep-cap/neo was digested with EcoRV/NotI and the rep-cap and neo<sup>f</sup> units were introduced into the NgoMI site of plasmid pAAV/CMV-β to generate plasmid pAAV/CMV-β/rep-cap/neo. For detailed construction of the plasmid see Materials and Methods and Figure 3.

3.3 Biological activity of pAAV/CMV-β/rep-cap/neo.

After construction of the tripartite plasmid pAAV/CMV-β/rep-cap/neo, we tested the ability of the plasmid to generate infectious rAAV/β-gal capable of expressing the gene for β-galactosidase. We demonstrated production of infectious virus and transgene expression using a virus passage assay. Plasmid pAAV/CMV-β/rep-cap/neo was introduced into adenovirus infected HeLa cells by transfection. The cells were grown to maximum CPE, cell lysate was harvested, and the adenovirus was inactivated by heat treatment. Cellular debri was removed from the lysate by centrifugation and the supernatant was used to infect HeLa cells. Infectious rAAV/β-gal was expected to enter cells and direct the expression of β-galactosidase. Forty eight hours after infection, HeLa cells were stained for β-galactosidase by X-gal histochemical staining. The observation of multiple blue cells suggested that infectious rAAV/β-gal was generated after the introduction of the tripartite plasmid into adenovirus infected HeLa cells.
3.4 Generation of the H44 stable cell line.

Since the tripartite plasmid could direct the production of infectious rAAV/β-gal capable of expressing β-galactosidase, we went on to generate neomycin resistant cell lines that maintained the tripartite plasmid in a stable form. pAAV/CMV-β/rep-cap/neo plasmid DNA (5 μg) was used to transfect HeLa cells (3x10⁶). Two days after transfection, the cells were plated on 100 mm plates at a concentration of 5x10⁴ cells per plate. G418 was added to the medium two days later and the cells were maintained until distinct neomycin resistant colonies were formed (about 2 weeks). Neomycin resistant colonies were expanded and screened for the presence of functional copies of the lacZ gene by directly staining the cells with X-gal. The stable cell line chosen for further characterization was designated H44.

3.5 Yield of infectious rAAV/β-gal from the H44 cell line.

Our next step was to quantify the amount of rAAV/β-gal produced from the H44 cell line. A given number of H44 cells were infected with adenovirus and rAAV/β-gal was isolated according to the protocol described in Materials and Methods. The titration of infectious rAAV/β-gal was accomplished using the C12 cell line. C12 is a stable cell line that contains the rep and cap genes of AAV and the neo" transcription unit integrated into chromosome 20 of HeLa cells (Clark et al., 1996b). Adenovirus infected C12 cells can support the replication and encapsidation of rAAV because they supply the missing replication and encapsidation functions in trans. C12 cells were infected with serial 1:10 dilutions of rAAV/β-gal in duplicate and in the presence of adenovirus. Two days after infection the cells were stained with X-gal and blue cells were counted in the end-point dilutions. The amount of rAAV/β-gal
generated was determined to be 23 to 36 infectious units/cell (Figure 7). These values are comparable to the number of infectious units produced by transient transfection of two plasmids, one containing the rAAV vector and the other containing the AAV rep and cap genes (Clark et al, 1995).

3.6 Particle-to-infectivity ratio of rAAV/β-gal and wild-type AAV.

After determining the infectious titer of our rAAV/β-gal stock, we were interested in estimating the particle titer of our stock and comparing it to the particle titers reported in the literature. In addition, the particle-to-infectivity ratio was calculated for rAAV/β-gal and compared to the particle-to-infectivity ratio of wild-type AAV.

First, we constructed standard curves for wild-type AAV and rAAV genomes using plasmids psub201 and pCMV-β respectively. psub201 is a molecular clone of wild-type AAV and pCMV-β is a plasmid containing a β-galactosidase expression cassette. Because AAV contains a single stranded DNA genome, each single DNA strand of the plasmids was considered to be equivalent to one viral genome. DNA was extracted from serial 1:10 dilutions of psub201 and pCMV-β genome equivalents and was applied to nitrocellulose. In parallel, DNA was also extracted from serial 1:10 dilutions of wild-type AAV and rAAV/β-gal infectious particles and applied to nitrocellulose. rAAV/β-gal and wild-type AAV genomes were detected by hybridization analysis using β-galactosidase and rep specific probes. The intensity of hybridization between the viral particles and their corresponding standard curves was quantified using an AMBIS 4000 phosphorimaging system (Figure 8). The particle-to-infectivity ratio was derived by dividing the number of calculated genome equivalents by the number of infectious particles. Our assay for determining the particle-to-
infectivity ratios of viral preparations is based on DNA hybridization and it does not detect empty capsids. Therefore, our estimated values represent a minimum estimate of the true ratio. The particle-to-infectivity ratio for wild-type AAV was determined to be 62:1 whereas for rAAV/β-gal was 13:1. Therefore, rAAV/β-gal generated from the H44 cell line has a particle-to-infectivity ratio similar to that of wild-type AAV. In addition, our particle titers are about one order of magnitude higher than our infectivity titers, values similar to the ones published by others (McLaughlin et al., 1988; Samulski et al., 1989).

3.7 AAV capsid production is restricted to 50% of the H44 cells.

To determine virus production on a per cell basis, capsid protein expression at the time of virus production was assayed by IFA. The assumption was that H44 cells that produce rAAV/β-gal would be positive for capsid protein expression. H44 cells were infected or mock infected with adenovirus and grown to maximum CPE. The cells were then collected on a microscope slide by the use of a cytopsin and stained for AAV capsid proteins by IFA. Approximately 50% of the adenovirus infected H44 cells were positive for capsid production indicating that at most, 50% of the H44 cells generate rAAV. The H44 cells in the absence of adenovirus were negative for capsid production indicating that rAAV/β-gal was produced only in the presence of adenovirus (data not shown).

The observation that 50% of the adenovirus infected H44 cells were positive for capsid production was comparable to what we have observed with other stable cell lines generated in our laboratory, such as A64 and A80. A64 and A80 are stable cell lines, very similar to the H44 cell line, that package the gp160 gene of simian immunodeficiency virus upon infection with adenovirus.
Approximately 30% of the adenovirus infected A64 cells and <1% of the adenovirus infected A80 cells were positive for capsid production, as shown by IFA (Figure 9). To verify the clonal nature of the A64 and A80 cell lines we performed limiting dilution experiments. Thirty seven subclones were isolated from the A64 cell line and 20 from the A80 cell line. After screening of the subclones for capsid production by IFA, we were unable to isolate a subclone in which capsid production was detected in more than 50% of the cells (Figure 9). Similar results have been reported in the literature on the isolation of stable cell lines that express the Rep protein of AAV (Yang et al., 1994a). The authors reported that they could not isolate a stable cell line where 100% of the cells expressed the Rep protein, even after limiting dilution. Although we have not verified the clonal nature of the H44 cell line by limiting dilution, we believe that the line is clonal since capsid expression in the H44 cell line follows the pattern of expression of the A64 and A80 cell lines.

3.8 Transgene expression is restricted to 50% of the H44 cells.

To demonstrate that H44 cells contain a functional copy of the lacZ gene we used X-gal histochemical staining. H44 cells were infected or mock infected with adenovirus and grown to maximum CPE. At maximum CPE, the cells were collected on a microscope slide by the use of a cyto spinning and stained for β-galactosidase. Approximately 50% of the adenovirus infected H44 cells were positive for β-galactosidase expression. Less than 1% of the H44 cells mock infected with adenovirus were positive for β-galactosidase (Figure 10) indicating that adenovirus infection augmented the expression of β-galactosidase in H44 cells. In our constructs, β-galactosidase is under the control of the CMV promoter. It has been shown that the CMV promoter
contains binding sites for the transcription factor YY1 which mediates its repression in non-permissive cells (Liu et al., 1994). The repression mediated by the YY1 transcription factor is relieved by the E1A protein of adenovirus (Shi et al., 1991). Therefore, it is possible, that in cells infected with adenovirus the CMV promoter becomes derepressed resulting in enhanced levels of β-galactosidase expression. In addition, in the presence of adenovirus rAAV/β-gal genomes replicate resulting in enhanced expression of β-galactosidase in H44 cells.

3.9 Wild-type AAV production from the H44 line.

One of the challenges in the production of rAAV is the generation of rAAV free of wild-type AAV. Production of rAAV/β-gal free of wild-type AAV is important for studying the biology of rAAV in vitro and in vivo and for the use of rAAV vectors in animal or human trials. We were interested in determining whether rAAV/β-gal produced from the H44 cell line was wild-type AAV free. Wild-type AAV generation during rAAV/β-gal production was analyzed using two methods. First, we used indirect immunofluorescent analysis to detect wild-type AAV virions in our rAAV/β-gal preparation. Second, we used the polymerase chain reaction to amplify wild-type AAV sequences in our rAAV/β-gal preparation.

3.9.1 Detection of wild-type AAV in rAAV/β-gal preparation by IFA.

To detect contaminating wild-type AAV in rAAV/β-gal preparations and to quantify the amount of wild-type AAV generated, we selectively titered wild-type AAV in the rAAV/β-gal preparation by IFA. HeLa cells were infected with serial 1:10 dilutions of rAAV/β-gal in quadruplicate and in the presence and absence
of adenovirus. Since HeLa cells are rep-cap negative, they could not support rAAV/β-gal replication in the presence of adenovirus. Therefore, contaminating wild-type AAV should have been selectively amplified after infection of HeLa cells with rAAV/β-gal and adenovirus. Two days after infection, HeLa cells were stained for AAV capsid proteins by IFA. The rAAV/β-gal infections in the absence of adenovirus were negative for capsid proteins indicating that input viral capsids were not detected by the antibody used. However, the rAAV/β-gal infections in the presence of adenovirus were positive for capsid proteins indicating the presence of wild-type AAV in our rAAV/β-gal preparation. A titer of 3.9x10^3 TCID50/ml of wild-type AAV was calculated using the Reed-Muench formula. The rAAV/β-gal titer of the same stock was 1x10^9 blue forming units per milliliter (bfu/ml) indicating that approximately one particle out of one million was a wild-type AAV particle (data not shown).

A wild-type AAV titration curve was established to determine the sensitivity of the assay. Briefly, serial 1:10 dilutions of a known titer of wild-type AAV were used to infect HeLa cells in the presence and absence of adenovirus. The wild-type AAV infections in the absence of adenovirus were negative for capsid proteins indicating that input capsids were not detected by the antibody used. With the assay described here we were able to detect 0.7 TCID50 (thus 1 infectious particle) of wild-type AAV (data not shown).

The IFA data suggested that wild-type AAV was generated during rAAV/β-gal production from the H44 cell line. To confirm these observations, we used an alternative method, (see below), to detect wild-type AAV genomes.
3.9.2 PCR amplification of wild-type AAV-like sequences in the H44 cell line.

To support the evidence that wild-type AAV was present in our rAAV/β-gal viral stock we used the polymerase chain reaction to amplify wild-type AAV sequences. We achieved the selective amplification of wild-type AAV sequences by designing PCR primers that would selectively amplify wild-type AAV. Figure 11 shows the primer pairs used in the PCR experiments described below. We used three primers that annealed on the AAV terminal repeats (TR) and five primers that annealed on various locations on the rep gene (rep). We also used three different templates in our PCR experiments: (i) total DNA isolated from the H44 cell line; (ii) the tripartite plasmid used to generate the H44 cell line (Figure 11, bottom); and, (iii) wild-type AAV (Figure 11, top). The TR/rep primer pairs were expected to amplify fragments with sizes ranging between 600 to 1200 bp when wild-type AAV sequences were used as template for PCR (Figure 11, top). The same TR/rep primer pairs were expected to amplify a fragment of about 10 Kb when pTP plasmid DNA was used as template for PCR (Figure 11, bottom). H44 total DNA was expected to amplify the same size fragment as the pTP plasmid DNA if the point of integration of the plasmid into the chromosome was not contained between the primer pairs.

All primer pairs and their combinations gave the expected fragments when psub201 was used as the PCR template, indicating that all primer pairs could efficiently amplify wild-type AAV sequences (Figure 12, lanes 1, 4, 7, 10, 13, 16, 19). Surprisingly, when pTP plasmid DNA was used as template for PCR, most primer pairs amplified a fragment approximately 200 bp longer than the fragment generated after amplification of wild-type AAV sequences with the same primers (Figure 12, lanes 2, 5, 8, 11, 14, 17, 20 compare to lanes 1, 4, 7, 10, 13, 16, 19). All PCR generated fragments showed positive hybridization to a
rep specific probe internal to the PCR primers indicating specific amplification of wild-type AAV sequences (data not shown). When H44 total DNA was used as template for PCR, wild-type AAV specific fragments were amplified in both adenovirus infected and non-infected cells. These fragments were also approximately 200 bp longer than the fragments generated when wild-type AAV sequences were amplified with the same primers (Figure 13, lanes 3, 4). Since the PCR primers were designed to amplify wild-type AAV sequences and not pTP plasmid sequences, we suspected that a wild-type AAV-like molecule may be amplified that was about 200 bp longer than the wild-type AAV genome.

To understand the mechanism of wild-type AAV generation in the H44 cell line, we decided to investigate the nature of the 200 bp fragment by cloning and sequencing the PCR fragments.

3.10 Cloning and sequencing of the PCR fragments.

Plasmid DNA (pAAV/CMV-β/rep-cap/neo) was PCR amplified using the TR/rep primer pairs 480/483 and 485/483 (Figure 12, lanes 14, 8). The PCR generated fragments were cloned into TA pCR II vector (Invitrogen) and two clones from each fragment (17-3, 17-4 and 8-1, 8-2) were sequenced using the dideoxy termination method. The sequencing strategy used is shown in Figure 14 and the nucleotide sequence obtained is shown in Figure 15. The sequencing data identified the translocation of one of the AAV terminal repeats next to the rep-cap sequence of AAV with the SV40 polyA signal (200 bp long) inserted between them (clones 17-3, 17-4, and 8-2). The insertion of the SV40 polyA signal between one of the AAV terminal repeats and the rep-cap open reading frames appeared to explain the 200 bp discrepancy observed during PCR amplification of pAAV/CMV-β/rep-cap/neo plasmid DNA or H44 total DNA.
Clone 8-1 contains the β-galactosidase sequence and SV40 polyA signal next to the AAV terminal repeat. Since clone 8-1 was the result of amplification of sequences between primers 485/483, we believe that clone 8-1 arose from misspriming of primer 483 on the β-galactosidase sequence. Clone 8-1 represents the sequence on plasmid pAAV/CMV-β/rep-cap/neo at the expected order (see Figure 11, bottom).

3.11 Amplification of wild-type AAV-like sequences by template switch of Taq DNA polymerase.

The sequencing data suggested a model that might explain how wild-type AAV-like sequences were amplified during PCR (Figure 16). Within plasmid pAAV/CMV-β/rep-cap/neo, two SV40 polyA signals exist, one in the β-galactosidase transcription unit and the second in the neo\textsuperscript{r} transcription unit. Figure 16, top, shows the pAAV/CMV-β/rep-cap/neo plasmid in a linear conformation with the locations of the SV40 polyA signals indicated. The two SV40 polyA signals could potentially anneal to each other and juxtapose one of the AAV terminal repeats to the rep-cap sequences of AAV by inserting the SV40 polyA signal between them (Figure 16, bottom). During PCR amplification of either plasmid DNA or H44 total DNA, the TR/rep primers could amplify a wild-type AAV-like sequence approximately 200 bp longer than the wild-type AAV sequence because of the inserted 200 bp SV40 polyA signal between the AAV terminal repeat and the rep-cap open reading frames. It seems likely that the PCR amplification of the wild-type AAV-like sequence was achieved by template switch of Taq polymerase during PCR. Template switch of Taq polymerase during DNA amplification has been reported in the literature (Odelberg et al., 1995).
To eliminate the possibility that a rearranged form of the pTP plasmid was amplified during PCR, we investigated the presence of rearranged forms of the pTP plasmid. If a rearranged form of the plasmid did exist within our pTP DNA preparation, it must have been in very low levels because it was not detected by restriction enzyme analysis of the plasmid (data not shown). In an effort to isolate intact plasmid DNA, single cell colonies from bacteria transformed with the pTP DNA were used to isolate DNA which was subjected to PCR analysis using the TR/rep primer pairs. If indeed what we amplified was a rearranged form of the plasmid, we expected to be able to isolate intact DNA that would not amplify the same size fragments as above. However, DNA isolated from several bacterial colonies amplified the exact same size fragments as above indicating that the PCR generated fragments were amplified from an intact DNA template by template switch of Taq polymerase (data not shown). The possibility still remains, however, that low levels of plasmid rearrangement take place within each bacterial colony and intact DNA cannot be isolated in a pure form.

If the presence of two identical sequences within the tripartite plasmid (SV40 polyA signal) could result in the amplification of wild-type AAV sequences during PCR, we argued that in the cell the same duplicated sequences could trigger a recombination event that may result in the replication and packaging of wild-type AAV-like sequences into virions. If that was true, we hypothesized that replacement of one of the SV40 polyA sequences with a different one should result in a cell line that generates rAAV/β-gal free of wild-type AAV. To assess this hypothesis we constructed a second tripartite plasmid that contained the tk polyA signal in place of the SV40 polyA signal in the neo\textsuperscript{r} transcription unit.

The construction of the tripartite plasmid pAAV/CMV-β/rep-cap/neo-tk is very similar to the construction of plasmid pAAV/CMV-β/rep-cap/neo described previously. The tripartite plasmid contains three elements: (i) the lacZ gene positioned between the AAV terminal repeats under the control of the CMV promoter and SV40 polyA signal; (ii) the rep and cap genes of AAV under the control of the AAV p5, p19, and p40 promoters and the AAV polyA signal; and, (iii) the neo^ gene under the control of the SV40 promoter and herpes simplex virus thymidine kinase (tk) polyA signal (Figure 4). Our cloning vector was plasmid psub201 (Samulski et al., 1987). Briefly, psub201 was digested with Xbal to remove the rep and cap genes of AAV and in their place the β-galactosidase expression cassette was introduced to generate plasmid pAAV/CMV-β. The rep-cap open reading frames of wild-type AAV were cloned into the Xbal site of plasmid PBS/neo-tk to generate plasmid PBS/rep-cap/neo-tk. Plasmid PBS/rep-cap/neo-tk was digested with NotI/ClaI and the rep-cap and neo^ transcription units were cloned into the NgoMI site of pAAV/CMV-β to generate plasmid pAAV/CMV-β/rep-cap/neo-tk. For detailed construction of the plasmid see Materials and Methods and Figure 4.


After construction of the tripartite plasmid pAAV/CMV-β/rep-cap/neo-tk, we tested the ability of the plasmid to support the production of infectious virions (rAAV/β-gal) capable of expressing the gene for β-galactosidase. We demonstrated production of infectious virus and transgene expression using a virus passage assay. Plasmid pAAV/CMV-β/rep-cap/neo-tk was introduced into
adenovirus infected HeLa cells by transfection. The cells were grown to maximum CPE, cell lysate was harvested, and the adenovirus was inactivated by heat treatment. Cellular debris was removed from the lysate by centrifugation and the supernatant was used to infect HeLa cells. Infectious rAAV/β-gal was expected to enter cells and direct the expression of β-galactosidase. Forty eight hours after infection HeLa cells were stained for β-galactosidase by X-gal histochemical staining. The observation of multiple blue cells suggested that infectious rAAV/β-gal was generated after the introduction of the tripartite plasmid into adenovirus infected HeLa cells.

3.14 Generation of the D6 stable cell line.

Since the tripartite plasmid could support the production of infectious rAAV/β-gal we went on to construct stable cell lines that maintained the tripartite plasmid in a stable form. pAAV/CMV-β/rep-cap/neo-tk plasmid DNA (5 μg), was introduced into HeLa cells (3x10⁶). Two days after transfection, the cells were plated on 100 mm plates at a concentration of 5x10⁴ cells per plate. G418 was added to the medium two days later and the cells were maintained until distinct neomycin resistant colonies were formed (about 2 weeks). Neomycin resistant colonies were expanded and screened for the presence of functional copies of the lacZ gene by directly staining the cells with X-gal. The stable cell line chosen for further characterization was designated D6.

3.15 rAAV/β-gal free of wild-type AAV.

After construction of the D6 cell line, the presence or absence of wild-type AAV in our rAAV/β-gal preparation was determined by two methods. First,
we used indirect immunofluorescent analysis to detect wild-type AAV in our rAAV/\(\beta\)-gal preparation. Second, we used the polymerase chain reaction to amplify wild-type AAV sequences in our rAAV/\(\beta\)-gal preparation.

3.15.1 Absence of wild-type AAV in rAAV/\(\beta\)-gal preparation.

To detect contaminating wild-type AAV in rAAV/\(\beta\)-gal preparation we selectively amplified wild-type AAV and stained for capsid proteins by IFA. Briefly, HeLa cells were infected with serial 1:10 dilutions of rAAV/\(\beta\)-gal in quadruplicate and in the presence and absence of adenovirus. Since HeLa cells are rep-cap negative, they could not support rAAV/\(\beta\)-gal replication in the presence of adenovirus. Therefore, only contaminating wild-type AAV would be amplified after infection of HeLa cells with rAAV/\(\beta\)-gal and adenovirus. Two days after infection, HeLa cells were stained for capsid proteins by IFA. The rAAV/\(\beta\)-gal infections in the absence of adenovirus were negative for capsid proteins indicating that input viral capsids were not detected by the antibody used. The rAAV/\(\beta\)-gal infections in the presence of adenovirus were also negative for capsid proteins indicating the absence of wild-type AAV in the rAAV/\(\beta\)-gal preparation (data not shown).

A wild-type AAV titration curve was also established to determine the sensitivity of the assay. Briefly, serial 1:10 dilutions of a known titer of wild-type AAV were used to infect HeLa cells in the presence and absence of adenovirus. The wild-type AAV infections in the absence of adenovirus were negative for capsid proteins indicating that input capsids were not detected by the antibody used. With the assay described here we were able to detect 5 TCID\textsubscript{50} of wild-type AAV (data not shown).
3.15.2 Lack of amplification of wild-type AAV sequences.

To increase the sensitivity of our detection we used a very sensitive virus passage assay. Briefly, adenovirus infected HeLa cells were infected with rAAV/β-gal isolated from the D6 cell line, at moi of 1000. This moi was equivalent to approximately 5x10^6 infectious units of rAAV/β-gal. Since HeLa cells are rep-cap negative they were unable to support replication of rAAV/β-gal in the presence of adenovirus. Only contaminating wild-type AAV would be replicated after infection of HeLa cells with rAAV/β-gal in the presence of adenovirus. At maximum CPE, cell lysate was isolated and used to infect fresh HeLa cells in the presence of adenovirus. The cells were grown to maximum CPE and Hirt DNA was isolated that potentially contained wild-type AAV replicating forms. Hirt DNA was fractionated on a 0.8% agarose gel and after hybridization to a rep specific probe, wild-type AAV replicating forms were not detected (data not shown). For higher sensitivity, Hirt DNA was also used in PCR amplification using rep specific primers that specifically amplified wild-type AAV sequences. The PCR generated fragments were fractionated on a 0.8% agarose gel and hybridized to a rep specific probe. Wild-type AAV specific fragments were not detected even after long exposures (Figure 17).

To establish the sensitivity of our assay we constructed a titration curve by infecting HeLa cells with serial 1:10 dilutions of a known titer of wild-type AAV in the presence of adenovirus. As above, the cells were incubated to maximum CPE, cell lysate was isolated and used to infect fresh HeLa cells in the presence of adenovirus. Hirt DNA was isolated and PCR amplified using rep specific primers that specifically amplified wild-type AAV sequences. The sensitivity of our assay approached 0.5 infectious units of wild-type AAV (Figure 17).
Our data suggest that identical sequences within the tripartite plasmids led to recombination that resulted in the generation of wild-type AAV. By removing complementarity within the tripartite plasmid we were able to generate a stable cell line (D6) that generates rAAV/β-gal free of wild-type AAV.

3.16 Yield of infectious rAAV/β-gal from the D6 cell line.

After demonstrating that the D6 cell line generated infectious rAAV/β-gal free of wild-type AAV, we set out to quantify the amount of rAAV produced. rAAV/β-gal was titered on C12 cells according to the protocol described in Materials and Methods. C12 cells were infected with serial 1:10 dilutions of rAAV/β-gal in duplicate and in the presence of adenovirus. Adenovirus infected C12 cells can support the replication and encapsidation of rAAV vectors because they supply the missing rep-cap functions of AAV in trans (Clark et al., 1995; Clark et al., 1996b). Two days after infection the cells were stained with X-gal and blue cells were counted in the end-point dilutions. The number of rAAV/β-gal generated was determined to be 30 to 50 infectious units/cell, very similar to the number of infectious units/cell generated from the H44 cell line (Figure 22). These values are comparable to the number of infectious units produced by transient transfection of two plasmids, one containing the rAAV vector and the other containing the AAV rep-cap open reading frames. Furthermore, virus production from the D6 stable cell line generates rAAV/β-gal efficiently and reproducibly.

3.17 Virus production is restricted to 50% of the D6 cells.

To assess virus production from the D6 cell line, we made use of two methodologies. First, indirect immunofluorescence analysis was employed to
detect capsid protein expression at the time of virus production. The assumption was that D6 cells that produced rAAV/β-gal would be positive for capsid protein expression. Second, *in situ* hybridization analysis was employed to directly detect viral genomes at the time of virus production.

3.17.1 AAV capsid production is restricted to 50% of the D6 cells.

D6 cells were infected with adenovirus and grown to maximum CPE at which time rAAV/β-gal production was maximal. At maximum CPE, the cells were collected on microscope slides by the use of a cytospin and stained for AAV capsid proteins by IFA. D6 cells mock infected with adenovirus were also prepared in parallel to assess virus production in the absence of adenovirus. Approximately 50% of the adenovirus infected D6 cells were positive for capsid proteins indicating that 50% of the D6 cells generated virus. The D6 cells mock infected with adenovirus were negative for capsid proteins indicating that rAAV/β-gal was produced only in the presence of adenovirus (Figure 18, 22).

The observation that approximately 50% of the D6 cells were positive for capsid proteins was comparable to previous observations with the H44, A64, and A80 cell lines (Figure 9).

3.17.2 The *in situ* hybridization probes are specific for β-galactosidase sequences.

DNA or RNA *in situ* hybridization methodologies involve the immobilization of nucleic acids into cells and hybridization *in situ*. It is fundamental to establish that the DNA or RNA probes are specific for the target sequence and do not lead to background hybridization which could mistakenly interpreted as specific signal. The specificity of our riboprobes was
demonstrated by Northern hybridization analysis of total RNA isolated from D6 cells. Briefly, D6 cells were infected or mock infected with adenovirus and grown to maximum CPE at which time total RNA was isolated. The RNA was fractionated on a formaldehyde/agarose gel and hybridized with the sense and antisense riboprobes prepared as described in Materials and Methods. The Northern blot analysis showed that in adenovirus infected D6 cells the antisense probe detected an RNA species of the right size (β-galactosidase mRNA is 3.5 Kb plus polyA tail) in the absence of background hybridization (Figure 19A, lanes 1, 2, 3, 4). In D6 cells mock infected with adenovirus the antisense probe did not detect a similar RNA species indicating that β-galactosidase mRNA expression was below the detection limit of our assay (Figure 19A, lane 6). D6 cells infected or mock infected with adenovirus showed negative hybridization with the sense probe as expected (Figure 19B, lanes 1, 2).

We were also interested in determining the sensitivity of our riboprobes and in parallel, to estimate the minimum number of target molecules our probes could detect. Sense and antisense β-galactosidase RNAs were synthesized in vitro and serial 1:10 dilutions were analyzed by Northern blot hybridization using the same sense and antisense probes as above. We were able to detect at least 10 pg of transcript with an over night exposure (data not shown). Ten pg of transcript is equivalent to approximately 5x10^6 copies of β-galactosidase mRNA. Since 10 μg of RNA (the amount loaded on the Northern blot) correspond to approximately 10^6 cells, we estimated that the level of our detection was at least 5 copies of mRNA per cell (Figure 19).
3.17.3 Replicating rAAV/β-gal genomes are detected in 50% of the D6 cells. In situ hybridization analysis allowed us to identify virus producing D6 cells by direct detection of rAAV/β-gal genomes. The probes used in the in situ hybridization experiments were sense and antisense RNA probes specific for β-galactosidase. Both probes were synthesized using plasmid pGEM/β-gal-1A which contained the first 2074 bp of the β-galactosidase sequence (Figure 5). For the preparation of the antisense probe, plasmid pGEM/β-gal-1A was digested with XbaI to linearize the plasmid at the 5' end of the β-galactosidase sequence. For the preparation of the sense probe, plasmid pGEM/β-gal-1A was digested with SacI to linearize the plasmid at the 3' end of the β-galactosidase sequence. The linearized plasmids were used to synthesize antisense and sense RNA probes by in vitro transcription from the bacteriophage promoters SP6 and T7 respectively: [33-P] UTP was incorporated during the RNA synthesis. The in vitro generated transcripts were about 2100 bases long and were hydrolyzed to fragments approximately 300 bases long prior to use.

For the in situ hybridization experiments, cells were prepared as follows. D6 and HeLa cells were infected or mock infected with adenovirus and grown to maximum CPE. The cells were concentrated on microscope slides by the use of a cytospin and fixed in 4% paraformaldehyde. After fixation and before in situ hybridization, the cells were treated with proteinase K. The cells were hybridized at 50°C over night according to the protocol described in Materials and Methods. The next day the slides were washed extensively and a nuclear emulsion was applied. The slides were exposed for 5 days.

Adenovirus infected D6 cells showed positive hybridization with both the sense and antisense probes. AAV is a single stranded DNA virus, and therefore, it was expected that the sense and antisense RNA probes would
hybridize to viral DNA of the opposite polarity, the antisense probe would also
hybridize to β-galactosidase mRNA. The number of adenovirus infected D6
cells that generated rAAV/β-gal genomes was approximately 50%, similar to the
number of adenovirus infected D6 cells that were positive for capsid and β-
galactosidase protein expression. D6 cells mock infected with adenovirus
showed positive hybridization with only the antisense probe which detects β-
galactosidase mRNA, indicating that D6 cells in the absence of adenovirus did
not generate virus. Less than 1% of the adenovirus mock infected D6 cells
hybridized with the antisense probe, similar to the number of D6 cells that were
positive for β-galactosidase protein expression. HeLa cells in the presence or
absence of adenovirus were negative after hybridization with both the sense
and antisense probes (Figure 20, 22).

3.18 Transgene expression is restricted to 50% of the D6 cells.

To demonstrate that D6 cells contained a functional copy of the lacZ
gene we made use of two methodologies. First, X-gal histochemical staining
was utilized to determine β-galactosidase protein expression in the D6 cell line.
Second, in situ hybridization experiments were used to detect β-galactosidase
mRNA in the D6 cell line.

3.18.1 β-galactosidase protein expression is restricted to 50% of the D6 cells.

To determine the number of D6 cells capable of transgene expression at
the time of virus production, we used X-gal histochemical staining. Briefly, D6
cells were infected or mock infected with adenovirus. At maximum CPE, the
cells were collected on microscope slides by the use of a cytospin and stained
for β-galactosidase. Approximately 50% of the adenovirus infected D6 cells
were positive for β-galactosidase expression. Less than 1% of the D6 cells mock infected with adenovirus were positive for β-galactosidase indicating that adenovirus infection augmented the expression of β-galactosidase in the D6 cells (Figure 21, 22). In our constructs, β-galactosidase is under the control of the CMV promoter and SV40 polyA signal. It has been shown that the CMV promoter contains binding sites for the transcription factor YY1 which mediates its repression in non-permissive cells (Liu et al., 1994). The repression mediated by the YY1 transcription factor is relieved by the E1A protein of adenovirus (Shi et al., 1991). It is therefore possible, that in adenovirus infected cells the CMV promoter becomes derepressed resulting in enhanced levels of β-galactosidase expression. We observed similar results with the H44 cell line (Figure 10). In addition, in the presence of adenovirus, rAAV/β genomes replicate resulting in enhanced expression of β-galactosidase in D6 cells.

3.18.2 β-galactosidase mRNA in D6 cells.

RNA in situ hybridizations demonstrated that 50% of the adenovirus infected D6 cells showed positive hybridization with both sense and antisense probes. DNase I digestion was performed to digest away viral DNA and allow for detection of β-galactosidase mRNA. Unfortunately, we were unable to achieve complete digestion of the viral DNA before degradation of the mRNA. We believe that the difficulty in digesting viral DNA was due to high levels of DNA generated in the virus producing cells. Less than 1% of the D6 cells mock infected with adenovirus hybridized with the antisense probe (detecting β-galactosidase mRNA) whereas, the sense probe showed negative hybridization
with the D6 cells. HeLa cells in the presence and absence of adenovirus were negative after hybridization with both the sense and antisense probes (Figure 20, 22).

3.19 Subcloning of D6 cells by limiting dilution.

The data described so far indicated that approximately 50% of the D6 cells generate rAAV upon exposure to adenovirus. To eliminate the possibility that the restricted virus production was due to D6 cells being non-clonal, limiting dilution experiments were performed. D6 cells were trypsinized and approximately one cell was seeded per three wells of a 96-well plate. Wells that contained single cells were selected by visual observation and further expanded. The subclones were then screened for β-galactosidase expression. The hypothesis was that if the D6 cell line was not clonal, then we should be able to isolate a subclone where more than 50% of the cells would be positive for β-galactosidase expression. Seven subclones were isolated and tested for β-galactosidase expression. One of the subclones was negative whereas approximately 50% of the cells were positive for β-galactosidase expression in the other six subclones. We were unable to isolate a subclone where β-galactosidase expression was detected in more than 50% of the cells, suggesting that D6 was in fact, clonal. Similar observations had been made earlier with the A64 and A80 cell lines. Limiting dilution experiments with those lines resulted in subclones whose expression ranged from 0-50% (Figure 9).

3.20 Genomic characterization of the D6 cell line.

The genomic organization of the D6 cell line was analyzed by Southern blot analysis and field inversion gel electrophoresis (FIGE) of high molecular
weight DNA. Total cellular DNA was isolated from D6 cells according to the protocol described in Materials and Methods, digested with SpeI (an enzyme that cuts into the plasmid once), and analyzed by Southern blot hybridization. Figure 23, lane 5, shows the presence of three high molecular weight bands, one corresponding to the full length linear form of the plasmid (14.5 Kb) and the others corresponding to potential junction fragments with HeLa cell DNA (>14.5 Kb). This hybridization pattern is suggestive of plasmid integration into the chromosome in a head-to-tail orientation. Two additional fragments of approximately 8 and 4 Kb are also detected indicative of a deleted form of the plasmid integrated into the chromosome as a single copy.

To determine the number of integration events, D6 high molecular weight DNA was digested with BsrGI, BglIII, and AflIII (enzymes that do not cut into the plasmid), and the DNA was fractionated on a FIGE gel and analyzed by Southern blot hybridization. The hybridization analysis verified the presence of two integration events in the D6 cell line. The first is the integration of the full length plasmid pAAV/CMV-β/rep-cap/neo-tk in a concatameric head-to-tail orientation. The second is the integration of a deleted form of the plasmid (~12Kb) in a single copy (data not shown).

3.21 The effect of methylation on β-galactosidase expression.

As mentioned above, transgene and AAV capsid protein expression are restricted to 50% of the D6 cells. To determine whether chromosome methylation was responsible for the restricted β-galactosidase expression, D6 cells were treated with 5-aza-cytidine. 5-aza-cytidine is a nucleoside analog modified in the 5' position of cytosine that inhibits the cytosine methylation of DNA (Creusot et al., 1982; Jones and Taylor, 1980). D6 and HeLa cells were
grown in the presence and absence of 5-aza-cytidine for 16 hr. After 16 hr the
cells were washed twice with drug free media and infected or mock infected
with adenovirus. Forty eight hours later, β-galactosidase expression was
assayed by X-gal histochemical staining according to established protocols.
We did not observe a difference in the expression of β-galactosidase indicating
that DNA methylation was not responsible for the restricted expression of β-
galactosidase in D6 cells (data not shown).

3.22 The effect of genotoxic agents on β-galactosidase expression.

Cells treated with a variety of genotoxic agents can support replication of
wild-type AAV in the absence of helper virus (Yakobson et al., 1989; Yakobson
et al., 1987; Yalkinoglu et al., 1988). We were interested in extending these
observations to the D6 cell line and determine whether treatment of D6 cells
with genotoxic agents would result in the replication of rAAV/β-gal. We chose a
DNA damaging agent (cis-platinum), a protein synthesis inhibitor
(cycloheximide), mitotic inhibitors (colchicine and nocodazole), and DNA
synthesis inhibitors (hydroxyurea, aphidicolin, and etoposide). D6 cells were
treated with the above agents according to the protocol described in Materials
and Methods. After treatment, the cells were washed twice with drug free media
and mock or adenovirus infected. Forty eight hours after infection the cells were
stained for β-galactosidase by X-gal histochemical staining.

β-galactosidase expression was unchanged after treatment of mock and
adenovirus infected D6 cells with the DNA damaging agent cis-platinum.
Similarly, the mitotic inhibitors colchicine and nocodazole exhibited no effect on
the expression of β-galactosidase in mock and adenovirus infected D6 cells. However, at concentrations of these drugs where cytotoxicity was observed, β-galactosidase expression was slightly increased (Figure 24).

The DNA synthesis inhibitors hydroxyurea, aphidicolin, and etoposide mediated an increase in β-galactosidase expression in adenovirus infected D6 cells. All agents except hydroxyurea also showed an increase in β-galactosidase expression in D6 cells not infected with adenovirus (Figure 25). Cycloheximide, a protein synthesis inhibitor, showed the highest increase in β-galactosidase expression in both mock and adenovirus infected D6 cells (Figure 26).

3.23 The effect of genotoxic agents on capsid protein expression.

To determine the effect of genotoxic agents on capsid protein expression, D6 cells were treated with genotoxic agents according to the protocol described in Materials and Methods. After treatment, the cells were washed twice with drug free media and mock or adenovirus infected. Forty eight hours after infection the cells were stained for capsid proteins by IFA.

The DNA damaging agent cis-platinum and the mitotic inhibitors colchicine and nocodazole had no effect on capsid protein expression at low concentrations of the drugs. However, at high drug concentrations, all agents inhibited capsid protein expression. This is in contrast to what we have observed for β-galactosidase protein expression, which becomes slightly enhanced at drug concentrations toxic to the cells. None of the agents induced capsid protein expression in the absence of adenovirus (Figure 24).

The DNA synthesis inhibitors inhibited capsid protein expression even at low drug concentrations. None of the agents induced capsid protein expression
in the absence of adenovirus (Figure 25). Interestingly, capsid protein expression was not affected by any concentration of cycloheximide (Figure 26).

3.24 The effect of DNA synthesis inhibitors on the production of infectious rAAV/β-gal.

To determine whether the observed increase in the expression of β-galactosidase correlated with an increase in production of infectious rAAV, we isolated rAAV/β-gal from D6 cells treated with hydroxyurea and aphidicolin. D6 cells were treated with 80 and 160 mM hydroxyurea and 30 and 35 μg/ml aphidicolin according to the protocol described in Materials and Methods. After treatment, the cells were mock or adenovirus infected and grown to maximum CPE at which time crude lysate was isolated. rAAV/β-gal was titered on C12 cells, the number of infectious units per cell was calculated, and the values compared between the different treatments. Adenovirus infected D6 cells treated with hydroxyurea and aphidicolin generated less infectious units of rAAV/β-gal per cell than untreated D6 cells. D6 cells not infected with adenovirus did not generate rAAV/β-gal in either the treated or untreated controls (data not shown).

3.25 The effect of cycloheximide (a protein synthesis inhibitor) on rAAV/β-gal production.

Since cycloheximide had the most dramatic effect on β-galactosidase expression and no obvious effect on capsid protein expression, we chose this agent to study its effect on rAAV/β-gal production from the D6 cell line. We took two approaches to demonstrate virus production. Infectivity titrations were
performed to demonstrate production of infectious rAAV/β-gal and, in situ hybridization analysis was used to determine the number of cycloheximide treated D6 cells generating rAAV/β-gal.

3.25.1 Infectivity titrations of cycloheximide treated D6 cells.

rAAV/β-gal isolated from a known number of D6 cells grown in the presence and absence of cycloheximide provided a direct assessment of the number of infectious units generated per cell. D6 cells were treated with 0, 50, 100, 200, and 400 μg/ml cycloheximide in the presence and absence of adenovirus according to the protocol described in Materials and Methods. After treatment, the cells were washed twice with drug free media and mock or adenovirus infected for 24 hr. At maximum CPE, crude lysate was prepared and rAAV/β-gal was isolated according to the protocol described in Materials and Methods. rAAV/β-gal was titered on C12 cells, the number of infectious units per cell was calculated, and the values compared between the different cycloheximide concentrations. We determined that at high concentrations of cycloheximide (200 and 400 μg/ml) the number of infectious rAAV/β-gal produced per cell was increased by 2-fold (Figure 26).

3.25.2 In situ hybridizations of cycloheximide treated D6 cells.

In situ hybridization analysis allowed us to identify virus producing D6 cells by direct detection of rAAV/β-gal genomes. Briefly, D6 cells were treated with 0, 50, 100, 200, and 400 μg/ml cycloheximide according to the protocol described in Materials and Methods. The cells were washed twice with drug free media and mock or adenovirus infected. Forty eight hours after infection, the cells were fixed with 4% paraformaldehyde and hybridized in situ as
described before. Approximately 50% of the adenovirus infected D6 cells exhibited positive hybridization with both the sense and antisense probes at all cycloheximide concentrations. These results correlated well with capsid protein expression in the same cells which was approximately 50% (Figure 26). Even though the percent of D6 cells generating virus remained the same (50%) under all concentrations of cycloheximide, we believe that the increased virus production was due to the increased production of rAAV/b-gal from each cell.
Production of rAAV in the laboratory has been cumbersome because of the lack of efficient packaging systems. To improve rAAV production, our laboratory has developed a novel strategy that involves the construction of stable cell lines that generate rAAV upon infection with adenovirus (Figure 6C). The advantage of such an approach is that 100% of the cells will contain such genetic elements, rather than a small subset of the cell population when transfection methodologies are used. These producer cell lines are generated by transfection of tissue culture cells with a plasmid that expresses three elements: the recombinant vector (the AAV terminal repeats flanking a gene expression cassette), the rep-cap open reading frames of AAV, and the neo\(^\text{R}\) gene. The recombinant vector is the element that will be packaged into virions upon infection of the cell line with adenovirus. The rep-cap open reading frames of AAV provide the missing replication and encapsidation functions of AAV in \textit{trans}. Finally, the neo\(^\text{R}\) gene is required for selecting stable cell lines. Adenovirus infection of stable cell lines leads to the generation of rAAV efficiently and in high titers (Clark \textit{et al.}, 1995).
4.1 Particle and infectivity titers of rAAV/β-gal generated from stable cell lines.

To study the biology of rAAV in vitro and in vivo we aimed to construct stable cell lines that package the lacZ gene of E. coli into recombinant virions. β-galactosidase is a reporter gene that can be conveniently detected in vitro using X-gal histochemical staining. H44 was the first cell line we constructed by stable transfection of HeLa cells with the tripartite plasmid pAAV/CMV-β/rep-cap/neo. The tripartite plasmid contained three elements: the AAV terminal repeats flanking the lacZ gene of E. coli (rAAV genome), the rep-cap open reading frames of AAV, and the neo^ gene (Figure 3). Infectivity titrations of rAAV/β-gal generated from the H44 cell line identified the production of 23 to 36 infectious units per cell (Figure 7). These values were comparable to the number of infectious units produced by transient transfection of two plasmids, one containing the rAAV vector and the other the AAV rep-cap open reading frames (Clark et al., 1995). Moreover, rAAV isolation using a stable cell line such as H44 negates the need of transfection methodologies and generates rAAV efficiently and reproducibly.

Wild-type AAV is characterized by high particle-to-infectivity ratio (Clark et al., 1995; de la Maza and Carter, 1980a; de la Maza and Carter, 1980b; Laughlin et al., 1979a). To determine whether rAAV/β-gal generated from the H44 cell line displayed a similar particle-to-infectivity ratio as wild-type AAV, the particle-to-infectivity ratio of rAAV/β-gal was estimated and compared to that of wild-type AAV. We established that wild-type AAV had a particle-to-infectivity ratio of 62:1 whereas rAAV/β-gal of 13:1 (Figure 8). Therefore, the particle titer
of rAAV/β-gal was about one order of magnitude higher than the infectivity titer, a value similar to that of wild-type AAV. Similar values have been reported for other rAAV vectors (McLaughlin et al., 1988; Samulski et al., 1989).

4.2 Stable cell lines that generate rAAV/β-gal free of wild-type AAV.

The use of rAAV as a gene transfer vector requires that rAAV be produced free of contaminating wild-type AAV. The presence of contaminating wild-type AAV in our rAAV/β-gal preparation from the H44 cell line was identified by selective amplification of wild-type AAV virions and by PCR amplification of wild-type AAV sequences (Figure 11, 12, 13). We estimated the amount of wild-type AAV in our rAAV/β-gal preparation to be approximately one wild-type AAV infectious particle per 10⁶ infectious particles of rAAV/β-gal. Since all the stable cell lines constructed in our laboratory generate rAAV free of wild-type AAV, we suspected that sequence elements within the pAAV/CMV-β/rep-cap/neo plasmid used to generate the H44 cell line might have been responsible for generating wild-type AAV.

PCR analysis identified the amplification of wild-type AAV-like sequences that were approximately 200 bp longer than wild-type AAV (Figure 12, 13). Sequence analysis of the PCR products identified the translocation of one of the AAV terminal repeats next to the rep-cap open reading frames with the SV40 polyA signal inserted between them (Figure 14, 15). Plasmid pAAV/CMV-β/rep-cap/neo contains two SV40 polyA signals, one on the β-galactosidase transcription unit and one on the neo' transcription unit (Figure 16, top). We suggest that the translocation of one of the AAV terminal repeats next to the rep-cap open reading frames occurred because of the annealing of the two SV40 polyA signals in the plasmid (Figure 16, bottom). We believe that during PCR
amplification, Taq polymerase switches templates at the point where the SV40 polyA signals anneal to each other and proceeds to synthesize the sequence on the other template (Figure 16, bottom). Template switch by Taq polymerase during DNA synthesis has been documented (Odelberg et al., 1995). We do not believe that wild-type AAV-like sequences were amplified from a rearranged form of the plasmid because we were unable to isolate intact plasmid DNA that failed to amplify wild-type AAV-like sequences (data not shown). However, we cannot exclude the possibility that low level of rearrangement occurs within each bacterial colony resulting in our inability to isolate intact DNA in a pure form. If rearrangement does occur, it must be indeed very low, because rearranged plasmid could not be detected by restriction enzyme analysis (data not shown).

Based on the information we obtained from the PCR experiments we propose that in the cell the duplicated SV40 polyA sequences may trigger a recombination event that leads to the generation of wild-type AAV-like genomes by juxtaposing the AAV terminal repeat(s) to the rep-cap open reading frames. This recombination event may or may not insert the SV40 polyA signal between the AAV terminal repeat and rep-cap open reading frames. However, if a sequence like the SV40 polyA signal does become inserted into the wild-type AAV genome, the resulting wild-type AAV-like molecule will only be 4% above the size of the wild-type AAV genome and therefore could be efficiently packaged into virions (Samulski et al., 1987). In addition, AAV genomes with deletions in the terminal repeats can be repaired to generate intact molecules (Samulski et al., 1983). Therefore, even if one of the terminal repeats was missing, the AAV genome would repair the missing terminal repeat and generate an intact molecule.
We hypothesized that if the presence of two SV40 polyA signals within plasmid pAAV/CMV-β/rep-cap/neo were responsible for wild-type AAV generation during production of rAAV/β-gal from the H44 cell line, replacing one of the SV40 polyA signals should result in the elimination of wild-type AAV production. Thus, we constructed a different tripartite plasmid, pAAV/CMV-β/rep-cap/neo-tk, where the SV40 polyA signal in the neo^ transcription unit was replaced with the tk polyA signal (Figure 4). We generated a stable cell line which we named D6 and analyzed rAAV/β-gal for wild-type AAV generation. We showed that rAAV/β-gal isolated from the D6 cell line was wild-type AAV free (Figure 17) supporting the supposition that duplicated sequences within the tripartite plasmid led to wild-type AAV generation. Finally, our data suggested that to produce rAAV free of wild-type AAV care should be taken during construction of the tripartite plasmids to eliminate complementary sequences. The presence of complementary sequences in the plasmids may lead to recombination events and subsequent generation of wild-type AAV.

4.3 AAV capsid and β-galactosidase protein expression from the stable cell lines.

An indirect assessment of the number of cells generating rAAV/β-gal is the presence of capsid proteins in those cells. The assumption is that cells which generate virus would be positive for capsid protein expression. For both H44 and D6 cell lines, capsid protein expression was restricted to about 50% of the cells (data not shown and Figure 18), and this was similar to other producer cell lines in our laboratory such as A64 and A80 (Figure 9). Because AAV capsid protein expression correlated with virus production, the data suggested that rAAV/β-gal was produced in 50% of the cells.
The number of H44 and D6 cells that contain a functional copy of the β-galactosidase gene is assessed in vitro using X-gal histochemical staining. Similar to the capsid protein expression, β-galactosidase expression was restricted to 50% of the cells for both H44 and D6 cell lines (Figure 10, 21). Limiting dilution experiments performed with the D6 cell line did not result in the isolation of subclones where more than 50% of the cells were positive for β-galactosidase expression, arguing towards the clonal nature of the line. Finally, D6 cells grown in the presence of cycloheximide exhibited β-galactosidase expression in almost 100% of the cells verifying the clonal nature of the D6 cell line (Figure 26). Supporting evidence also comes from limiting dilution experiments performed with the A64 and A80 cell lines. For both lines, we could not isolate a subclone where more than 50% of the cells were positive for capsid production arguing towards the clonal nature of the cell lines (Figure 9). Similar results have been reported in the literature on the isolation of stable cell lines that express the Rep protein of AAV (Yang et al., 1994a). The authors reported that they could not isolate a stable cell line where 100% of the cells expressed the Rep protein, even after limiting dilution. A similar situation is seen during infection of tissue culture cells with wild-type AAV and adenovirus. At moi's where most of the cells are expected to express the AAV capsid protein, only a small percentage of the cells express these proteins. This phenomenon has been attributed to AAV autointerference (Carter et al., 1979).

4.4 Detection of rAAV/β-gal genomes by in situ hybridizations.

The restricted pattern of AAV capsid protein expression observed in the stable cell lines suggested that rAAV/β-gal was generated in 50% of the cells. We used in situ hybridization analysis with RNA probes to analyze virus
production from the D6 cell line by directly detecting viral genomes. We were not able to show virus production in more than 50% of the adenovirus infected D6 cells (Figure 20). These results were in agreement with what we had observed using protein expression assays, such as IFA and X-gal staining (Figure 18, 21, 22).

The data obtained suggested an association between protein expression and rAAV/β-gal production in the stable cell lines H44 and D6. Although we have not shown directly that the same cells expressing capsid proteins also express β-galactosidase and package virus, we believe that this is likely the case.

4.5 The effect of DNA methylation on protein expression.

Genomic characterization of the D6 cell line showed that plasmid pAAV/CMV-β/rep-cap/neo-tk was integrated into the chromosome of HeLa cells in a concatameric head-to-tail orientation (Figure 23). It is well established that hypomethylation of DNA is associated with increased gene expression (Jones and Taylor, 1980). We tested the possibility that DNA methylation was inhibiting β-galactosidase protein expression by treatment of D6 cells with 5-aza-cytidine. The nucleoside analog 5-aza-cytidine is modified in the 5' position of cytosine and inhibits the cytosine methylation of DNA (Creusot et al., 1982; Jones and Taylor, 1980). We observed no effect on β-galactosidase expression indicating that methylation of DNA was not responsible for restricted β-galactosidase protein expression in the D6 cell line. Fluorescent in situ hybridization (FISH) analysis performed on a number of different cell lines constructed in our laboratory revealed that integration of plasmid DNA takes place at different loci on the chromosome of HeLa cells (Clark et al., 1996a). In spite of the different
integration loci, all the stable cell lines constructed so far exhibit a similar pattern of expression, arguing against DNA methylation being the cause of the restricted protein expression observed in these cell lines.

4.6 The effect of genotoxic agents on protein expression.

To understand the mechanism responsible for the restricted protein expression and virus production in the D6 cell line, we tested the effect of genotoxic agents on mock and adenovirus infected D6 cells. It has been shown that genotoxic agents such as carcinogens, viruses (herpes viruses, vaccinia virus, and adenoviruses), and metabolic inhibitors induce amplification of viral DNA sequences in a number of cell lines transformed with SV40, polyoma, papova, and hepatitis B viruses (Baran et al., 1983; Lambert et al., 1983; Schlehofer et al., 1986; Schlehofer et al., 1983a; Schlehofer et al., 1983b). In addition, cells treated with carcinogens or metabolic inhibitors can support replication of wild-type AAV in the absence of helper virus (Baran et al., 1983; Lambert et al., 1983; Schlehofer et al., 1986; Schlehofer et al., 1983a; Schlehofer et al., 1983b; Yakobson et al., 1989; Yakobson et al., 1987; Yalkinoglu et al., 1988). In view of these observations, we were interested in determining the effect of genotoxic agents on protein expression in D6 cells. We chose to test a DNA damaging agent (cis-platinum), a protein synthesis inhibitor (cycloheximide), mitotic inhibitors (colchicine and nocodazole), and DNA synthesis inhibitors (hydroxyurea, aphidicolin, and etoposide).

β-galactosidase expression was unchanged after treatment of mock and adenovirus infected D6 cells with the DNA damaging agent cis-platinum or the mitotic inhibitors colchicine and nocodazole. All agents caused a slight increase in β-galactosidase protein expression at drug concentrations toxic to
the cells (Figure 24). However, the DNA synthesis inhibitors hydroxyurea, aphidicolin, and etoposide caused a more significant increase in β-galactosidase expression in adenovirus infected D6 cells. All agents except hydroxyurea also showed an increase in β-galactosidase expression in D6 cells mock infected with adenovirus (Figure 25). Cycloheximide, a protein synthesis inhibitor, showed the highest increase in β-galactosidase expression in both mock and adenovirus infected D6 cells (Figure 26).

Capsid protein expression exhibited a pattern of expression opposite to what has been observed for β-galactosidase expression. The DNA damaging agent cis-platinum and the mitotic inhibitors colchicine and nocodazole had no effect on capsid protein expression at low concentrations of the drugs. However, at high drug concentrations, all agents inhibited capsid protein expression. This is in contrast to what we have observed for β-galactosidase protein expression, which becomes slightly enhanced at drug concentrations toxic to the cells. None of the agents induced capsid protein expression in the absence of adenovirus (Figure 24). The DNA synthesis inhibitors (hydroxyurea, aphidicolin, and etoposide) also inhibited capsid protein expression at high drug concentrations. Aphidicolin and etoposide exhibited a marked inhibition on the expression of AAV capsid proteins over concentrations of the drug where β-galactosidase protein expression was greatly enhanced. In addition, aphidicolin and etoposide inhibited capsid protein expression even at low drug concentrations. None of the agents induced capsid protein expression in the absence of adenovirus (Figure 25). Interestingly, the protein synthesis inhibitor cycloheximide, did not inhibit capsid protein expression at all drug concentrations (Figure 26).
Thus, the effect of various genotoxic agents on AAV capsid and β-galactosidase protein expression varies depending on the agent. In untreated cells, AAV capsid and β-galactosidase expression are tightly correlate; however, under conditions of genotoxic stress, AAV capsid and β-galactosidase protein expression are differentially regulated (Figure 24, 25, 26).

4.7 The effect of genotoxic agents on rAAV/β-gal production.

Since AAV capsid protein expression correlates with virus production, we were interested in determining the effect of genotoxic agents on rAAV/β-gal production and whether the correlation between capsid protein expression and virus production persists. We tested the effect of hydroxyurea and aphidicolin on rAAV/β-gal production and determined that treatment of D6 cells with the above agents did not lead to increased production of rAAV/β-gal. Interestingly, we obtained different results when we studied the effect of cycloheximide on rAAV/β-gal production from the D6 cell line. We analyzed virus production using two methods: infectivity titrations to quantitate production of infectious rAAV/β-gal and in situ hybridizations to determine the percent of D6 cells that generate rAAV/β-gal. We determined that at high cycloheximide concentrations (200 and 400 μg/ml), adenovirus infected D6 cells produced approximately 2-fold higher levels of rAAV/β-gal, even though the percent of D6 cells generating virus remained the same (approximately 50%). To increase virus production even further, we took advantage of the inhibitory effect of cycloheximide on cell growth. Since cells stop growing at high cycloheximide concentrations, we were able to seed 2-3 times the number of cells in each flask, increasing the virus yield per flask up to 5-fold.
In conclusion, we constructed and characterized a stable cell line (D6) that generates rAAV/β-gal upon infection with adenovirus. Importantly, rAAV generated from the D6 cell line is apparently free of wild-type AAV. Our studies with several genotoxic agents suggested that it might be possible in the future to modulate rAAV production in producer cell lines. The ability to produce rAAV easily and efficiently in the laboratory will now enable us to expand investigation on the biology of gene transfer mediated by rAAV in animal models.


Figure 1: Map of the AAV genome. Filled arrows represent the AAV inverted terminal repeats (TR); open boxes and arrows, the AAV coding regions; p5, p19, and p40, the transcriptional start sites; polyA, the AAV polyadenylation signal; and carets, the AAV intron. The left open reading frame encodes the four Rep proteins, Rep78, Rep68, Rep52, and Rep40. The right open reading frame encodes the three structural proteins, VP1, VP2, and VP3. See text for details.
Figure 1: Map of the AAV genome.
Figure 2: AAV DNA replication. Intermediates formed during AAV DNA replication. Arrows indicate the 3' OH ends; solid lines represent the parental DNA strand; dotted lines represent the daughter strand; and trs represents the terminal resolution site. See text for details.
Figure 2: AAV DNA replication.
Figure 3: Schematic diagram for the construction of the tripartite plasmid pAAV/CMV-β-gal/rep-cap/neo. The plasmid contains the lacZ gene of *E. coli* positioned between the AAV terminal repeats, the rep-cap open reading frames of AAV and the neo\(^r\) transcription unit. Plasmid psub201, a molecular clone of AAV, was digested with Xbal to generate plasmid psub201/Xbal. The 4.5 Kb PstI fragment containing the lacZ gene of *E. coli* under the control of the CMV promoter and SV40 polyA signal was cloned into the Xbal site of psub201/Xbal to result plasmid pAAV/CMV-β. The rep-cap open reading frames of AAV were introduced into the Xbal site of plasmid pBS/SVneo to generate plasmid pBS/rep-cap/neo. The rep-cap and neo\(^r\) transcription units were removed from plasmid pBS/rep-cap/neo by NotI/EcoRV digestion and cloned into the NgoMI site of plasmid pAAV/CMV-β to result the tripartite plasmid pAAV/CMV-β-gal/rep-cap/neo. The rep-cap transcription units are under the control of the native AAV promoters whereas the neo\(^r\) transcription unit is under the control of the SV40 promoter and SV40 polyA signal. For detailed construction of the plasmid see Materials and Methods. X, Xbal; MI, NgoMI; P, PstI; RI, EcoRI; B, BamHI; RV, EcoRV; N, NotI.
Figure 3: Schematic diagram for the construction of the tripartite plasmid pAAV/CMV-β/rep-cap/neo.
Figure 4: Schematic diagram for the construction of the tripartite plasmid pAAV/CMV-β-gal/rep-cap/neo-tk. The plasmid contains the lacZ gene of *E. coli* positioned between the AAV terminal repeats, the rep-cap open reading frames of AAV and the neo" transcription unit. Plasmid psub201, a molecular clone of AAV was digested with Xbal to generate plasmid psub201/Xbal. The 4.5 Kb PstI fragment containing the lacZ gene of *E. coli* under the control of the CMV promoter and SV40 polyA signal was cloned into the Xbal site of psub201/Xbal to generate plasmid pAAV/CMV-β. The rep-cap open reading frames of AAV were introduced into the Xbal site of plasmid pBS/neo-tk to generate plasmid pBS/rep-cap/neo-tk. The rep-cap and neo" transcription units were removed from plasmid pBS/rep-cap/neo-tk by NotI/Clal digestion and cloned into the NgoMI site of plasmid pAAV/CMV-β to result the tripartite plasmid pAAV/CMV-β-gal/rep-cap/neo-tk. The rep-cap transcription units are under the control of the native AAV promoters whereas the neo" transcription unit is under the control of the SV40 promoter and tk polyA signal. For detailed construction of the plasmid see Materials and Methods. X, Xbal; Ml, NgoMI; P, PstI; N, NotI; C, Clal.
Figure 4: Schematic diagram for the construction of the tripartite plasmid pAAV/CMV-β/rep-cap/neo-tk.
Figure 5: Plasmid maps of pGEM/β-gal-1 and pGEM/β-gal-1A. pGEM/β-gal-1 was used for the \textit{in vitro} synthesis of full length β-galactosidase antisense and sense transcripts (3.5 Kb) after digestion with Xbal and BamHI, respectively. pGEM/β-gal-1A was used for the \textit{in vitro} synthesis of β-galactosidase antisense and sense riboprobes (2.1 Kb) after digestion with Xbal and Sacl, respectively. Shaded arrows show the direction of the β-galactosidase open reading frame. Open boxes designate the bacteriophage promoters SP6 and T7.
Figure 5: Plasmid maps of pGEM/β-gal-1 and pGEM/β-gal-1A.
Figure 6: Strategies for rAAV production. A. "Three-hit" packaging system. 
 rAAV is generated by co-transfecting cells with the recombinant vector and helper plasmid (rep-cap) and subsequently infecting the cell with adenovirus. 
 B. "Two-hit" packaging system. Stable cell lines are generated that maintain the helper plasmid in a stable form. rAAV is generated by transfecting the rep-cap expressing cells with the recombinant vector and subsequently infecting the cells with adenovirus. 
 C. "One-hit" packaging system. Stable cell lines are generated that maintain the recombinant vector and helper plasmid in a stable form. rAAV is generated by simply infecting the cell line with adenovirus.
A. "Three-hit" packaging system.

B. "Two-hit" packaging system.

C. "One-hit" packaging system.

Figure 6: Strategies for rAAV production.
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a. Refers to 3 separate rAAV lot preparations, one of which was performed by the author.
b. Cell counts in mock infected cells were made immediately before adenovirus infection.
c. Virus yield was derived by taking the rAAV/β-gal titer/ml and multiplying by the total lysate volume.
d. Total virus yield/total cells.

Figure 7: Production of rAAV/β-gal from H44 cells.
Figure 8: Estimation of particle-to-infectivity ratio for wild-type AAV and rAAV/β-gal. A. Serial 1:10 dilutions of a known titer of wild-type AAV and a known number of psub201 genome equivalents were incubated in the presence or absence of DNase I. DNA was extracted, immobilized on nitrocellulose, and hybridized to a rep specific probe. The particle-to-infectivity ratio was derived by dividing the number of calculated particles by the infectious titer of the virus preparation. For the virus preparations, calculations were performed using counts from the DNase I treated samples. B. The same procedure described above was applied to a preparation of a known titer of rAAV/β-gal and a known number of pCMV-β genome equivalents.
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Figure 9: AAV capsid protein expression in A64 and A80 subclones. A64 and A80 cells were subcloned by limiting dilution. Individual subclones were infected with adenovirus and stained for AAV capsid proteins by IFA.
Figure 10: β-galactosidase protein expression in H44 cells. H44 cells were infected (+Ad) or mock infected (-Ad) with adenovirus and approximately 72 hr later 0.5-1x10^5 cells were collected on a microscope slide by the use of a cytopsin. HeLa cells were infected (+Ad) or mock infected (-Ad) with adenovirus and treated as above. The cells were stained for β-galactosidase by X-gal histochemical staining according to the protocol described in Materials and Methods. Approximately 50% of the adenovirus infected H44 cells were positive for β-galactosidase expression whereas, <1% of the H44 cells mock infected with adenovirus exhibited positive staining. HeLa cells in the presence or absence of adenovirus were negative for β-galactosidase expression.
Figure 11: PCR strategy for the selective amplification of wild-type AAV sequences in rAAV/β-gal preparations. To selectively amplify wild-type AAV in our rAAV/β-gal viral preparations we designed PCR primers such that only wild-type AAV sequences could be amplified. We designed three primers that annealed on the AAV terminal repeat sequences (TR primers 446, 485, 480) and five primers that annealed on various positions on the AAV rep gene (rep primers 208, 215, 482, 483, 484). The top portion of Figure 11 shows a schematic representation of wild-type AAV sequences and the positions where the TR and rep primers were expected to anneal. The TR/rep primer pairs were expected to amplify fragments with sizes ranging between 600 to 1200 bp when wild-type AAV sequences were used as template for PCR. We used plasmid psub201, a molecular clone of wild-type AAV, in all experiments involving the amplification of wild-type AAV sequences. The bottom portion of Figure 11 shows the circular map of the tripartite plasmid pAAV/CMV-β-gal/rep-cap/neo which was used to generate the stable cell line H44. The positions where the TR and rep primers were expected to anneal on the plasmid are shown. Based on the positions on the plasmid where the primers were expected to anneal at, we expected either no amplification or amplification of a fragment about 10 Kb long.
Figure 11: PCR strategy for the selective amplification of wild-type AAV sequences in rAAV/β-gal preparations.
Figure 12: Ethidium bromide staining of PCR amplified fragments. Lanes 1, 4, 7, 10, 13, 16 and 19 show the expected fragments after PCR amplification of psub201 plasmid DNA with the TR/rep primer pairs: 485-215, 485-482, 485-483, 485-484, 480-483, 480-484, and 480-215 respectively. The corresponding sizes of the above fragments are: 622 bp, 809 bp, 1072 bp, 1179 bp, 1052 bp, 1159 bp, and 602 bp respectively. Lanes 2, 5, 8, 11, 14, 17, and 20 show the fragments generated after PCR amplification of plasmid pAAV/CMV-β-gal/rep-cap/neo with the same TR/rep primer pairs. Based on the positions on the plasmid the primers were expected to anneal to (Figure 9), we expected either no amplification or amplification of fragments about 10 Kb in size. The fragments we amplified instead were approximately of the following sizes: 2000 bp, 1000 bp, 1300 bp, 1400 bp, 1300 bp, 1400 bp, and 2200 bp respectively. All rep primers except one, rep primer 215 (lanes 2, and 20), amplified fragments about 200 bp longer than wild-type AAV (compare lanes 1, 4, 7, 10, 13, 16, and 19 to lanes 2, 5, 8, 11, 14, 17, and 20). Lanes 3, 6, 9, 12, 15, 18, and 21 are the water controls and all show the absence of contaminating bands.
Figure 13: Southern hybridization of PCR amplified H44 total DNA. Total DNA was isolated from HeLa and H44 cells mock or adenovirus infected. One μg of total DNA was PCR amplified using the TR/rep primer pair 446/208 and fractionated on a 0.8% agarose gel. The 446/208 TR/rep primer pair gave the expected fragment (626 bp) when wild-type AAV sequences were used as the template for PCR (data not shown). The same primer pair amplified a fragment about 200 bp longer than wild-type AAV (850 bp) when total DNA isolated from adenovirus infected H44 cells (lane 4) was used as the template for PCR. Total DNA isolated from H44 cells mock infected with adenovirus also amplified a 850 bp fragment (lane 3) which became greatly intensified after adenovirus infection (compare lanes 3 and 4). Total DNA isolated from HeLa cells mock or adenovirus infected was negative for the presence of wild-type AAV sequences (lanes 1, 2). The probe used for hybridization was a PCR generated 360 bp fragment prepared using primers 214/215, internal to the primers 446/208.
Figure 14: Sequencing strategy of the PCR fragments. Plasmid pAAV/CMV-β-gal/rep-cap.neo was PCR amplified using the TR/rep primer pairs 485/483 and 480/483 (Figure 12, lanes 8, 14). The PCR generated fragments were gel purified, cloned, and two clones from each PCR fragment were sequenced. The top diagram shows a schematic representation of wild-type AAV sequences and outlines the positions where the PCR primers 485, 480, and 483 were expected to anneal. Below, a diagramatic representation of the four PCR clones isolated (17-3, 17-4, 8-1, 8-2) is shown. The universal primers SP6 and T7 were used to sequence the 5' and 3' ends of the clones, whereas primers 480 and 500 were used to sequence from the terminal repeats into the rep sequence and from the rep sequence into the terminal repeats, respectively. The dotted lines show the sequence obtained for each clone. The sequencing results identified the insertion of the SV40 polyA signal downstream the AAV terminal repeat in all four clones. In addition, in three of the clones, 17-3, 17-4, and 8-2, the rep sequence of AAV was juxta posed to the SV40 polyA signal. In clone 8-1 the β-galactosidase sequence was juxta posed to the SV40 polyA signal. The nucleotide position of primer 500 on the AAV-2 sequence published by Ruffing et al., (1994) is nt. 230-nt. 250.
Figure 14: Sequencing strategy of the PCR fragments.
Figure 15: Nucleotide sequence of the PCR clones 17-3, 17-4, 8-1, and 8-2. Sequence in bold represents the SV40 polyadenylation signal; underlined sequence, the AAV terminal repeats; sequence in italics, the rep gene of AAV; lower case letters, the β-galactosidase sequence; and regular print, vector sequences.
Clone 17-3

T7: g c a g m t t c g g c t t g t a g t t a a t g a t t a a c c c g c c a t g c t a c t t a t c t a c g t a g c c a t c g t c t g g t c g a c t c t a g a

TT ATT GC ACT TTA AAT GGT TAA CAA AAT AA AAG CTA AT TCA TAC A AAG CA TAC ACA TAC ACA ACA ACA A GCT T TAAAAAATGCTTTTATTTAGTCTAGCATGCT ACT CTCT

SP6: c c a g t g t g a t g g a t a t c t g c a g a a t t c g g c t t c a c g g g c t g c t g g c c c a c c a g g t a g t c g g g g g c g g t t t t a g t c a g g

CTCGATAACTTTCCCGCATTGTCCAAGGCAGCCTTGATTTGGGACCGCGAGTTGGAGGCCGCACTGGTGGTCCTGCTGGATCCACTGCTTCTCCGAGGTAATCCCCTTGTCCACGAGCCACCCGACCAGCTCCATGTACCTGGCTGAAG

SOOlAAAACACTCACGTGACCrCTAATACAGGACCTCTAGAACTAGTGGATCCAGACATGATAAGATACATTGATGA

GTTTGGACAAACCACAAACTGCTAAATAGCAATGCAATGAAATGGTTGTTGTTAACTTGT

TT ATT GC ACT TTA AAT GGT TAA CAA AAT AA AAG CTA AT TCA TAC A AAG CA TAC ACA TAC ACA ACA ACA A GCT T TAAAAAATGCTTTTATTTAGTCTAGCATGCT ACT CTCT

Clone 17-4

T7: c c g c c a g t g t g c t g g a a t t c g g c t t c a c g g g c t g c t g g c c c a c c a g g t a g t c g g g g g c g g t t t t a g t c a g g

CTCGATAACTTTCCCGCATTGTCCAAGGCAGCCTTGATTTGGGACCGCGAGTTGGAGGCCGCACTGGTGGTCCTGCTGGATCCACTGCTTCTCCGAGGTAATCCCCTTGTCCACGAGCCACCCGACCAGCTCCATGTACCTGGCTGAAG

SOOlAAAACACTCACGTGACCTCTAGAACTAGTGGATCCAGACATGATAAGATACATTGATGA

GTTTGGACAAACCACAAACTGCTAAATAGCAATGCAATGAAATGGTTGTTGTTAACTTGT

TT ATT GC ACT TTA AAT GGT TAA CAA AAT AA AAG CTA AT TCA TAC A AAG CA TAC ACA TAC ACA ACA ACA A GCT T TAAAAAATGCTTTTATTTAGTCTAGCATGCT ACT CTCT

Clone 8-1

SP6: c c a c t a g t a a c g g c c g c c a g t g t g c t g g a a t t c g g c t t c a c g g g c t g c t g g c c c a c c a g g t a g t c g g g g g c g g t t t t a g t c a g g

CTCGATAACTTTCCCGCATTGTCCAAGGCAGCCTTGATTTGGGACCGCGAGTTGGAGGCCGCACTGGTGGTCCTGCTGGATCCACTGCTTCTCCGAGGTAATCCCCTTGTCCACGAGCCACCCGACCAGCTCCATGTACCTGGCTGAAG

SOOlAAAACACTCACGTGACCTCTAGAACTAGTGGATCCAGACATGATAAGATACATTGATGA

GTTTGGACAAACCACAAACTGCTAAATAGCAATGCAATGAAATGGTTGTTGTTAACTTGT

TT ATT GC ACT TTA AAT GGT TAA CAA AAT AA AAG CTA AT TCA TAC A AAG CA TAC ACA TAC ACA ACA ACA A GCT T TAAAAAATGCTTTTATTTAGTCTAGCATGCT ACT CTCT

Clone 8-2

SP6: c c a c t a g t a a c g g c c g c c a g t g t g c t g g a a t t c g g c t t c a c g g g c t g c t g g c c c a c c a g g t a g t c g g g g g c g g t t t t a g t c a g g

CTCGATAACTTTCCCGCATTGTCCAAGGCAGCCTTGATTTGGGACCGCGAGTTGGAGGCCGCACTGGTGGTCCTGCTGGATCCACTGCTTCTCCGAGGTAATCCCCTTGTCCACGAGCCACCCGACCAGCTCCATGTACCTGGCTGAAG

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GTTTGGACAAACCACAAACTGCTAAATAGCAATGCAATGAAATGGTTGTTGTTAACTTGT

TT ATT GC ACT TTA AAT GGT TAA CAA AAT AA AAG CTA AT TCA TAC A AAG CA TAC ACA TAC ACA ACA ACA A GCT T TAAAAAATGCTTTTATTTAGTCTAGCATGCT ACT CTCT

Figure 15: Nucleotide sequence of the PCR clones 17-3, 17-4, 8-1, and 8-2.
Figure 16: Model for the amplification of wild-type AAV-like sequences. The sequencing data shown on Figure 14 suggested the following model to explain the amplification of wild-type AAV-like sequences during PCR on plasmid DNA. The top half of Figure 16 shows plasmid pAAV/CMV-β-gal/rep-cap/neo in a linear conformation. Within the plasmid two SV40 polyA signals exist, one on the β-galactosidase transcription unit and the second on the neoR transcription unit. The bottom half of Figure 16 shows the potential annealing of the two SV40 polyA signals which would juxtapose one of the AAV terminal repeats to the rep-cap open reading frames by inserting the SV40 polyA signal between them. We suggest that Taq polymerase switches templates at the point where the SV40 polyA signals anneal. During PCR amplification using the TR and rep primer pairs the SV40 polyA signal is inserted between the AAV rep-cap open reading frames and terminal repeats.
Figure 16: Model for the amplification of wild-type AAV-like sequences.
Figure 17: Southern blot of PCR amplified Hirt DNA. HeLa cells were mock infected (lane 9) or infected with rAAV/β-gal at moi of 1000 (lane 10) in the presence of adenovirus. At maximum CPE, cell lysate was isolated and used to infect fresh HeLa cells in the presence of adenovirus. The cells were grown to maximum CPE and Hirt DNA was isolated. One μg of Hirt DNA was PCR amplified using the TR/rep primer pair 485/482 and fractionated on a 0.8% agarose gel. The probe used in hybridization was a 360 bp PCR generated fragment using the rep primers 214/215 internal to the PCR primers 485/482. To establish the sensitivity of the assay, we constructed a titration curve by infecting HeLa cells with wild-type AAV at moi of 10^{-7}-10^{-2} in the presence of adenovirus. The above moi's correspond to approximately 0.005-550 TCID_{50} (lane 1, moi of 0.005; lane 2, moi of 0.05; lane 3, moi of 0.5, lane 4, moi of 5; lane 5, moi of 55; lane 6, moi of 550). HeLa cells were incubated to maximum CPE and cell lysate was isolated and used to infect fresh HeLa cells in the presence of adenovirus. At maximum CPE, Hirt DNA was isolated and used in PCR amplifications as above. psub201 is plasmid DNA used as a positive control (lane 7). Both wild-type AAV and psub201 amplified the expected 810 bp band. The water control confirms the absence of any contaminating fragments (lane 8).
Figure 18: AAV capsid protein expression in D6 cells. D6 cells were infected (+Ad) or mock infected (-Ad) with adenovirus and approximately 72 hr later, 0.5-1x10^5 cells were collected on a microscope slide by the use of a cytospin. HeLa cells were infected with wild-type AAV (moi of 20) in the presence (+Ad) or absence (-Ad) of adenovirus and treated as above. HeLa cells were infected (+Ad) or mock infected (-Ad) with adenovirus and treated as above. The cells were stained for AAV capsid proteins by IFA according to the protocol described in Materials and Methods. Approximately 50% of the adenovirus infected D6 cells were positive for capsid proteins, whereas D6 cells mock infected with adenovirus exhibited negative staining. HeLa cells infected with wild-type AAV and adenovirus were positive for capsid proteins, whereas HeLa cells in the presence or absence of adenovirus exhibited negative staining.
Figure 19: Northern hybridization of total RNA isolated from D6 cells. 

A. Total RNA was isolated from adenovirus infected D6 cells (D6+Ad) and 10 μg, 1 μg, 100 ng, and 10 ng were fractionated on a formaldehyde/agarose gel and hybridized with the β-galactosidase antisense riboprobe (lanes 1-4). Similarly, 10 μg of total RNA isolated from D6 cells mock infected with adenovirus (D6-Ad) were hybridized to the β-galactosidase antisense probe (lane 6). The antisense probe detected an RNA species of the size of β-galactosidase mRNA (3.5 Kb plus polyA tail) in adenovirus infected D6 cells whereas, in mock infected D6 cells the probe did not detect a similar RNA species. β-galactosidase sense RNA was synthesized in vitro and serial dilutions were hybridized with the antisense probe to establish the detection limits of our probe. We could detect 10 pg of β-galactosidase sense RNA with an overnight exposure. Here we show 100 pg of β-galactosidase sense RNA hybridized with the antisense probe.

B. Total RNA isolated from mock and adenovirus infected D6 cells was hybridized with the β-galactosidase sense probe as above. The sense probe did not detect an RNA species as expected (lanes 1, 2). The sense probe hybridized to 100 pg of antisense β-galactosidase RNA synthesized in vitro (lane 3).
Figure 20: In situ hybridizations of D6 cells. D6 cells were infected (+Ad) or mock infected (-Ad) with adenovirus and approximately 72 hr later, 0.5-1x10^5 cells were collected on a microscope slide by the use of a cytopsin. HeLa cells were mock infected with adenovirus and treated as above. The cells were hybridized with β-galactosidase-specific sense and antisense riboprobes according to the protocol described in Materials and Methods. Approximately 50% of the adenovirus infected D6 cells hybridized with both the sense and antisense riboprobes. Less than 1% of the D6 cells mock infected with adenovirus hybridized with the antisense probe whereas, D6 cells mock infected with adenovirus exhibited negative hybridization with the sense probe. HeLa cells showed negative hybridization with both the sense and antisense probes.
Figure 21: β-galactosidase protein expression in D6 cells. D6 cells were infected (+Ad) or mock infected (-Ad) with adenovirus and approximately 72 hr later 0.5-1x10^5 cells were collected on a microscope slide by the use of a cytopsin. HeLa cells were infected (+Ad) or mock infected (-Ad) with adenovirus and treated as above. The cells were stained for β-galactosidase by X-gal histochemical staining according to the protocol described in Materials and Methods. Approximately 50% of the adenovirus infected D6 cells were positive for β-galactosidase expression whereas, <1% of the D6 cells mock infected with adenovirus exhibited positive staining. HeLa cells in the presence or absence of adenovirus were negative for β-galactosidase expression.
<table>
<thead>
<tr>
<th></th>
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<th>-Ad</th>
</tr>
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<tbody>
<tr>
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<td>0</td>
</tr>
<tr>
<td>% β-gal&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>&lt;1%</td>
</tr>
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a. Percent of D6 cells that stained positive for AAV capsid proteins using IFA.
b. Percent of D6 cells that stained positive for β-galactosidase using X-gal histochemical staining.
c. Percent of D6 cells that hybridized with the sense probe specific for β-galactosidase.
d. Percent of D6 cells that hybridized with antisense probe specific for β-galactosidase.
e. Yield of infectious virus/total cells.

Figure 22: Protein expression and rAAV/β-gal production from the D6 cell line.
Figure 23: Southern hybridization of total DNA isolated from D6 cells. Total DNA was isolated from HeLa and D6 cells and 7 μg was incubated in the presence or absence of SpeI (lanes 1, 3, 5, 6). HeLa DNA was spiked with 100 pg of pAAV/CMV-β-gal/rep-cap/neo-tk plasmid DNA (pTP) and incubated in the presence or absence of SpeI (lanes 2, 4). The digestion fragments were fractionated on a 0.7% agarose gel and hybridized with linearized pAAV/CMV-β-gal/rep-cap/neo-tk plasmid DNA according to the protocol described in Materials and Methods. SpeI is a restriction enzyme that cuts the plasmid pAAV/CMV-β-gal/rep-cap/neo-tk once and results in a linear 14.5 Kb band (lane 4). D6 DNA digested with SpeI gave the following bands: a 14.5 Kb band, corresponding to the linear form of the plasmid integrated into the chromosome in a head-to-tail orientation and two bands that migrate higher than 14.5 Kb that correspond to the junction fragments. Two additional bands of the approximate sizes of 8 and 4 Kb are also present that we speculated correspond to a deleted form of the plasmid (approximately 12 Kb) integrated as a single copy into the chromosome (lane 5).
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<th>-Ad</th>
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<sup>a</sup> D6 cells were treated with cis-platinum, colchicine, or nocodazole and analyzed 48 hr after adenovirus infection.

<sup>b</sup> Percent of D6 cells that stained positive for β-galactosidase using X-gal histochemical staining.

<sup>c</sup> Percent of D6 cells that stained positive for AAV capsid antibodies using IFA.

Figure 24: Treatment of D6 cells with DNA damaging agents (cis-platinum) and mitotic inhibitors (colchicine, nocodazole).
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a. D6 cells were treated with hydroxyurea, aphidicolin, or etoposide and analyzed 48 hr after adenovirus infection.
b. Percent of D6 cells that stained positive for β-galactosidase using X-gal histochemical staining.
c. Percent of D6 cells that stained positive for AAV capsid antibodies using IFA.

Figure 25: Treatment of D6 cells with DNA synthesis inhibitors (hydroxyurea, aphidicolin, etoposide).
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<th>β-gal (%)(^c)</th>
<th>Virus producers (%)(^d)</th>
<th>iu/cell(^e)</th>
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a. D6 cells were treated with cycloheximide (CHX) at concentrations of 0, 50, 100, 200, and 400 µg/ml, infected with adenovirus, and analyzed at maximum CPE.
b. Percent of D6 cells that stained positive for AAV capsid antibodies using IFA.
c. Percent of D6 cells that stained positive for β-galactosidase using X-gal histochemical staining.
d. Percent of D6 cells that were positive for rAAV/β-gal genomes by in situ hybridizations.
e. Yield of infectious virus/total cells.

Figure 26: Protein expression and virus production in D6 cells treated with the protein synthesis inhibitor cycloheximide and infected with adenovirus.