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ADENOVIRAL VECTORS FOR PROTEIN EXPRESSION

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Gene Expression Systems: Using Nature for the Art of Expression

Adenovirus Biology

Introduction

Adenoviruses (Ad) are a large family of nonenveloped, double-stranded DNA viruses. At least 47 serotypes of human adenovirus have been isolated, as well as related animal viruses including avian, canine, and bovine types. In humans, the most common serotypes generally cause mild, self-limiting infections of the respiratory tract or gut (Horwitz, 1996). Adenovirus has served as an important model system for studying many aspects of molecular biology, and a large body of knowledge has accumulated that allows it to be readily manipulated in the laboratory (for an extensive review of Ad biology, see Shenk, 1996). Many features of this viral system, including the ability to infect a wide variety of nondividing cells, a large capacity for insertion of DNA, ease of production and stability of the viral particles, and the high viral titers that can be produced, make Ad-based vectors especially useful in gene transfer. Vectors based on several Ad serotypes have been used for gene expression work both *in vitro* and *in vivo*.

Virus Structure and Receptor Interactions

The adenoviral particle consists of an icosahedral protein capsid encasing a double-stranded DNA molecule of approximately 36 kb (see Fig. 1). The most abundant viral protein is hexon, which makes up most of the outer shell of the virus (van Oostrum and Burnett, 1985). At each vertex is a complex composed of the penton base and fiber proteins, both of which interact with cellular receptors during the process of virus infection (Philipson *et al.*, 1968; Bergelson *et al.*, 1997; Tomko *et al.*, 1997; Wickham *et al.*, 1993). Several minor viral proteins also contribute to the capsid and may play roles in capsid assembly and in viral chromosome packaging. Ad DNA is packaged in a complex with several viral proteins (Mirza and Weber, 1982), and each end of the chromosome is covalently attached to a single molecule of terminal protein (TP) (Rekosh *et*

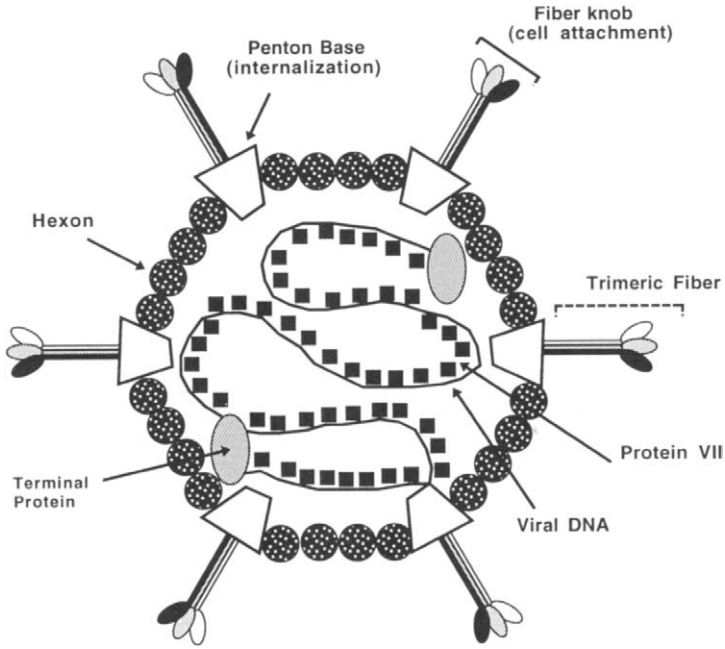

Figure 1 Idealized cross section of the adenovirus particle. Hexon protein makes up the majority of the capsid by mass. A complex of penton base and fiber proteins is located at each vertex. The 36-kb double-stranded DNA chromosome is packaged as a complex with protein VII, and one molecule of terminal protein is attached covalently at each end. For clarity, a number of minor proteins have been omitted from the diagram.

al., 1977). TP acts as a primer for DNA synthesis (Van der Vliet, 1995) and also serves to anchor the viral chromosome to the nuclear matrix (Schaack *et al.*, 1990).

Genome Structure and Expression

Most gene transfer and expression experiments have been done with adenovirus serotypes 2 and 5. The entire genomic sequences of these viruses are known (Chroboczek *et al.*, 1992; Roberts *et al.*, 1984), and many years of work have been spent elucidating a well-defined program of viral gene expression and defining functions for most of the viral reading frames (Shenk, 1996). Historically, the Ad chromosome has been divided into 100 map units (1 m.u. is roughly equivalent to 360 bp). The positions of the major transcriptional units in the virus, which can be broadly divided into two classes, are shown in Fig. 2. Early genes, encoding mainly regulatory functions, are those transcribed shortly after infection. Late genes, including those encoding the major structural proteins, are highly expressed only after viral DNA replication begins. Both early and late Ad promoters have also been used to express heterologous genes.

Early Events in Infection Shortly after infection, transcription from viral early promoters begins. The most important regulator of viral growth is the immediate-early E1a protein, a promiscuous transcriptional activator that interacts with many cellular transcription factors (reviewed by Jones, 1995). E1a both activates further viral gene expression and modifies host cell regulation to drive the cell into S phase, which ensures that sufficient precursors for viral DNA and protein will be available. Deletion of the E1a gene blocks most viral gene expression as well as DNA synthesis and therefore renders Ad replication defective. Infection by E1-deleted Ad vectors often does not cause cytotoxic effects or grossly perturb the underlying cell biology; indeed, such vectors have been approved for administration to human subjects in many gene therapy protocols. These vectors are therefore useful in studies of protein function in intact cells, and most recombinant Ads now in use are E1 deleted.

E2 transcripts are spliced to produce several proteins that are involved in viral DNA synthesis. These include the viral DNA polymerase and terminal protein precursor encoded by E2b, and the

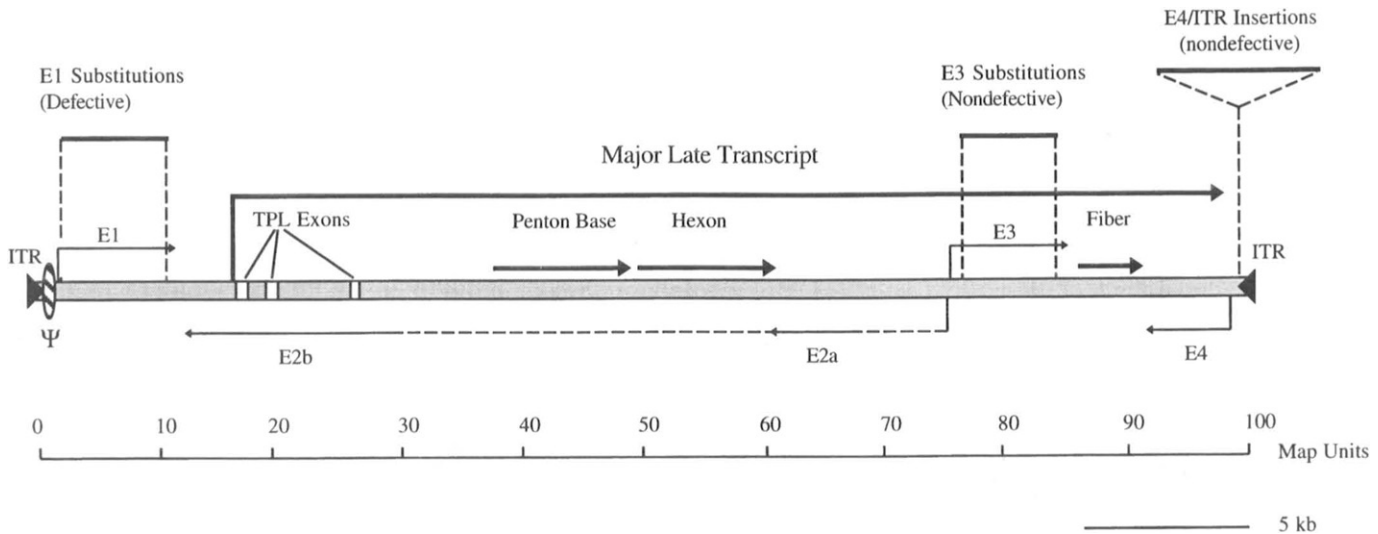

Figure 2 Diagram of the adenovirus type 5 (Ad5) genome. Locations of the E1, E2, E3, and E4 early transcriptional units are indicated. The major late transcript is represented by a heavy arrow, and the positions of the penton, hexon, and fiber open reading frames within this transcript are shown. The three small exons making up the tripartite leader (TPL) are shown as open boxes. The inverted terminal repeats (ITR) at each end of the chromosome are represented by black triangles, and the location of the essential packaging sequences is indicated by Ψ. The E1 and E3 regions can be substituted with foreign DNA as shown to generate defective and nondefective vectors, respectively. Nondefective vectors can also be produced by insertions between the right ITR and the E4 promoter. One map unit (m.u.) is equal to approximately 360 bp.

single-stranded DNA-binding protein produced from E2a. For a review of the roles of E2 products in DNA replication, see Van der Vliet (1995). E4 contains several open reading frames (ORFs) encoding proteins that regulate processes including viral transcription, the shut off of host cell macromolecular synthesis, and correct synthesis of unit length viral chromosomes (Falgout and Ketner, 1987; Weiden and Ginsberg, 1994; Halbert *et al.*, 1985).

Unlike other early regions, E3 products are not required for the replication of virus in cultured cells (Ginsberg *et al.*, 1989). Instead, these proteins are involved in evading the host antiviral immune response. E3 encodes a 19-kDa glycoprotein that downregulates the transport of major histocompatibility complex I molecules to the surface of an infected cell and reduces the inflammatory response generated against the vector (Ginsberg *et al.*, 1989). Other E3 products block tumor necrosis factor-induced lysis or apoptosis of infected cells (see Wold, 1993; Tollefson, 1998). Because the E3 ORF can be deleted without affecting viability, a foreign gene can be substituted into this region and expressed under control of the E3 promoter.

Late Events in Infection Viral DNA replication is initiated by about 8 hr postinfection and is followed by the activation of late viral gene expression. Genes encoding the major structural proteins, such as penton, hexon, and fiber, are all transcribed from the major late promoter (MLP), which initiates a long primary transcript spanning most of the virus genome. After the onset of viral DNA replication, the MLP is transcribed at very high levels and its transcripts are efficiently spliced into mRNAs encoding the various structural proteins (Shenk, 1996).

Three small exons totaling 200 nucleotides (the tripartite leader, TPL) are spliced onto the 5′ ends of all the late mRNAs (Chow *et al.*, 1997; Akusjärvi and Pettersson, 1979; Zain *et al.*, 1979). Late in infection, host cell protein synthesis is shut off due to inhibition of eIF-4E, a cellular translation initiation factor (Zhang *et al.*, 1994). The presence of the TPL confers eIF-4E independence and ensures that the viral mRNAs are translated preferentially (Dolph *et al.*, 1988, 1990). This allows viral proteins to accumulate to very high levels late in infection. These viral transcriptional and translational elements have also been used in recombinant Ad to express foreign proteins at high levels and are discussed further below.

As the late gene products accumulate, virus particles are assembled in the nucleus and released by lysis of the cell, which may in-

volve virus-induced apoptosis (Tollefson *et al.*, 1996). An infected cell can produce 1000 or more progeny virus, allowing large numbers of virions to be isolated.

Adaptation of Adenovirus as a Gene Transfer Vector

Recombinant adenoviruses have been used to express foreign proteins for a number of years, and the recent explosion of interest in gene therapy has led to the development both of improved adenoviral vectors and of more efficient techniques for generating them. Adenovirus-mediated gene therapy is not the focus of this work and has previously been reviewed (Haddada *et al.*, 1995; Kozarsky and Wilson, 1993; Ali *et al.*, 1994). However, technology developed for gene therapy is, in many cases, applicable to the problem of protein expression in general, and improved vectors are likely to make the adenovirus system even more versatile.

Nondefective (Replication-Competent) Adenoviral Vectors

Provided that the total length of the recombinant DNA molecule does not exceed about 105% of wild type (Bett *et al.*, 1993), DNA can be inserted at several sites in the adenovirus chromosome to create a nondefective recombinant (see Fig. 2). Such vectors have been used to express foreign proteins in many studies, particularly for vaccine development studies where the ability of the virus to replicate postadministration may be advantageous. In addition to the commonly used serotypes 2 and 5, adenoviruses of other subgroups such as Ad4 and Ad7 have been used for protein expression (Imler, 1995). A property of nondefective vectors that may be useful in some situations is their ability to shut off host cell macromolecular synthesis. This effectively enriches the expression of a recombinant protein regulated by late viral transcriptional and translational elements.

In many studies, the nonessential E3 region has been replaced by a foreign sequence of interest (Schneider *et al.*, 1989; Morin *et al.*, 1987; Dewar *et al.*, 1989; Johnson *et al.*, 1988), and the plasmid-

based systems now available for construction of recombinant viruses (see later) contain unique restriction sites to simplify cloning into the E3 locus. However, it may be advantageous to retain these sequences in viruses that are to be used for experiments *in vivo* because of the role of E3 in reducing the antiviral immune response (Wold, 1993; Ginsberg *et al.*, 1989; Tollefson, 1998). Helper-independent viruses can also be created by inserting DNA between the E4 promoter and the inverted terminal repeat at the right end of the chromosome (Saito *et al.*, 1985; Mason *et al.*, 1990). However, unless another viral sequence (such as E3) is deleted, the packaging size constraint limits such insertions to about 1.8 kb.

Defective (Replication-Incompetent) Adenoviral Vectors

A replication-defective adenovirus is produced by the removal of essential sequences (such as E1a or E4) from the viral chromosome. As it is possible for cells to be infected simultaneously with two different adenoviruses, defective mutants can be grown by coinfection with a nondefective helper that *trans* complements the missing function. Pure stocks of defective vectors can also be propagated by growth in cell lines that provide the missing viral function(s). The first such cell line, 293, was constructed by the transformation of human embryonic kidney cells with sheared Ad5 DNA and contains an integrated copy of the E1 region (Graham *et al.*, 1977). This allows growth of viral mutants in which the E1a region has been replaced by foreign sequences. Complementation by 293 cells is very efficient and allows growth of E1-deleted adenoviruses to near wild-type levels. A subline of 293, which was adapted for growth in suspension culture, has been described (Graham, 1987). This should be of use in producing large quantities of recombinant protein in high-density cultures.

Regions of the viral chromosome required for replication and packaging of DNA have been identified and make up only a small part of the chromosome. The ends of the Ad chromosome are composed of 105-bp inverted terminal repeat (ITR) sequence. Binding sites for a number of proteins within the ITR are necessary and sufficient for the initiation of viral DNA synthesis (Bernstein *et al.*, 1986; Hay, 1985). A 164 nucleotide sequence adjacent to the left ITR is also required for the packaging of viral DNA into particles (Hearing *et al.*, 1987). In principle, these are the only elements required in *cis*, and the rest of the 36-kb chromosome could be replaced with foreign DNA.

Defective vectors lacking essentially all of the Ad genome have been described (Haecker *et al.*, 1996; Parks *et al.*, 1996; Kumar-Singh and Chamberlain, 1997; Kochanek *et al.*, 1997; Fisher *et al.*, 1996). These vectors are propagated by coinfection with a helper virus and have very high capacities to accept DNA inserts. In one example of this "empty" vector strategy, the Cre/Lox recombinase system was used to remove the packaging signals from the helper virus chromosome during vector growth, rendering it unpackageable (Parks *et al.*, 1996). However, so far there are no such vectors that are truly helper independent, and it is currently impossible to remove all the helper virus from a preparation of vector. This may be a disadvantage, depending on the intended application of the vector. As helper viruses themselves are usually replication defective, their presence at low levels may not be an issue for *in vitro* experiments. It also appears that the minimum allowable size of DNA for efficient packaging is about 27 kb, as shorter chromosomes are not propagated stably (Parks and Graham, 1997).

Because E1a products are essential for progression through the viral life cycle, E1-deleted vectors are almost completely defective for growth in most cells and can generally be expected to have only minimal effects on host cell function. This has made them valuable tools for delivering genes in cell biology studies. However, the E1 deletion may not completely block the expression of viral genes following administration *in vivo*. This leads to an antivector immune response and contributes to the short-term gene expression seen in most gene therapy experiments to date (Yang *et al.*, 1994, 1995). This has prompted the development of vector systems deleted for additional viral genes such as E4 or the E2a DNA-binding protein, which are expected to be less immunogenic and safer *in vivo* (Wang *et al.*, 1995; Brough *et al.*, 1996; Yeh *et al.*, 1996; Krougliak and Graham, 1995; Zhou *et al.*, 1996; Brough *et al.*, 1996; Gorziglia *et al.*, 1996). These multiply deleted vector systems also have higher capacities for insertion of a gene of interest and should be more versatile transfer vectors for protein expression.

Construction of Adenovirus Expression Vectors

Adenovirus DNA is infectious, in that transfection of viral DNA alone into an appropriate cell line results in its replication, viral

protein synthesis, and production of complete viral particles. This process is much more efficient if the DNA–TP complex is isolated intact from virions, probably due to the role of TP in priming viral DNA synthesis (Sharp *et al.*, 1976). A number of methods for generating recombinant virus by the manipulation of viral DNA and retransfection have been developed (for an excellent recent review of recombinant Ad construction techniques, including protocols, see Graham and Prevec, 1995). The large size of the adenovirus chromosome means that it contains few convenient unique restriction sites for insertion of DNA. Most protocols for the generation of recombinant viruses involve manipulation of the vector in two or more fragments, followed either by ligation *in vitro* or by cotransfection and recombination in host cells to regenerate a full-length chromosome. Use of the most common methods to construct an Ad recombinant with a cDNA inserted in place of the E1a gene is illustrated in Fig. 3.

Generation of Adenoviruses by Manipulation of Viral DNA

Recombinant adenoviruses were originally created by isolating fragments of the viral chromosome, followed by ligation or recombination to regenerate a complete viral backbone. Generally, DNA isolated from virions is digested with a restriction enzyme that removes the left end of the chromosome, including the viral packaging signals. This renders the large DNA fragment noninfectious. The unique ClaI site located at 2.6 m.u. is commonly used for this purpose (Fig. 3A). Undigested viral DNA and the smaller DNA fragments are often removed by sucrose gradient ultracentrifugation. The large fragment is ligated *in vitro* to a DNA fragment containing the left end of the chromosome and containing the desired modifications (Stow, 1981). If the viral DNA contained in this fragment has a deletion of the E1a region, the resulting recombinant Ad will be a replication-defective E1 mutant. Inclusion of unique restriction sites in the left end-containing plasmid (generally in the place of the E1a gene) simplifies insertion of a cDNA. The ligation products are then transfected into host cells to regenerate infectious virus (Fig. 3A).

In a variation of this method (Fig. 3B), the purified large fragment is transfected into host cells along with a plasmid that contains overlapping viral DNA, including the left end (Gluzman et al., 1982; Graham and Prevec, 1995; Haj-Ahmad and Graham, 1986).

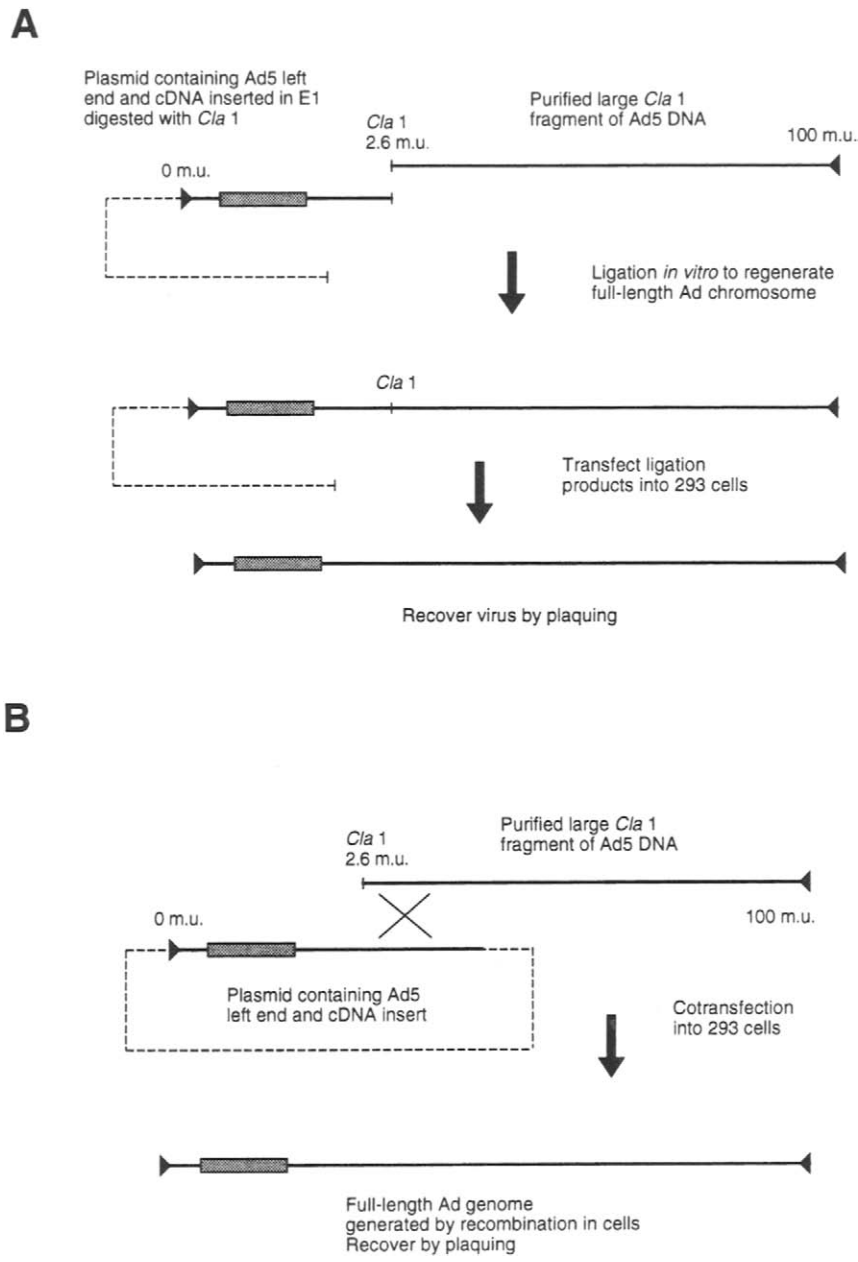

Figure 3 Commonly used methods for the construction of recombinant Ad. The use of each method to construct a replication-defective recombinant with a cDNA inserted into the E1 region is shown. Adenoviral DNA and plasmid sequences are indicated as solid and dashed lines, respectively. (A) *In vitro* ligation of a purified restriction fragment of Ad DNA containing most of the genome (here, the *Cla*I fragment containing the Ad5 sequence from 2.6 to 100 m.u.) to plasmid DNA representing the remaining part of the genome and modified by the insertion of the desired sequence. Ligation products are then transfected to 293

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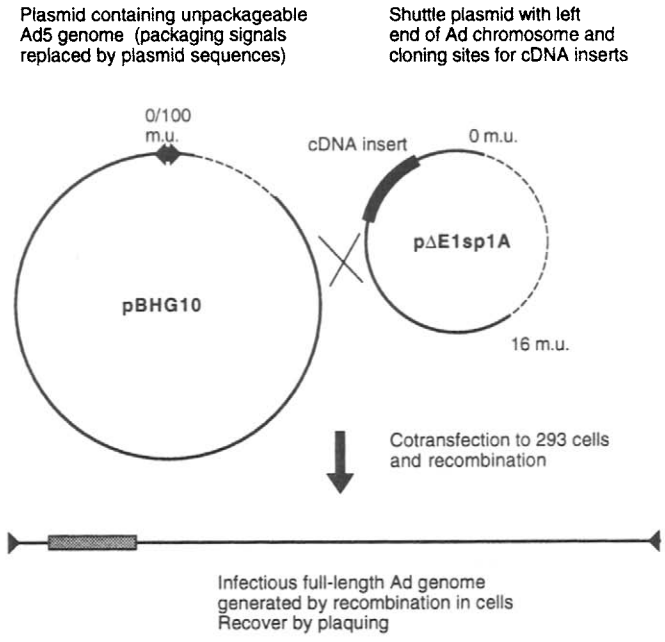
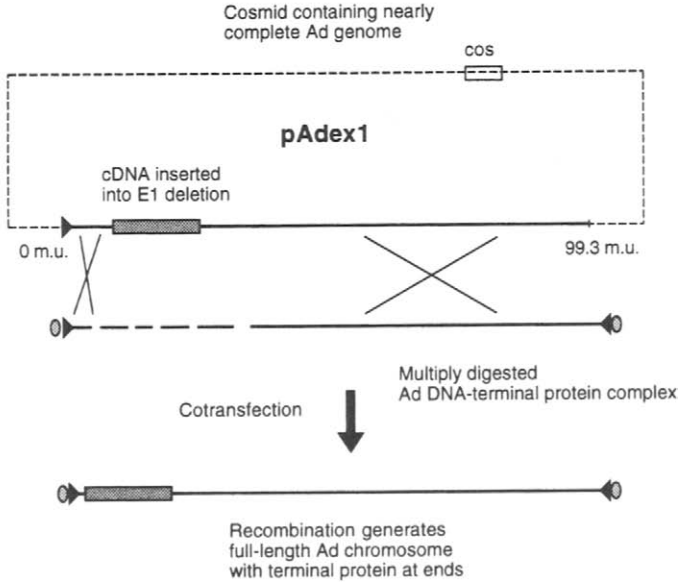

Plasmid containing unpackageable Ad5 genome (packaging signals replaced by plasmid sequences)
Shuttle plasmid with left end of Ad chromosome and cloning sites for cDNA inserts
0/100 m.u.
cDNA insert
0 m.u.
pΔE1sp1A
pBHG10
16 m.u.
Cotransfection to 293 cells and recombination
Infectious full-length Ad genome generated by recombination in cells Recover by plaquing

D


Cosmid containing nearly complete Ad genome
cos
pAdex1
cDNA inserted into E1 deletion
0 m.u.
99.3 m.u.
Multiply digested Ad DNA-terminal protein complex
Cotransfection
Recombination generates full-length Ad chromosome with terminal protein at ends

An infectious Ad chromosome can then be generated by homologous recombination between the viral fragment and the plasmid sequences, resulting in the production of infectious virus. Similar methods have been used to create viral genomes with modifications or insertions near the right end of the chromosome (e.g., insertions into the E3 region).

These methods of generating recombinant Ads may produce a background of wild-type virus due to contamination with undigested viral DNA or religation of the wild-type fragments. This increases the number of plaques that must be isolated and screened to identify the desired recombinant. Imler *et al.* (1995) found that the background can be reduced if the donor virus from which the large restriction fragment is derived contains a copy of the HSV thymidine kinase gene inserted into the E1 region. This parental virus cannot form plaques in the presence of gancyclovir. Growth of any residual donor virus is therefore suppressed by the drug selection, and isolation of the desired recombinant is simplified. Schaack *et al.* (1995a) have described a donor virus containing a β-galactosidase gene near the left end of the genome. Plaques resulting from undigested parental DNA or religation of the two fragments in the host cells will express β-galactosidase and can be identified by incorporation of X-Gal (a chromogenic β-galactosidase substrate) into the agarose overlay.

Figure 3 *(continued)*

cells to generate replicating virus. (B) Overlap recombination in 293 cells between the purified adenoviral fragment as in (A) and a plasmid containing the left part of the viral genome following cotransfection into cells. (C) Use of the two-plasmid system described in the text. The cDNA of interest is inserted into one of the unique cloning sites in the plasmid pΔE1sp1a (which contains Ad DNA corresponding to 0–16 m.u. with an E1 deletion), and the resulting construct is cotransfected with a plasmid such as pBHG10, which contains an unpackageable Ad genome. A complete, packageable Ad chromosome is generated only after recombination takes place between the two DNAs. (D) The cosmid-terminal protein system. pAdex1 is a cosmid containing most (0–99.3 m.u.) of the Ad5 genome and is modified to contain a unique cloning site in place of the E1a gene. Following insertion of the desired cDNA into pAdex1, it is transfected into cells along with an Ad5 DNA-terminal protein complex that has been digested by a restriction enzyme that cuts at several sites in the genome. Recombination between pAdex1 and the terminal fragments of the Ad5 DNA generates a full-length chromosome with a molecule of terminal protein covalently attached at each end, which is efficiently packaged as virus.

Generation of Adenoviruses by Recombination of Plasmid DNA

An adenovirus chromosome, ligated into a circular *Escherichia coli* plasmid vector, can be excised from the plasmid and propagated as virus after transfection into an appropriate host cell line (Graham, 1984; Hanahan and Gluzman, 1984). Berkner and Sharp (1983) also demonstrated that two or more plasmids containing overlapping adenoviral DNA fragments could undergo recombination in cells to regenerate a complete viral chromosome.

Taking advantage of these findings, a two-plasmid system (Fig. 3C) has been developed to simplify the construction of recombinant viral genomes (McGrory *et al.*, 1988; Bett *et al.*, 1994). An essentially complete but unpackageable viral genome is cloned into one plasmid, such as pJM17 or pBHG10. A construct such as pJM17 is noninfectious due to the insertion of plasmid sequences into the viral region, rendering it too large to package into viral particles (McGrory *et al.*, 1988). Deletion of the viral packaging sequences from plasmids such as pBHG10, as shown in Fig. 3C, prevents replication of the nonrecombinant plasmid (Bett *et al.*, 1994). A second plasmid contains the left end of the viral chromosome, including an intact packaging sequence. The E1a region is generally deleted from this second plasmid (pΔE1Sp1A in Fig. 3C) and replaced by a linker with one or more unique restriction sites. The presence of unique cloning sites, and the smaller size of this second plasmid, greatly simplifies the process of inserting a gene of interest into the viral backbone. Another unique restriction site has been engineered into the E3 deletion of pBHG10, which allows insertion of foreign sequence here as well. After cloning of the desired sequences into either or both plasmids, they are cotransfected into a packaging cell line such as 293. Only after recombination takes place between the two plasmids will a packageable viral chromosome be generated.

This method allows propagation of all viral DNA segments in *E. coli*, which is technically much simpler than isolating and purifying fragments from virion DNA. The background of nonrecombinant virus produced following plasmid transfection can be very low. Using the pJM17 system, background plaques arising from rearrangement of the oversized vector plasmid have been reported (McGrory *et al.*, 1988; Graham and Prevec, 1995). Using a pBHG10-type plasmid (which lacks packaging signals) reduces the background of nonrecombinant plaques to essentially zero.

Use of an Adenovirus DNA–Terminal Protein Complex

As noted earlier, the Ad DNA–TP complex is much more infectious on a per microgram basis than plasmid DNA or proteinase-treated viral DNA alone. A method developed by Saito and coworkers (Fig. 3D) takes advantage of this to simplify the recovery of recombinant viruses (Miyake *et al.*, 1996). Most of the Ad genome is contained in the cosmid pAdex1, which has been modified to contain a unique restriction site for the insertion of a foreign sequence in place of the viral E1 region. Cosmid DNA is transfected into host cells along with an adenovirus DNA–TP complex that has been digested by a restriction enzyme that cuts the viral backbone many times. Fragmentation of the viral backbone reduces background due to the carryover of donor virus or to religation of its fragments. Homologous recombination between the pAdex cosmid and the terminal fragments of the donor viral DNA regenerates a full-length chromosome that has a molecule of TP covalently attached at each end. These workers report that recovery of the desired recombinant from this system is very efficient and much simpler than using purified viral DNA alone.

Other Systems for Adenovirus Vector Construction

Two methods have been described for generating and modifying full-length adenoviral chromosomes in lower organisms. This strategy has the theoretical advantage that regeneration of the desired Ad chromosome does not depend on any viral functions. This might simplify the recovery of poorly growing Ad mutants, which may be difficult to recover using the recombination strategies outlined earlier.

Ketner *et al.*, (1994) have succeeded in constructing a yeast artificial chromosome (YAC) containing the entire Ad2 genome. The Ad chromosome can be excised from the YAC by restriction digestion and transfected to mammalian cells. This system allows any desired alterations of the viral chromosome to be performed using the very powerful homologous recombination techniques available in the yeast system, without the need to isolate partial adenovirus clones. Methods for virus construction using recombination in *E. coli* have recently been described (Chartier *et al.*, 1996; He *et al.*, 1998). As in the yeast system, DNA isolated from bacteria after recombination is infectious and can be used to produce virus by

transfection of mammalian cells. These vector systems have not yet come into widespread use, however.

Purification of Recombinant Adenoviruses

The presence of recombinant Ad following transfection of cells by appropriate DNA is readily detectable by the presence of plaques or cytopathic effect (CPE). Regardless of the method used for generating a recombinant Ad, the recovered virus should be plaque purified before use. Nondefective virus will form plaques on monolayers of essentially any cell type that is permissive for viral growth, whereas defective vectors must be grown on a complementing cell line (such as 293 for E1-deleted vectors). If the cell monolayers are overlaid with culture medium containing agarose following transfection, the resulting plaques can be picked directly for further purification (for Ad plaquing and propagation protocols, see Graham and Prevec, 1995).

Alternatively, transfected cells can be maintained under liquid medium and the wells or plates observed for the spreading cell death characteristic of an active viral infection (Zhang *et al.*, 1993). The virus present in a crude extract from the infected plate is then purified by plaquing. It is sometimes easier to detect the production of virus using this method, as detection of the relatively few plaques that result from transfection is dependent on the cell monolayer maintaining good morphology throughout the experiment. This is especially important if the recombinant virus grows slowly.

Production of Viral Stocks

A major advantage of the adenovirus system is the ease with which large quantities of high-titer viral stocks can be produced. Nondefective vectors can be propagated in a number of cell lines, most commonly epithelial cells such as HeLa. Defective vectors are either grown as mixed stocks, including a helper virus, or as pure stocks using complementing cell lines. For propagation of E1-deleted Ads, the standard has been the human embryonic kidney-derived cell line 293 (Graham *et al.*, 1977). Additional E1-complementing lines based on retinal epithelial cells or A549 lung carcinoma cells have been reported (Gorziglia *et al.*, 1996; Fallaux

et al., 1996). The newer generation of vectors deleted for viral genes such as E2, E4, or fiber are grown in packaging lines expressing the corresponding viral proteins (Yeh *et al.*, 1996; Gorziglia *et al.*, 1996; Wang *et al.*, 1995; Brough *et al.*, 1996; Zhou *et al.*, 1996; Langer and Schaack, 1996; Amalfitano *et al.*, 1996; Caravokyri and Leppard, 1995; Schaack *et al.*, 1995b; Weinberg and Ketner, 1983; Krougliak and Graham, 1995; Von Seggern *et al.*, 1998). In most cases, high-titer stocks of these viruses can be prepared readily.

Growth of Ad vectors requires no special facilities beyond those normally used for mammalian tissue culture (NIH Biosafety Level 2). The majority of virions produced remain associated with the cell, allowing concentration by pelleting infected cells prior to spontaneous lysis. A simple freeze–thaw lysis of infected cells is then performed, followed by banding of virus on CsCl gradients (Graham and Prevec, 1995; Everitt *et al.*, 1977). For some uses, such as infection of cells *in vitro* to produce a stock of recombinant protein, CsCl purification may be unnecessary. Purified virus is dialyzed into buffer containing 10% glycerol and is quite stable for long-term storage at –70 °C (Graham and Prevec, 1995). Storing the purified virus in 10 m*M* Tris, pH 8.1, 0.9% NaCl, and 10% glycerol has been found to improve stability. Titers of a standard E1-deleted adenovirus in excess of 10^{11} plaque-forming units (PFU)/ml can be obtained readily by this method. Chromatographic methods for virus isolation have been developed that may further simplify production and scaleup (Huyghe *et al.*, 1995).

Virus concentration has been determined by a number of methods. For a detailed study of several methods for Ad quantitation and of factors that affect results, see Mittereder *et al.* (1996). The number of virus particles in solution is often determined by absorbance at 280 nm or by a standard protein assay (1 μg of Ad2 proteins corresponds to 4×10^9 viral particles). An HPLC-based method for quantifying Ad particles has also been described (Shabram *et al.*, 1997). However, measuring the virus particle number does not provide information about the biological activity of a preparation, and infectious titers are generally reported in PFU. Plaque assays are straightforward to carry out, but require from 10 to 14 days from start to finish, depending on the rate of growth of an individual virus. A more rapid assay is the measurement of fluorescent focus units (FFU) (Thiel and Smith, 1967). Results of the FFU assay are available 1 to 2 days after infection of cells. Because of their high level of synthesis, viral structural proteins are good targets for immunostaining of infected cells. The authors have used polyclonal

antibodies raised against either penton base or fiber to measure FFU.

Time Line for Vector Construction

The steps involved in producing a recombinant Ad vector with the standard two-plasmid system (Fig. 3C) are (1) cloning a foreign gene or genes into the appropriate Ad plasmid(s), (2) transfection into host cells followed by recombination, and (3) purification of the resulting virus. As shown in Fig. 4, a standard first-generation vector (e.g., insertion of a new expression cassette into the E1 region) can be generated in approximately 7–10 weeks. Much of the time involved is spent waiting for plaques or CPE to appear in the transfected cells and in the successive rounds of plaque purification, which do not require much "hands-on" work.

Applications of Adenoviral Vectors in Gene Expression

Proteins successfully produced from recombinant Ad (see below) have included heterologous viral proteins, intracellular enzymes, cell surface proteins, and secreted proteins such as cytokines and

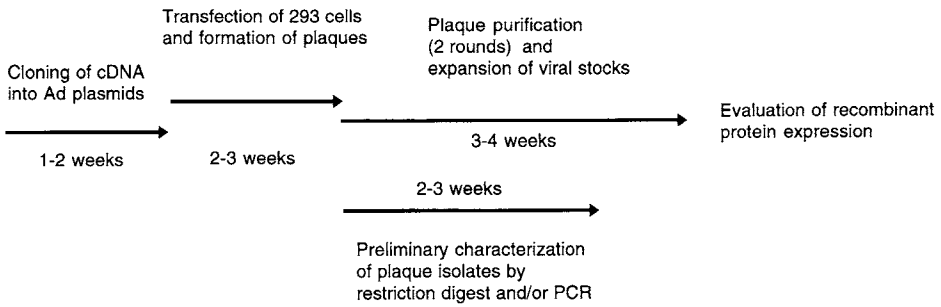


Figure 4 Representative time line for construction of a standard E1-deleted Ad recombinant. Note that after the primary plaques appear, both plaque purification and characterization of the recombinant virus (by restriction digestion or PCR analysis) can be carried out simultaneously.

growth factors. An area in which Ad-mediated expression is particularly useful is the study of protein function in the cell. Because of the interest in Ad-mediated gene therapy, much of this work has been aimed at expression in clinically relevant cell types rather than at producing large amounts of purified protein. Because of this, relatively few workers have reported the mass yields of their adenovirus-produced protein. A number of examples where the yields of expressed protein were reported are summarized in Table 1.

Schaffhausen *et al.* (1987) purified polyomavirus middle T antigen from cells infected with their Ad5 vector and reported yields of up to 100 μg protein/100-mm dish of infected 293 cells. Zhao *et al.* (1993) inserted the gene encoding a protein tyrosine phosphatase (PTP1C), driven by the MLP and TPL sequences, into the E1 region of an Ad5 vector. They purified the recombinant protein from infected 293 cells and reported isolating more than 100 μg of purified enzyme per 150-mm dish of cells (Zhao *et al.*, 1993). Garnier *et al.* (1994) used the same recombinant virus to explore scaleup of protein production using a 293 suspension cell line. They were able to produce the PTP1C protein at levels of 15% of the total cellular protein content (equivalent to 90 mg protein/liter of culture) by growing cells at high density (2×10^6 cells/ml). This type of strategy should be generally applicable for the large-scale production of other proteins.

Adenovirus-Mediated Expression of Heterologous Viral Proteins

An important application for recombinant adenoviruses has been the expression of heterologous viral proteins (Berkner, 1992), either for structure/function studies or for their possible use as vaccines. A discussion of Ad-based vaccines is outside the scope of this chapter, and this topic has previously been reviewed (Imler, 1995). However, this work illustrates some of the myriad potential strategies for Ad-mediated protein expression.

Hepatitis B virus surface antigen (HBsAg) has been produced from Ad-infected cells using a wide variety of strategies. In one case the HBsAg gene, driven by the MLP either with or without inclusion of TPL sequences, was inserted in place of the Ad5 E1 region (Davis *et al.*, 1985), and addition of the TPL resulted in approximately a 70-fold increase in protein synthesis. As expected for expression driven by late viral regulatory elements, the appearance of HBsAg in the medium was observed at late times (10–20 hr

Table 1
Recombinant Proteins Expressed Using Ad Vectors and Yields Obtained

Protein expressed	Position	Serotype	Promoter	Cell line[a]	Yield[b]	Reference
Human α_1-antitrypsin[c]	E1	Ad5	MLP + TPL	HeLa	6 μg/ml/10^6 cells	Gilardi *et al.* (1990)
Human α_1-antitrypsin[c]	E1	Ad5	MLP	HUVEC	0.3–0.6 μg/10^6 cells/day	Lemarchand *et al.* (1992)
β-Galactosidase	E1	Ad5	CMV	MRC5	27 μg/6 cm dish[d]	Wilkinson and Akrigs (1992)
Luciferase	E3	Ad5[e]	SV40 early	HeLa	20 μg/10^6 cells	Mittal *et al.* (1993)
PTP1C protein tyrosine phosphatase	E1	Ad5	MLP + TPL	293 S	90 mg/liter	Garnier *et al.* (1994)
PTP1C protein tyrosine phosphatase	E1	Ad5	MLP + TPL	293	100 μg/150 mm dish	Zhao *et al.* (1993)
HBsAg[c]	E1	Ad5	E1a	Vero	0.5–1 μg/10^6 cells	Ballay *et al.* (1985)
HBsAg	E4/ITR	Ad4[e], Ad7[e]	MLP + TPL	A549	3–4 μg/10^6 cells	Mason *et al.* (1990)
HbsAg[c] and IL-6[c]	E4/ITR, E3	Ad7[e]	MLP + TPL, E3	A549	8–10 μg/10^6 cells, 0.1–0.2 μg/10^6 cells	Lindley *et al.* (1994)
Hepatitis B precore antigen,[c] HbSAg[c]	E3, E4/ITR	Ad7[e]	E3, MLP + TPL	A549	1.4 μg, 6 μg /10^6 cells	Ye *et al.* (1991)
Measles virus N protein	E1	Ad5	CMV	MRC5	20% of soluble cell protein[b]	Fooks *et al.* (1995)
Porcine respiratory coronavirus spike glycoprotein	E3	Ad5	E3	ST	33 μg/10^6 cells	Callebaut and Pensaert (1995)

HIV-1 env	E3	Ad5[e]	E3	HeLa S	1 mg/liter of culture	Dewar *et al.* (1989)
HIV-1 env	E4/ITR	Ad7[e]	MLP + TPL	A549	4 μg/10^6 cells	Chanda *et al.* (1990)
Polyomavirus middle T antigen	E1	Ad5	MLP + TPL	293	100 μg/100-mm dish	Schaffhausen *et al.* (1987)
Murine IL-12 (both subunits)[c]	E1, E3	Ad5	CMV, CMV	293	42,000 U/10^6 cells/day	Bramson *et al.* (1996)
Soluble ciliary neurotrophic factor[c]	E1	Ad5	RSV LTR	1° rat astrocytes	120 pg/10^6 cells/hr	Smith *et al.* (1996)
Neurotrophin-3[c]	E1	Ad5	RSV LTR	1° rat astrocytes	350 pg/10^6 cells/hr	Smith *et al.* (1996)
Fibroblast growth factor 4	E1	Ad5	Sr α	Dami	2–3 μg/10^6 cells	Konishi *et al.* (1996)
Human IL-2[c]	E1	Ad5	CMV	Various human	1–2 μg/10^6 cells	Addison *et al.* (1995)
VEGF[c]	E1	Ad5	CMV	HUVEC	2.2 μg/10^6 cells/day	Mühlhauser *et al.* (1995)
GM-CSF[c]	E1	Ad5	CMV	293	8 μg/10^6 cells	Xing *et al.* (1996)

[a]HeLa, human cervical carcinoma cells; A549, human lung carcinoma; HUVEC, primary human vascular endothelial cells; MRC5, human fibroblasts; 293, Ad5-transformed human embryonic kidney cell line; 293S, suspension subline of 293; Vero, monkey kidney cell line; ST, swine testicle cell line; Dami, human megakaryocytic cell line.

[b]Yields reported by the authors were converted to μg recombinant protein/10^6 cells.

[c]Secreted to medium.

[d]Forskolin induced.

[e]Nondefective vector.

postinfection). HBsAg has also been placed into the E1 region downstream of the Ad5 E1a promoter (Ballay *et al.*, 1985) and inserted into the E3 region of a nondefective Ad5 (Morin *et al.*, 1987). In the latter case, the hepatitis B gene was inserted without a promoter, and its expression appeared to be driven by both early (probably E3) and late (possibly the MLP) Ad promoters.

Mason *et al.* (1990) inserted a cassette consisting of the Ad4 MLP, TPL, and HBsAg coding sequences between the right ITR and the E4 promoter of a nondefective Ad4. In addition to the TPL exons, their constructs retained varying amounts of the intronic sequence located between the first and the second TPL segments. As had been noted previously for the analogous Ad5 intron (Mansour *et al.*, 1986), these workers reported that sequences within this intron could dramatically upregulate mRNA levels driven by the MLP. HBsAg levels produced by infected A549 cells reached levels of almost 4 $\mu g/10^6$ cells in these experiments. Saito *et al.* (1985) also created a nondefective vector by inserting the HBsAg gene (this time driven by the HBV promoter) between E4 and the ITR of Ad5. This construct lacked a TPL and although HBV mRNA was readily detectable, HBsAg was expressed only at very low levels (less than 1 $ng/10^6$ cells), emphasizing the importance of translational regulation after infection with nondefective Ad vectors.

Finally, the ability of Ad vectors to accommodate multiple DNA insertions has been used to coexpress HBsAg along with other proteins. A nondefective Ad7 vector was constructed with MLP, TPL (including the first intron), and HBsAg sequences inserted between the E4 and right ITR, and either the HBV core or precore protein gene inserted in place of the E3 region and driven by the E3 promoter (Ye *et al.*, 1991). HBsAg was produced by these viruses with the late timing expected for MLP transcription and reached levels of 6 $\mu g/10^6$ cells in the medium. The E3-driven core and precore antigens were expressed by 12 hr after infection (as expected for Ad early genes) and were reported to reach levels of 1.46 and 0.33 $\mu g/10^6$ cells, respectively.

Fooks and co-workers (1995) used the CMV promoter to direct expression of the measles nucleocapsid (N) protein from an E1-deleted Ad5 vector. By treating infected cells with forskolin (to increase CMV promoter transcription), they were able to produce recombinant N protein at levels representing 20% of total soluble cellular protein (Fooks *et al.*, 1995). These workers compared the adenovirus-produced protein to recombinant N protein produced using a baculovirus system or expressed in *E. coli* (Warnes *et al.*,

1994). The approximate level of expression of the baculovirus- and adenovirus-expressed N proteins was found to be similar and considerably higher than in the *E. coli* system (the yields reported in this case were 40, 25, and 3% of total cellular protein, respectively).

The *env* gene of HIV-1 has been expressed using nondefective Ad5 (Dewar *et al.*, 1989) and Ad7 (Chanda *et al.*, 1990) vectors, with proper cleavage of the gp160 protein into the gp120 and gp41 fragments and syncitia formation by infected cells. Dewar *et al.* (1989) found that with their Ad5-based vector, gp160 was produced in HeLa suspension cultures at approximately 1 mg/liter. Chanda *et al.* (1990) reported production of very high levels (2–5 mg/liter of culture) of gp160 in A549 cells infected with their Ad7 vector if the HIV-1 *rev* gene product was provided, either by coinfection with a rev-expressing Ad vector or from a *rev* gene fragment expressed from the Ad E3 promoter. A genomic fragment of SIV containing the overlapping *env* and *rev* genes was inserted into the E3 region of Ad5 (Cheng *et al.*, 1992), and proper splicing of the mRNA to produce both SIV proteins was observed. The presence of the *rev* gene upregulated *env* protein production in this context as well, indicating that a complex foreign transcript incorporated into an Ad vector can be spliced into two different functional mRNAs that produce appropriately interacting proteins.

Adenovirus-Mediated Expression of Cytokines and Growth Factors

The emphasis on potential therapeutic uses of adenovirus has led a number of investigators to insert cytokine or growth factor genes into Ad vectors. Biologically active interleukin (IL)-6 was expressed using a recombinant Ad that also contained the HBsAg gene (Lindley *et al.*, 1994). A CMV-driven human IL-2 gene was inserted into the E1 region of an Ad5 vector, and expression was evaluated in several human cell lines (Addison *et al.*, 1995). Secretion of IL-2 was measured at 25–110 ng/10^6 cells over a period of several days. The recombinant protein was active, as measured by the ability to stimulate growth of an IL-2-dependent cell line. Smith and coworkers (1996) inserted the genes for secretable ciliary neurotrophic factor (sCNTF) or for neurotrophin-3 into replication-defective Ad and reported that both viruses allowed secretion of their respective proteins into the medium at concentrations sufficient to promote neuronal survival *in vitro*. Other growth factors that have

been expressed using Ad vectors include fibroblast growth factor-4 (HST-1/FGF-4) and vascular endothelial growth factor (VEGF) (Konishi *et al.*, 1996; Mühlhauser *et al.*, 1995).

Expression of heterodimeric proteins such as IL-12 is complicated by the need to deliver the genes encoding both subunits to the same cells in approximately equal dosages. Here, the ability to insert more than one foreign DNA segment into the adenovirus chromosome is valuable. Bramson *et al.* (1996) inserted a gene encoding the p35 subunit of murine IL-12 into the E1 region and that encoding the p40 subunit into E3, both driven by the strong CMV promoter. This strategy ensured that all infected cells received both genes at the same copy number. Infection of 293 cells or the human fibroblast line MRC5 with the IL-12-bearing Ad vector resulted in the secretion of biologically active IL-12 into the medium at levels up to 40,000 units/10^6 cells (Bramson *et al.*, 1996).

Adenovirus-Mediated Protein Expression for Cell Biology Studies

A major advantage of the adenovirus system is that highly efficient infection of a population of cells can be achieved in a relatively synchronous way. A protein of interest can be introduced readily into most or all cells in an experimental setting, and infection of primary cultures, biopsy samples, or cell lines that are difficult to transfect using standard methods has been reported. By using a replication-defective Ad vector, it is often possible to infect cells without grossly perturbing normal cell functions, thus allowing analysis of the functional properties of the recombinant protein.

Askanas *et al.* (1996) used an Ad vector to express the β-amyloid precursor protein (β-APP) in primary cells derived from biopsy specimens of normal human muscle in order to determine whether β-APP could cause degenerative changes seen in inclusion body myositisis, a degenerative muscle disease. By using the strong CMV immediate-early promoter, this group was able to express high levels of the recombinant β-APP in essentially all cells and to demonstrate that its overexpression caused morphological changes similar to those seen in the disease state.

Huang *et al.* (1997) recently investigated the role of different MAP kinase isoforms in Fas-induced apoptosis by expressing several different MAP kinase molecules, as well as dominant negative, constitutively active, and chimeric forms of these proteins, in the

Jurkat T-cell line. This line is refractory to transfection by standard methods, and the ability of Ad to synchronously transfect essentially all of the cells was instrumental in defining the role of a specific MAP kinase molecule in the apoptosis pathway.

In a study of glucose metabolism, an Ad vector allowed expression of hexokinase 1 in the majority of cells of isolated (but intact) rat islets of Langerhans (Becker *et al.*, 1994). Here, the Ad system allowed transfection of a much higher percentage of cells (60–70%) than could be transfected by nonviral methods. These investigators were able to gather information about hexokinase overexpression without the use of transgenic animals.

In endothelial cells, Ad-mediated expression of a nuclear-localized form of the NF-κB inhibitor, Iκ-Bα, inhibited NF-κB activity and blocked expression of several NF-κB-dependent markers of cell activation in response to lipopolysaccharide. NF-κB-independent markers such as secretion of von Willebrand's factor or of prostacyclin were unaffected (Wrighton *et al.*, 1996). In another study, Ad vectors were used to investigate signal transduction by the type II transforming growth factor-β (TGF-β) receptor (Yamamoto *et al.*, 1996). Expression of a truncated receptor in cultured cells completely blocked both the antiproliferative and the transcriptional activation effects of TGF-β treatment, without affecting the response to two other growth factors.

The ability of more than one adenovirus to infect the same cell has also been used to coexpress multiple protein subunits. Herpes simplex virus 1 encodes a heterodimeric immunoglobulin receptor that is expressed on the surface of an infected cell. After cloning the genes for the two subunits into separate Ad vectors, Hanke *et al.* (1990) expressed either or both subunits simply by infecting target cells with either or both recombinant Ads. This allowed determination of the IgG-binding properties of the individual subunits as well as of the heterodimeric complex on the surface of the infected cells.

In Vivo Expression of Recombinant Adenoviral-Encoded Proteins

Ad vectors can efficiently transfect a large number of organs or tissues *in vivo*, including liver (Jaffe *et al.*, 1992), kidney (Moullier *et al.*, 1994), skin (Setoguchi *et al.*, 1994b), brain (Akli *et al.*, 1993; Doran *et al.*, 1997), skeletal muscle (Ragot *et al.*, 1993), heart (Stratford-Perricaudet *et al.*, 1992), lung epithelium (Rosenfeld *et al.*,

1991), and several ocular tissues (Mashhour *et al.*, 1994) (for further review, see Ali *et al.*, 1994, or Kozarsky and Wilson, 1993). This property allows protein function to be investigated *in vivo* without generating transgenic animals.

Adenoviral gene delivery is particularly useful if a large number of variant proteins are to be evaluated. For example, to identify the domains of the closely related lipoprotein lipase and hepatic lipase proteins responsible for determining their substrate specificity *in vivo*, Kobayashi *et al.* (1996) made several chimeric proteins by exchanging domains between the two and cloned the resulting genes into E1-deleted Ad vectors. They systemically administered these viruses to hepatic lipase-deficient mice and examined changes in serum lipids. These experiments showed that the greater ability of hepatic lipase to hydrolyze phospholipids and to reduce total serum cholesterol is largely determined by the "lid" domain of the proteins, which is thought to restrict access to the active site of the enzyme.

Cytokine and chemokine functions *in vivo* have been studied using Ad vectors to locally produce factors, including GM-CSF, RANTES, and IL-6 in rat lung (Xing *et al.*, 1994, 1996; Braciak *et al.*, 1996). Expression of these molecules in a defined tissue has allowed investigation of their roles on inflammatory processes and recruitment of cells such as monocytes and T lymphocytes. Local expression of Ad-delivered genes may be more relevant to their normal physiological roles than systemic administration of the purified protein.

Systemically administered Ad vectors can also provide high-level temporary expression of a protein that is secreted into the bloodstream. Much of this work has focused on preclinical applications, as in expression of factor VIII for the treatment of hemophilia A (Connelly *et al.*, 1996) or of erythropoietin to stimulate erythropoiesis (Setoguchi *et al.*, 1994a), but this property can also be useful in understanding the *in vivo* roles of secreted proteins. For example, rats treated intravenously with a viral vector directing expression of the rat leptin protein exhibited reduced food intake and weight gain, as well as a disappearance of fat deposits (Chen *et al.*, 1996). Intraperitoneal administration of an Ad vector containing the gene encoding HST-1/FGF-4 to mice stimulated platelet production (Sakamoto *et al.*, 1994). This group then used the same viral vector in an *in vitro* study to demonstrate that the increased platelet count was due to FGF-4 stimulation of megakaryocyte maturation (Konishi *et al.*, 1996).

Miscellaneous Applications

As noted previously, the large size of the Ad chromosome should allow for the transfer of very large genes. Vectors containing only the minimum Ad packaging sequences have been used to express full-length human (Haecker *et al.*, 1996) or murine (Kochanek *et al.*, 1997; Kumar-Singh and Chamberlain, 1997) dystrophin in cultured myotubes and *in vivo*. The 14-kb dystrophin cDNA far exceeds the capacity of other viral vectors now in widespread use.

Several groups have constructed Ad vectors that express the Cre recombinase protein of bacteriophage P1. This enzyme catalyzes recombination between 34-bp target sites (*LoxP*) with excision of the intervening DNA. Expression of Cre from an Ad recombinant could remove an inactivating sequence flanked by *LoxP* sites and switch on expression of a reporter gene either located on a second Ad coinfected along with the Ad–Cre recombinant or stably integrated into the host cell chromosome (Anton and Graham, 1995; Miyake *et al.*, 1996; Sakai *et al.*, 1995). Wang *et al.* (1996) extended these findings *in vivo* and demonstrated that the Ad–Cre system was capable of causing efficient recombination between *LoxP* sites on the chromosomes of transgenic mice. This approach should be useful in conditionally expressing proteins or in producing gene knockouts that are spatially and/or temporally restricted.

Another application for Ad-mediated gene delivery is modulation of the receptor repertoire expressed on the surface of infected cells. Lieber *et al.* (1995) used an Ad vector to deliver the amphotrophic retrovirus receptor (RAM) cDNA to cells that are normally resistant to infection with the amphotropic retroviral vectors commonly used in gene therapy work. Following Ad infection, target cells were markedly more infectible with a recombinant retrovirus. An Ad recombinant was used to express human CD4 on the surface of a number of CD4-negative cell lines (Yasukawa *et al.*, 1997). In several of these lines, CD4 expression allowed infection by the herpesvirus HHV–7, confirming the role of CD4 in HHV-7 entry.

As replication-defective Ad vectors can be introduced to a relatively small area of tissue by local injection or application, they have utility as markers for the study of cell migration and development. When *LacZ*-containing Ad vectors were administered to the external surface of early stage chicken hearts, β-galactosidase-expressing cells in some, but not all, regions of the heart were detectable at later times (Fisher and Watanabe, 1996), providing infor-

mation about the origin of the cells making up various heart structures. Similar experiments have demonstrated that Ad can be used to mark cells in the developing rat central nervous system and in preimplantation mouse embryos (Lisovoski *et al.*, 1994; Tsukui *et al.*, 1995).

Considerations in the Use of Adenovirus Expression Vectors

Choice of Insertion Site: E1 vs E3

Insertion of foreign DNA into either or both of the E1 and E3 regions is compatible with high-titer virus production. The ability to insert two genes into the same vector chromosome has obvious advantages if a heterodimeric protein (Bramson *et al.*, 1996) or two proteins designed to work together (Hanke *et al.*, 1990) are to be expressed in the same cells. There are functional differences in the use of E3 vs E1 insertions, however, and one site may be preferable over the other for a particular application.

Substitution of the nonessential E3 region by foreign DNA has the advantage that replication-competent viruses can be generated readily. This property is of special interest for vaccine development, and many E3 substitution vectors have been designed for this purpose (Imler, 1995) . The most common method of generating E3 insertions in Ad5 vectors has been replacement of the *Xba*I fragment between 78.8 and 85.7 m.u. by foreign sequence. This removes most E3 coding sequences but leaves the E3 promoter intact, which allows it to direct expression of the transgene. A number of studies using such substitutions have reported that transgene expression was largely E3 driven, even when the inserted DNA contained its own promoter sequences (Both *et al.*, 1993; Schneider *et al.*, 1989; Johnson *et al.*, 1988). Expression of genes inserted into the E3 region of a nondefective vector may also be affected by late viral promoters, probably the MLP, late in infection (Dewar *et al.*, 1989; Morin *et al.*, 1987). This viral regulation of transgene expression may be a disadvantage if cell type or temporally restricted expression is desired.

In contrast, the E1 deletions generally used remove the E1a pro-

moter sequences. In many cases, cellular promoters incorporated into the E1 region of adenoviral vectors have been shown to be regulated in a manner similar to their endogenous genes, allowing expression of a gene of interest in a cell-type or tissue-specific manner. As E1-deleted vectors are almost completely replication defective and can have minimal effects on normal cellular processes, they have been chosen for most work involving cell biology studies or *in vivo* experiments.

Use of Viral Regulatory Elements

Because viral proteins are translated preferentially at the expense of host proteins late in infection, it can be advantageous to use viral late gene control elements for applications where very high level expression is desired (for a detailed discussion of this topic, see Berkner, 1992). As noted earlier, expression of a foreign gene placed under the control of the viral MLP and TPL can be enhanced along with that of bona fide viral gene products (Berkner *et al.*, 1987; Alkhatib and Briedis, 1988; Berkner, 1992; Davis *et al.*, 1985; Garnier *et al.*, 1994). Sequences in the first intron of the TPL have also been found to increase MLP transcription in infected cells, contributing to the very high level expression seen in some of these studies (Mansour *et al.*, 1986; Mason *et al.*, 1990). The highest yields of recombinant protein reported from Ad vectors (Table 1) have been from constructs using both the MLP and the TPL for transgene expression in cells that support productive viral infections (e.g., E1-deleted vectors in 293 cells or nondefective vectors in other cell types).

Inclusion of the TPL in expression constructs has been reported previously to boost the production of a variety of proteins, even in uninfected cells (Sheay *et al.*, 1993), and TPL can increase greatly the expression of the Ad5 fiber protein in 293-based cell lines in the absence of any viral infection (Von Seggern *et al.*, 1998). These sequences may therefore improve protein yield even in the context of a nonpermissive cell type infected by a defective virus and may have general value in protein expression work.

As noted earlier, the E3 promoter has been used (sometimes inadvertently) to express many foreign genes. E3 is transcribed in many cell types, and transcription is activated within a few hours postinfection.

Use of Heterologous Promoters

Many different promoters have been inserted into Ad vectors and in most cases have retained their activity. Widely used viral promoters include the long terminal repeat of RSV, the SV40 early promoter, and the cytomegalovirus (CMV) major immediate-early promoter. The CMV promoter provides very strong expression of a transgene in many cell types, at least in the short term (Jiang *et al.*, 1996; Guo *et al.*, 1996), and has become perhaps the most widely used promoter in adenoviral vectors. Bartlett *et al.* (1996) have reported that the constitutively active U1 small nuclear RNA promoter, which is transcribed in essentially all cell types, has activity similar to CMV and is active when placed into an E1-deleted Ad vector.

When placed in the adenovirus context, a number of tissue-specific promoters have been shown to be regulated much like their cellular counterparts. Friedman and co-workers (1986) found that the liver-specific rat albumin promoter in an Ad5 vector was expressed in rat or human hepatoma cells or in primary hepatocytes, but not in a myeloma cell line, whereas the B-cell-specific immunoglobulin heavy chain promoter was expressed strongly in myeloma but not hepatoma cells. More recently, studies of a modified mouse albumin promoter driving expression of the human factor VIII gene demonstrated specificity both for hepatocytes *in vitro* and for liver *in vivo* (Connelly *et al.*, 1996).

A number of groups have sought to target adenovirus-based gene expression to tumor cells. Hepatoma cells often express elevated levels of α-fetoprotein (AFP) relative to normal liver. A recombinant Ad carrying AFP promoter and enhancer sequences driving the herpes simplex virus thymidine kinase (TK) gene expressed high levels of TK in AFP-expressing tumor cell lines such as HuH-7 or HepG2, but not in non-AFP-expressing liver cell lines such as HLF (Kanai *et al.*, 1996). Other workers have reported that expression of foreign genes placed under the control of the carcinoembryonic antigen (CEA), osteocalcin (OC), or DF-3 promoters in Ad vectors is specific for CEA-, OC-, or DF-3-expressing tumor cells, respectively (Lan *et al.*, 1996; Ko *et al.*, 1996; Chen *et al.*, 1995). Hashimoto *et al.* (1996) found that Ad-mediated expression using two different neural cell-specific promoters was restricted to the expected cell types both in culture and after administration of the respective Ad vectors to rat brain *in vivo*.

The ability to control the level or timing of transgene expression

may be desirable in a particular experimental setting. A number of inducible or regulated promoters have been placed into the Ad backbone. For example, a synthetic construct consisting of minimal thymidine kinase promoter sequences and several retinoic acid response elements was inserted into the E1 region of an Ad5 vector. Transcription of a reporter gene in infected cells could be induced by the addition of retinoids, both in culture and *in vivo* (Hayashi *et al.*, 1994). The mouse metallothionine promoter also retained its zinc-inducible expression in an Ad context (Yajima *et al.*, 1996). Varley *et al.* (1995) made Ad constructs containing a luciferase gene driven by either of two inflammation-responsive promoters. Cells infected with either virus were found to express high levels of luciferase in response to the cytokines present in conditioned culture medium. *In vivo* studies demonstrated that luciferase expression was upregulated in tissues of mice infected with these vectors and challenged with inflammatory agents such as lipopolysaccharide toxins or turpentine (Varley *et al.*, 1995).

The pattern that emerges from these studies is that cellular promoters in an Ad chromosome often behave as would be predicted from their normal activities, although it may be worth noting that all of these specific promoter activities were generated using insertions into the viral E1 region. The ability to reproduce cell-specific patterns of gene expression after adenovirus-mediated transfer will be invaluable in targeting transgene function to specific cells and may be useful in optimizing expression of a recombinant protein in the cell type of interest.

Advantages and Disadvantages of Adenoviral Vectors

The unique advantage of the adenoviral expression system is its versatility. Once constructed, a single vector can be used for *in vitro* protein expression and purification, studies of the effect of the gene product on cell biology, or *in vivo* studies in many tissue types and a number of different species. For these reasons, adenovirus is becoming the system of choice for work involving both *in vitro* and *in vivo* studies.

The investigator has the option of using either well-characterized

viral regulatory sequences or a heterologous promoter, which should allow expression to be tailored to the requirements of the particular system of interest. The large (and growing) capacity of Ad vectors allows expression of large genes or of a combination of genes, as in expression of both subunits of a heterodimeric protein or of an antigenic protein together with a cytokine. Finally, the wide tropism and ability to infect nondividing cell types allows Ad vectors to be used in many cases where other vectors, such as retroviruses, cannot be used.

The major disadvantage of this system is the complexity of vector construction. A recombinant vector for Ad-based expression of a protein can take considerably longer to produce than a plasmid for use in a prokaryotic expression system or simple transfection into eukaryotic cells. However, techniques for adenovirus construction have improved dramatically. The plasmid-based systems now available for construction of standard E1- or E3-deleted vectors are quite straightforward, and the effort required for the construction of recombinant adenoviruses is similar to that needed to construct recombinant baculoviruses.

As Ad DNA does not normally integrate into host cell chromosomes, the expression of the transgene is transient. This is of little consequence in short-term or *in vitro* experiments, but may limit some long-term *in vivo* studies.

Although Ad vectors are being used successfully *in vivo* to study protein function, there are still problems associated with the host response to the vector itself. Following the administration of currently available Ad vectors, antivector immune responses are generated that can lead to inflammation, elimination of the infected cells, and short duration of transgene expression. This limits long-term studies in immunocompetent animals. Infection even by replication-defective Ads also affects the endogenous signal transduction pathways (Li *et al.*, (1998) and can lead to responses in the infected cells, such as the production of cytokines. This can potentially complicate the interpretation of studies of protein expression *in vivo*. This type of effect can often be controlled for by administration of an Ad vector lacking an insert.

Finally, although Ad vectors have been found to infect a wide variety of cells in several species, including human, mouse, rat, and chicken, not all cells of interest may express the appropriate integrin and CAR proteins to allow infection. The repertoire of integrin and fiber receptor expression on the target cell type should therefore be considered when planning *in vivo* work using Ad vectors (Table 2).

Table 2
Advantages and Disadvantages of Adenoviral Vectors

Advantages	Disadvantages
Versatility; recombinant protein expressed from same vector both *in vitro* and *in vivo*	Complexity of recombinant Ad construction
High capacity for (multiple) DNA insertion	Expression is generally transient
Ability to use wide variety of cellular promoters in Ad recombinants	*In vivo* antivector immune response
High-level protein expression *via* late Ad regulatory elements	Current Ad vectors cannot be specifically targeted *in vivo*
Ease of producing high-titer viral stocks	
Wide tropism of Ad includes many nondividing cells	

Future Directions

As interest in adenovirus as a gene therapy vector remains high, further improvements in vector design and techniques for their construction can be expected. Packaging systems for the complementation of additional Ad genes such as E2, E4, terminal protein, or fiber are now available (Krougliak and Graham, 1995; Yeh *et al.*, 1996; Caravokyri and Leppard, 1995; Schaack *et al.*, 1995b; Gorziglia *et al.*, 1996; Wang *et al.*, 1995; Brough *et al.*, 1996; Weinberg and Ketner, 1983; Amalfitano *et al.*, 1996; Zhou *et al.*, 1996, Von Seggern *et al.*, 1998). Viral vectors deleted for the corresponding sequences will have a higher capacity for insertion of DNA and should be useful for expressing large proteins such as dystrophin or combinations of genes (perhaps multisubunit enzyme complexes). As they are expected to be less immunogenic and to persist longer *in vivo*, they should also improve the prospects for the use of Ad to study protein function in the context of an intact animal.

Ongoing work is also extending the already impressive list of cell types that can be infected. Some cell types (most notably many hematopoietic cells) are difficult to infect using Ad. This is largely due to these cells lacking expression of either the CAR protein, which serves as the fiber receptor (Bergelson *et al.*, 1997; Tomko *et al.*, 1997), or integrins appropriate for the internalization of virus (Wickham *et al.*, 1993). For example, in human airway organ culture, regenerating cells were found to be much more infectible than

normal cells (Dupuit *et al.*, 1995). Another study showed that immature cells in the airway were much more susceptible to infection by Ad and that these cells expressed higher levels of the integrins needed for virus internalization (Goldman and Wilson, 1995). In skeletal muscle, the infectibility of different cell populations has also been shown to correlate with α_v integrin expression (Acsadi *et al.*, 1994). Huang and colleagues have demonstrated that lymphoid cells can be infected efficiently if they are treated with growth factors that upregulate the synthesis of α_v integrins (Huang *et al.*, 1995) and that there is an alternate pathway of infection using α_m integrins in at least some lymphoid cell types (Huang *et al.*, 1996).

Several groups are now developing adenoviruses that have modified tropism by altering the fiber or penton base proteins that interact with cellular receptors. Wickham *et al.* (1996) have described Ads with epitope tags incorporated into the penton base, allowing the virus to be redirected using a bispecific antibody approach. The modified virus was shown to infect endothelial and smooth muscle cells, which are normally difficult to infect due to low CAR expression (Wickham *et al.*, 1996).

Replacement of the fiber gene in a vector of one serotype by that of another has been shown to confer the cell-binding specificity associated with the new fiber serotype (Gall *et al.*, 1996). Chimeric fiber genes containing the receptor-binding domain of a different Ad serotype have also been used to alter viral tropism (Stevenson *et al.*, 1997; Krasnykh *et al.*, 1996). Michael and colleagues added a short peptide sequence from gastrin-releasing peptide to the fiber (Michael *et al.*, 1995) and demonstrated that this peptide epitope is accessible for antibody binding at the surface of the fiber protein.

A high-resolution structure for the receptor-binding knob domain of the fiber protein is now available, which should lead to improved strategies for redirecting fiber binding (Xia *et al.*, 1994). This type of work should lead to Ad vectors that can be targeted to particular cell types, extending the utility of the system for studies *in vivo* or using tissue samples.

Conclusions

Adenoviral gene transfer vectors are extremely versatile and are becoming widely used, both for protein expression *in vitro* and for

studies of protein function *in vivo*. The high insert capacity of these vectors means that large genes or combinations of different genes can be transferred to cells. By using transcriptional and translational control elements from adenoviral late genes, a very high level expression of recombinant proteins has been demonstrated. Many tissue-specific or regulatable promoters have been shown to retain their function when placed on Ad chromosomes, allowing foreign genes to be expressed in a predictable way in the desired cell type.

As Ad vectors are becoming more widely used in gene therapy work, there has been much interest in improving the technology. Plasmid-based systems for vector construction have simplified the generation of recombinant Ad greatly, and the new Ad vector/packaging cell systems becoming available will provide further increases in capacity and in the effectiveness of *in vivo* gene transfer. Targetable vectors are being developed that will eventually allow expression of a protein of interest in specific cell types *in vivo*.

Acknowledgments

The authors thank members of the Nemerow Laboratory for their assistance, Colleen McKiernan for comments on the manuscript, and Joan Gausepohl for her help with the manuscript.

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