Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
ADENOVIRAL VECTORS FOR PROTEIN EXPRESSION

Dan J. Von Seggern and Glen R. Nemerow
Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

Adenovirus Biology
   Introduction
   Virus Structure and Receptor Interactions
   Genome Structure and Expression
Adaptation of Adenovirus as a Gene Transfer Vector
   Nondefective (Replication-Competent) Adenoviral Vectors
   Defective (Replication-Incompetent) Adenoviral Vectors
Construction of Adenovirus Expression Vectors
   Generation of Adenoviruses by Manipulation of Viral DNA
   Generation of Adenoviruses by Recombination of Plasmid DNA
   Use of an Adenovirus DNA-Terminal Protein Complex
   Other Systems for Adenovirus Vector Construction
   Purification of Recombinant Adenoviruses
   Production of Viral Stocks
   Time Line for Vector Construction
Applications of Adenoviral Vectors in Gene Expression

Gene Expression Systems: Using Nature for the Art of Expression
Copyright © 1999 by Academic Press. All rights of reproduction in any form reserved.
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 $\Psi$. 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 re-
view of the roles of E2 products in DNA replication, see Van der
Vliet (1995). E4 contains several open reading frames (ORFs) encod-
ing proteins that regulate processes including viral transcription,
the shut off of host cell macromolecular synthesis, and correct syn-
thesis 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 prod-
ucts block tumor necrosis factor-induced lysis or apoptosis of in-
fected 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 vi-
ral 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 tran-
scripts 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 translation-
al elements have also been used in recombinant Ad to express for-
eign proteins at high levels and are discussed further below.

As the late gene products accumulate, virus particles are assem-
bled 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 unpackagable (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* 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 (continues on next page)
C

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

D

Cosmid containing nearly complete Ad genome

pAdex1

cDNA inserted into E1 deletion

Cotransfection

Multiply digested Ad DNA-terminal protein complex

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 co-workers [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 mM 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 $4 \times 10^9$ 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
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 \(2 \times 10^6\) 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 | Yield | Reference                        |
|----------------------------------------|----------|----------|-------------|-----------|-------|----------------------------------|
| Human α₁-antitrypsin                   | E1       | Ad5      | MLP + TPL   | HeLa      | 6 μg/ml/10⁶ cells                | Gilardi et al. (1990) |
| Human α₁-antitrypsin                   | E1       | Ad5      | MLP         | HUVEC     | 0.3–0.6 μg/10⁶ cells/day         | Lemarchand et al. (1992) |
| β-Galactosidase                        | E1       | Ad5      | CMV         | MRC5      | 27 μg/6 cm dish                 | Wilkinson and Akrigs (1992) |
| Luciferase                             | E3       | Ad5      | SV40 early  | HeLa      | 20 μg/10⁶ 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                                  | E1       | Ad5      | E1a         | Vero      | 0.5–1 μg/10⁶ cells              | Ballay et al. (1985) |
| HBsAg and IL-6                         | E4/ITR   | Ad4, Ad7  | MLP + TPL   | A549      | 3–4 μg/10⁶ cells                | Mason et al. (1990) |
| Hepatitis B precore antigen, HBsAg     | E4/ITR, E3 | Ad7  | MLP + TPL, E3 | A549      | 8–10 μg/10⁶ cells, 0.1–0.2 μg/10⁶ cells | Lindley et al. (1994) |
| Measles virus N protein                 | E3, E4/ITR | Ad7  | E3, MLP + TPL | A549      | 1.4 μg, 6 μg /10⁶ cells         | Ye et al. (1991) |
| Porcine respiratory coronavirus spike  | E3       | Ad5      | E3          | ST        | 20% of soluble cell protein     | Fooks et al. (1995) |
| glycoprotein                            |          |          |             |           | 33 μg/10⁶ cells                 | Callebaut and Pensaert (1995) |
| Protein/Vector                        | Producer Cell Line | Transgene(s) | Expression in | Quantity/Unit |
|--------------------------------------|--------------------|--------------|---------------|---------------|
| HIV-1 env                            | E3                 | Ad5          | HeLa S        | 1 mg/liter of culture |
| HIV-1 env                            | E4/ITR             | Ad7          | A549          | 4 μg/10⁶ cells |
| Polyomavirus middle T antigen        | E1                 | Ad5          | MLP + TPL     | 100 μg/100-mm dish |
| Soluble ciliary neurotrophic factor  | E1                 | Ad5          | CMV, CMV      | 42,000 U/10⁶ cells/day |
| Neurotrophin-3c                      | E1                 | Ad5          | RSV LTR       | 120 pg/10⁶ cells/hr |
| Fibroblast growth factor 4           | E1                 | Ad5          | Sr α          | 350 pg/10⁶ cells/hr |
| Human IL-2c                          | E1                 | Ad5          | CMV           | 2–3 μg/10⁶ cells |
| VEGFc                                | E1                 | Ad5          | CMV           | 1–2 μg/10⁶ cells |
| GM-CSFc                              | E1                 | Ad5          | CMV           | 2.2 μg/10⁶ cells/day |

| Reference                             |                     |              |               |               |
|--------------------------------------|----------------------|--------------|---------------|---------------|
| Dewar et al. (1989)                  |                     |              |               |               |
| Chanda et al. (1990)                 |                     |              |               |               |
| Schaffhausen et al. (1987)           |                     |              |               |               |
| Bramson et al. (1996)                |                     |              |               |               |
| Smith et al. (1996)                  |                     |              |               |               |
| Smith et al. (1996)                  |                     |              |               |               |
| Konishi et al. (1996)                |                     |              |               |               |
| Addison et al. (1995)                |                     |              |               |               |
| Mühlhauser et al. (1995)             |                     |              |               |               |
| Xing et al. (1996)                   |                     |              |               |               |

*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.

bYields reported by the authors were converted to μg recombinant protein/10⁶ cells.

cSecreted to medium.

dForskolin induced.

eNondefective 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 μg/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 μg/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 μg/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.,
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 co-workers (1996) inserted the genes for secrutable 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; Mühlauser 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 myositis, 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).
5. Adenoviral Vectors for Protein Expression

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-
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 XbaI 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 inadventently) 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 $\alpha_v$ 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 $\alpha_v$ integrins (Huang et al., 1995) and that there is an alternate pathway of infection using $\alpha_m$ 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.

References

Acsadi, G., Jani, A., Massie, B., Simoneau, M., Holland, P., Blaschuk, K., and Karpati, G. [1994]. A differential efficiency of adenovirus-mediated in vivo gene transfer into skeletal muscle cells of different maturity. Hum. Mol. Genet. 3, 579–584.

Addison, C. L., Braciak, T., Ralston, R., Muller, W. J., Gauldie, J., and Graham, F. L. [1995]. Intratumoral injection of an adenovirus expressing interleukin 2 induces regression and immunity in a murine breast cancer model. Proc. Natl. Acad. Sci. U.S.A. 69, 8522–8526.

Akli, S., Caillaud, C., Vigne, E., Stratford-Perricaudet, L. D., Poenaru, L., Perricaudet, M., Kahn, A., and Peschanski, M. R. [1993]. Transfer of a foreign gene into the brain using adenovirus vectors. Nat. Genet. 3, 224–228.

Akusjärvi, G., and Pettersson, U. [1979]. Sequence analysis of adenovirus DNA: Complete nucleotide sequence of the spliced 5’ noncoding region of adenovirus 2 hexon messenger RNA. Cell (Cambridge, Mass.) 16, 841–850.

Ali, M., Lemoine, N. R., and Ring, C. J. A. [1994]. The use of DNA viruses as vectors for gene therapy. Gene Ther. 1, 367–384.
Alkhatib, G., and Briedis, D. J. (1988). High-level eucaryotic in vivo expression of biologically active measles virus hemagglutinin by using an adenovirus Type 5 helper-free vector system. *J. Virol.* 62, 2718–2727.

Amalfitano, A., Begy, C. R., and Chamberlain, J. S. (1996). Improved adenovirus packaging cell lines to support the growth of replication-defective gene-delivery vectors. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3352–3356.

Anton, M., and Graham, F. L. (1995). Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: A molecular switch for control of gene expression. *J. Virol.* 69, 4600–4606.

Amalfitano, A., Begy, C. R., and Chamberlain, J. S. (1996). Improved adenovirus packaging cell lines to support the growth of replication-defective gene-delivery vectors. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3352–3356.

Anton, M., and Graham, F. L. (1995). Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: A molecular switch for control of gene expression. *J. Virol.* 69, 4600–4606.

Ballay, A., Leverero, M., Buendia, M.-A., Tiollais, P., and Perricaudet, M. (1985). *In vitro and in vivo* synthesis of the hepatitis B virus surface antigen and of the receptor for polymerized human serum albumin from recombinant human adenoviruses. *EMBO J.* 4, 3861–3865.

Bartlett, J. S., Sethna, M., Ramamurthy, L., Gowen, S. A., Samulski, R. J., and Marzluff, W. F. (1996). Efficient expression of protein coding genes from the murine U1 small nuclear RNA promoter. *Proc. Natl. Acad. Sci. U.S.A.* 93, 8852–8857.

Becker, T. C., BeltrandelRio, H., Noel, R. J., Johnson, J. H., and Newgard, C. B. (1994). Overexpression of hexokinase I in isolated islets of Langerhans via recombinant adenovirus. *J. Biol. Chem.* 269, 21234–21238.

Beckers, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. (1997). Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* 275, 1320–1323.

Berkner, K. L. (1992). Expression of heterologous sequences in adenoviral vectors. *Curr. Top. Microbiol. Immunol.* 158, 39–66.

Berkner, K. L., and Sharp, P. A. (1983). Generation of adenovirus by transfection of plasmids. *Nucleic Acids Res.* 11, 6003–6020.

Berkner, K. L., Schaffhausen, B. S., Roberts, T. M., and Sharp, P. A. (1987). Abundant expression of polyomavirus middle T antigen and dihydrofolate reductase in an adenovirus recombinant. *J. Virol.* 61, 1213–1220.

Bernstein, J. A., Porter, J. M., and Challberg, M. D. (1986). Template requirements for *in vivo* replication of adenovirus DNA. *Mol. Cell. Biol.* 6, 2115–2124.

Bett, A. J., Prevec, L., and Graham, F. L. (1993). Packaging capacity and stability of human adenovirus type 5 vectors. *J. Virol.* 67, 5911–5921.

Bett, A. J., Haddara, W., Prevec, L., and Graham, F. L. (1994). An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. U.S.A.* 91, 8802–8806.

Both, G. W., Lockett, L. J., Janardhana, V., Edwards, S. J., Bellamy, A. R., Graham, F. L., Prevec, L., and Andrew, M. E. (1993). Protective immunity to rotavirus-induced diarrhoea is passively transferred to newborn mice from naive dams vaccinated with a single dose of a recombinant adenovirus expressing rotavirus VP7sc. *Virology* 193, 940–950.

Braciak, T. A., Bacon, K., Xing, Z., Torry, D. J., Graham, F. L., Schall, T. J., Richards, C.D., Croitoru, K., and Gauldie, J. (1996). Overexpression of RANTES using a recombinant adenovirus vector induces the tissue-directed recruitment of monocytes to the lung. *J. Immunol.* 157, 5076–5084.
Bramson, J., Hitt, M., Gallichan, W. S., Rosenthal, K. L., Gauldie, J., and Graham, F. L. [1996]. Construction of a double recombinant adenovirus vector expressing a heterodimeric cytokine: In vitro and in vivo production of biologically active interleukin-12. *Hum. Gene Ther.* 7, 333-342.

Brough, D. E., Lizonova, A., Hsu, C., Kulesa, V. A., and Kovesdi, I. [1996]. A gene transfer vector-cell line system for complete functional complementation of adenovirus early regions E1 and E4. *J. Virol.* 70, 6497-6501.

Callebaut, P. and Pensaert, M. [1995]. Expression and immunogenicity of the spike glycoprotein of porcine respiratory coronavirus encoded in the E3 region of adenovirus. In "Corona- and Related Viruses" (P.J. Talbot and G.A. Levy, eds.), pp. 265-270. Plenum, New York.

Caravokyri, C. and Leppard, K. N. [1995]. Constitutive episomal expression of polypeptide IX [pIX] in a 293-based cell line complements the deficiency of pIX mutant adenovirus type 5. *J. Virol.* 69, 6627-6633.

Chanda, P. K., Natuk, R. J., Mason, B. B., Bhat, B. M., Greenberg, L., Dheer, S. K., Molnar-Kimber, K. L., Mizutani, S., Lubeck, M. D., Davis, A. R., and Hung, P. P. [1990]. High level expression of the envelope glycoproteins of the human immunodeficiency virus type 1 in presence of rev gene using helper-independent adenovirus Type 7 recombinants. *Virology* 175, 535-547.

Chartier, C., Degryse, E., Gantzer, M., Dieterlé, A., Pavirani, A., and Mehtali, M. [1996]. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J. Virol.* 70, 4805-4810.

Chen, G., Koyama, K., Yuan, Lee, Y., Zhou, Y.-T., O'Doherty, R. O., Newgard, C. B., and Unger, R. H. [1996]. Disappearance of body fat in normal rats induced by adenovirus-mediated leptin gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* 93, 14795-14799.

Chen, L., Chen, D., Manome, Y., Dong, Y., Fine, H. A., and Kufe, D. W. [1995]. Breast cancer selective gene expression and therapy mediated by recombinant adenoviruses containing the DF3/MUC1 promoter. *J. Clin. Invest.* 96, 2775-2782.

Cheng, S.-M., Lee, S.-G., Ronchetti-Blume, M., Virk, K. P., Mizutani, S., Eichberg, J. W., Davis, A., Hung, P. P., Hirsch, V. M., Chanock, R. M., Purcell, R. H., and Johnson, P. R. [1992]. Coexpression of the simian immunodeficiency virus Env and Rev proteins by a recombinant human adenovirus host range mutant. *J. Virol.* 66, 6721-6727.

Chow, L. T., Gelinas, R. E., Broker, T. R., and Roberts, R. J. [1997]. An amazing sequence arrangement at the S' ends of adenovirus 2 messenger RNA. *Cell (Cambridge, Mass.)* 12, 1-8.

Chroboczek, J., Bieber, F., and Jacrot, B. [1992]. The sequence of the genome of adenovirus type 5 and its comparison with the genome of adenovirus type 2. *Virology* 186, 280-285.

Connelly, S., Gardner, J.M., McClelland, A., and Kaleko, M. [1996]. High-level tissue-specific expression of functional human factor VIII in mice. *Hum. Gene Ther.* 7, 183-195.

Davis, A. R., Kostek, B., Mason, B. B., Hsiao, C. L., Morin, J., Dheer, S. K., and Hung, P. P. [1985]. Expression of hepatitis B surface antigen with a recombinant adenovirus. *Proc. Natl. Acad. Sci. U.S.A.* 82, 7560-7564.

Dewar, R. L., Natarajan, V., Vasudevacchari, M. B., and Salzman, N. P. [1989]. Synthesis and processing of human immunodeficiency virus type 1 envelope proteins encoded by a recombinant human adenovirus. *J. Virol.* 63, 129-136.
Dolph, P. J., Racaniello, V., Villamarin, A., Palladino, F., and Schneider, R.J. (1988). The adenovirus tripartite leader may eliminate the requirement for cap-binding protein complex during translation initiation. *J. Virol.* **62**, 2059-2066.

Dolph, P. J., Huang, J., and Schneider, R.J. (1990). Translation by the adenovirus tripartite leader: Elements which determine independence from cap-binding protein complex. *J. Virol.* **64**, 2669-2677.

Doran, S. E., Ren, X. D., Betz, A. L., Pagel, M. A., Neuwelt, E. A., Roessler, B. J., and Davidson, B. L. (1997). Gene expression from recombinant viral vectors in the central nervous system after blood-brain barrier disruption. *Neurosurgery* **36**, 965-970.

Dupuit, F., Zahm, J.-M., Pierrot, D., Brezillon, S., Bonnet, N., Imler, J.-L., Pavirani, A., and Puchelle, E. (1995). Regenerating cells in human airway surface epithelium represent preferential targets for recombinant adenovirus. *Hum. Gene Ther.* **6**, 1185-1193.

Everitt, E., Meador, S. A., and Levine, A. S. (1977). Synthesis and processing of the precursor to the major core protein of adenovirus type 2. *J. Virol.* **21**, 199-214.

Falgout, B., and Ketner, G. (1987). Adenovirus early region 4 is required for efficient virus particle assembly. *J. Virol.* **61**, 3759-3768.

Fallaux, F. J., Kranenburg, O., Cramer, S. J., Houweling, A., Van Ormondt, H., Hoeben, R. C., and van der Eb, A. J. (1996). Characterization of 911: A new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum. Gene Ther.* **7**, 215-222.

Fisher, K. J., Choi, H., Buda, J., Chen, S.-J., and Wilson, J. M. (1996). Recombinant adenovirus deleted of all viral genes for gene therapy of cystic fibrosis. *Virology* **217**, 11-22.

Fisher, S. A., and Watanabe, M. (1996). Expression of exogenous protein and analysis of morphogenesis in the developing chicken heart using an adenoviral vector. *Cardiovasc. Res.* **31**, E86-E95.

Fooks, A. R., Schadeke, E., Liebert, U. G., Dowsett, A. B., Rima, B. K., Steward, M., Stephenson, J.R., and Wilkinson, G. W. G. (1995). High-level expression of the measles virus nucleocapsid protein by using a replication-deficient adenovirus vector: Induction of an MHC-I-restricted CTL response and protection in a murine model. *Virology* **210**, 456-465.

Friedman, J. M., Babiss, L. E., Clayton, D. F., and Darnell, J. E., Jr. (1986). Cellular promoters incorporated into the adenovirus genome: Cell specificity of albumin and immunoglobulin expression. *Mol. Cell. Biol.* **6**, 3791-3797.

Gall, J., Kass-Eisler, A., Leinwand, L., and Falck-Pedersen, E. (1996). Adenovirus type 5 and 7 capsid chimera: Fiber replacement alters receptor tropism without affecting primary immune neutralization epitopes. *J. Virol.* **70**, 2116-2123.

Garner, A., Coté, J., Nadeau, I., Kamen, A., and Massie, B. (1994). Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells. *Cytotechnology* **15**, 145-155.

Gilardi, P., Courtney, M., Pavirani, A., and Perricaudet, M. (1990). Expression of human $\alpha_1$-antitrypsin using a recombinant adenovirus vector. *FEBS Lett.* **1**, 60-62.

Ginsberg, H. S., Lundholm-Beauchamp, U., Horswood, R. L., Pernis, B., Wold, W. S. M., Chanock, R. M., and Prince, G. A. (1989). Role of early region 3 (E3) in pathogenesis of adenovirus disease. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3823-3827.

Gluzman, Y., Reichl, H., and Solnick, D. (1982). Helper-free adenovirus type-5 vec-
tors. In “Eukaryotic Viral Vectors” [Y. Gluzman, ed.], pp. 187–192. Cold Spring Harbor Lab., Cold Spring Harbor, NY.

Goldman, M. J., and Wilson, J. M. (1995). Expression of αvβ5 integrin is necessary for efficient adenovirus-mediated gene transfer in the human airway. J. Virol. 69, 5951–5958.

Gorziglia, M. I., Kadan, M. J., Ye, S., Lim, J., Lee, G. M., Luthra, R., and Trapnell, B. C. (1996). Elimination of both E1 and E2a from adenovirus vectors further improves prospects for in vivo human gene therapy. J. Virol. 70, 4173–4178.

Graham, F. L. (1984). Covalently closed circles of human adenovirus DNA are infectious. EMBO J. 3, 2917–2922.

Graham, F. L. (1987). Growth of 293 cells in suspension culture. J. Gen. Virol. 68, 937–940.

Graham, F. L., and Prevec, L. (1995). Methods for construction of adenovirus vectors. Mol. Biotechnol. 3, 207–220.

Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. 36, 59–72.

Guo, Z. S., Wang, L.-H., Eisensmith, R. C., and Woo, S. L. C. (1996). Evaluation of promoter strength for hepatic gene expression in vivo following adenovirus-mediated gene transfer. Gene Therapy 3(9), 802–810.

Haddada, H., Cordier, L., and Perricaudet, M. [1995]. Gene therapy using adenovirus vectors. Curr. Top. Microbiol. Immunol. 199(3), 297–306.

Haecker, S. E., Stedman, H. H., Balice-Gordon, R. J., Smith, D. B. J., Grelleish, J. P., Mitchell, M. A., Wells, A., Sweeney, H. L., and Wilson, J. M. (1996). In vivo expression of full-length human dystrophin from adenoviral vectors deleted of all viral genes. Hum. Gene Ther. 7, 1907–1914.

Haj-Ahmad, Y. and Graham, F. L. (1986). Development of a helper-independent human adenovirus vector and its use in the transfer of the herpes simplex virus thymidine kinase gene. J. Virol. 57, 267–274.

Halbert, D. N., Cutt, J. R., and Shenk, T. (1985). Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. J. Virol. 56, 250–257.

Hanahan, D., and Gluzman, Y. (1984). Rescue of functional replication origins from embedded configurations in a plasmid carrying the adenovirus genome. Mol. Cell. Biol. 4, 302–309.

Hanke, T., Graham, F. L., Lulitanond, V., and Johnson, D. C. (1990). Herpes simplex virus IgG receptors induced using recombinant adenovirus vectors expressing glycoproteins E and I. Virology 177, 437–444.

Hashimoto, M., Aruga, J., Hosoya, Y., Kanegae, Y., Saito, I., and Mikoshiba, K. (1996). A neural cell-type-specific expression system using recombinant adenovirus vectors. Hum. Gene Ther. 7, 149–158.

Hay, R. T. (1985). The origin of adenovirus DNA replication: Minimal DNA sequence requirement in vivo. EMBO J. 4, 421–426.

Hayashi, Y., DePaoli, A. M., Burant, C. F., and Refetoff, S. (1994). Expression of a thyroid hormone-responsive recombinant gene introduced into adult mouse liver by replication-defective adenovirus can be regulated by endogenous thyroid hormone receptor. J. Biol. Chem. 269, 23872–23875.

He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1988). A simplified system for generating recombinant adenoviruses. Proc. Natl. Acad. Sci. U.S.A. 95, 2509–2514.
Hearing, P., Samulski, R. J., Wishart, W. L., and Shenk, T. [1987]. Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. *J. Virol.* 61, 2555-2558.

Horwitz, M. S. [1996]. Adenoviruses. In "Virology" [B. N. Fields, D. M. Knipe, and P. M. Howley, eds.], pp. 2149-2170. Lippincott-Raven, Philadelphia.

Huang, S., Endo, R. I., and Nemerow, G. R. [1995]. Upregulation of integrins αvβ3 and αvβ5 on human monocytes and T lymphocytes facilitates adenovirus-mediated gene delivery. *J. Virol.* 69, 2257-2263.

Huang, S., Kamata, T., Takada, Y., Ruggeri, Z. M., and Nemerow, G. R. [1996]. Adenovirus interaction with distinct integrins mediates separate events in cell entry and gene delivery to hematopoietic cells. *J. Virol.* 70, 4502-4508.

Huang, S., Jiang, Y., Li, Z., Nishida, E., Mathias, P., Lin, S., Ulevitch, R. J., Nemerow, G. R., and Han, J. [1997]. Apoptosis signaling pathway in T cells is composed of ICE/Ced-3 family proteases and MAP kinase kinase 6b. *Immunology* 6, 739-749.

Huyghe, B. G., Liu, X., Sutjipto, S., Sugarman, B.J., Horn, M. T., Shepard, M., Scandella, C. J., and Shabram, P. [1995]. Purification of type 5 recombinant adenovirus encoding human p53 by column chromatography. *Hum. Gene Ther.* 6, 1403-1416.

Imler, J.-L. [1995]. Adenovirus vectors as recombinant viral vaccines. *Vaccine* 13, 1143-1151.

Imler, J.-L., Chartier, C., Dieterlé, A., Dreyer, D., Mehtali, M., and Pavirani, A. [1995]. An efficient procedure to select and recover recombinant adenovirus vectors. *Gene Ther.* 2, 263-268.

Jaffe, H. A., Danel, C., Longenecker, G., Metzger, M., Setoguchi, Y., Rosenfeld, M. A., Gant, T. W., Thorgeirsson, S. S., Stratford-Perricaudet, L. D., Perricaudet, M., Pavirani, A., Lecocq, J.-P., and Crystal, R. G. [1992]. Adenovirus-mediated in vivo gene transfer and expression in normal rat liver. *Nat. Genet.* 1, 372-378.

Jiang, C., O'Connor, S.P., Armentano, D., Berthelette, P. B., Schiavi, S. C., Jefferson, D.M., Smith, A.E., Wadsworth, S.C., and Cheng, S.H. [1996]. Ability of adenovirus vectors containing different CFTR transcriptional cassettes to correct ion transport defects in CF cells. *Am. J. Physiol.* 4 [Pt. 1], L527-L537.

Johnson, D. C., Ghosh-Choudhury, G., Smiley, J. R., Fallis, L., and Graham, F. L. [1988]. Abundant expression of herpes simplex virus glycoprotein gB using an adenovirus vector. *Virology* 164, 1-14.

Jones, N. [1995]. Transcriptional modulation by the adenovirus E1A gene. *Curr. Top. Microbiol. Immunol.* 199(3), 59-80.

Kanai, F., Shiratori, Y., Yoshida, Y., Wakimoto, H., Hamada, H., Kanegae, Y., Saito, I., Nakabayashi, H., Tamaoki, T., Tanaka, T., Lan, K.-H., Kato, N., Shina, S., and Omata, M. [1996]. Gene therapy for α-fetoprotein-producing human hepatoma cells by adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene. *Hepatology* 23, 1359-1368.

Ketner, G., Spencer, F., Tugendreich, S., Connelly, C., and Hieter, P. [1994]. Efficient manipulation of the human adenovirus genome as an infectious yeast artificial chromosome clone. *Proc. Natl. Acad. Sci. U.S.A.* 91, 6186-6190.

Ko, S.-C., Choen, J., Kao, C., Gotoh, A., Shirakawa, T., Sikes, R.A., Karsenty, G., and Chung, L. W. K. [1996]. Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. *Cancer Res.* 56, 4614-4619.

Kobayashi, J., Applebaum-Bowden, D., Dugii, K. A., Brown, D. R., Kashyap, V. S.,
5. Adenoviral Vectors for Protein Expression

Parrott, C., Duarte, C., Maeda, N., and Santamarina-Fojo, S. [1996]. Analysis of protein structure-function in vivo. J. Biol. Chem. 271, 26296–26301.

Kochanek, S., Clemens, P. R., Mitani, K., Chen, H.-H., and Chan, S. [1997]. A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and β-galactosidase. Proc. Natl. Acad. Sci. U.S.A. 93, 5731–5736.

Konishi, H., Ochiya, T., Yasuda, Y., Sakamoto, H., Muto, T., Sugimura, T., and Terada, M. [1996]. HST-1/FGF-4 stimulates proliferation of megakaryocyte progenitors synergistically and promotes megakaryocyte maturation. Oncogene 13, 9–19.

Kozarsky, K. F., and Wilson, J. M. [1993]. Gene therapy: Adenovirus vectors. Curr. Opin. Genet. Dev. 3, 499–503.

Krasnykh, V. N., Mikheeva, G. V., Douglas, J. T., and Curiel, D. T. [1996]. Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. J. Virol. 70, 6839–6846.

Krougliak, V., and Graham, F. L. [1995]. Development of cell lines capable of complementing E1, E4, and protein IX defective adenovirus type 5 mutants. Hum. Gene Ther. 6, 1575–1586.

Kumar-Singh, R., and Chamberlain, J. S. [1997]. Encapsidated adenovirus minichromosomes allow delivery and expression of a 14 kb dystrophin cDNA to muscle cells. Hum. Mol. Genet. 5, 913–921.

Lan, K.-H., Kanai, F., Shiratori, Y., Okabe, S., Yoshida, Y., Wakimoto, H., Hamada, H., Tanaka, T., Ohashi, M., and Omata, M. [1996]. Tumor-specific gene expression in carcinoembryonic antigen-producing gastric cancer cells using adenovirus vectors. Gastroenterology 111, 1241–1251.

Langer, S. J., and Schaack, J. [1996]. 293 cell lines that inducibly express high levels of adenovirus type 5 precursor terminal protein. Virology 221, 172–179.

Lemarchand, P., Jaffe, H. A., Danel, C., Cid, M. D., Kleinman, H. K., Stratford-Perricaudet, L. D., Perricaudet, M., Pavirani, A., Lecocq, J.-P., and Crystal, R. G. [1992]. Adenovirus-mediated transfer of a recombinant human α1-antitrypsin cDNA to human endothelial cells. Proc. Natl. Acad. Sci. U.S.A. 89, 6482–6486.

Li, E., Stupack, D., Klemke, R., Cheresh, D. A., and Nemerson, G. R. [1998]. Adenovirus endocytosis via α1 integrins requires phosphoinositide-3-OH kinase. J. Virolgy 72, 2055–2061.

Lieber, A., Vrancken Peeters, M. J., and Kay, M. A. [1995]. Adenovirus-mediated transfer of the amphotropic retrovirus receptor cDNA increases retroviral transduction in cultured cells. Hum. Gene Ther. 6, 5–11.

Lindley, T., Virk, K. P., Ronchetti-Blume, M., Goldberg, K., Lee, S.-G., Eichberg, J. W., Hung, P. P., and Cheng, S.-M. [1994]. Construction and characterization of adenovirus co-expressing hepatitis B virus surface antigen and interleukin-6. Gene 138, 165–170.

Lisovoski, F., Cadusseau, J., Akli, S., Caillaud, C., Vigne, E., Poenaru, L., Stratford-Perricaudet, L., Perricaudet, M., Kahn, A., and Peschanski, M. [1994]. In vivo transfer of a marker gene to study motoneuronal development. NeuroReport 5, 1069–1072.

Mansour, S. L., Grodzicker, T., and Tjian, R. [1986]. Downstream sequences affect transcription initiation from the adenovirus major late promoter. Mol. Cell. Biol. 6, 2684–2694.

Mashhour, B., Couton, D., Perricaudet, M., and Briand, P. [1994]. In vivo adenovirus-mediated gene transfer into ocular tissues. Gene Ther. 1, 122–126.
Mason, B. B., Davis, A. R., Bhat, B. M., Hengalvala, M., Lubeck, M. D., Zandle, G., Kostek, B., Chodofsky, S., Dheer, S., Molnar-Kimber, K., Mizutani, S., and Hung, P. P. (1990). Adenovirus vaccine vectors expressing hepatitis B surface antigen: Importance of regulatory elements in the adenovirus major late intron. *Virology* **177**, 452–461.

McGrory, W. J., Bautista, D. S., and Graham, F. L. (1988). A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology* **163**, 614–617.

Michael, S. I., Hong, J. S., Curie1, D. T., and Engler, J. A. (1995). Addition of a short peptide ligand to the adenovirus fiber protein. *Gene Ther.* **2**, 660–668.

Mirza, M. A., and Weber, J. (1982). Structure of adenovirus chromatin. *Biochim. Biophys. Acta* **696**, 76–86.

Mittal, S. K., McDermott, M. R., Johnson, D. C., Prevec, L., and Graham, F. L. (1993). Monitoring foreign gene expression by a human adenovirus-based vector using the firefly luciferase gene as a reporter. *Virus Res.* **28**, 67–90.

Mittereder, N., March, K. L., and Trappnell, B. C. (1996). Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J. Virol.* **70**, 7498–7509.

Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., Takamori, K., Tokuda, C., and Saito, I. (1996). Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1320–1324.

Morin, J. E., Lubeck, M. D., Barton, J. E., Conley, A. J., Davis, A. R., and Hung, P. P. (1987). Recombinant adenovirus induces antibody response to hepatitis B virus surface antigen in hamsters. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4626–4630.

Moullier, P., Friedlander, G., Calise, D., Ranco, P., Perricaudet, M., and Ferry, N. (1994). Adenoviral-mediated gene transfer to renal tubular cells in vivo. *Kidney Int.* **45**, 1220–1225.

Mühlhauser, J., Merrill, M. J., Pili, R., Maeda, H., Bacic, M., Bewig, B., Passaniti, A., Edwards, N. A., Crystal, R. G., and Capogrossi, M. C. (1995). VEGF165 expressed by a replication-deficient recombinant adenovirus vector induces angiogenesis in vivo. *Circ. Res.* **77**, 1078–1086.

Parks, R. J., and Graham, F. L. (1997). A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. *J. Virol.* **71**, 3293–3298.

Parks, R. J., Chen, L., Anton, M., Sankar, U., Rudnicki, M. A., and Graham, F. L. (1996). A helper-dependent adenovirus vector system: Removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 13565–13570.

Philipson, L., Lonberg-Holm, K., and Petterson, U. (1968). Virus-receptor interaction in an adenovirus system. *J. Virol.* **2**, 1064–1075.

Ragot, T., Vincent, N., Chafey, P., Vigne, E., Gilgenkrantz, H., Contou, D., Cartand, J., Briand, P., Kaplan, J.-C., Perricaudet, M., and Kahn, A. (1993). Efficient adenovirus-mediated transfer of a human minidystrophin gene to skeletal muscle of *mdx* mice. *Nature (London)* **361**, 647–650.

Rekosh, D. M. K., Russell, W. C., and Bellet, A. J. D. (1977). Identification of a protein linked to the ends of adenovirus DNA. *Cell (Cambridge, Mass.)* **11**, 283–295.

Roberts, R. J., O'Neill, K. E., and Yen, C. T. (1984). DNA sequences from the adenovirus 2 genome. *J. Biol. Chem.* **259**, 13968–13975.
Rosenfeld, M. A., Siegfried, W., Yoshimura, K., Yoneyama, K., Fukayama, M., Stier, L. E., Pääkkö, P. K., Gilardi, P., Stratford-Perricaudet, L. D., Perricaudet, M., Jalilat, S., Pavirani, A., Lecocq, J.-P., and Crystal, R. G. (1991). Adenovirus-mediated transfer of a recombinant α1-antitrypsin gene to the lung epithelium in vivo. *Science* **252**, 431-434.

Saito, I., Oya, Y., Yamamoto, K., Yuasa, T., and Shimojo, H. (1985). Construction of nondefective adenovirus type 5 bearing a 2.8-kilobase hepatitis B virus DNA near the right end of its genome. *J. Virol.* **54**, 711–719.

Sakai, K., Mitani, K., and Miyazaki, J. (1995). Efficient regulation of gene expression by adenovirus vector-mediated delivery of the CRE recombinase. *Biochem. Biophys. Res. Commun.* **217**, 393–401.

Sakamoto, H., Ochiiya, T., Sato, Y., Tsukamoto, M., Konishi, H., Saito, I., Sugimura, T., and Terada, M. (1994). Adenovirus-mediated transfer of the HST-1 (FGF4) gene induces increased levels of platelet count in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12368–12372.

Schaack, J., Yew-Wai Ho, W., Freimuth, P., and Shenk, T. (1990). Adenovirus terminal protein mediates both nuclear matrix association and efficient transcription of adenovirus DNA. *Genes Dev.* **4**, 1197–1208.

Schaack, J., Langer, S., and Guo, X. (1995a). Efficient selection of recombinant adenoviruses by vectors that express β-galactosidase. *J. Virol.* **69**, 3920–3923.

Schaack, J., Guo, X., Yew-Wai Ho, W., Kariok, M., Chen, C., and Ornelles, D. (1995b). Adenovirus type 5 precursor terminal protein-expressing 293 and HeLa cell lines. *J. Virol.* **69**, 4079–4085.

Schaffhausen, B. S., Bockus, B. J., Berkner, K. L., Kaplan, D., and Roberts, T. M. (1987). Characterization of middle T antigen expressed by using an adenovirus expression system. *J. Virol.* **61**, 1221–1225.

Schneider, M., Graham, F. L., and Prevec, L. (1989). Expression of the glycoprotein of vesicular stomatitis virus by infectious adenovirus vectors. *J. Gen. Virol.* **70**, 417–427.

Setoguchi, Y., Danel, C., and Crystal, R. G. (1994a). Stimulation of erythropoiesis by in vivo gene therapy: Physiologic consequences of transfer of the human erythropoietin gene to experimental animals using an adenovirus vector. *Blood* **84**, 2946–2953.

Setoguchi, Y., Jaffe, H. A., Danel, C., and Crystal, R. G. (1994b). Ex vivo and in vivo gene transfer to the skin using replication-deficient recombinant adenovirus vectors. *J. Invest. Dermatol.* **102**, 415–421.

Shabram, P. W., Giroux, D. D., Goudreau, A. M., Gregory, R. J., Horn, M. T., Huyghe, B. G., Liu, X., Nunnally, M. H., Sugarman, B. J., and Sutjipto, S. (1997). Analytical anion-exchange HPLC of recombinant type-5 adenoviral particles. *Hum. Gene Ther.* **8**, 453–465.

Sharp, P. A., Moore, C., and Haverty, J. L. (1976). The infectivity of adenovirus 5 DNA-protein complex. *Virology* **75**, 442–456.

Sheay, W., Nelson, S., Martinez, I., Chu, T.-H. T., Bhatia, S., and Dornburg, R. (1993). Downstream insertion of the adenovirus tripartite leader sequence enhances expression in universal eukaryotic vectors. *BioTechniques* **15**, 856–862.

Shenk, T. (1996). Adenoviridae: The viruses and their replication. In “Virology” (B.N. Fields, D.M. Knipe, and P.M. Howley, eds.), pp. 2111–2148. Lippincott-Raven, Philadelphia.

Smith, G. M., Hale, J., Pasnikowski, E. M., Lindsay, R. M., Wong, V., and Rudge, J. S. (1996). Astrocytes infected with replication-defective adenovirus containing a se-
creted form of CNTF or NT3 show enhanced support of neuronal populations in vitro. Exp. Neurol. 139, 156-166.

Stevenson, S. C., Rollence, M., Marshall-Neff, J., and McClelland, A. (1997). Selective targeting of human cells by a chimeric adenovirus vector containing a modified fiber protein. J. Virol. 71, 4782-4790.

Stow, N. D. (1981). Cloning of a DNA fragment from the left-hand terminus of the adenovirus type 2 genome and its use in site-directed mutagenesis. J. Virol. 37, 171-180.

Stratford-Perricaudet, L. D., Makeh, I., Perricaudet, M., and Briand, P. (1992). Widespread long-term gene transfer to mouse skeletal muscles and heart. J. Clin. Invest. 90, 626-630.

Thiel, J. F. and Smith, K. O. (1967). Fluorescent focus assay of viruses on cell monolayers in plastic petri plates. Proc. Soc. Exp. Biol. Med. 125, 892-895.

Tollefson, A. E., Ryerse, J. S., Scaria, A., Hermiston, T. W., and Wold, W. S. M. (1996). The E3 11.6 KDa adenovirus death protein (ADP) is required for efficient cell death: Characterization of cells infected with adp mutants. Virology 220, 152-162.

Tollefson, A. E., Hermiston, T. W., Lichtenstein, D. L., Colle, C. F., Tripp, R. A., Dimitrov, T., Toth, K., Wells, C. E., Doherty, P. C., and Wold, W. S. N. (1998). Forced degradation of Fas inhibits apoptosis in adenovirus-infected cells. Nature 392, 726-730.

Tomko, R. P., Xu, R., and Philipson, L. (1997). HCAR and MAR: The human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc. Natl. Acad. Sci. U.S.A. 94, 3352-3356.

Tsukui, T., Miyake, S., Azuma, S., Ichise, H., and Saito, I. (1995). Gene transfer and expression in mouse preimplantation embryos by recombinant adenovirus vector. Mol. Reprod. Develop. 42, 291-297.

Van der Vliet, P. C. (1995). Adenovirus DNA replication. Curr. Top. Microbiol. Immunol. 199(2), 1-30.

van Oostrum, J. and Burnett, R. M. (1985). Molecular composition of the adenovirus type 2 virion. J. Virol. 56, 439-448.

Varley, A. W., Coulthard, M.G., Meidell, R. S., Gerard, R. D., and Munford, R. S. (1995). Inflammation-induced recombinant protein expression in vivo using promoters from acute-phase protein genes. Proc. Natl. Acad. Sci. U.S.A. 92, 5346-5350.

Von Seggern, D. J., Kehler, J., Endo, R. I., and Nemerow, G. R. (1998). Complementation of a fiber mutant adenovirus by packaging cell lines stably expressing the Ad5 fiber protein. J. Gen. Virol. 79, 1461-1468.

Wang, Q., Jia, X.-C., and Finer, M. H. (1995). A packaging cell line for propagation of recombinant adenovirus vectors containing two lethal gene-region deletions. Gene Ther. 2, 775-783.

Wang, Y., Krushel, L. A., and Edelman, G. M. (1996). Targeted DNA recombination in vivo using an adenovirus carrying the cre recombinase gene. Proc. Natl. Acad. Sci. U.S.A. 93, 3932-3936.

Warnes, A., Fooks, A. R., and Stephenson, J. R. (1994). Production of measles nucleoprotein in different expression systems and its use as a diagnostic reagent. J. Virol. Methods 49, 257-268.

Weiden, M. D., and Ginsberg, H. S. (1994). Deletion of the E4 region of the genome produces adenovirus DNA concatemers. Proc. Natl. Acad. Sci. U.S.A. 91, 153-157.

Weinberg, D. H., and Ketner, G. (1983). A cell line that supports the growth of a de-
fective early region 4 deletion mutant of human adenovirus type 2. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5383–5386.

Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. [1993]. Integrins αβ3 and αβ5 promote adenovirus internalization but not virus attachment. *Cell (Cambridge, Mass.)* **73**, 309–319.

Wickham, T. J., Segal, D. M., Roelvink, P. W., Carrion, M. E., Lizonova, A., Lee, G. M., and Kovesdi, I. [1996]. Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. *J. Virol.* **70**, 6831–6838.

Wilkinson, G. W. G., and Akrigg, A. [1992]. Constitutive and enhanced expression from the CMV major IE promoter in a defective adenovirus vector. *Nucleic Acids Res.* **20**, 2233–2239.

Wold, W. S. M. [1993]. Adenovirus genes that modulate the sensitivity of virus-infected cells to lysis by TNF. *J. Cell. Biochem.* **53**, 329–335.

Wrighton, C. J., Hofer-Warbinek, R., Moll, T., Eytner, R., Bach, F. H., and deMartin, R. [1996]. Inhibition of endothelial cell activation by adenovirus-mediated expression of IκBα, an inhibitor of the transcription factor NF-κB. *J. Exp. Med.* **183**, 1013–1022.

Xia, D., Henry, L. J., Gerard, R. D., and Deisenhofer, J. [1994]. Crystal structure of the receptor-binding domain of adenovirus type 5 fiber protein at 1.7 Å resolution. *Structure* **2**, 1259–1270.

Xing, Z., Braciak, T., Jordana, M., Croitoru, K., Graham, F. L., and Gauldie, J. [1994]. Adenovirus-mediated cytokine gene transfer at tissue sites. *J. Immunol.* **153**, 4059–4069.

Xing, Z., Ohkawara, Y., Jordana, M., Graham, F. L., and Gauldie, J. [1996]. Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *J. Clin. Invest.* **97**, 1102–1110.

Yajima, H., Kosukegawa, A., Hoque, M. M., Shimojima, T., Ishizu, K., Takayama, M., Sasaki, Y., Sakai, H., Otsuka, M., Inokuchi, S., and Handa, H. [1996]. Construction and characterization of a recombinant adenovirus vector carrying the human preproinsulin gene under the control of the metallothionein gene promoter. *Biochem. Biophys. Res. Commun.* **229**, 778–787.

Yamamoto, H., Ueno, H., Ooshima, A., and Takeshita, A. [1996]. Adenovirus-mediated transfer of a truncated transforming growth factor-β (TGF-β) Type II receptor completely and specifically abolished diverse signaling by TGF-β in vascular wall cells in primary culture. *J. Biol. Chem.* **271**, 16253–16259.

Yang, Y., Nunes, F., Berencsi, K., Furth, E. E., Gőnczöl, E., and Wilson, J. M. [1994]. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4407–4411.

Yang, Y., Li, Q., Ertl, H. C., and Wilson, J. M. [1995]. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J. Virol.* **69**, 2004–2015.

Yasukawa, M., Inoue, Y., Ohminami, H., Sada, E., Miyake, K., Tohyama, T., Shima-da, T., and Fujita, S. [1997]. Human herpesvirus 7 infection of lymphoid and myeloid cell lines transduced with an adenovirus vector containing the CD4 gene. *J. Virol.* **71**, 1708–1712.

Ye, W. W., Mason, B. B., Chengalvala, M., Cheng, S.-M., Zandle, G., Lubeck, M. D., Lee, S.-G., Mizutani, S., Davis, A. R., and Hung, P. P. [1991]. Co-expression of hepatitis B virus antigens by a non-defective adenovirus vaccine vector. *Arch. Virol.* **118**, 11–27.

Yeh, P., Dedieu, J.-F., Orsini, C., Vigne, E., Denefle, P., and Perricaudet, M. [1996]. Efficient dual transcomplementation of adenovirus E1 and E4 regions from a
293-derived cell line expressing a minimal E4 functional unit. *J. Virol.* **70**, 559–565.

Zain, S., Sambrook, J., Roberts, R. J., Keller, W., Fried, M., and Dunn, A. R. (1979). Nucleotide sequence analysis of the leader segments in a cloned copy of adenovirus 2 fiber mRNA. *Cell (Cambridge, Mass.)* **16**, 851–861.

Zhang, W.-W., Fang, X., Branch, C. D., Mazur, W., French, B. A., and Roth, J. A. (1993). Generation and identification of recombinant adenovirus by liposome-mediated transfection and PCR analysis. *BioTechniques* **15**, 868–872.

Zhang, Y., Feigenblum, D., and Schneider, R. J. (1994). A late adenovirus factor induces eIF-4E dephosphorylation and inhibition of cell protein synthesis. *J. Virol.* **68**, 7040–7050.

Zhao, Z., Bouchard, P., Diltz, C. D., Shen, S.-H., and Fischer, E. H. (1993). Purification and characterization of a protein tyrosine phosphatase containing SH2 domains. *J. Biol. Chem.* **268**, 2816–2820.

Zhou, H., O'Neal, W., Morral, N., and Beaudet, A. L. (1996). Development of a complementing cell line and a system for construction of adenovirus vectors with E1 and E2a deleted. *J. Virol.* **70**, 7030–7038.