Gene Therapy Technologies, Applications and Regulations. Edited by Anthony Meager

Print ISBN 0-471-976709-2 Electronic ISBN 0-470-84238-5

5 Adenoviral Vectors

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5.1 INTRODUCTION

Adenoviral vectors are among the most promising gene transfer vehicles for direct, *in vivo* gene therapy for the treatment of a diverse array of human diseases, including monogenic inherited disorders such as cystic fibrosis, Duchenne muscular dystrophy, and hemophilias A and B, as well as acquired diseases such as cancer (for review see Trapnell and Gorziglia, 1994; Wilson, 1995). In addition, the use of adenoviral vectors for *ex vivo* therapies also has been evaluated. Adenoviral vectors offer many advantages over other gene delivery systems. Adenoviral vectors can transduce a wide spectrum of cell types and do not require division of the target cell for gene transfer or expression. The adenovirus chromosome remains episomal in the transduced cell, thus avoiding the possibility of insertional mutagenesis (Gingsberg, 1984; Horowitz, 1990). Adenoviruses can be rendered replication deficient by deletion of critical viral regulatory genes (Berkner, 1988; Gorziglia *et al*., 1996), which allow the vectors to accommodate heterologous DNA inserts of more than 8 kb, depending on the extent of the viral gene deletions. Replication-deficient vectors can be produced, in a variety of complementing cell lines, easily and at high titers ($\sim 10^{11}$ infectious units per milliter). Finally, adenovirus infection has never been associated with any type of tumor in humans, as adenoviruses have been evaluated extensively as live vaccines in millions of individuals (Straus, 1984). The main disadvantage of adenoviral vectors is that the host immune response, in general, appears to limit the duration of transgene expression and the ability to re-administer the vector. Efforts currently are directed toward reducing the immunogenicity of the vectors and to developing strategies to circumvent the host immune response. Within the past several years, numerous reports of successful adenoviral vector-mediated delivery and expression of a wide variety of heterologous genes in several mammalian species have appeared (Trapnell and Gorziglia, 1994; Wilson, 1995). To date, adenoviral vectors are employed in or have been proposed for use in human clinical trials for the treatment of cystic

fibrosis, ornithine transcarbamolyase deficiency, and a diverse array of cancers, including breast, colon, glioblastoma, head and neck, liver, melanoma, neuroblastoma, ovarian, prostate (Marcel and Grausz, 1996), and lung (Cohen-Haguenauer, 1996). However, adenoviral vector-mediated gene therapy is at an early stage and nearly all of the studies consist of phase I trials, with the goal of establishing safety, rather than efficacy, of the procedures.

5.2 STRUCTURE AND GENOMIC ORGANIZATION OF HUMAN ADENOVIRUSES

Human adenoviruses are non-enveloped DNA viruses belonging to the parvovirideae family (reviewed by Ginsburg, 1984; Horowitz , 1990). The virion is 80–90 nm in diameter with a spiked, icosohedral morphology and a molecular mass of 175–185 $\times$ 10^6 Da. Adenoviruses have been classified serologically into nearly 50 distinct serotypes, subgrouped A through G (Wadell, 1984). While viruses of the subgroups A and B have been shown to have oncogenic potential in newborn rodents, viruses used as gene transfer vectors belong to the non-tumorigenic subgroup C. Subgroup C adenoviruses cause mild respiratory disease in humans, and account for 5–15% of occurrences of the 'common cold' (Straus, 1984).

Since the isolation of adenoviruses over 40 years ago (Rowe *et al.*, 1953), knowledge of the adenovirus genetic system has increased dramatically, in part due to early interest in adenovirus as a model of eukaryotic gene expression (Ginsburg, 1984; Horowitz, 1990). Adenovirus infects target cells by attachment to the coxsackie and adenovirus receptor (CAR) on the cell surface (Bergelson *et al.*, 1997), internalization via clatharine-coated pits into endosomes, escape of the virion into the cytoplasm by endosomolysis, translocation to the nuclear membrane via nuclear targeting signals within the capsid polypeptides, and transport of the viral genome into the cell nucleus, where the viral genome remains episomal.

The genome consists of a 36 kb linear, double-stranded DNA molecule, which contains, covalently attached at each 5′ end, a 55 kDa terminal protein important for viral DNA replication. The viral DNA contains short, inverted terminal repeats (ITRs) at each end of the genome that are required for DNA replication. The viral genome is organized into four distinct early regions, termed E1–E4, and five alternatively spliced late regions, L1–L5, based on expression before or after the initiation of viral DNA synthesis, in addition to several minor intermediate and/or late transcription units (Figure 5.1). In general, early gene products alter host cell biology to support virus production, while the late genes encode most of the structural proteins which comprise the virion and aid in viral assembly. Immediately upon entry of the viral genome into the nucleus, the El region is actively transcribed by cellular

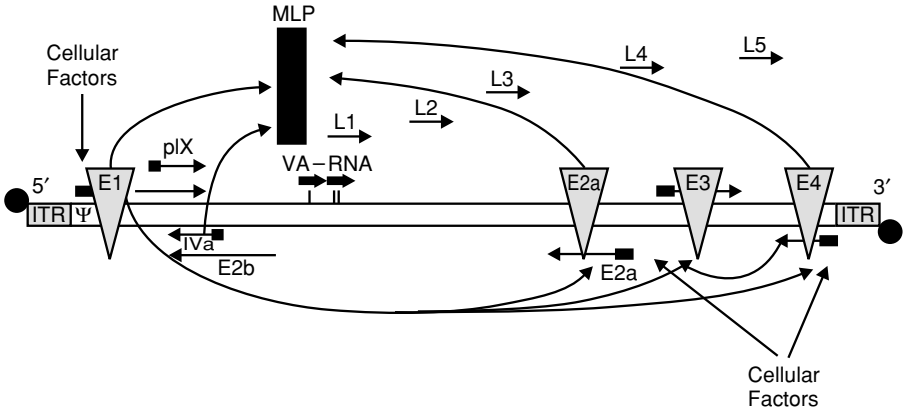

Figure 5.1. Schematic representation of the adenovirus genome and regulation of transcription. The adenoviral genome is represented by the double parallel lines, and transcription units are represented by the horizontal arrows. The arrowheads indicate the direction of transcription. Curved arrows represent transactivation pathways. Intermediate and/or minor transcription units, pIX, IVa, and VA-RNA (I-III), are displayed. The viral inverted terminal repeat regions are indicated (ITR) as well as the 55 kDa terminal proteins covalently attached to the 5′ ends of the genome. Both ITR regions and the presence of terminal protein are required for efficient viral replication. The majority of first-generation adenoviral (Av1) vectors contain deletions in both the E1 and E3 regions; however, a few Av1 vectors contain only the E1 region deletion. Removal of the E1 region renders the virus replication impaired, as the E1 gene products upregulate the majority of adenoviral transcription units, such as E2, E3, E4, and the major late promoter (MLP), indicated by the curved arrows. E1, E2, and E4 are activated by host cell endogenous transcription factors. Third-generation vectors (Av3) contain, in addition to deletions of E1 and E3, a partial deletion of the E2a coding region. Vectors with combined deletions of E1/E3/E4 have been generated also. These further attenuated vectors reduce expression of the late transcription units by preventing upregulation of the MLP by the E2a or E4 gene products, respectively. Large triangles represent deletions of adenoviral regions; (■), promoters; (●), terminal protein.

transcription factors, and codes for proteins directly involved in the activation of the remaining early regions, E2, E3, and E4. The E1 gene products, encoded in the E1a and E1b regions, have been implicated in virus-induced transformation of cultured cells (Van der Eb *et al.*, 1977). The E2 region encodes proteins required for viral DNA replication, including a single-stranded DNA binding protein (E2a) and both the viral DNA polymerase and the 55 kDa terminal protein (E2b). The E3 region is composed of a series of transcription units involved in evading host defense mechanisms that act to eliminate virus infected cells, and is dispensable for virus replication (Wold and Gooding, 1991). E4 gene products are involved in the regulation of viral and cellular protein expression, viral DNA replication, viral late

mRNA accumulation and protein synthesis, and the corresponding down-regulation of host protein synthesis. A recent report has demonstrated that an E4 protein, encoded in open reading frame 6, has oncogenic potential, similar to that described for the E1b gene product (Moore *et al.*, 1996). Expression of the early genes leads to DNA replication, approximately eight hours after infection, and subsequent activation of the late genes under the transcriptional control of the major late promoter, production of virus progeny, and finally, death of the host cell and virus release.

5.3 DESIGN AND CONSTRUCTION OF REPLICATION-DEFECTIVE HUMAN ADENOVIRAL VECTORS

Replication-deficient adenoviral vectors, similar to other viral vectors, are composed of the virion structure surrounding a modified viral genome. To date, most vector particles are based on the wild-type capsid structure which, in addition to protecting the viral DNA, provides the means to bind and enter (transduce) target cells. However, the viral genome has been modified substantially. These changes are designed to disable growth of the virus in target cells, by deleting viral functions critical to the regulation of DNA replication and viral gene expression, while maintaining the ability to grow in available packaging or helper cells. Deletion of such sequences provides space within the viral genome for insertion of exogenous DNA that encodes and enables appropriate expression of the gene of interest (transgene).

The subgroup C adenoviruses, serotypes 2 and 5 (Ad2 and Ad5), are among the best studied adenoviruses, and the viruses used most commonly as gene transfer vectors. The vast majority of adenoviral vectors for gene therapy are E1 replacement vectors, where the transgene is inserted in place of the E1 region. This E1 region deletion includes the entire E1a gene and approximately 60% of the E1b gene. The vectors retain the immediate 5′ end of the viral genome, including the left inverted terminal repeat (ITR) and encapsidation signal (ψ), sequences required for packaging, and the overlapping E1 enhancer region, in addition to the remainder of the viral genome (Figure 5.1). As the E1 gene products lead to sequential activation of the major transcription units, deletion of this region greatly reduces early and late gene expression and renders the virus severely replication impaired (Berkner, 1988). To provide more space within the adenoviral vectors for insertion of the transgene, the E3 region, not required for viral replication or growth, is also frequently deleted. Occasionally, the transgene is inserted into this E3 region deletion. Adenoviral vectors lacking only E1 and E3 regions are referred to as first generation, or Av1, vectors. Adenoviruses can effectively package DNA up to 105% of the genome size (Bett *et al.*, 1993), allowing the accommodation of up to 8 kb of exogenous DNA in E1/E3 deleted Av1 vectors.

Av1 vectors have been constructed in several ways. The most commonly employed technique is through homologous recombination of the viral genome with a plasmid bearing the transgene (Berkner, 1988). Briefly, the transgene transcription unit is inserted into a plasmid containing a segment of the viral genome. This plasmid is co-transfected into a permissive cell line (see below), with appropriately prepared viral DNA, by conventional DNA transfer techniques. In the cells, homologous recombination results in the rescue of the cloned viral sequences and the transgene into the viral genome, thus generating the *recombinant* adenoviral vector (Figure 5.2). To generate Av1 vectors with the transgene insertion in the E1 region, the plasmid must contain the left end of the viral genome, including the ITR and encapsidation signal, the transgene expression cassette, and approximately 1 kb or more (Berkner and Sharp, 1983) of downstream viral DNA sequence. The viral DNA used in the co-transfection is prepared by restriction enzyme cleavage to remove the left end of the viral genome. Preparation of the viral DNA in this manner reduces the infectivity of the parental viral DNA and therefore enhances the efficiency of isolation of recombinant vectors resulting from *in vitro* recombination. The large DNA fragment (~ 34 kb) isolated from the Ad5 E3 deletion mutant, dl327 (Thimmappaya *et al.*, 1982), cleaved with the restriction enzyme Cla I and gel-purified, is used routinely for this purpose (Smith *et al.*, 1993). In a similar manner, heterologous DNA can be inserted into the E3 region. In this case, however, the plasmid contains the right end portion of the adenovirus genome, and the viral DNA is prepared by restriction enzyme digestion to remove the right end of the genome. Alternatively, restriction enzyme digested viral DNA isolated to retain the 55 kDa terminal protein covalently attached to the 5′ end of the viral genome has been used in co-transfection procedures (Sharp *et al.*, 1976). While the infectivity of viral DNA containing terminal protein is at least 10-fold higher than protease-digested genomic DNA (Sharp *et al.*, 1976), a high background of non-recombinant virus due to the presence of small amounts of highly infectious uncleaved viral DNA may be detected (Berkner and Sharp, 1983). Following transfection, the resulting viral plaques are isolated, expanded, and screened by restriction analysis, Southern blotting, and/or transgene expression to identify the desired recombinant vector (for detailed protocols, see Graham and Prevec, 1991).

Other methods employed to generate adenoviral vectors include the direct cloning of plasmid sequences into the adenoviral genome via appropriate restriction sites, with subsequent transfection of permissive cells with the *in vitro* ligated DNA. Such a procedure was one of the original methods described for the generation of adenovirus host-range mutants (Stow, 1981). However, due to the large size of the adenovirus genome, the availability of unique or infrequent restriction sites is limited.

A significant problem associated with the generation of adenoviral vectors

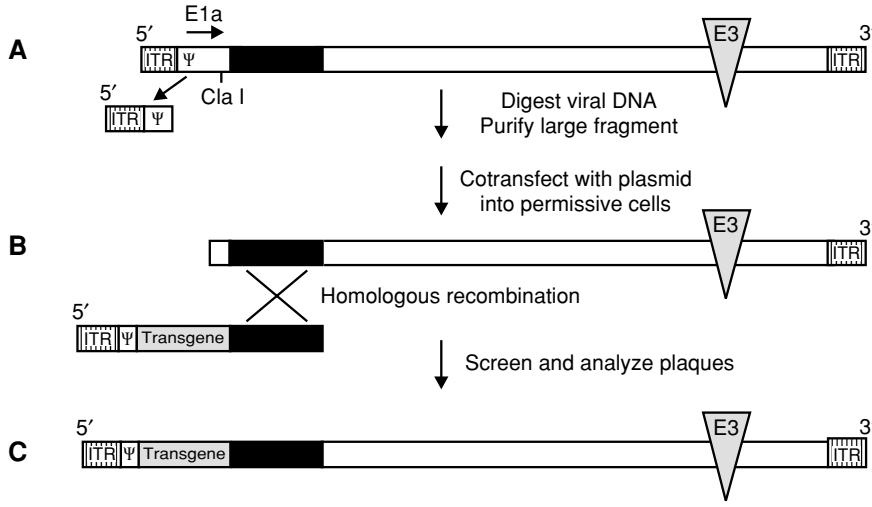

Figure 5.2. Generation of recombinant adenoviruses. (A) Adenoviral genomic DNA is prepared by Cla I digestion of the E3 region deletion mutant Ad5-dl327 to remove the left end of the viral genome, and to render the viral DNA non-infectious. (B) A plasmid containing a viral ITR and packaging signal, the transgene transcription unit, and one kilobase or more of adenoviral DNA is co-transfected with the prepared viral DNA into permissive cells. (C) As a result of intercellular homologous recombination, a recombinant E1/E3 deleted adenoviral vector containing the transgene replacing the viral E1 genes is generated. The horizontal arrow represents the E1 transcription unit. (▥), viral ITR; (Ψ), viral packaging signal; (▩), exogenous transgene transcription unit; (■), homologous recombination region; (□),adenoviral genomic DNA. Large triangles represent deletion of the adenoviral E3 region.

by direct ligation or homologous recombination of plasmid and viral DNA is the rescue of parental virus caused by the presence of contaminating infectious parental viral DNA. The recovery of the recombinant virus, in the presence of such background, can be difficult, especially if the engineered vector grows more slowly than the competing, parental virus. Several clever approaches have been utilized to solve this problem. For example, parental vectors with impaired replication have been employed to eliminate the possibility of a growth advantage over the desired recombinant (Berkner, 1988). Likewise, an Av1 vector expressing a conditional lethal phenotype was developed by the inclusion of the herpes simplex virus thymidine kinase (TK) gene into the viral genome (Imler *et al.*, 1995). The expression of the TK gene product prevented viral replication in the presence of the nucleoside analog ganciclovir, allowing for the selection of only the recombinant vectors in which the TK gene had been replaced (Imler *et al.*, 1995). Another approach is to circumvent the need for infectious viral DNA with

the use of plasmids that comprise the entire adenovirus genome (Ghosh-Choudhury *et al.*, 1986; Bett *et al.*, 1994). Infectious virus can be generated upon transfection of one or more adenoviral DNA-containing plasmids. However, the transfection efficiency of plasmid DNA is much lower than that of viral DNA (Ghosh-Choudhury *et al.*, 1986; Bett *et al.*, 1994). Similarly, the complete genome of Ad2 was constructed as a yeast artificial chromosome (YAC), and shown to be infectious (Ketner *et al.*, 1994). Using conventional yeast genetic techniques, the viral sequences contained in the YAC can be modified, and adenoviral vectors recovered from the YAC clones (Ketner *et al.*, 1994).

The transgene transcriptional unit consists of the elements required to enable appropriate expression of the transgene such as the promoter, the gene of interest, and a polyadenylation signal, and, in most instances, is designed to maximize the expression of the exogenous gene. A large variety of promoters have been utilized for transgene expression, the choice of which depends on the application and the target tissue. Strong, constitutively expressed viral promoters such as the adenovirus major later promoter (Stratford-Perricaudet *et al.*, 1990), the Rous sarcoma virus promoter (Stratford-Perricaudet *et al.*, 1992; Smith *et al.*, 1993), the cytomegalovirus (CMV) promoter (Herz and Gerard, 1993) and a hybrid CMV enhancer/β-actin promoter (Kozarsky *et al.*, 1993) have been incorporated into recombinant adenoviral vectors. More recently, the use of cellular, tissue-specific promoters such as the liver-specific albumin promoter (Connelly *et al.*, 1995, 1996a,b,c), lung-specific cystic fibrosis transmembrane conductance regulator promoter (Imler *et al.*, 1996; Suzuki *et al.*, 1996), the cardiac muscle-specific myosin light chain-2 promoter (Rothmann *et al.*, 1996), and the hepatoma-specific α-fetoprotein promoter (Kaneko *et al.*, 1995; Arbuthnot *et al.*, 1996) has been described. Finally, regulatable promoters responsive to hormonal (Hayashi *et al.*, 1994) or pharmacological agents (Suzuki *et al.*, 1996) have been incorporated into adenoviral vectors. The inclusion of tissue-specific and/or regulatable promoters to the transgene expression cassette avoids the unknown consequences of overexpression of genes in tissues other than the targeted organ, and may, therefore, increase the safety of such vectors. An additional approach shown to increase the potency of the transgene in adenoviral vectors is the introduction of genomic elements into the expression cassette. For example, the addition of an intron to the human factor VIII (FVIII) cDNA boosted *in vivo* expression approximately 10-fold (Connelly *et al.*, 1996b), and the inclusion of the human factor IX (FIX) truncated first intron and 5′ and 3′ untranslated regions to the human FIX cDNA functioned synergistically to increase human FIX plasma levels in transduced mice approximately 2000-fold (M. Kaleko, unpublished). Finally, a variety of signals have been used to direct polyadenylation such as the simian virus 40 polyadenylation signal.

5.4 PROPAGATION AND PURIFICATION OF ADENOVIRAL VECTORS

The propagation of Av1 adenoviral vectors, rendered almost completely replication defective by the deletion of the E1 region, requires the generation of cell lines to complement the E1 functions in *trans*. Several human cell lines that constitutively express the E1 proteins have been established. To date, the most widely used cell line, 293, consists of human embryonic kidney cells transformed with sheared Ad5 DNA that express the left 11% of the Ad5 genome (Graham *et al.*, 1977). While 293 cells allow replication of Av1 vectors to high titers, this cell line is not ideal for large-scale vector production. Recombination between homologous E1 region sequences encoded in the vectors with those inserted in the 293 cell genome has the potential to generate replication-competent adenoviruses (RCA) (Lochmüller *et al.*, 1994). RCA are to be avoided as uncontrolled replication of the 'reverse recombinant' also would allow replication of the vector. Furthermore, the presence of RCA in preparations of adenoviral vectors was shown to induce significant tissue damage *in vivo* (Lochmüller *et al.*, 1994). The generation of RCA may be prevented by elimination of sequence homology between the vector DNA and the adenovirus sequences in the genome of the complementing cells. Recently, the development of Av1 vectors containing more extensive deletions of the E1 region, and alternative cell lines for the propagation of these vectors have been described (Fallaux *et al.*, 1996; Imler *et al.*, 1996). Cell lines that express, in addition to the adenovirus E1 region, E2a or an E4 protein encoded in open reading frame 6 (orf6) of the E4 region have been generated and used to propagate adenoviral vectors containing deletions of the E2a or the E4 regions (see below) (Armentano *et al.*, 1995, Wang *et al.*, 1995; Gao *et al.*, 1996; Gorziglia *et al.*, 1996; Yeh *et al.*, 1996). As constitutive expression of adenoviral proteins, in many instances, is toxic, the development of cell lines that express multiple adenoviral proteins is difficult (Wang and Finer, 1996). Unlike retroviral vectors, the stability of the adenovirus virion allows extensive purification and concentration without significant loss of activity. Procedures for adenoviral vector purification involve harvest and disruption of infected cells using multiple freeze and thaw cycles (Smith, 1995), or sonication (Kanegae *et al.*, 1994), and removal of the cell debris by centrifugation. Vector is purified and concentrated in one to three CsCl centrifugation steps, followed by dialysis or chromatography. However, for large-scale manufacturing, chromatographic methods which avoid CsCl centrifugation are desirable. Vector concentration is determined spectrophotometrically, to evaluate particle number, and biologically, to measure infectivity by gene transfer or plaque assay (Mittereder *et al.*, 1996). For use in human clinical trials, preparations must meet a Food and Drug Administration (FDA) requirement of a particle to infectious unit ratio of less than 100

(Smith, 1995; Mittereder *et al.*, 1996). Concentrated vector preparations containing 10^{11} plaque forming units per milliliter can be obtained routinely, several orders of magnitude greater than possible with retroviral or adeno-associated viral vectors. Vector preparations are tested extensively for RCA by E1-specific polymerase chain reaction (PCR) analysis (Tolstoshev *et al.*, 1994) and plaque assay. The current FDA requirement for clinical lots of adenovirus is one or fewer RCA per vector dose (Smith, 1995).

5.5 *IN VIVO* ADENOVIRUS-MEDIATED GENE TRANSFER

Within the past several years, extensive evaluation of the efficacy and safety of Av1 vectors administered *in vivo* to a wide spectrum of animal species and humans has resulted in increased understanding of adenoviral vector biology (Trapnell and Gorziglia, 1994; Wilson, 1995). Adenoviral vector-mediated expression of a diverse array of transgenes, including reporter genes, such as β-galactosidase and luciferase, has been detected in numerous tissues, the most highly studied of which are the lung and the liver. Adenovirus-mediated gene expression is dependent upon the animal model, the vector, and dose utilized, but in general initial expression is high. The duration of gene expression, however, varies widely. In immunocompromised or immunologically immature mice, expression is generally longer term than that detected in adult mice, where expression declines greatly within two to three weeks, suggesting that the immune system limits vector persistence (Trapnell and Gorziglia, 1994; Wilson, 1995). However, after intravenous delivery of Av1 vectors to normal adult mice, long-term expression of human blood clotting FVIII (Connelly *et al.*, 1996a) and FIX (M. Kaleko, unpublished) has been reported. Transduction of muscle with Av1 vectors also, in some cases, resulted in persistent expression (Stratford-Perricaudet *et al.*, 1992; Ragot *et al.*, 1993; Vincent *et al.*, 1993; Tripathy *et al.*, 1996).

Interest in developing gene therapy for the two most common hereditary lung diseases, α_1-antitrypsin (α_1-AT) deficiency and cystic fibrosis (CF), has resulted in extensive analysis of adenoviral-mediated delivery of reporter genes, α_1-AT, and the human cystic fibrosis transmembrane conductance regulator (CFTR) cDNA to the lung. The initial studies were performed using the cotton rat lung as the animal model, as the cotton rat has been shown to display a sensitivity to infection with adenovirus similar to that seen in humans. Tracheal instillation of an α_1-AT-encoding Av1 vector resulted in expression of α_1-AT (Rosenfeld *et al.*, 1991). Similarly, *in vivo* administration of a CFTR-encoding adenoviral vector into the airway of the cotton rat resulted in CFTR expression and CFTR RNA detectable for six weeks (Rosenfeld *et al.*, 1992). Despite the high efficiency of transgene delivery to the lung, the duration of gene expression was short term, and associated with an acute

cellular inflammation within the pulmonary parenchyma in the cotton rat (Yei *et al.*, 1994b). Studies in non-human primates (Simon *et al.*, 1993) have demonstrated similar host immune responses, but with a more protracted time course. Administration of the CFTR-encoding Av1 vector to the nasal epithelia of human CF patients resulted in short-term correction of the chloride secretory defect with no evidence of vector-associated toxicity (Zabner *et al.*, 1993). In a separate study, an Av1 CFTR vector was administered to the nose and lung of CF patients; expression of CFTR RNA was detected in the nose two days after treatment, and one patient who received a higher vector dose to the lung developed an acute self-limited febrile response associated with a rise in adenovirus antibody titer and lung interleukin-6 levels (Crystal *et al.*, 1994).

A second major target organ for *in vivo* gene therapy is the liver. Many genes, including those encoding blood clotting factors, metabolic enzymes, and lipoproteins, are candidates for liver gene therapy. Notably, peripheral vein administration of adenoviral vectors to mice (Smith *et al.*, 1993), dogs (Connelly *et al.*, 1996c), and non-human primates (T.A.G. Smith, unpublished) results in efficient transduction of hepatocytes, demonstrating the feasibility of non-invasive, systemic adenoviral vector delivery for the treatment of liver disorders. Much progress has been made recently in adenoviral vector-mediated gene therapy for the hemophilias A and B, deficiencies of blood coagulation FVIII and FIX. Intravenous administration of low doses of potent Av1 vectors encoding the human blood clotting FVIII and FIX to normal adult mice resulted in expression of therapeutic levels of the clotting factors for at least five months (Connelly *et al.*, 1996a), and over one year (M. Kaleko, unpublished), respectively. However, when high, hepatotoxic, doses of the vectors were administered, the initial high levels of clotting factor expression declined rapidly to background suggesting that dose-dependent vector toxicity limited vector persistence (Smith *et al.*, 1993; Connelly *et al.*, 1995, 1996a). Administration of the FVIII adenoviral vector to FVIII-deficient dogs resulted in complete correction of the hemophiliac phenotype and high-level expression of human FVIII (Connelly, 1996c). However, FVIII expression in the dogs was short term due to the development of human FVIII inhibitory antibodies (Connelly *et al.*, 1996c). Treatment of hemophilia B dogs with a canine FIX-encoding Av1 vector resulted in transient phenotypic correction of the bleeding disorder, although, in this case, the presence of canine FIX-specific antibodies was not detected (Kay *et al.*, 1994).

In other models of liver-targeted disease, Av1 vectors including those encoding an ornithine transcarbamylase gene (Stratford-Perricaudet, 1990; Morsy *et al.*, 1993), a phenylalanine hydroxylase gene (Fang *et al.*, 1994), an apolipoprotein E gene (Stevenson *et al.*, 1995), and the human low density liproprotein (LDL) receptor gene (Ishibashi *et al.*, 1993) have been used to treat genetically engineered mice containing knockouts of the studied gene.

In all cases, phenotypic correction of the defect after adenoviral vector treatment was reported. However, in most instances, the therapeutic effect was transient. Similarly, treatment of the hyperlipidemic Watanabe rabbit with the LDL receptor-encoding Av1 vector resulted in significant lowering of the cholesterol levels lasting for three weeks (Kozarsky *et al.*, 1994).

Av1 vectors have been evaluated and shown to efficiently transduce a wide variety of tissues and organs in addition to the lung and liver, such as heart (Kass-Eisler *et al.*, 1993), skeletal and cardiac muscle (Stratford-Perricaudet *et al.*, 1992; Kass-Eisler *et al.*, 1993; Ragot *et al.*, 1993; Vincent *et al.*, 1993), bone marrow (Mitani *et al.*, 1994), brain (Le Gal La Salle *et al.*, 1993), CNS (Bajocchi *et al.*, 1993; Davidson *et al.*, 1993), endothelial cells (Lee *et al.*, 1993; Lemarchand *et al.*, 1993), kidney (Moullier *et al.*, 1994), retinal cells (Jomary *et al.*, 1994), and solid tumors (Haddada *et al.*, 1993; Brody *et al.*, 1994; Wills *et al.*, 1994).

Adenoviral vector-mediated therapy for acquired disorders, such as cancer, have also been explored using two different strategies, inducement of tumor cell-specific cytotoxicity and enhancement of existing host antitumor immunity. Tumor cell cytotoxicity has been induced by treatment of tumor tissue with adenoviral vectors encoding p53 (Wills *et al.*, 1994; Zhang *et al.*, 1995), or utilization of a combination of a herpes thymidine kinase gene and ganciclovir to successfully treat pre-established tumors in murine models (Chen *et al.*, 1995; Yee *et al.*, 1996). Tumor vaccination approaches include the use of an Av1 vector encoding an interleukin-2 cDNA, which was shown to enhance antitumor immunity in mice (Haddada *et al.*, 1993; Addison *et al.*, 1995; Cordier *et al.*, 1995; Huang *et al.*, 1996).

5.6 CIRCUMVENTING THE HOST IMMUNE RESPONSE TO *IN VIVO* ADENOVIRAL GENE TRANSFER

Despite the high transduction and transgene expression efficiency obtained with first-generation adenoviral vectors, the duration of gene expression, in many cases, is transient. This loss of expression is associated with direct toxicity of the vector (Connelly *et al.*, 1996a) and infiltration of inflammatory and immune cells (Yei *et al.*, 1994a; Yang *et al.*, 1994a,b, 1995a), resulting in destruction of the transduced cells. Characterization of cells transduced with Av1 vectors has revealed that although expression of most adenoviral genes is severely attenuated, a low level of expression of viral proteins (viral backbone gene products) can be detected (Mittereder *et al.*, 1994; Yang *et al.*, 1994a,b). These observations led to the suggestion that a cytotoxic T lymphocyte (CTL) response directed against cells expressing viral backbone genes resulted in elimination of the genetically modified cells (Yang *et al.*, 1994ab, 1995a, 1996c). Therefore, further attenuation of viral gene expression

may reduce host immune responses to transduced cells and increase the duration of transgene expression. Indeed, the development of a second-generation, Av2, adenoviral vector with a temperature-sensitive 72-kDa DNA-binding protein, encoded in the E2a region of the virus backbone, allowed prolonged expression in the livers of immune competent adult mice and was associated with a reduced CTL response (Engelhardt *et al.*, 1994ab, Yang *et al.*, 1994c; Ye *et al.*, 1996). However, analysis of the Av2 vector in a different mouse strain and in hemophilia B dogs revealed no difference in transgene expression persistence compared to similar Av1 vectors (Fang *et al.*, 1996). Recently, a third-generation, Av3, vector, containing deletions in the E1, E2a, and E3 regions (Figure 5.1), has been generated and characterized *in vitro* and shown to be improved over Av1 and Av2 vectors with respect to the potential for vector DNA replication and viral late protein expression (Gorziglia *et al.*, 1996). In addition, adenoviral vectors containing combined deletions in the E1 and E4 regions have been described (Armentano *et al.*, 1995; Wang *et al.*, 1995; Gao *et al.*, 1996; Yeh *et al.*, 1996). Subsequent generation adenoviral vectors containing more extensive backbone gene deletions are, no doubt, in progress, and require, in addition, the development of complementing cell lines to supply the missing gene products in *trans*. Ultimately, the use of adenoviral vectors containing only the essential adenovirus packaging signals and the transgene expression cassette may become feasible, and such vectors have been reported (Clemens *et al.*, 1996; Haecker *et al.*, 1996; Kochanek *et al.*, 1996; Lieber *et al.*, 1996). However, these vectors are grown in the presence of a helper virus which is difficult to separate completely from the vector. A packaging cell line that possesses all the viral complementing functions, although difficult to generate, would allow production of the vector without helper contamination. These 'gutless' adenoviral vectors have the potential for minimizing cytotoxicity and cellular immune responses as well as allowing the accommodation of larger (up to 35 kb) exogenous DNA fragments.

Alternative methods for reducing the cellular immune response to adenoviral transduced cells resulting in sustained transgene expression include the use of immunosuppressive reagents such as cyclosporin A (Fang *et al.*, 1995), FK506 (Lochmüller *et al.*, 1995, 1996; Vilquin *et al.*, 1995), and cyclophosphamide (Jooss *et al.*, 1996). However, the prolonged use of immunosuppressants may induce severe side effects. As an alternative to immunosuppressant drugs, monoclonal antibodies, directed against proteins involved in T cell activation, also were effective in reducing cell-mediated immunity (Kay *et al.*, 1995; Guérette *et al.*, 1996; Yang *et al.*, 1996b). Therefore, the use of improved adenoviral vectors containing more extended deletions of viral backbone genes in combination with immunosuppressive regimens designed to block cell-mediated immunity may allow more persistent transgene expression.

Another host immune-mediated limitation to the use of adenoviral vectors is that repeated vector administration has been unsuccessful, as a result of a humoral immune response to the viral capsid proteins (Smith *et al.*, 1993; Kay *et al.*, 1994; Kozarsky *et al.*, 1994; Yei *et al.*, 1994a). The generally transient nature of adenoviral vector-mediated transgene expression will require multiple administrations of the vector, especially for the treatment of chronic illnesses such as cystic fibrosis and hemophilia, where life-long therapy will be required. Studies by Smith et al. (1996) have demonstrated that the immune response to a systemically administered adenoviral vector is dose dependent and can be modulated by transient immunosuppression with cyclophosphamide or deoxyspergualin (DSG) at the time of initial vector treatment to allow effective repeated treatment. More recently, using low-dose combination immunotherapy, at least three efficacious adenoviral vector treatments were achieved (T.A.G. Smith, unpublished). In addition, repeated vector delivery has been achieved via immunosuppression strategies designed to block T and B cell activation (Yang *et al.*, 1995b, 1996a), and by the induction of tolerance by vector administration to neonatal mice (Walter *et al.*, 1996). Finally, the use of adenoviral vectors derived from different virus serotypes has been proposed, as adenovirus neutralizing antibodies developed with the administration of one virus serotype do not cross-react or prevent transduction with adenovirus of a second serotype (Kass-Eisler *et al.*, 1996; Mastrangeli *et al.*, 1996). However, such a strategy would involve the generation and characterization of multiple vectors encoding the gene of interest.

5.7 SUMMARY

Adenoviral vectors currently represent the most efficient means to transfer an exogenous gene to a large spectrum of target cells *in vivo*. Numerous demonstrations of efficacious adenoviral vector-mediated delivery of a diverse array of transgenes, in several animal species and humans have been reported. In general, initial transgene expression is extremely efficient, but transient, in many cases lasting less than one month. Much effort has been directed at overcoming the obstacles that may restrict use of this vector system to treat human disease effectively. The major hurdles are (i) the cellular immune response to transduced cells that express low levels of viral backbone genes resulting in cell elimination; (ii) humoral immunity to the viral capsid that limits repeated vector administration; and (iii) the potential for generation of contaminating RCA in the production of vectors for clinical use. While much work still needs to be done, many advances have been made toward overcoming each of these obstacles. Subsequent generation vectors containing more extended deletions (Av2, Av3, gutless) and cell lines to

propagate these vectors have been generated which may reduce host immune responses to transduced cells and therefore increase the duration of transgene expression. A variety of novel strategies have been developed to subdue the immune system, which, taken together with improved vector design, may allow persistent and efficacious repeated vector administration. Finally, the generation of new cell lines and improved vector purification and wild-type adenovirus detection techniques have greatly reduced the possibility of the generation of contaminating RCA. To date, human clinical trials utilizing the early generation, Av1, adenoviral vectors, have revealed that human gene transfer is feasible. The Av1 vectors may find successful application in the treatment of human disease where short-term expression and single dosing is adequate, such as cancer vaccine therapies. Alternatively, future clinical trials using the improved gene transfer strategies and more attenuated vectors may demonstrate efficacious, long-term gene therapy for treatment of a diverse range of human diseases.

ACKNOWLEDGMENTS

The author is grateful to Drs Michael Kaleko and Mario Gorziglia for critical reading of the manuscript, Drs Michael Kaleko and Theodore A.G. Smith for communication of results prior to publication, and Dr Mario Gorziglia and Claudia Lapcevich for the design and contribution of Figure 5.1.

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