CHAPTER 8

Use of Recombinant Adenovirus for Metabolic Engineering of Mammalian Cells


Departments of Biochemistry and Internal Medicine and Gifford Laboratories for Diabetes Research
University of Texas Southwestern Medical Center
Dallas, Texas 75235

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* Current Address: Departament de Bioquimica i Fisiologia, Facultat de Quimica, Universitat de Barcelona, 08028 Barcelona, Spain.
The ability to transfer DNA rapidly and efficiently into bacteria and yeast via physical techniques has allowed researchers to generate insights into the roles of particular genes in metabolic regulation and has also led to exploitation of certain recombinant strains for industrial purposes (Kispal et al., 1989; Bailey, 1991; Liao and Butow, 1993). In contrast, alteration of dynamic metabolic processes in mammalian cells by introduction of particular genes has traditionally been hindered by the relative inefficiency of available techniques for gene transfer. These problems have also resulted in reduced enthusiasm for the prospects for gene therapy for inherited metabolic diseases. Recently, however, optimism has been rekindled by the rapid development of a number of gene transfer vectors and techniques based on the properties of DNA viruses such as adenovirus, herpesvirus, vaccinia virus and adeno-associated virus (AAV) (Berkner, 1988; Graham and Prevec, 1991; Miller, 1992; Morgan and French Anderson, 1993; Nienhuis et al., 1993). In this chapter, we will describe the utility of these viral systems for transfer of genes involved in metabolic regulation into mammalian cells, with particular reference to primary cell types with low replicative activity such as hepatocytes and cells of the islets of Langerhans. Since most of the work in this area to date has been performed with recombinant adenoviruses, we will focus on this system.

I. Historical Perspective on the Development and Application of Viral Vectors for DNA Transfer

Researchers have long recognized that viruses exist by virtue of their capacity to transfer genetic information into host cells. With the advent of recombinant DNA technology, it became apparent that this essential property of viruses might be exploited for the creation of efficient gene transfer vectors. The viral genome can consist of RNA, in the case of retroviruses, or of DNA. Research into the life cycle and biological properties of RNA and DNA viruses proceeded in a parallel fashion, beginning with the discovery of these distinct classes several decades ago (Berkner, 1988; Varmus, 1988; Graham and Prevec, 1991; Miller, 1992; Morgan and French Anderson, 1993; Nienhuis et al., 1993). Despite the near-simultaneous development of gene transfer vectors derived from DNA and RNA viruses in the early 1980s, most of the studies on the utility of viral vectors for gene transfer into mammalian cells were initially focused on retroviruses. The reasons for the bias toward retroviruses are not entirely clear, but may have been related to the fact that retroviruses are capable of reverse transcribing their RNA genome to proviral DNA that then integrates into the genomic DNA of mammalian cells. Although integration of the recombinant gene into the cellular genomic DNA insures transfer of genetic information during cell division, it also enhances the risk for cellular transformation caused by insertional mutagenesis or activation of oncogenes. Furthermore, the retroviral genome must be integrated for the transferred gene to be expressed,
and integration only occurs in dividing cells, apparently because the process requires the participation of cellular factors present only during cell division (Varmus, 1988). Nevertheless, the intense focus on retroviruses has led to significant improvements in vector design, especially in the area of production of helper viruses that prevent the production of potentially dangerous replication-competent recombinant strains (for review, see Varmus, 1988; Miller, 1992; Morgan and French Anderson, 1993; Nienhuis et al., 1993). In fact, nearly all currently approved gene therapy protocols employ disabled murine retroviruses (Miller, 1992; Morgan and French Anderson, 1993). Unfortunately for investigators interested in alteration of metabolic regulation in cell types involved in whole-animal fuel homeostasis such as hepatocytes or cells of the islets of Langerhans, adipose tissue, or muscle, the generally low mitotic activity of these cells precludes the use of retroviruses for efficient gene transfer. Furthermore, retrovirus-mediated gene therapy for metabolic disorders can only be efficiently applied to tissues that can be surgically resected, manipulated in vitro, and replaced; these procedures may be viable for liver (Wilson et al., 1990; Ponder et al., 1991), but certainly not for inaccessible tissues such as the islets of Langerhans.

DNA viruses have emerged as potentially attractive gene transfer vectors in recent years (Berkner, 1988; Graham and Prevec, 1991; Miller, 1992; Morgan and French Anderson, 1993; Nienhuis et al., 1993). Each virus under study has some distinct advantages and disadvantages. For example, vaccinia viruses are capable of accommodating extremely large inserts (25 kb or more), but the large size of the viral genome (190 kb) dictates that recombinant viruses can only be produced by homologous recombination in virally infected host cells. This process is so inefficient that it requires genetic methods for selection of recombinant viruses (Smith, 1991). AAV appears to integrate in a site-specific manner in human chromosome 19 (19q13.3qter) (Kotin et al., 1990, 1991). This site-specific integration appears to be lost, however, in recombinant AAV virions. Furthermore, the small size of the AAV genome (4680 bp) limits the size of recombinant inserts. AAV is actually a parvovirus that requires the activities of adenovirus genes for replication. The AAV vectors also have essential portions of the genome deleted, requiring the propagation of recombinant AAV virions in adenovirus-infected packaging cell lines that provide activities encoded by the deleted genes in trans. Given these complications, it is not surprising that viral stocks with titers approaching those of other DNA viruses have not yet been produced.

Of the available DNA virus systems, adenovirus is the best studied, and probably the most accessible and generally useful, although its applicability for long-term gene therapy is still very much unclear. Positive features of adenovirus include the capacity for expression of relatively large DNA inserts (up to 7 kb in currently available helper-independent viral genomes), the ability to propagate high-titer viral stocks, an extremely broad range of infectivity of mammalian cell types, and the availability of an ever-increasing number of
vectors containing different promoters. Although early clinical trials on the use of recombinant adenovirus for delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) are underway, the long-term prospects for this approach may be hindered by the fact that adenovirus integrates into genomic DNA with very low efficiency and exists predominantly in an episomal mode, suggesting that duration of expression of the introduced gene may be limited. Activation of the immune system by the initial administration of virus will likely preclude the use of multiple injections as a strategy to circumvent the lack of permanent expression. An interesting problem for the near future will be to determine if the positive features embodied in each of the DNA viruses can be incorporated in combination into a “second generation” of DNA viral vectors by genetic engineering.

II. Examples of the Utility of Recombinant Adenovirus for Studies on Metabolic Regulation

Although the future of adenoviral vectors for human gene therapy is uncertain, their utility for rapid and efficient gene delivery to mammalian cells, including nonreplicating primary cells, has been clearly demonstrated. This property of the adenoviral vectors opens a new range of experimental possibilities. Before providing details about the specific properties of adenoviral vectors and procedures for their use, a short review is in order of successful experiments performed to date with this system, particularly those in the area of metabolic regulation.

A. Pioneering Studies

The first adenovirus vectors began to appear in the early 1980s (Solnick, 1981; Thummel et al., 1981). Initially, they were mainly utilized for transfer of genes that could induce transformation, for example, SV40 or polyomavirus T antigens (Solnick, 1981; Thummel et al., 1981; Van Doren and Gluzman, 1984; Berkner et al., 1987; Davidson and Hassell, 1987; Sen et al., 1988). Some of the early vectors had antibiotic selection markers, and were used to show that adenovirus has a rather low efficiency of transformation (0.4 to 0.75%), probably because of its low propensity for integration into genomic DNA (Van Doren et al., 1984). Adenovirus was initially touted as a system for high-level expression of recombinant proteins, following reports that cloning of genes next to the major late promoter of adenovirus could lead to their expression at levels representing 10–20% of newly synthesized cellular protein (Stillman et al., 1985; Yamada et al., 1985; Berkner et al., 1987; Davidson and Hassell, 1987; Alkhatib and Briedis, 1988; Johnson et al., 1988). Dihydrofolate reductase (Berkner et al., 1987) and thymidine kinase (Yamada et al., 1985) were the first
enzymes expressed in cells with recombinant adenovirus, but these studies were motivated more by evaluation of the system for selectability and overexpression than by interest in impacting metabolic pathways.

The first use of adenovirus in the context of metabolic regulation was described by Stratford-Perricaudet et al. (1990). These investigators constructed a recombinant adenovirus containing the ornithine transcarbamoylase (OTC) cDNA expressed from the adenoviral major late promoter. OTC catalyzes the condensation of ornithine and carbamoyl phosphate to yield citrulline in the mammalian urea cycle. Mice with a partial deficiency in OTC (Spf-ash strain) exhibit elevated levels of ammonia and orotic acid in the circulation and a phenotype of growth retardation and sparse fur (Doolittle et al., 1974). Injection of a single bolus of 2–4 × 10⁷ plaque-forming units (pfu) of the recombinant adenovirus into newborn mice resulted in high levels of OTC expression sufficient to reverse the sparse fur phenotype in 2 of 8 mice studied 1 mo after virus injection, 2 of 7 mice studied 2 mo after injection, and 1 of 2 mice after 15 mo. Among those mice that did not display a full reversal of phenotype, several had levels of hepatic OTC enzymatic activity that were higher than those observed in control animals injected with adenovirus lacking the OTC insert. Finally, the authors were able to demonstrate a beneficial metabolic impact of adenovirus-mediated gene transfer because orotic aciduria was significantly reduced in 2 mice for periods of up to 13 mo. Despite the lack of consistency in the efficiency of gene delivery among different animals, this study provided the first suggestion that adenovirus-mediated gene transfer could be used to deliver genes directly to important metabolic tissues in vivo.

B. Adenovirus-Mediated Gene Transfer to Mammalian Cells in Culture

1. Hepatocytes

Recombinant adenoviruses also allow manipulation of metabolic regulation in isolated primary cells in tissue culture that would normally be refractory to gene transfer by physical methods or retroviral vectors because of their poor replicative capacity. Of particular interest in our laboratory are studies on metabolic function in liver and the islets of Langerhans, the two tissues that are most intimately involved in the control of fuel homeostasis in mammals. An example of the potential of the adenoviral technique for alteration of metabolic events in cells is provided in the study by Gómez-Foix et al. (1992). We constructed a recombinant adenovirus containing the muscle glycogen phosphorylase cDNA linked to the cytomegalovirus (CMV) immediate early promoter (AdCMV-MGP) and used this system to transduce primary rat hepatocytes in culture. The study was undertaken to evaluate whether regulation of hepatic glycogen metabolism could be altered by overexpression of the muscle isozyme of glycogen phosphorylase which, unlike the naturally expressed liver phosphorylase isozyme, is potently activated by the allosteric ligand AMP (Newgard et al., 1989). AMP-activatable glycogen phosphorylase activity was increased
46-fold after infection of the liver cells with AdCMV-MGP. *In situ* hybridization with an antisense cRNA probe specific for muscle phosphorylase revealed that 86% of the cells expressed muscle phosphorylase mRNA. Despite large increases in phosphorylase activity, glycogen levels were only slightly reduced in AdCMV-MGP-infected liver cells relative to uninfected cells or cells infected with wild-type adenovirus. The lack of correlation of phosphorylase activity and glycogen content suggested that the liver cell environment inhibited the muscle phosphorylase isozyme. This inhibition could be overcome, however, by addition of carbonyl cyanide m-chlorophenylhydrazone (CCCP), which increased AMP levels 30-fold and caused a much larger decrease in glycogen levels in AdCMV-MGP-infected cells than in control hepatocytes. Introduction of muscle phosphorylase into hepatocytes therefore enables a glycogenolytic response to external effectors that is not provided by the endogenous liver phosphorylase isozyme.

The remarkable efficiency of adenovirus-mediated gene transfer to hepatocytes both *in vitro* (Stratford-Perricaudet *et al.*, 1990; Gómez-Foix *et al.*, 1992) and *in vivo* (Stratford-Perricaudet *et al.*, 1990; see subsequent discussion) suggests that the technique may be applicable for studies on gene therapy for glycogen storage disorders, at least in animal models. An example of a suitable model is the gsd/gsd rat, which appears to lack hepatic phosphorylase kinase activity (Malthus *et al.*, 1980). The obvious strategy of replacement of phosphorylase kinase activity by adenovirus-mediated gene transfer is complicated by the fact that phosphorylase kinase exists as a heterotetramer and that the missing subunit in the gsd/gsd animals has not been defined. An alternative to replacement of phosphorylase kinase might be to overexpress muscle phosphorylase or engineered liver phosphorylase proteins that gain some of the regulatory features of muscle phosphorylase (Coats *et al.*, 1991) in gsd/gsd hepatocytes. Such maneuvers may allow the block in glycogenolysis to be circumvented by alternative modes of phosphorylase activation. Other studies indicate that adenovirus vectors may also be applicable to gene therapy of McArdle’s disease, a genetic deficiency in expression of glycogen phosphorylase in skeletal muscle. Baque *et al.* (1993) have shown that muscle phosphorylase cDNA is transferred to muscle cells in culture with the AdCMV-MGP recombinant virus, and with greater efficiency to differentiated myotubes than to myoblasts. These data suggest that deficient muscle phosphorylase activity may be replaceable in patients with McArdle’s disease via adenovirus or derivative vector systems.

2. Pancreatic Islets of Langerhans

Adenovirus also has utility for metabolic engineering of pancreatic islets. Using a recombinant adenovirus containing a nuclear-localizing variant of the *Escherichia coli* β-galactosidase gene inserted adjacent to the CMV promoter (AdCMV-βGal), Becker *et al.* (1993) demonstrated highly efficient gene transfer
into freshly isolated rat islets of Langerhans. Infection of isolated islets with the AdCMV-βGal virus resulted in expression of β-galactosidase in islets for at least 21 days postinfection. Analysis of multiple islet sections showed that the recombinant virus transferred the β-galactosidase gene into 60–70% of the islet cells compared to 10–20% transfer efficiencies with more traditional methods. Importantly, perifusion studies demonstrated that the normal insulin secretory responses to glucose or glucose + arginine were not affected by infection with the AdCMV-βGal virus. Since glucose-stimulated insulin secretion is the signature functional attribute of the normal islet and is a process that is dependent on active glucose metabolism (Meglasson and Matchinsky, 1986; Newgard, 1992), we concluded that recombinant adenovirus is an attractive and highly efficient system for studying the impact of overexpression of specific genes in the islets.

Changes in the rate of glucose metabolism in islet β cells in response to changes in the external glucose concentration are thought to be mediated by the GLUT-2 facilitated glucose transporter and the glucose phosphorylating enzyme glucokinase; the latter protein is likely to play the true rate-limiting role because of its relatively low level of expression in islets (for review see Meglasson and Matchinsky, 1986; Newgard, 1992). Since the rate of glucose metabolism in β cells appears to be proportional to the magnitude of the glucose-stimulated insulin secretion response, glucokinase and GLUT-2 are thought of as essential components of the “glucose sensing apparatus” of the β cells (Newgard, 1992). Although the circumstantial evidence supporting this contention is compelling, support from molecular studies has been slowly forthcoming because of the difficulties inherent in achieving high-efficiency gene transfer into islet cells.

Previous gene transfer studies on isolated islets have employed physical techniques such as lipofection or electroporation (German et al., 1990; Welsh et al., 1990; German, 1993). In one of these studies, immunofluoresence analysis revealed that the gene being transferred, chloramphenicol acetyl transferase (CAT), was expressed in approximately 11% of the islet cells (German et al., 1990). Such efficiencies allow meaningful studies on expression of promoter-reporter chimeric gene constructs (German et al., 1990; German, 1993). In fact, co-transfection of fetal islets by electroporation with plasmids containing hexokinase I and the rat insulin I promoter linked to CAT resulted in a reduced glucose concentration threshold for activation of the insulin promoter (German, 1993). This effect is presumably due to the enhancement in glucose metabolism occurring at low glucose concentration, since hexokinase has a $K_m$ for glucose of 50 µM, whereas glucokinase has a $K_m$ for glucose (8 mM) that falls within the physiological range (4–10 mM). The important knowledge gained about regulation of insulin expression in the foregoing study is reliant on the high incidence of co-transfection of two plasmids into a single cell. Clearly, however, an overall transfection efficiency of only 10–20% will not provide meaningful insights into the impact of overexpression of genes on acute regulation of insulin
release, because most of the cells will not have received the gene of interest. To circumvent this problem we have prepared recombinant adenoviruses containing various isoforms of glucokinase (AdCMV-GK) and hexokinase I (AdCMV-HKI) and have used these constructs for gene transfer into normal rat islets (Becker et al., 1994). Expression of these proteins was increased approximately 10–30-fold 4 days after infection of islets with AdCMV-GK or AdCMV-HKI. Islets overexpressing glucokinase exhibited an enhanced glucose-stimulated insulin secretion response relative to control islets that were either untreated or treated with the AdCMV-βGal virus. In contrast, islets overexpressing hexokinase I exhibited no increase in the magnitude of response to stimulatory glucose, but showed a doubling in basal insulin release in the presence of the nonstimulatory glucose concentration. These results validate recombinant adenoviruses as an effective means of altering metabolic regulation in isolated pancreatic islets, and open the door for studies on a number of other potential regulatory loci, including phosphofructokinase and enzymes controlling islet lipid metabolism.

3. Clonal Cell Lines

Much of the early work with recombinant adenovirus was performed with clonal cell lines. Abundant evidence suggests that the virus efficiently transfers genes to a wide range of cell types (Solnick, 1981; Van Doren and Gluzman, 1984; Van Doren et al., 1984; Stillman et al., 1985; Yamada et al., 1985; Berkner et al., 1987; Davidson and Hassell, 1987; Alkhatib and Briedis, 1988; Johnson et al., 1988; Sen et al., 1988; Stratford-Perricaudet et al., 1990). This property of adenovirus virions can be used to advantage for rapid evaluation of the effects of introduced genes in clonal cells. For example, our laboratory is engaged in studies on engineering of glucose-stimulated insulin secretion in insulin-secreting cell lines that are normally insensitive to the sugar (reviewed by Newgard, 1992; Newgard et al., 1993). These studies involve transfer of genes such as GLUT-2 and glucokinase that have been implicated in the “glucose sensing” function of the islets of Langerhans. Until recently, these studies relied on stable transfection and selection of transfected clones by antibiotic resistance. Although such approaches do produce clonal lines with stable expression of the genes of interest, the transfection and selection of clones is time consuming, and it is necessary to evaluate multiple clones to be certain that phenotypic changes are due to the transferred gene rather than to spurious effects of clonal selection, while adenovirus DNA does not integrate efficiently and thus is lost over time as clonal cells replicate; the system can be extremely valuable for rapid testing of concepts. For example, we have recently demonstrated that rat insulinoma (RIN) cells gain a glucose-stimulated insulin secretion response on stable transfection with a plasmid containing GLUT-2, but the response is found to be maximal at subphysiological glucose concentrations (Ferber et al., 1994). We hypothesized that the hypersensitive response to
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C. glucose is due to high levels of expression of hexokinase in RIN cells, a contention supported by studies with the hexokinase inhibitor 2-deoxyglucose, which caused the insulin secretion response to occur at higher glucose concentrations (Ferber et al., 1994). We have now prepared a recombinant adenovirus with the hexokinase I cDNA in antisense orientation (H. BeltrandelRio, T. C. Becker, and C. B. Newgard, unpublished work), which will allow a rapid test of the concept that reduction in expression of hexokinase I will provide the correct glucose-sensing threshold.

C. In Vivo Studies

Recombinant adenovirus also appear to have great applicability for gene delivery in vivo (Gerard and Meidell, 1993). Although the impetus for such work is often to evaluate adenovirus as a means of replacing genes that are absent in genetic disorders, the technique also has fascinating potential for elucidating the effects of perturbation of metabolic pathways in whole animals. After the pioneering study involving gene transfer of OTC (Stratford, Perricaudet et al., 1990) the efficacy of adenovirus for in vivo gene delivery was confirmed and elaborated by several groups. Initial efforts were directed at delivery of genes to the airway epithelia of lung, since these cells can be specifically targeted by intratracheal instillation (Rosenfeld et al., 1991, 1992). Recombinant adenoviruses have been used successfully to deliver α1-antitrypsin (Rosenfeld et al., 1991) and the CFTR gene (Rosenfeld et al., 1992) to the airway epithelium of cotton rats; expression of the introduced human proteins persisted for a period of weeks. These promising results have led rapidly to multiple clinical trials of recombinant adenovirus for delivery of the CFTR to patients, with as yet unknown results. More recently, Herz and Gerard (1993) and co-workers, (Ishibashi et al., 1993) have elegantly demonstrated the utility of recombinant adenovirus for whole animal metabolic studies. In an initial study (Herz and Gerard, 1993) AdCMV-βGal virus (approximately $10^9$ pfu) was injected into the jugular vein of normal mice resulting in gene transfer to approximately 90% of the hepatic parenchymal cells. Similar efficiencies of gene transfer to the liver were measured by immunofluorescent staining following injection of a recombinant virus containing the cDNA encoding the human low density lipoprotein (LDL) receptor (AdCMV-LDLR). After confirming hepatic overexpression of the LDL receptor by Western blotting, the authors demonstrated as much as a 10-fold increase in the rate of $^{125}$I-labeled LDL clearance, with rates being roughly proportional to the amount of AdCMV-LDLR virus administered (Herz and Gerard, 1993). Among tissues studied (liver, kidney, lung, spleen, and heart), only the liver exhibited enhanced accumulation of labeled LDL, indicating that the increased rate of clearance was due to hepatic expression of the LDL receptor. In a subsequent study, Ishibashi et al. (1993) employed homologous recombination to create mice lacking a functional LDL receptor gene. These mice exhibited a doubling of total plasma cholesterol that
was due to a 7- to 9-fold increase in intermediate density lipoproteins (IDL) and LDL, with little change in high density lipoproteins (HDL). Injection of the AdCMV-LDLR virus into such mice resulted in a return of the IDL/LDL levels to normal, and mice with restored LDL receptor levels also cleared 125I-labeled very low density lipoproteins (VLDL) much more rapidly than LDL receptor-deficient mice injected with the AdCMV-βGal virus as a control (Ishibashi et al., 1993).

The utility of recombinant adenovirus for gene delivery to tissues other than lung or liver is not yet clear. Injection of a recombinant virus containing the firefly luciferase gene (AdCMV-Luc) resulted in accumulation of the vast majority of luciferase activity in liver, exceeding the activities found in lung, skeletal muscle, spleen, kidney, or heart by several orders of magnitude (Herz and Gerard, 1993). Other investigators have also observed highly efficient gene transfer to the liver by systemic delivery of recombinant adenovirus (Stratford-Perricaudet et al., 1990, 1992; W. Coats and C. B. Newgard, unpublished observations), but claims of efficient systemic delivery to tissues such as skeletal muscle and heart (Stratford-Perricaudet et al., 1992), or brain (Le Gal Le Salle et al., 1993) remain to be confirmed. We have infused the AdCMV-βGal virus for several days at a rate of 1 × 10^8 pfu/hr and found preferential expression of β-galactosidase in the islets of Langerhans among pancreatic cells (W. Coats and C. B. Newgard, unpublished observations). The result has been confirmed by isolation of the islets after virus infusion, but reliable estimates of gene transfer efficiency will require further investigation. Preferential targeting of recombinant adenovirus to tissues such as liver and the islets of Langerhans may be due to the fact that blood can easily come in direct contact with such cells, via the sinusoidal vasculature of the liver or through the fenestrations that exist in the intense vasculature of the islets (Henderson and Moss, 1985). Targeting tissues that are not as well served by the systemic circulation might ultimately be achieved by direct injection or inoculation with recombinant virus, as has been demonstrated for the hippocampus and the substantia nigra of the brain (Le Gal Le Salle et al., 1993) and for skeletal muscle fibers (Quantin et al., 1992; Ragot et al., 1993). Further studies in these areas will be required.

### III. Adenovirus Biology

Wild-type adenovirus exists as an icosahedral particle approximately 75 nm in diameter with a dense core containing the DNA genome (Horwitz, 1990). The virus was originally isolated from human adenoidal tissue in 1953 (Rowe et al., 1953). It has been associated with a number of human respiratory illnesses and was the first human virus demonstrated to cause malignant tumors in animals, although, interestingly, no human malignancies have yet been described that are due to adenovirus transformation. Human adenovirus serotypes 2 (Ad2) and 5 (Ad5) are the most extensively studied, and their DNA genome
has a size of 36 kb. The interested reader is referred to several excellent reviews on adenovirus biology that provide detailed information about the viral genome (Berkner, 1988; Horwitz, 1990; Graham and Prevec, 1991). The lytic life cycle of the wild-type virions can be divided into early and late phases, which are defined as occurring before and after the onset of viral DNA replication, respectively. Genes expressed during the early phase are noncontiguous in the genome and include the E1 regions E1A and E1B, the E2 regions E2A and E2B, E3, and E4 (see Figure 1). After the onset of DNA replication triggered by expression of the early genes, the major late promoter (MLP) is activated and is responsible for most of the transcriptional activity in the late phase. MLP-driven transcripts contain multiple open reading frames that are alternatively spliced to yield mRNAs containing identical 5' untranslated regions known as the tripartite leader (Berkner, 1988; Horwitz, 1990; Graham and Prevec, 1991). Late in infection, 20–40% of total cellular RNA is viral and cell lysis begins to occur 48–72 hr after infection.

Current strategies for adenovirus vector design focus on deleting portions of the genome to allow packaging of relatively large DNA inserts. Wild-type adenovirus can only accommodate 2 kb of foreign DNA, but deletion of one or more of the early genes of the virus can allow recombinants with inserts up to 7 kb in size. Before describing some of the currently available vectors in detail, it seems useful to describe the function of those genes that have been targeted for deletion. The E1 genes are required for viral transformation. E1A is particularly important because it appears to be required for activation of all other early genes (E1B, E2, E3, and E4). Although the mechanism(s) responsible for global activation by E1A is not yet understood, an intriguing structural relationship between certain E1A and myc and myb gene products has been noted (Ralston and Bishop, 1983; McLachlan and Boswell, 1985). An E1A protein has also been shown to be similar to SV40 T antigen and the human papillomavirus-16 E7 transforming proteins; all bind to the retinoblastoma (RB) growth suppressor gene (DeCaprio et al., 1988; Whyte et al., 1988), suggesting a common mechanism of action for these transforming genes. E1A and E1B

\[
\begin{array}{c}
\text{E1} \\
\text{E2} (a,b) \\
\text{MLP, TL} \\
\text{L1-L5} \\
\text{E3} \\
\text{E4}
\end{array}
\]

**Fig. 1** Transcriptional map of adenovirus genome. The 36-kb adenovirus genome is a linear molecule that can be divided into 100 map units of 360 bp each. Early gene transcripts (E1–E4) are represented by thin arrows. Late gene transcripts (L1–L5) are initiated at the major late promoter (MLP) and all contain the tripartite leader (TL) sequence.
appear to work together in mediating cellular transformation by adenovirus. E2A and E2B encode DNA binding and DNA polymerase activities, respectively, and are essential for DNA replication. In contrast, E3 appears to have no essential function for adenovirus growth or DNA replication, and may instead be involved in modulating host responsiveness to adenovirus infection. A 19-kDa protein encoded by the E3 region has been shown to bind to major histocompatibility complex (MHC) polypeptides in the endoplasmic reticulum, thereby inhibiting appearance of MHC peptides at the cell surface (Persson et al., 1979; Paabo et al., 1986). The E4 region is currently not well understood, although a 34-kDa protein encoded by this region is thought to interact with an E1B 55-kDa protein in the nucleus. Apparently six of the seven open reading frames within E4 can be deleted without affecting viral replication or infectivity (Halbert et al., 1985).

IV. Development of Adenovirus Vectors

The strategy employed for construction of a particular recombinant adenovirus depends in part on the regions of the adenovirus genome that are deleted to accommodate DNA inserts. To date, the most common strategies have been to delete portions of the E1 or the E3 regions of the viral genome, or both. Wild-type Ad5 can accommodate only 2 kb of insert DNA. Deletion of E3 with two naturally occurring Xba I restriction enzyme sites removes 1.9 kb from the viral genome, thereby accommodating inserts approaching 4 kb in length (Berkner, 1988; Graham and Previc, 1991). Up to 3 kb can be deleted from the E1 region, allowing a maximum insert size in doubly deleted viral genomes of approximately 7 kb. Theoretically, it should also be possible to delete nonessential regions of E4, allowing inserts of up to 10 kb, but such vectors are not yet available. Recombinant viruses can be generated either by direct ligation of insert sequences into the deleted E1 or E3 regions or, more commonly, by homologous recombination of overlapping fragments of the viral genome in cell lines. If the recombinant genome is deleted only in E3, the virus is described as nonconditional since E3 is not essential for viral replication and growth. In such constructs, recombinant virions can be grown in a wide array of cell lines, including HeLa or KB cells. In the case of deletion of the essential E1 gene, viral propagation is conditional because the function of the E1 gene must be provided in trans. This is achieved by growing E1-deleted recombinant virus in the human 293 cell line, which was originally transformed with Ad5 and contains the left 14% of the adenovirus genome integrated into cellular DNA, including the E1 region (Graham et al., 1977).

Since the adenovirus genome is very large, the chances of finding unique restriction sites that allow direct ligation into the E1 or E3 regions are small. One strategy that has been used successfully is to remove the far-left 2.6 map units of Ad5 by digestion at a unique Cla I site. For example, recombinant
adenovirus containing the OTC cDNA was prepared by direct ligation of a cassette consisting of the adenovirus MLP, the tripartite leader, the OTC cDNA, and SV40 3' sequences via a 3' Acc I site, which can ligate to Cla I-restricted DNA (Stratford-Perricaudet et al., 1990). This DNA construct is then transfected into 293 cells for viral propagation. A current focus of investigators in the field is to attempt to prepare new viral vectors that add or eliminate restriction sites to allow more flexibility in direct ligation strategies. The discovery of restriction endonucleases that recognize 8-bp palindromic sequences will facilitate this process. In the interim, most recombinant viruses are generated by homologous recombination. A particularly useful strategy in our hands has been adapted from procedures devised by Graham and co-workers (1991). An important discovery that has contributed to current methods was that adenovirus DNA circularizes in infected cells, allowing isolation of the viral DNA as infectious plasmids (Ruben et al., 1983; Graham, 1984). Recombinant virions can therefore be produced by co-transfection with the circular viral genome and a plasmid containing a fragment of the viral genome and the recombinant gene of interest. A problem with this approach is that significant contamination with wild-type virus can occur; in fact, the wild-type virus will often have a growth advantage over the recombinant. This problem has been circumvented by preparation of a new plasmid called pJM17, which contains a DNA segment of 4.3 kb inserted at 3.7 map units that includes the ampicillin and tetracycline resistance genes and a bacterial origin of replication (McGrory et al., 1988; see Fig. 2 for map). The size of this insert exceeds the packaging limit of wild-type virus, thus preventing propagation of wild-type virions and selecting for recombinants.

More recently, new vectors have been designed that will allow the use of a variety of promoter–enhancer elements, including those with tissue-specific properties such as the insulin promoter. A very useful vector, pACCMVpLpA, has been developed by modification of the pAC vector (Gluzman et al., 1982) by R. Gerard, by replacement of a region of the adenovirus genome between map units 1.3 and 9.1 with the CMV early promoter (Fig. 3). A convenient cloning cassette has also been inserted immediately downstream from the CMV promoter, which in turn is followed by a fragment of the SV40 genome that includes the small t antigen intron and the polyadenylation signal (see map, Fig. 1). A large number of cDNAs including muscle glycogen phosphorylase (Gómez-Foix et al., 1992), the LDL receptor (Herz and Gerard, 1993), glucokinase and hexokinase I (Becker et al., 1994), the GLUT-2 facilitated glucose transporter (Ferber et al., 1994), and apolipoprotein A1 (R. S. Meidell and R. D. Gerard, unpublished work) have been cloned into pACCMVpLpA and have been used to generate recombinant adenoviruses by recombination with pJM17, as shown in Fig 4. In our hands, generation of virions by co-transfection of 293 cells with purified pAC constructs and pJM17 requires 2–4 wk, allowing for the relatively rare recombination events to occur (see also subsequent discussion). Future development of this vector system will focus on two features. (1) The CMV promoter will be substituted with other nonviral promoters
in the pAC plasmid. For example, we have recently succeeded in replacing the CMV promoter with the human insulin promoter and are beginning to test the efficacy of this virus for tissue-specific expression in insulin-producing islet β-cells (L. Moss, R. Noel, T. Becker, P. Antinozzi, and C. B. Newgard, unpublished observations). (2) Restriction site engineering must occur so the DNA insert of interest can be cloned into one of the series of pAC plasmids, which can then be linearized and ligated directly to the remainder of the adenovirus genome, thus obviating the need for the homologous recombination step.

V. Specific Procedures for Preparation of Recombinant Viruses

For simplicity, we will focus in this section on construction of recombinant adenovirus by co-transfection of 293 cells with the plasmids pJM17 and
pACCMV.pLpA. Viruses derived from these particular reagents lack the E1 but not the E3 region of the virus, so there is an inherent limitation on the size of the cDNA that can be inserted. Note that the pJM17 plasmid (McGrory et al., 1988) was derived from Ad5 containing insertions and deletions in the E3 gene, so the E3 gene is largely intact but nonfunctional (Jones and Shenk, 1978). Using pACCMV.pLpA and pJM17, we have been able to prepare a recombinant virus containing the 3.6-kb hexokinase I cDNA (Becker et al., 1994), close to the theoretical upper limit of 3.8 kb. This system of preparing recombinant adenoviruses requires a rare recombination event to occur between

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**Fig. 3** Map of pACCMV.pLpA plasmid. This plasmid was derived from the pAC plasmid (Gluzman et al., 1982) by insertion of the CMV promoter/enhancer element, a pUC19 cloning cassette, and the SV40 polyadenylation signal. The plasmid consists of Ad5 sequence (shown as the shaded bars) and bacterial plasmid sequence that includes the ampicillin resistance gene.
Fig. 4 General strategy for preparing recombinant adenovirus by homologous recombination. The cDNA of interest is cloned into the pACCMVpLpA vector. The recombinant pAC and pJM17 are purified and co-transfected into 293 cells as described in detail in the text. Since 293 cells were originally produced by adenovirus transformation, the missing E1 gene function of pJM17 is provided in trans. Virus is isolated, titered, and amplified as described in the text. The final product is a recombinant adenovirus that is replication defective (at least in cells lacking the E1 region of adenovirus) but fully infectious.
two plasmids co-transfected at a low efficiency into 293 cells. In spite of this additional limitation, we have successfully prepared a large number of recombinant viruses using the following protocols. Note that production of a recombinant virus may require as little as a single co-transfection, or may require several co-transfections. A flow chart providing an overview of the procedure used to generate recombinant virions is provided in Fig. 5.

A. Purification of Plasmid DNA

Obtaining high quality DNA for co-transfections is a prerequisite for preparing recombinant viruses. Preparation of the large (40 kb) pJM17 plasmid poses
particular problems because of its propensity for shearing during handling. Bacteria containing pJM17 or pAC-derived plasmids are grown on LB plates or in LB or TB media (Sambrook et al., 1989) containing 100 μg/ml ampicillin. For large-scale plasmid preparations, 1-liter cultures of LB or TB with ampicillin are inoculated and grown overnight. Plasmid DNA is isolated using the QIAGEN MaxiPrep system (QIAGEN, Chatsworth, CA). Using this method, we obtain 300–500 μg pACCMVpLpA DNA per 75 ml TB/ampicillin and 150–200 μg pJM17 per liter LB/ampicillin. Note that the yield of pJM17 DNA is typically lower than the yield of pACCMVpLpA.

To assess the quality of the purified pJM17 DNA, an aliquot of the DNA is typically digested with Hind III (see Fig. 1). If the DNA was purified with a minimum of shearing, this digest will resolve as a distinct "ladder" of 7–8 bands between 2 and 8 kb on a 0.8% agarose gel. Uncut DNA resolves as several high MW bands, presumably corresponding to various supercoiled forms of the DNA. Some pJM17 preparations may include a 4-kb band corresponding to a pBRX sequence that is spontaneously excised from pJM17 and that has the capacity to replicate autonomously because of retention of the bacterial origin of replication and the antibiotic resistance genes. Although recombinant virions can be produced from pJM17 preparations containing this contaminant, preparations in which it represents the major DNA species should be discarded.

B. Co-transfection of 293 Cells

The method of choice for co-transfection is calcium phosphate co-precipitation. This method is chosen because it allows for relatively efficient co-transfection of two plasmids, even though other methods such as electroporation or lipofection may be more efficient for single plasmids (Sambrook et al., 1989). Although the importance of passage number has yet to be firmly established, we routinely co-transfect 293 cells before they reach passage 30. The following specific procedure is followed for co-transfection in our laboratories.

1. The E1-tranformed 293 cell line is maintained in 60-mm plates at 37°C in an atmosphere of 5% CO₂ in high-glucose (4.5 g/liter) Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin.
2. The 293 cells should be co-transfected when they are subconfluent, typically at approximately 80% confluence.
3. For each co-transfection, mix 500 μl sterile 2× HEPES-buffered saline (2× HBS; 280 mM NaCl, 42 mM HEPES, 10 mM KCl, 1.4 mM Na₂HPO₄·7H₂O, 0.2% dextrose, pH 7.05–7.15), 10 μg pACCMVpLpA DNA containing the cloned cDNA fragment, 10 μg pJM17 DNA, and sterile water to a volume of 950 μl. To avoid shearing the pJM17 DNA, mix by inversion rather than using a vortex mixer.
4. Slowly add 50 μl 2.5 M CaCl₂ and mix by inversion. Allow the DNA/calcium phosphate precipitate to form for 45–60 min at room temperature. The precipitate should appear as a very fine, slightly turbid suspension.

5. Add 500 μl precipitate to each of two 60-mm plates of semiconfluent 293 cells containing 5 ml complete DMEM. Incubate at 37°C and 5% CO₂ for 4–6 hr.

6. Aspirate the medium and precipitate and wash once with phosphate-buffered saline (PBS).

7. Since a brief treatment of cells with glycerol is believed to increase the efficiency of transfection (Halbert et al., 1985), the cells are treated for 1–2 min with 15% glycerol in serum-free DMEM, followed by an additional PBS wash and replacement of complete medium.

8. Cells are maintained by replacing the majority (but not all) of the medium on a weekly basis. We and others have noted a transient "yellowing" or acidification of the medium in some plates following refeeding. We have found this to be neither harmful to the cells nor a predictor of virus formation.

9. When a recombination event occurs in a co-transfected 293 cell, the resulting recombinant virus completes the life cycle in the permissive host, resulting in cell lysis and the formation of plaques of dead cells. Plaque formation may occur as early as 2 wk and as late as 4 wk after co-transfection and first appears as a visible "hole" in the monolayer. This initial plaque will enlarge as the viral infection progresses, so by 3–5 days after the appearance of the plaque, the monolayer should be completely lysed.

10. After the monolayer lyses, collect the medium and lyse any remaining intact cells by several freeze/thawing cycles. Pellet the cell debris by centrifugation and store the viral supernatant at −20°C.

11. After isolating viral DNA from infected 293 cells, the presence of the cDNA insert can be confirmed by preparation of viral DNA (see subsequent section) and Southern blotting. In addition, a single clone must be isolated and amplified to be used as the working viral stock.

12. Alternatively, single clones can be isolated from the original transfection by replacement of the medium (see Step 7) with 0.65% Noble agar in DMEM with 2.5% FBS.

C. Extraction of Viral DNA from 293 Cells

The procedure for isolating viral DNA from 293 cells is adapted from an established method (Hirt, 1967).

1. On Day 1, infect a subconfluent 60-mm plate of 293 cells with recombinant virus. For initial lysates obtained after co-transfection, infections are typically done using 10–100 μl viral lysate in a final volume of 2 ml complete medium for 1 hr at 37°C, followed by a PBS wash and replacement of complete medium.
When the titer of the viral stock is known, a multiplicity of infection of 10 pfu/cell is recommended.

2. Approximately 36 hr later, the cells should appear rounded and refractile, yet should still be adherent to the plate. At this point, aspirate the medium and gently wash twice with PBS.

3. Aspirate the PBS wash, add 800 μl freshly prepared lysis buffer (0.6% SDS, 10 mM EDTA, 100 μg/ml proteinase K), and incubate at 37°C for 1 hr.

4. Add 200 μl 5 M NaCl dropwise while swirling the plate to uniformly mix the lysate; then incubate on ice for 1 hr.

5. Transfer the viscous lysate to a 1.5-ml eppendorf tube and centrifuge at ≥12,000 g for 30-45 min at 4°C.

6. The “pellet” at this point is likely to be very loose and undefined, and should be removed with an inoculating loop, leaving behind the supernatant.

7. Remove proteinase K activity from the supernatant by extracting once with an equal volume of phenol: chloroform. To avoid shearing the viral DNA, mix by inversion rather than using a vortex mixer. After a brief centrifugation (≥12,000 g), recover the upper phase and precipitate the viral DNA at −20°C in the presence of 0.25 M sodium acetate, pH 5.2, and 2 volumes of ethanol.

8. Pellet the viral DNA by centrifugation (≥12,000 g), wash the pellet with 70% ethanol, and gently resuspend the viral DNA in 25–50 μl TE containing 10 μg/ml RNase A. The DNA is now ready for restriction enzyme digestion and Southern blot analysis.

D. Isolation and Amplification of Viral Clones

One of the great advantages of recombinant adenovirus is that high-titer viral stocks (≥10⁹ pfu/ml) can easily be obtained. The initial lysate obtained after co-transfection, however, typically has a titer one or more orders of magnitude lower and, in addition, is not necessarily a clonal stock. For these reasons, it is desirable to isolate single discrete plaques and to use them for amplification to generate pure high-titer recombinant stocks. A convenient technique for titering viral stocks and for isolating individual viral plaques is plating by agar overlay.

1. Prepare serial dilutions of the viral stock in 2 ml complete medium. Typically, dilutions between 10⁻³ and 10⁻⁹ are prepared.

2. Infect subconfluent 293 cells with the 2 ml containing the various viral dilutions for 1 hr at 37°C.

3. Aspirate the virus-containing media and wash the monolayers twice with PBS.

4. In a separate tube, premix equal volumes of melted 1.3% agarose in water with 2× DMEM (prepared from DMEM powder; GIBCO/BRL, Grand Island, NY) containing 4% PBS, 200 U/ml penicillin/streptomycin + 0.5 μg/ml Fungizone, and 30 mg/liter of phenol red. It is important that the melted agarose
be neither too hot nor too cool. For this reason, the melted agarose should be incubated in a 56°C water bath prior to use.

5. After aspirating the final PBS wash from the infected 293 cells, slowly add 6 ml of the warm agarose/DMEM mixture and allow the mixture to solidify at room temperature for several minutes before returning the plates to the incubator.

6. Viral plaques should form 7–10 days after plating.

To amplify a clonal viral stock, single plaques are isolated by taking a core with a pipet tip and transferring to a tube containing 2 ml complete medium. The following procedure is then used to amplify the pure virus.

1. Elute the virus from the agarose by several freeze/thaw cycles of the medium containing the viral plug.

2. Infect a plate of subconfluent 293 cells with the entire 2 ml of relatively dilute viral sample for 1 hr at 37°C, followed by a PBS wash and addition of complete medium.

3. On cell lysis, which can be anticipated approximately 1 wk after infection, the lysate is collected, cell debris is removed by centrifugation, and the supernatant is collected as the initial or “P1” viral stock. This stock, which will generally have a titer in the range of 10^7 pfu/ml, should be checked by Southern blotting as described in Section V,C.

4. To increase the titer of the clonal stock further, infect one or more 10-cm plates of subconfluent 293 cells with a minimal volume (5–25 μl) of the clonal stock in a final volume of 3 ml complete medium for 1 hr at 37°C.

5. Wash with PBS and replace complete medium.

6. After the monolayer lyses, collect the media in a sterile 50-ml polypropylene tube and lyse any remaining intact cells with several rounds of freezing and thawing. Pellet cell debris by centrifugation and store the viral supernatant at -20°C. The titer of this high-titer stock can be determined as described in Section V,E.

In some instances, particularly when planning experiments involving chronic infusion of recombinant adenoviruses, it is desirable to obtain large quantities of purified recombinant virus.

1. Inoculate 30 dishes (150 × 25 mm) of 293 cells at 80% confluence with enough adenovirus to achieve a multiplicity of infection of at least 10 pfu/cell. Allow approximately 36–48 hrs for cells to lyse completely.

2. Harvest lysate and add 0.5% Nonidet P–40. Place on orbital shaker at room temperature for 10 min to ensure thorough mixing and then clear debris by centrifugation at 20,000 g.

3. Recover supernatant and add 0.5 volume of 20% polyethylene glycol 8000/2.5 M NaCl. Incubate on ice 30 min to overnight with frequent shaking. Centrifuge 10 min at 20,000 g to pellet adenovirus. Discard supernatant into bleach.
4. Resuspend adenovirus pellets in 3–6 mL phosphate-buffered saline and centrifuge to clear debris (10 min at 4000 g in a table-top centrifuge). Recover supernatant and add solid CsCl until 1 mL of the solution weighs 1.32–1.34 g (approximately 0.5 g CsCl for each mL of supernatant). Load solution into 13 × 32 mm sealable ultracentrifuge tubes and spin 3 hrs at 361,000 g (90,000 rpm in a Beckman TLA-100 rotor).

5. Extract white adenovirus band from tubes with needle and syringe in a total volume of 0.5–1.0 mL and load onto a Pharmacia PD-10 Sephadex column equilibrated with 137 mM NaCl, 5 mM KCl, 10 mM Tris-HCl pH 7.4, and 1 mM MgCl₂. Collect 8–10 drop fractions and determine adenovirus peak by measuring absorbance at 260 nm (use 1/50 dilutions). Pool fractions and determine titer by plaque assay or using the formula 1 OD₂₆₀ = 10¹² pfu/mL. Add 0.1% BSA and store in small aliquots at −80 degrees centigrade.

E. Transduction of Mammalian Cells with Recombinant Virions in Vitro

Recombinant adenovirus can be used for transduction of isolated primary cells such as hepatocytes, small cell clusters such as the islets of Langerhans, or clonal cell lines. In all cases, the basic procedure is the same. For cells that adhere to tissue culture plates, the recombinant viral stock is added directly to the plates in a minimal volume of complete medium (usually 3 ml per 10-cm plate). Generally, a multiplicity of infection of 5–10 pfu/cell applied for 1 hr insures high-efficiency gene transfer (≥80%). Use of higher ratios (as high as 500:1) can enhance the level of expression of the recombinant gene, since each cell can be multiply infected. The potential for overexpression is limited, however, by eventual toxic effects of the virus; the upper limit appears to be different for different cell types. For each new cell type that is considered for adenoviral gene transfer, we recommend a “functional titration” to determine the range of expression that is possible.

Isolated islets are generally maintained intact in suspension culture. Transduction with recombinant virus is achieved by collection of the islets by mild centrifugation, aspiration of their media, and replacement with the viral stock for 1 hr. The virus is then removed by aspiration, the cells are washed with PBS, and the islets are returned to suspension culture in complete media.

Although detailed time-course studies have not been performed, high levels of expression of recombinant genes are evident 48 hr after exposure to recombinant adenovirus that persists for at least 6 days in hepatocytes and 21 days in islets (Gómez-Foix et al., 1992; Becker et al., 1994).

F. Delivery of Recombinant Virions in Vivo

As detailed earlier, recombinant adenoviruses have been useful for delivering genes to whole animals. In general, viruses have been administered as
single injections via accessible blood vessels such as the external jugular vein (Stratford-Perricauclet et al., 1990; Rosenfeld et al., 1992; Herz and Gerard, 1993). High efficiency gene delivery to hepatocytes has been reported with a protocol in which a single injection containing approximately $2 \times 10^9$ pfu was administered systemically into mice weighing 20–40 g (Rosenfeld et al., 1992; Herz and Gerard, 1993). Other groups have reported widespread gene delivery to tissues of neonatal animals with a comparable dose (Ishibashi et al., 1993). An alternative method for delivery of large numbers of virions is to infuse the virus slowly over time. We have achieved this in rats by chronic cannulation of the left lateral ventricle with silastic tubing (Dow Corning, Midland, Michigan) under sodium pentothal anesthesia (W. Coats and C. B. Newgard, unpublished observations). The tubing is exteriorized between the shoulders and held in place and protected by a device consisting of a swivel, a harness, and a spring tether, as previously described (Komiya et al., 1990). This configuration allowed the animals to be housed in an unrestrained state with free access to food and water. AdCMV-βGal virus containing approximately $1 \times 10^{10}$ pfu/ml was prepared by CsCl gradient ultracentrifugation of a crude viral stock. The purified stock was diluted to a concentration of $2.5 \times 10^8$ pfu/ml in a solution of 0.9% NaCl, 10% glycerol and was infused continuously via the ventricular cannula for several days at a rate of 0.4 ml/hr. This protocol allowed gene delivery to the islets of Langerhans of the pancreas, whereas a single bolus injection did not. Whether viral infusion allows enhanced gene delivery to other tissues as well remains to be determined.

**VI. Future Directions**

As indicated earlier, adenovirus is one of several DNA viruses currently being exploited as a gene transfer vector for mammalian cells, each with its own advantages and disadvantages. In closing, we will speculate on future directions for this rapidly expanding area of research, including in our discussion the applicability of new physical gene transfer methods, some of which have been combined with viral technologies to create “hybrid” approaches.

As summarized earlier, recombinant adenovirus provides distinct advantages over most physical DNA transformation techniques such as Ca$_3$(PO$_4$)$_2$ coprecipitation, lipofection, or electroporation in terms of efficiency of gene transfer (generally 10–20% with the physical methods versus 60–100% with recombinant adenovirus). Adenovirus-mediated DNA delivery in vivo is also clearly possible, albeit with varying efficiency in different tissues. Recent developments have suggested other alternative or potentially complementary approaches. One approach is to prepare the DNA to be transferred as a chemically linked conjugate with ligands such as galactose-bearing asialo-orosomucoid (Wu and Wu, 1987, 1988) or transferrin (Wagner et al., 1990) that targets the complex to the receptor-mediated endocytosis pathway of cells. Two distinct advantages of
the DNA-conjugate approach over the recombinant adenovirus approach are (1) the absence of theoretical size limitations for the DNA to be transferred and (2) the tissue-specific delivery of the gene of interest to those tissues that express receptors recognizing the coupled ligand. An apparent disadvantage of this approach is that it appears to work with limited efficiency, probably because a substantial percentage of the DNA that is internalized via receptor-mediated endocytosis is degraded within lysosomes.

Recently, combination viral/physical transformation protocols have been described that show considerable promise. Replication-defective adenovirus greatly enhances the transfer of conjugated DNA by virtue of its ability to lyse endosomes, thereby releasing the internalized DNA before it is degraded (Curiel et al., 1991). The efficacy of simple co-infection of cells that are transfected with conjugated DNA is limited by the relatively low probability of internalization of the conjugate and a virus into the same endosome. To circumvent this problem, more recent approaches have emphasized physical coupling of the adenovirus to the DNA conjugate. This has been achieved either by chemically linking an anti-adenovirus antibody to the DNA conjugate (Curiel et al., 1992) or, more simply, by coupling adenovirus to poly-L-lysine through the action of transglutaminase or the use of biotin/streptavidin (Wagner et al., 1992). The poly-L-lysine-conjugated adenovirus is then combined with the DNA to be transferred and the poly-L-lysine-conjugated ligand (i.e., transferrin) to form a virus–DNA–ligand complex. Such complexes enhance the efficiency of gene transfer into cell lines and primary cells by several orders of magnitude compared with conjugated DNA lacking adenovirus; efficiencies of 100% have been reported for HeLa cells (Wagner et al., 1992) and rodent hepatocytes (Wagner et al., 1992; Christiano et al., 1993). Although adenovirus conjugation overcomes the problem of low efficiency of gene transfer with DNA conjugates, it removes tissue specificity since adenovirus receptors are found on most mammalian cells. A technique for overcoming this final hurdle has been presented recently, involving treatment of a transferrin–DNA–adenovirus complex with a monoclonal antibody against the adenoviral fiber protein (Michael et al., 1993). Such treatment was found to block the permissive effect of free adenovirus on conjugate-mediated DNA uptake into HeLa cells but had no effect on complexes with physically coupled adenovirus. Whether these multifunctional molecular conjugate vectors will retain their efficacy and specificity of gene delivery in whole animals remains to be determined.

Another issue is whether even simpler methods may ultimately prevail. Thus, it has been appreciated for some time that efficient DNA transfer can be achieved in vitro with lipofection, a technique in which DNA is packaged into cationic liposomes consisting of the synthetic lipid \( N-[1-(2,3\text{-dioleloxy})\text{propyl}]\text{-N,N,N-trimethylammonium chloride (DOTMA) Felgner et al., 1987}. Optimization of the DNA:liposome ratio has allowed efficient DNA delivery to a wide array of tissues in rodents injected with a single dose of the conjugate (Zhu et al., 1993). Expression was found to persist in many tissues for as long as 9 wk, although
the status of the plasmid DNA (integrated versus episomal) was not clearly established. Importantly, re-administration of the conjugate caused a second peak of transgene expression, indicating that the liposomes may be different from recombinant adenoviruses in the degree to which they are tolerated by the immune system. Further studies will be required to evaluate the safety and consistency of this exciting new method.

VII. Conclusions

Rapid progress has been made in recent years in the area of techniques of gene transfer to mammalian cells. In this chapter we have selected recombinant adenovirus as one of the best-studied examples of the new technology, and have attempted to provide the rationale and precedent for its use as an exciting new tool for studying metabolic regulation. We have also provided methods and procedures for constructing and propagating new recombinant virions in the laboratory. Adenovirus in its current form is unlikely to represent the ultimate transfer vector for human gene therapy, given problems such as the lack of integration of the viral genome and the potential for immunological response to injected virus. It is therefore likely that new research initiatives will focus on attempting to engineer “second generation” virions that combine functional features of different viruses, that is, the site-specific integration function of AAV with the growth and infectivity characteristics of adenovirus. In the interim, however, recombinant adenovirus greatly enhances our power to investigate the impact of gene transfer in normal animals as well as in animal models of disease, thereby providing the knowledge base that will allow us to take maximal advantage of the safer and more efficacious gene therapy strategies that are likely to emerge in the future.

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References


8. Recombinant Adenovirus for Metabolic Engineering of Cells


