GENE THERAPY: TRIALS AND TRIBULATIONS

Nikunj Somia and Inder M. Verma

The art and science of gene therapy has received much attention of late. The tragic death of 18-year-old Jesse Gelsinger, a volunteer in a Phase I clinical trial, has overshadowed the successful treatment of three children suffering from a rare but fatal immunological disease. In the light of the success and tragedy, it is timely to consider the challenges faced by gene therapy — a novel form of molecular medicine that may be poised to have an important impact on human health in the new millennium.

LIPOSOMES
Artificial lipid vesicles. Liposomes fuse with the cell membrane to deliver their contents, such as DNA for gene therapy.

EPISOMES
DNA molecules that are maintained in the nucleus without integrating into the chromosomal DNA.

The Laboratory of Genetics, The Salk Institute, 10,010 North Torrey Pines Road, La Jolla, California 92037, USA. e-mail: verma@salk.edu

The basic concept of gene therapy is disarmingly simple — introduce the gene, and its product should cure or slow down the progression of a disease. Encompassed within this idea are a number of goals, including the treatment of both inherited and acquired disease. This approach requires a technology capable of gene transfer in a wide variety of cells, tissues and whole organs, but the delivery vehicles needed to ferry genetic material into a cell still represent the 'Achilles heel' of gene therapy. An ideal vector should have the attributes outlined in BOX 1. At present, not all of these attributes can be found in any one vector, although distinct classes of vector have different combinations of attributes.

The vectors available now fall into two broad categories — the non-viral and viral vectors. Non-viral vectors include naked DNA and LIPOSOMES[1]. Although non-viral vectors can be produced in relatively large amounts, and are likely to present fewer toxic or immunological problems, they suffer from inefficient gene transfer at present. Furthermore, expression of the foreign gene is transient. Given the need, in many diseases, for sustained and often high-level expression of the transgene, viral vectors are the most suitable vehicles for efficient gene delivery. Therefore the purpose of this article is to review the current status of the most commonly used viral vectors (for comprehensive reviews on gene therapy vectors, see REFS 2,3). We discuss viral vectors within the framework of the 'ideal vector', and consider the implications of recent experimental data and gene therapy trials on the uses and future development of these vectors.

A survey of viral vectors

All viruses have a genetic component that is essential for further propagation. Viral vectors are derived from viruses by replacing these genetic components with the therapeutic gene. In a cell (typically called a packaging cell), the essential components can be provided *in trans*, which enables the viral vector to be packaged and to deliver genes to the target cell. But this is a dead-end infection, because the vector lacks the essential components for viral propagation. Recombination between the vector and the viral genes encoding the essential components in the packaging cells can lead to the generation of infectious parental virus. Therefore the removal or the separation of genes encoding viral components essential for viral propagation reduces the risk of generating infectious virus — a principle frequently used in gene therapy vector design.

The viral vectors can be divided into two general categories — integrating and non-integrating — the former holding the promise of life-long expression of the deficient gene product. At present, there are three main vectors (retroviral, lentiviral and adeno-associated viral) that can integrate in recipient cells, and one vector type (adenoviral) that is maintained as an EPISOME.

Retroviral vectors. Vectors based on retroviruses were among the first to be designed, and they have been important in the technical and conceptual development of viral vectors as a whole[4–7]. Retroviruses have three essential genes, which can be provided separately in

© 2000 Macmillan Magazines Ltd

TRANSDUCTION
The introduction of a gene into a target cell by a viral vector.

INTERNAL RIBOSOME ENTRY SITE
A sequence that is inserted between the coding regions for two proteins and allows efficient assembly of the ribosome complex in the middle of a transcript, leading to translation of the second protein.

HAEMATOPOIESIS
The programme of cellular differentiation leading to the formation of blood cells.

packaging cells: *gag* encodes viral structural proteins, *pol* encodes reverse transcriptase/integrase and *env* encodes viral envelope glycoprotein. The idea of separating *gag* and *pol* from *env* offered a packaging cell line in which the chances of generating replication-competent retroviruses (RCR) were significantly reduced (FIG. 1) — a concept in vector design first applied to retroviruses. Retroviral vectors were also important for the development of strategies, such as changing the envelope protein, to modify the range of target cells from ecotropic (infecting only rodent cells) to xenotropic (infecting most mammalian cells except rodent cells), amphotropic (infecting all mammalian cells) and pantropic (infecting various species)[8–10]. Another conceptual breakthrough in generating safe and transcriptionally regulable vectors was the development of SIN (self-inactivating) vectors[11], in which the viral regulatory elements have been deleted. On integration, all viral promoter/enhancer activity is lost and the transcription of the transgene is under the control of a heterologous promoter.

The use of retroviral vectors has also lead the way in the technological production, storage and distribution of commercial vectors on the scale required for human clinical trials. A wide variety of packaging cell lines and vectors with improved TRANSDUCTION efficiencies are now used that include features such as tissue-specific promoters, inducible promoters, INTERNAL RIBOSOMAL ENTRY SITES (IRESs) and *env* proteins with modified target specificity[3,12].

The main limitation of retroviral vectors has been their inability to infect non-dividing cells, meaning that tissues such as brain, eye, lungs and pancreas are not amenable to direct *in vivo* gene delivery. Furthermore, on transplantation of transduced cells in the host, transcription of the transgene is often extinguished[13,14]. These two serious limitations have lead many scientists to search for vectors that can infect non-dividing cells, as well as integrate into the host chromosome. Retroviral vectors continue to be extensively used for introducing genes into dividing cells, such as tumour cells and dividing HAEMATOPOIETIC cells.

Lentiviral vectors. Lentiviruses, such as human immunodeficiency virus (HIV), are part of the retrovirus family, but have acquired the unusual property of transducing non-dividing cells[15,16]. The mechanism of this KARYOPHILIC attribute is still in contention, but it clearly offers an opportunity missing in its distant cousins, the prototypic retroviruses such as Moloney murine leukaemia virus (M-MLV). The HIV genome, in addition to coding for the usual Gag, Pol and Env proteins, also codes for the accessory proteins[17] Tat, Rev, Nef, Vif, Vpu and Vpr. Although the precise involvement of each of these accessory proteins in the aetiology of AIDS is still a matter of debate, none of them is obligatory (except for Rev and Tat) for virus propagation *in vitro*.

The first-generation lentiviral vectors relied largely on substitution of viral Env protein with vesicular stomatitis virus G protein (VSVG), which relieved them of their dependence on CD4, the T-cell receptor protein required for lentivirus infection[18]. Instead, the vectors showed a wider TROPISM by infecting cells known not to express CD4 protein, including neurons, hepatocytes, muscle fibres and retinal cells. Although the first-generation vectors fulfilled many of the criteria of an ideal vector, they were viewed with some suspicion because of the possibility of recombination and generation of infectious HIV. To minimize some of these concerns, several research groups have taken the initiative of systematically deleting as many viral accessory genes as possible while maintaining the key feature of infection of non-dividing cells[19,20]. The latest lentiviral vector system retains less than 25% of the viral genome in the packaging constructs and less than 5% of the viral genome in the vector construct[20]. Extra features that have improved vectors include the central polypurine

Box 1 | Properties of the ideal gene therapy vector

Easy production
The vector should be easy to produce at high titre on a commercial scale. This consideration stems from the wide range of cell numbers that must be transduced — from a handful of stem cells capable of reconstituting the entire haematopoietic repertoire to 10^{11} or more cells to infect 5–10% of the liver. For widespread use, the vector should be amenable to commercial production and processing (such as concentration technology for delivery in small volumes), and should have a reasonable shelf-life for transport and distribution.

Sustained production
The vector, once delivered, should be able to express its genetic cargo over a sustained period or expression should be regulable in a precise way. Different disease states have different requirements (for example, regulated expression in diabetes and lifetime expression in haemophilia).

Immunologically inert
The vector components should not elicit an immune response after delivery. A humoral antibody response will make a second injection of the vector ineffective, whereas a cellular response will eliminate the transduced cells.

Tissue targeting
Delivery to only certain cell types is highly desirable, especially where the target cells are dispersed throughout the body (such as in the haematopoietic system), or if the cells are part of a heterogeneous population (such as in the brain). It is also important to avoid certain cells, such as dendritic cells, the 'professional' antigen-presenting cells of the body, because of their role in mediating the immune response. Cell or tissue-targeted vectors present a great challenge, but also offer rich dividends for gene therapy approaches.

Size capacity
The vector should have no size limit to the genetic material it can deliver. The coding sequence of a therapeutic gene can vary from 350 base pairs for insulin, to over 12,000 base pairs for dystrophin. Furthermore, addition of appropriate regulatory sequences may be required for efficient transduction and expression of the foreign genetic material.

Replication, segregation or integration
The vector should allow for site-specific integration of the gene into the chromosome of the target cell, or should reside in the nucleus as an episome that will faithfully divide and segregate on cell division. Site-specific integration is a very desirable attribute because it eliminates the uncertainty of random integration into the host chromosome, and endogenous regulatory regions will control its expression under physiological conditions. The ability of the vector to be maintained as an episome could make the genetic elements independent of local chromatin environments, but faithful replication and segregation is needed if the vector is to be effective in systems such as stem cells.

Infection of dividing and non-dividing cells
As large numbers of cells (such as neurons, hepatocytes and myocytes) are postmitotic, vectors capable of efficiently transducing non-dividing cells are very desirable.

© 2000 Macmillan Magazines Ltd

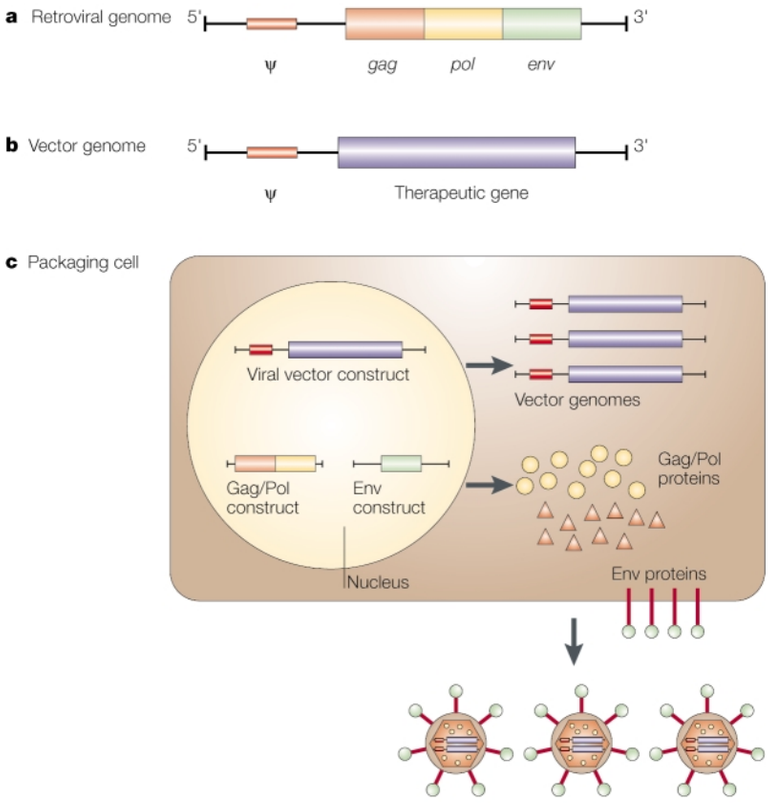

Figure 1 | **Retrovirus-based vectors. a** | The retroviral genome contains the *gag, pol* and *env* genes. The ψ sequence is the packaging sequence (in red) that differentiates the viral RNA from all other RNA in the cell, and is recognized by the viral proteins for packaging. **b** | The vector genome. The *gag, pol* and *env* genes are replaced by the therapeutic gene. **c** | The packaging cell. The *gag* and *pol* genes are separated from the *env* gene, making regeneration of a replication competent virus unlikely. The retrovirus is a membrane-bound virus, and the Env protein is expressed on the cell surface. The vector genomes, by virtue of the ψ sequence, are encapsulated along with the Pol and Gag proteins and are assembled under the membrane. The virus buds off from the packaging cell, resulting in the retroviral vector, which is used to infect the target cell.

Animated online

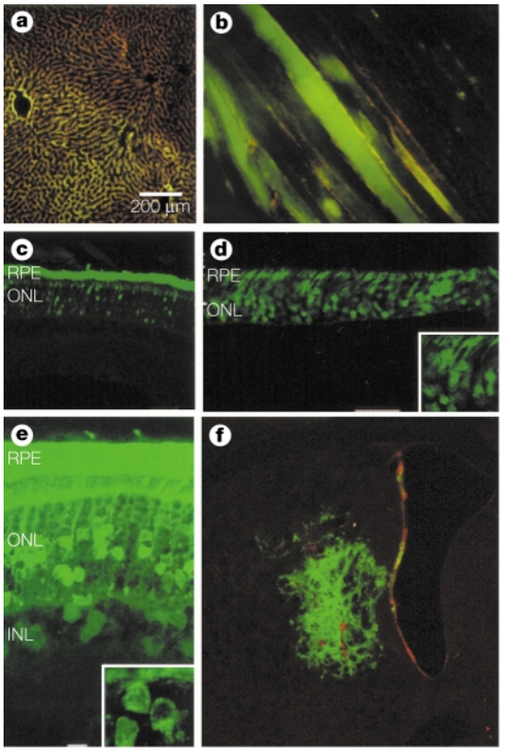

Figure 2 | ***In vivo* gene delivery into mice and rats using lentiviral vectors.** The gene coding for green fluorescent protein (GFP) was placed into lentiviral vectors, and these were injected into various tissues. **a,b** | Efficient gene transduction (green cells) to **a** | liver, and **b** | muscle. **c,d** | Different promoters driving transgene expression. **c** | The cytomegalovirus (CMV) promoter or **d** | the rhodopsin promoter driving *GFP* expression. Note the high-level expression of *GFP* in the retinal pigment epithelium (RPE) with the CMV promoter, and in photoreceptor cells with the rhodopsin promoter. **e,f** | A third-generation SIN lentivirus vector expresses *GFP* under the control of the CMV promoter **e** | in the retina and **f** | in the brain. ONL, outer nuclear layer; INL, inner nuclear layer. (See REFS 19,77.)

tract (cPPT), which allows internal initiation of second-strand DNA synthesis and probably aids in the transport of the pre-integration complex to the nucleus[21,22]; and the woodchuck post-transcriptional regulatory element (WPRE), which further improves the transduction and translational efficiency of lentiviral vectors[23]. Use of SIN lentiviral vectors further reduces the chance that recombination will generate replication-competent HIV[24,25]. Recently, packaging-cell lines in which most of the accessory gene products have been deleted have also been developed (D. Trono and T. Kafri, personal communication). The improved minimal lentiviral vectors can efficiently infect most cell types tested (FIG. 2).

Some investigators have used non-human lentiviruses, such as simian immunodeficiency virus, feline immunodeficiency virus and equine infectious anaemia virus, to generate efficient vectors capable of transducing non-dividing cells[26–29]. There are no clinical trials with lentiviral vectors at present, but several groups are discussing their use with regulatory authorities. Like other integrating vectors, the lentiviral vectors will have the disadvantage of non-specific integration in the chromosome. The duration of expression of the transgene in lentiviral vectors also needs further testing.

Adeno-associated viral vectors. Adeno-associated virus (AAV) is a small, non-pathogenic, single-stranded DNA virus that has turned out to be an efficient and useful delivery vehicle[30]. The virus is a member of the dependoviruses and, as the name suggests, requires extra genes to replicate[31]. These genes are provided by adenovirus (hence the name, as AAV was found associated with this virus) or herpes virus. AAV itself has two genes: *rep*, which codes for replication and integration functions of the virus; and *cap*, which codes for the structural components of the virus. On either side of *rep* and *cap* are two inverted terminal repeats (ITRs), which define the beginning and the end of the virus, and contain DNA sequences needed to pack the viral genome into CAPSIDS.

KARYOPHILIC
Literally, attracted to the nucleus — a nuclear localization signal in a protein is karyophilic.

TROPISM
The range of cells that can be productively infected by a virus.

CAPSID
The proteinaceous coat surrounding a virus.

© 2000 Macmillan Magazines Ltd

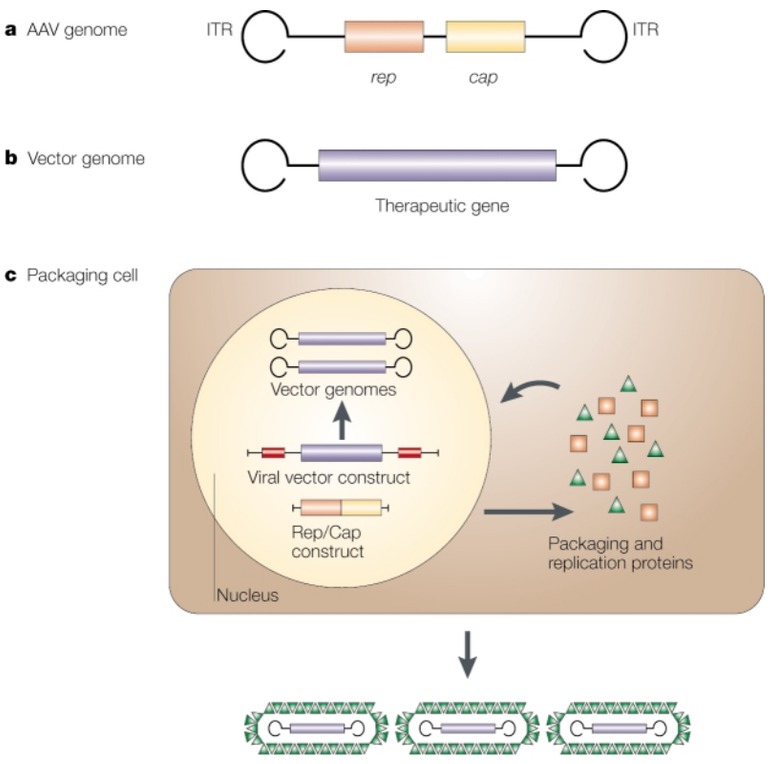

Figure 3 | **Adeno-associated viral vectors. a** | The adeno-associated virus (AAV) genome contains the sequences essential for transduction — the inverted terminal repeats (ITRs) and the genes *rep* and *cap*. **b** | In the vector genome, *rep* and *cap* are replaced by the therapeutic gene. **c** | The Rep and Cap gene proteins provided by the packaging constructs are required to produce single-stranded DNA genomes that are encapsulated by the coat proteins. AAV — a non-enveloped virus — is assembled in the nucleus. The helper proteins from adenovirus (Ad) needed for replication (E1A, E1B, E2A, E4orf6 and VA RNA) are not shown here. Ad replication is lytic, and this is the exit route for AAV. It has been difficult to separate Rep and Cap coding regions and still get production of high titre virus. As a result generation of replication competent virus is still possible. However, the target cell still needs helper functions from adenovirus for replication of the replication competent virus.

The viral vector is produced by replacing the *rep* and *cap* genes with the therapeutic gene. The Rep and Cap proteins are produced *in trans* in the packaging cell, as are the adenoviral proteins needed for replicating the virus (FIG. 3). AAV can integrate in a site-specific location on chromosome 19 through the action of the Rep protein. However, as the vector does not code for Rep, it does not have this highly desirable attribute after entry into the target cell[32]. Another interesting feature of AAV vectors with respect to chromosomal integration is their propensity for homologous recombination. The vector has been shown to correct point mutations and deletions in selectable reporter genes integrated into the chromosome, although this occurs at a very low frequency[33].

The cell tropism of the virus, and hence the vector, is relatively broad, and most cell types are receptive to gene transfer to some extent. Efforts are underway to define the receptors and residues in the capsid proteins responsible for interaction with the cell surface, and this could lead to the design of new capsids with restricted or targeted tropism[34,35]. When tested in mice, dogs and monkeys, gene expression from AAV vectors is sustained in tissues with long-lived cells, such as muscle, liver and brain[30]. This long-term expression results from randomly integrated vectors and some vector DNA that persists as extra-chromosomal DNA. At present, it is not clear what proportion of expression originates from integrated or extra-chromosomal DNA[36].

The main problem with AAV vectors is that the *rep* gene and some of the adenoviral helper genes are cytostatic and cytotoxic to the packaging cells, and so it has been difficult to scale up production of these viral vectors. No cell lines have been reported that stably produce high-titre AAV vectors carrying a therapeutic gene. Current clinical trials with AAV vectors rely on transient production systems, which may be suitable for proof of principle, but a more efficient production system is urgently required[37]. Another problem is the coding capacity of the vector, which is restricted to around 4.5 kilobases. However, two groups have extended the packaging capacity of these vectors by using the observation that AAV genomes concatomerize after transduction. When two vectors, one encoding the first half and the other encoding the second half of a protein, were transduced into cells, head-to-tail stitching of the viral genomes resulted in the reconstitution of a functional gene, effectively increasing the size of the gene that can be delivered. It remains to be seen if concatomerized vectors will be stable and have sustained expression[38,39].

Adenoviral vectors. The adenoviruses are a family of DNA tumour viruses that cause benign respiratory tract infections in humans[40]. The genome contains over a dozen genes, and on infection the virus remains episomal in the nucleus, and can transduce genetic material into both dividing and non-dividing cells. Replication-defective recombinant adenoviral vectors can be generated at high titres by deleting several viral genes, including *E1A*, *E1B*, *E3*, *E4* and *E2A* (REF. 41). Most recently, 'gutless' adenoviral vectors (in which all the viral genes are removed and provided *in trans*) have also been generated[42,43]. All adenoviral vectors retain the ability to transduce dividing and non-dividing cells efficiently, and it is relatively easy to generate high-titre commercial-grade recombinant vectors.

The challenge for adenoviral vectors concerns the persistence of expression of the transgene. All adenoviral vectors so far, with the exception of gutless vectors (for which data are still preliminary)[44], express the transgene in adult animals for only a short time (between 5 and 20 days post-infection)[45]. In immuno-compromised animals, expression in long-lived cells, such as muscle cells and neurons, is observed for long periods. It is now generally recognized that the short-term expression from recombinant adenoviral vectors is because of the immune response (see below). These vectors will, however, continue to be used in situations in which high-level but transient expression of the foreign gene is required, for example in RESTENOSIS and cancer.

Immune response: the bane of gene therapy

The biggest challenge facing all viral vectors is the immune response of the host. The host defence mechanism functions at both the cellular level, by generating

RESTENOSIS
Stenosis is the blocking of a blood vessel that can be cleared by mechanical disruption. Restenosis is the recurrence of the blockage caused, for example, by unchecked proliferation and migration of vascular smooth muscle cells.

© 2000 Macmillan Magazines Ltd

cytotoxic T cells, and at the humoral level, by generating antibodies to viral proteins. Cellular immunity eliminates the transduced cells, whereas humoral immunity precludes the repeat administration of the vector because the subsequent antibody response will be boosted by MEMORY CELLS[45,46]. The host immune system may also recognize the transgene product as foreign, and induce both cellular and humoral immunity[47].

To minimize the cellular response, most vectors have been designed to prevent the synthesis of viral proteins following transduction. However, adenoviral vectors present a unique problem, because even the inactivated recombinant adenoviral vectors can elicit potent cytotoxic T-cell responses against viral proteins[46]. Therefore it is difficult to see how gutless vectors, which still require the full complement of viral structural proteins for efficient transduction, can bypass this host immune response. The humoral response is also most pertinent to adenoviral vectors because they do not integrate, and so suffer loss by cell division and by DNA degradation, necessitating a repeat infection with the vector. The host raises neutralizing antibodies against viral proteins, thereby precluding any further infection. As there are scores of adenoviral SEROTYPES, one strategy to overcome this problem might be to use different serotypes[40].

Retroviral, lentiviral and AAV vectors do not seem to suffer from cytotoxic-T cell responses. It could be that the vectors are completely replication-defective, and that the incoming viral proteins do not elicit a cytotoxic T-cell response. Alternatively, the titres of at least the recombinant retro- or lentiviral vectors tested so far might not be sufficiently high to elicit an immune response. Antibody responses are also less of a concern, as retro- and lentiviral vectors integrate into the host genome and may not require subsequent transduction. Furthermore, for vectors engineered with the VSVG protein, there are several strains of VSV that have different serotypes[48]. Antibodies to AAV-based vectors have been detected, which has prevented transduction by a second injection of the vector[49], but this may also be overcome by using another serotype of AAV[50].

Why some vectors are more immunogenic than others is a matter of considerable interest, and early hints indicate that antigen-presenting DENDRITIC CELLS may be important. In contrast to AAV-based vectors, it seems that adenoviral vectors efficiently transduce dendritic cells[51]. The route of administration also influences the immunological outcome[52], and there is the question of pre-immunity in the host. Over 70% of the population may be carrying antibodies to adenovirus and AAV. What function might these pre-existing antibodies have in the efficiency of transduction or the toxicity of viral vectors? Are there sites in the body where the humoral response can be bypassed by the introduced vectors? Finally, the transgene itself may be highly immunogenic, particularly in hosts in which the transgene product was never made, due to either complete gene deletion or aberrant expression[53]. So, the gene therapy strategy in such hosts will also require the induction of TOLERANCE. Ultimately, individual patients may well require a therapeutic regimen tailored to their specific pathology, in the context of a genetic background that influences their immune response.

MEMORY CELLS
Immune cells that are primed, after an initial exposure to an antigen, to make a rapid response to subsequent exposure to the same antigen.

SEROTYPES
Antigenically distinct forms that elicit different antibody responses by the immune system.

DENDRITIC CELLS
These cells present antigen to T cells, and stimulate cell proliferation and the immune response.

TOLERANCE
The lack of an immune response to a specific foreign protein.

The Gelsinger tragedy

Jesse Gelsinger was barely 18 years old when he became the first patient to die in a Phase I gene therapy clinical trial. Although many patients have experienced severe adverse effects and even death during Phase I safety and toxicity studies, Jesse was the first patient in whom death could be directly attributed to the vector — an adenoviral vector.

Jesse suffered from deficiency of ornithine transcarbamylase (OTC), a metabolic enzyme required to break down ammonia. The total lack of this enzyme leads to death shortly after birth, owing to a build-up of ammonia. The partial presence of enzyme activity also leads to an accumulation of ammonia, but can be controlled by drugs and dietary intake. The aetiology of the disease, its associated morbidity, and the need for rapid production of the enzyme suggested that transient production of OTC by adenoviral vectors could extend the lifespan of OTC-deficient newborns, to allow implementation of a drug and dietary regime[54].

The Phase I trial consisted of a study in which a cohort of patients with partial OTC activity were given escalating doses of second-generation (deleted for the *E1* and *E4* genes) adenoviral vectors. Jesse was in the last cohort, receiving up to 6×10^{13} recombinant adenoviruses particles containing the *OTC* gene. Within hours of intra-hepatic administration, he began to experience severe complications and died two days later. What went wrong? Was too much virus infused? Perhaps not, as another patient getting the same dose of the same vector did not suffer the same consequences. Were Jesse's adenoviral antibody titres higher? Again, there is no clear answer, as other patients with higher antibody titres did not have the same reaction. Perhaps there were other mitigating causes, like other viral infections or higher levels of ammonia before vector transduction. Are the animal models really reliable? Should we be screening patients for genetic variations, as immune responses are so heterogeneous? These are just some of the questions that have been raised, and several expert committees are now in the process of defining and refining new measures for gene therapy trials (LINKS).

The first successes

The field of gene therapy also has cause to celebrate. Alain Fischer and colleagues in Paris have successfully treated three young babies (1–11 months old), who suffer from a fatal form of severe combined immunodeficiency (SCID) syndrome[55]. SCID-XI is an X-linked disorder characterized by an early block in T- and natural killer (NK) cell differentiation, due to mutations of the gene encoding the γC cytokine receptor subunit common to several interleukin receptors. A mutation in the γC subunit leads to disruption of signals required for growth, survival and differentiation of lymphoid progenitor cells.

Haematopoietic stem cells from the patients were transduced *ex vivo* with a recombinant mouse

© 2000 Macmillan Magazines Ltd

leukaemia viral vector containing the γC receptor gene and infused back into the young patients. After ten months, γC transgene expression in T- and NK cells was detected in the patients but, more importantly, T-, B- and NK-cell counts and function were comparable to those of age-matched controls. To all appearances, the recipients are clinically cured, and the fantastic promise of gene therapy is realized. Some concerns remain: only ten months of data are available, and expression of the transgene may be 'shut-off'. Also, very few patients have been treated so far. It remains to be seen if this approach will work for other diseases, because the success with SCID-XI is probably owing to the strong selective advantage provided to the transduced lymphoid progenitors. Only those haematopoietic cells that express the γC receptor subunit can survive and differentiate. In earlier trials of SCID patients suffering from adenosine deaminase deficiency (ADA), PEG–ADA (a protein preparation with enzymatic activity) was administered to the patients in addition to the vector expressing the *ADA* gene. This may have prevented the selective advantage observed in the successful French trial[56–58]. The use of modified MLV vectors and the extensive manipulation of the stem cells (use of cytokines to stimulate cell proliferation) before transduction are a testimony to the continuous and incremental progress made in the field. We believe that with the availability of lentiviral vectors capable of transducing resting stem cells, the efficiency of transduction will improve even further[59,60].

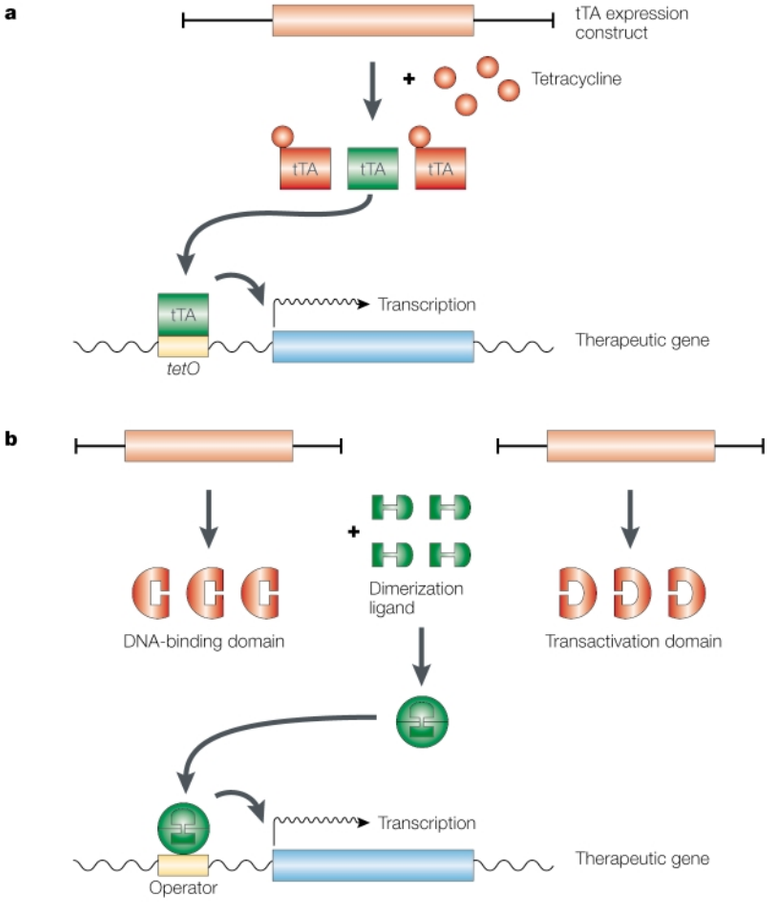

Figure 4 | **Regulation of gene expression. a** | The Tet system. Two expression cassettes need to be delivered to the target cell. One expresses the tTA protein, which either binds to tetracycline (red) or the *tet* operator (*tetO,* yellow), and the other expresses the therapeutic gene under the control of *tetO*. Gene expression is conditional on the binding of tTA to *tetO*, and hence on the absence of tetracycline. **b** | A dimerization-regulated system. Three expression constructs need to be delivered to the target cells that code for a DNA-binding domain, a transactivation domain and the therapeutic gene under the control of operator sequences of the DNA-binding domain. The DNA-binding domain binds to the operator sequence, but cannot trigger gene expression without the transactivation domain. These two domains can be dimerized by a ligand, and the dimer activates gene expression.

Haemophilia (A and B) is another excellent model system for gene therapy because the deficient protein does not have to be provided from its normal cellular source. Therefore, several vectors have been designed that transduce a range of cells to produce and secrete factor IX protein. Both the factor IX knockout mice and haemophilic dogs have turned out to be extremely beneficial pre-clinical model systems[53,61,62]. Further model systems will continue to be useful for pre-clinical studies, and promising results have recently been obtained in a mouse model of β-thalassaemia[63]. In another exciting human study, Kathryn High and colleagues at the University of Pennsylvania have treated several haemophilia B (factor IX-deficient) patients in a Phase I clinical trial with AAV vectors that contain the human *factor IX* gene[37]. The recombinant vector was injected intramuscularly, and preliminary results indicate that factor IX protein can be found in the serum of a patient. Although the levels of the factor IX protein expected to be produced by the low doses of injected AAV are not curative[44], the treated patients did show some clinical benefits. No factor IX inhibitors were found, but neither could they be expected, because very low amounts of factor IX were being secreted. This is still a preliminary study, but nevertheless it bodes well for success in treating haemophilia.

The next phase

It was not long ago that the 'battlecry' of the gene therapy community was 'titres, titres, titres'. Then it switched to 'delivery, delivery, delivery', and now it is 'expression, expression, expression'. We have the appropriate titres of desirable vectors for delivering genes to patients. The emphasis now is on efficiency, safety and duration of expression. The issue of safety will always remain predominant, and the trend is to generate 'minimal vectors' carrying the least amount of viral information needed for successful transduction.

Significant progress in vector development is occurring in the area of tissue- or cell-specific expression. For example, there have been encouraging advances in the targeting of vectors to specific tissues. The ideal here is that the original tropism of the virus is ablated and a new specificity generated. To this end, two strategies have generally been pursued. The first strategy involves engineering viral proteins responsible for binding the cellular receptors that subsequently mediate viral entry. For retroviruses, these are the envelope proteins. They have been altered to add new binding domains to the envelope, and although this does target

© 2000 Macmillan Magazines Ltd

the binding of the retroviral viral vectors to the desired cell type, it has been difficult to engineer a high-titre targeted vector. There is a coordination of binding and subsequent fusion between the virus and cell membranes, which has been difficult to achieve for the modified envelopes, resulting in low titres[64]. This type of engineering has also been reported for adenoviral[65] and for AAV vectors[35], and although there can be a loss of titre for these vectors, the approach is promising. The second strategy relies on adaptor molecules that bridge the virus and the cell, and has been applied to retroviral[66], adenoviral[65] and AAV vectors[67]. The adaptors are modular and comprise a component that binds the virus (either a fragment of the cellular receptor or part of a monoclonal antibody) and a component that recognizes the target molecule (a ligand or antibody). However, generating these vectors at high titres and testing their utility *in vivo* are problems that need to be overcome.

Although tissue- and cell-specific expression will continue to command interest, we suspect that regulated expression of the transgene will become an important focus for practitioners of gene therapy. Fortunately, several promising systems are available and are being explored. All are based on the idea that the expression of the therapeutic gene can be regulated by an inducible, co-expressed transcription factor. The induction should be reversible, the inducer must be non-toxic (and preferably active on oral administration), and the inducer should not activate other genes. Furthermore, the components of the regulatory system should not be immunogenic in the host.

The most widely used regulation system is based on bacterial tetracycline resistance regulation (the Tet system). The bacterial protein, TetR, binds to its target DNA, the *tet* operator (*tetO*), only in the absence of tetracycline or its non-antibiotic analogue, doxycycline (Doc). Bujard and colleagues[68] have engineered TetR and appended it to a eukaryotic transcriptional activator (tTA). The transgene, downstream of *tetO*, has to be delivered to the target cell along with the coding sequence for tTA. In the presence of tetracycline, the target gene is 'off', and in the absence of tetracycline, the target gene is 'on' (FIG. 4, FIG. 5). This system has also been extended, by ingenious manipulation, to generate rtTA, which binds DNA and activates transcription in the presence of tetracycline[69]. Modulation of the amount of tetracycline also modulates the amount of protein produced, and if tTA is produced from a tissue-specific promoter, this can enable spatial control of gene expression. As tTA is based on a bacterial gene, it may be recognized as foreign by the human immune system, but so far it has been tolerated in animal models. It has proved to be applicable to a wide variety of systems, including those using AAV and lentiviral vectors[70,71].

A second system is based on steroid hormones and relies on the observation that binding of the hormone to its receptor activates gene transcription. An early system was based on a truncated version of the progesterone receptor[72]. This mutant receptor binds progesterone antagonists, such as RU486, and, paradoxically, activates transcription. This mutant has been engineered with a DNA-binding domain from yeast (GAL4) and a transcriptional transactivation domain from a viral protein. Activation of a transgene downstream of the GAL4-binding sequence is conditional on the presence of RU486, which can be administered orally. However, RU486 is not inert — it is an antagonist of the progesterone receptor and is used as a method of birth control. Variants of this system use a steroid hormone receptor from a different organism — for example, the ecdysone receptors of insects such as *Drosophila*[73] and *Bombyx*[74]. In the presence of the insect hormone ecdysone, expression of the receptors in target cells activates transcription of genes placed downstream of ecdysone receptor DNA-binding sites. Agonists of these receptors, such as muristerone A, are commercially available and seem to be non-toxic[75].

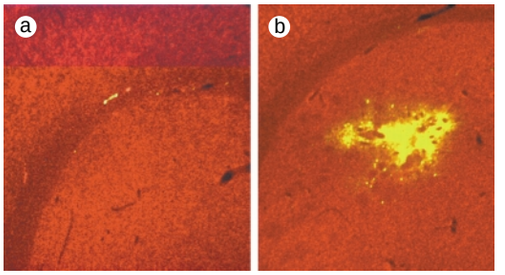

Figure 5 | **Regulated expression of a gene therapy vector.** Regulated expression is illustrated with a lentiviral vector in which the gene coding for green fluorescent protein (GFP) is under the control of the Tet system. *GFP* expression in the brain is **a** | predominately 'off' in the presence of tetracycline and **b** | 'on' in its absence[71].

Finally, a synthetic dimerizer strategy[75] has been successfully used *in vivo* to produce regulated amounts of erythropoietin[76] in an AAV vector system. The system involves ligand-binding sites on two proteins that can be brought together by a small molecule, resulting in dimerization (FIG. 4). The protein FKBP (FK506 binding protein) can be linked to a transactivation domain (FKBP–TA) and a DNA-binding domain (FKBP–DB). The target gene is linked to DNA-binding sites specific for the DNA-binding domain. The FKBP–DB will bind to the DNA-binding sites, and in the presence of the linker (a synthetic dimer of FK506, FK1012), FKBP–TA will form dimers with FKBP–DB and turn on transcription of the reporter gene. The immunogenicity of these proteins requires further investigation.

Perspectives

The last two decades have witnessed the birth of the field of gene therapy, which has generated great hopes and great hypes. The promise of influencing the outcome of a vast array of diseases, ranging from birth defects to neurological disorders and from cancer to infectious diseases, although far-reaching, is not beyond reach. With the completion of the sequence of the human genome, over 50,000 genes will be available to the practitioners of gene therapy. The potential benefits

© 2000 Macmillan Magazines Ltd

for human health are vast, so how can the biomedical community move forward to realize this potential?

Geneticists will continue to identify the genetic contribution to disease. Virologists will generate safe and efficient viral vectors, and molecular biologists will help to design vectors capable of cell- and tissue-specific expression of the foreign genes carried by the transducing vectors. Immunologists will work out ways to prevent unwanted immunological consequences of the delivery vehicles and their cargo. Cell biologists will devise ways to facilitate gene transfer to various tissues and will take the lead in identifying stem cells. Clinicians will carry out clinical trials on humans with the best vectors that the scientists can supply. To achieve successful gene therapy, all branches of biology will have to contribute to this endeavour.

Society has an enormous stake in science, and scientists have an obligation not to promise more than they can deliver. Gene therapy is a young science that has undergone extreme scrutiny in the recent past. It is our responsibility to assure the public that the patient's health and welfare is of paramount concern. Adherence to accepted guidelines is incumbent on all investigators participating in clinical trials, and those wilfully violating the recommended practices will have to pay the consequences. The field of gene therapy has also been rocked by charges of conflict of interest, an area relatively new in biomedical science. Harmonized guidelines need to be put in place to allay the public's concern of real or perceived conflicts of interest. The science of gene therapy has many hurdles ahead, but they are surmountable.

Links

DATABASE LINKS CD4 | OTC | *OTC* gene | SCID-XI | γC cytokine receptor subunit | ADA | *ADA* gene | Haemophilia A | Haemophilia B | factor IX | factor IX knockout mice | haemophilic dogs | β-thalassaemia | ecdysone receptor

FURTHER INFORMATION NIH recombinant DNA advisory committee meeting 8–10 March, 2000 | The Institute for Human Gene Therapy | Verma laboratory homepage

1. Li, S. & Huang, L. Nonviral gene therapy: promises and challenges. *Gene Ther.* **7**, 31–34 (2000).
2. Templeton, N. S. & Lasic, D. D. (eds) *Gene Therapy: Therapeutic Mechanisms and Strategies* (Marcel Dekker, Inc., New York, 2000).
   This book has several excellent chapters on viral and non-viral vectors written by experts in the field. It also describes a number of therapeutic approaches to diseases and requirements for regulatory affairs.
3. Friedmann, T. (ed.) *The Development of Human Gene Therapy* (Cold Spring Harbor Laboratory Press, New York, 1999).
4. Verma, I. M. Gene therapy. *Sci. Am.* **263**, 68–72 (1990).
5. Anderson, W. F. Human gene therapy. *Nature* **392**, 25–30 (1998).
6. Mulligan, R. C. The basic science of gene therapy. *Science* **260**, 926–932 (1993).
7. Miller, A. D. Human gene therapy comes of age. *Nature* **357**, 455–460 (1992).
8. Markowitz, D., Goff, S. & Bank, A. A safe packaging line for gene transfer: separating viral genes on two different plasmids. *J. Virol.* **62**, 1120–1124 (1988).
9. Danos, O. & Mulligan, R. C. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc. Natl Acad. Sci. USA* **85**, 6460–6464 (1988).
10. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M. & Yee, J. K. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and non-mammalian cells. *Proc. Natl Acad. Sci. USA* **90**, 8033–8037 (1993).
    Use of vesicular stomatitis virus glycoprotein (VSVG) to replace the envelope protein in MLV vectors. The resulting chimeric virus, containing VSVG protein, is pantropic and can be concentrated by ultracentrifugation.
11. Yu, S. F. *et al.* Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. *Proc. Natl Acad. Sci. USA* **83**, 3194–3198 (1986).
12. Morgan, R. A. & Anderson, W. F. Human gene therapy. *Annu. Rev. Biochem.* **62**, 191–217 (1993).
13. St Louis, D. & Verma, I. M. An alternative approach to somatic cell gene therapy. *Proc. Natl Acad. Sci. USA* **85**, 3150–3154 (1988).
    On transplantation of retrovirally-transduced mouse fibroblasts, producing and secreting human factor IX protein, the transcription of the transgene is 'shut off'.
14. Palmer, T. D., Rosman, G. J., Osborne, W. R. & Miller, A. D. Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. *Proc. Natl Acad. Sci. USA* **88**, 1330–1334 (1991).
15. Lewis, P., Hensel, M. & Emerman, M. Human immunodeficiency virus infection of cells arrested in the cell cycle. *EMBO J.* **11**, 3053–3058 (1992).
16. Bukrinsky, M. I. *et al.* A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature* **365**, 666–669 (1993).
17. Frankel, A. D. & Young, J. A. HIV-1: fifteen proteins and an RNA. *Annu. Rev. Biochem.* **67**, 1–25 (1998).
18. Naldini, L. *et al. In vivo* gene delivery and stable transduction of non-dividing cells by a lentiviral vector. *Science* **272**, 263–267 (1996).
    The first use of a lentiviral vector to transduce genes *in vitro* and *in vivo* in non-dividing cells. The lentiviral vector was packaged in VSVG envelope protein to expand the host range.
19. Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H. & Verma, I. M. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nature Genet.* **17**, 314–317 (1997).
20. Zufferey, R., Nagy, D., Mandel, R. J., Naldini, L. & Trono, D. Multiply attenuated lentiviral vector achieves efficient gene delivery *in vivo*. *Nature Biotechnol.* **15**, 871–875 (1997).
21. Zennou, V. *et al.* HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* **101**, 173–185 (2000).
22. Follenzi, A., Ailles, L. E., Bakovic, S., Geuna, M. & Naldini, L. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 *pol* sequences. *Nature Genet.* **25**, 217–222 (2000).
23. Zufferey, R., Donello, J. E., Trono, D. & Hope, T. J. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73**, 2886–2892 (1999).
24. Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H. & Verma, I. M. Development of a self-inactivating lentivirus vector. *J. Virol.* **72**, 8150–8157 (1998).
25. Zufferey, R. *et al.* Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. *J. Virol.* **72**, 9873–9880 (1998).
26. Johnston, J. C. *et al.* Minimum requirements for efficient transduction of dividing and nondividing cells by feline immunodeficiency virus vectors. *J. Virol.* **73**, 4991–5000 (1999).
27. Schnell, T., Foley, P., Wirth, M., Munch, J. & Uberla, K. Development of a self-inactivating, minimal lentivirus vector based on simian immunodeficiency virus. *Hum. Gene Ther.* **11**, 439–447 (2000).
28. Poeschla, E. M., Wong-Staal, F. & Looney, D. J. Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. *Nature Med.* **4**, 354–357 (1998).
29. Mitrophanous, K. *et al.* Stable gene transfer to the nervous system using a non-primate lentiviral vector. *Gene Ther.* **6**, 1808–1818 (1999).
30. Carter, P. J. & Samulski, R. J. Adeno-associated viral vectors as gene delivery vehicles. *Int. J. Mol. Med.* **6**, 17–27 (2000).
31. Berns, K. I. in *Fields Virology* (eds Fields, B. N., Knipe, D. M. & Howley P. M.,) 2173–2198 (Lippincott–Raven, Philadelphia, 1996).
32. Kotin, R. M. *et al.* Site-specific integration by adeno-associated virus. *Proc. Natl Acad. Sci. USA* **87**, 2211–2215 (1990).
33. Inoue, N., Hirata, R. K. & Russell, D. W. High-fidelity correction of mutations at multiple chromosomal positions by adeno-associated virus vectors. *J. Virol.* **73**, 7376–7380 (1999).
34. Summerford, C. & Samulski, R. J. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J. Virol.* **72**, 1438–1445 (1998).
35. Girod, A. *et al.* Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. *Nature Med.* **5**, 1052–1056 (1999).
36. Malik, P. *et al.* Recombinant adeno-associated virus mediates a high level of gene transfer but less efficient integration in the K562 human hematopoietic cell line. *J. Virol.* **71**, 1776–1783 (1997).
37. Kay, M. A. *et al.* Evidence for gene transfer and expression of *factor IX* in haemophilia B patients treated with an AAV vector. *Nature Genet.* **24**, 257–261 (2000).
    Gives the first hints of successful gene therapy in haemophilia B patients by using recombinant adeno-associated viral vectors. Expression of transduced *factor IX* could be detected in one patient.
38. Yan, Z., Zhang, Y., Duan, D. & Engelhardt, J. F. Trans-splicing vectors expand the utility of adeno-associated virus for gene therapy. *Proc. Natl Acad. Sci. USA* **97**, 6716–6721 (2000).
39. Nakai, H., Storm, T. A. & Kay, M. A. Increasing the size of rAAV-mediated expression cassettes *in vivo* by intermolecular joining of two complementary vectors. *Nature Biotechnol.* **18**, 527–532 (2000).
40. Shenk, T. in *Fields Virology* (eds Fields, B. N., Knipe D. M. & Howley, P. M.) 2111–2148 (Lippincott–Raven, Philadelphia, 1996).
41. Yeh, P. & Perricaudet, M. Advances in adenoviral vectors: from genetic engineering to their biology. *FASEB J.* **11**, 615–623 (1997).
42. Kochanek, S. *et al.* A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length *dystrophin* and β-galactosidase. *Proc. Natl Acad. Sci. USA* **93**, 5731–5736 (1996).
43. Parks, R. J. *et al.* A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc. Natl Acad. Sci. USA* **93**, 13565–13570 (1996).
    References 42 and 43 describe the generation of new adenoviral, 'gutless', vectors in which all the viral genes required for viral propagation are provided *in trans*. The gutless vectors show long-term expression of the transgene.

© 2000 Macmillan Magazines Ltd

44. Morral, N. *et al.* Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. *Proc. Natl Acad. Sci. USA* **96**, 12816–12821 (1999).
45. Dai, Y. *et al.* Cellular and humoral immune responses to adenoviral vectors containing *factor IX* gene: tolerization of *factor IX* and vector antigens allows for long-term expression. *Proc. Natl Acad. Sci. USA* **92**, 1401–1405 (1995).
46. Kafri, T. *et al.* Cellular immune response to adenoviral vector infected cells does not require *de novo* viral gene expression: implications for gene therapy. *Proc. Natl Acad. Sci. USA* **95**, 11377–11382 (1998).
Even physically inactivated adenoviral particles can generate a cytotoxic T-cell response, raising concerns about adenoviral vectors as suitable tools for long-term gene therapy.
47. Tripathy, S. K., Black, H. B., Goldwasser, E. & Leiden, J. M. Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. *Nature Med.* **2**, 545–550 (1996).
48. Wagner, R. R., & Rose, J. K. in *Fields Virology* (eds Fields, B. N., Knipe D. M. & Howley, P. M.) 1121–1136 (Lippincott–Raven, Philadelphia, 1996).
49. Chirmule, N. *et al.* Humoral immunity to adeno-associated virus type 2 vectors following administration to murine and nonhuman primate muscle. *J. Virol.* **74**, 2420–2425 (2000).
50. Halbert, C. L., Rutledge, E. A., Allen, J. M., Russell, D. W. & Miller, A. D. Repeat transduction in the mouse lung by using adeno-associated virus vectors with different serotypes. *J. Virol.* **74**, 1524–1532 (2000).
51. Fields, P. A. *et al*. Role of vector in activation of T cell subsets in immune responses against the secreted transgene product *factor IX*. *Mol. Ther.* **1**, 225–235 (2000).
52. Xiao, W. *et al*. Route of adminstration determines induction of T-cell-independent humoral responses to adeno-associated virus vectors. *Mol. Ther.* **1**, 323–329 (2000).
53. Wang, L., Takabe, K., Bidlingmaier, S. M., Ill, C. R. & Verma, I. M. Sustained correction of bleeding disorder in hemophilia B mice by gene therapy. *Proc. Natl Acad. Sci. USA* **96**, 3906–3910 (1999).
54. Scriver, C. R. S., Beaudet, A. L., Sly, W. S. & Valle, D. V. (eds) *The Metabolic Basis of Inherited Disease* (McGraw–Hill, New York, 1989).
55. Cavazzana-Calvo, M. *et al.* Gene therapy of human severe combined immunodeficiency (SCID)-XI disease. *Science* **288**, 669–672 (2000).
The first definitive example of successful gene therapy, in three children suffering from SCID-XI. The haematopoietic stem cells from the patients were transduced by recombinant retroviruses expressing the γc–subunit, which is common to many interleukin receptors.
56. Kohn, D. B. *et al.* T lymphocytes with a normal *ADA* gene accumulate after transplantation of transduced autologous umbilical cord blood $CD34^+$ cells in ADA-deficient SCID neonates. *Nature Med.* **4**, 775–780 (1998).
57. Blaese, R. M. *et al.* T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. *Science* **270**, 475–480 (1995).
58. Bordignon, C. *et al.* Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients. *Science* **270**, 470–475 (1995).
59. Miyoshi, H., Smith, K. A., Mosier, D. E., Verma, I. M. & Torbett, B. E. Transduction of human $CD34^+$ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* **283**, 682–686 (1999).
60. Guenechea, G. *et al*. Transduction of human $CD34^+CD38^-$ bone marrow and cord-derived SCID-repopulating cells with third-generation lentiviral vectors. *Mol. Ther.* **1**, 566–573 (2000).
References 59 and 60 show successful long-term transduction of human haematopoietic stem cells by lentiviral vectors, without the use of agents such as growth factors and cytokines.
61. Snyder, R. O. *et al.* Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nature Genet.* **16**, 270–276 (1997).
62. Wang, L., Nichols, T. C., Read, M. S., Bellinger, D. A. & Verma, I. M. Sustained expression of therapeutic level of *factor IX* in hemophilia B dogs by AAV-mediated gene therapy in liver. *Mol. Ther.* **1**, 154–158 (2000).
63. May, C. *et al.* Therapeutic haemoglobin synthesis in thalassaemic mice expressing lentivirus-encoded human-globin. *Nature* **406**, 82–86 (2000).
64. Cosset, F. L. & Russell, S. J. Targeting retrovirus entry. *Gene Ther.* **3**, 946–956 (1996).
65. Wickham, T. J. Targeting adenovirus. *Gene Ther.* **7**, 110–114 (2000).
66. Boerger, A. L., Snitkovsky, S. & Young, J. A. Retroviral vectors preloaded with a viral receptor-ligand bridge protein are targeted to specific cell types. *Proc. Natl Acad. Sci. USA* **96**, 9867–9872 (1999).
67. Bartlett, J. S., Kleinschmidt, J., Boucher, R. C. & Samulski, R. J. Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab'γ)2 antibody. *Nature Biotechnol.* **17**, 181–186 (1999); erratum **17**, 393 (1999).
68. Gossen, M. & Bujard, H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl Acad. Sci. USA* **89**, 5547–5551 (1992).
First description of a regulable gene expression system that uses tetracycline. The transgene is 'turned off' in the presence of tetracycline and 'turned on' in its absence.
69. Urlinger, S. *et al.* Exploring the sequence space for tetracycline-dependent transcriptional activators: Novel mutations yield expanded range and sensitivity. *Proc. Natl Acad. Sci. USA* **97**, 7963–7968 (2000).
70. Maxwell, I. H., Spitzer, A. L., Long, C. J. & Maxwell, F. Autonomous parvovirus transduction of a gene under control of tissue-specific or inducible promoters. *Gene Ther.* **3**, 28–36 (1996).
71. Kafri, T., Van Praag, H., Gage, F. H. & Verma, I. M. Lentiviral vectors: Regulated gene expression. *Mol. Ther.* **1**, 516–521 (2000).
72. Wang, Y., O'Malley, B. W. Jr, Tsai, S. Y. & O'Malley, B. W. A regulatory system for use in gene transfer. *Proc. Natl Acad. Sci. USA* **91**, 8180–8184 (1994).
73. No, D., Yao, T. P. & Evans, R. M. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc. Natl Acad. Sci. USA* **93**, 3346–3351 (1996).
74. Suhr, S. T., Gil, E. B., Senut, M. C. & Gage, F. H. High level transactivation by a modified *Bombyx* ecdysone receptor in mammalian cells without exogenous retinoid X receptor. *Proc. Natl Acad. Sci. USA* **95**, 7999–8004 (1998).
75. Spencer, D. M., Wandless, T. J., Schreiber, S. L. & Crabtree, G. R. Controlling signal transduction with synthetic ligands. *Science* **262**, 1019–1024 (1993).
76. Ye, X. *et al.* Regulated delivery of therapeutic proteins after *in vivo* somatic cell gene transfer. *Science* **283**, 88–91 (1999).
77. Miyoshi, H., Takahashi, M., Gage, F. H. & Verma, I. M. Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. *Proc. Natl Acad. Sci. USA* **16**, 10319–10323 (1997).

© 2000 Macmillan Magazines Ltd