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SPECIAL ARTICLE

Non-replicating expression vectors: applications in vaccine development and gene therapy

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SUMMARY

This review presents experimental, preclinical and clinical data illustrating the multiple uses of recombinant non-replicating virus vectors in the fields of immunoprophylaxis and gene therapy.

INTRODUCTION

A variety of non-replicating virus vectors have been developed for vaccine and gene therapy applications. This review will focus on the development of three virus vectors, poxviruses, adenoviruses and retroviruses, and will discuss some of the recent advances made with these vectors in the fields of vaccine immunoprophylaxis and gene therapy.

POXVIRUSES

On 26 October 1979, the World Health Organization announced that smallpox had been eradicated. This monumental achievement was made possible by the immunization of much of the world's population with vaccinia virus, a virus antigenically similar to variola virus, the epizootic agent of smallpox. The success of this vaccine prompted the development of poxviruses as recombinant expression vectors [1, 2]. Poxviruses are very large, double-stranded DNA viruses. Individual species can either have a very broad or very restricted host range. For example, vaccinia virus can replicate in a number of vertebrate species, whilst variola virus only replicates in man; a characteristic that facilitated the eradication of smallpox.

The nucleotide sequence of the Copenhagen strain of vaccinia virus has been determined [3]. The genome is approximately 191 kb and encodes 198 major open reading frames (ORFs). Large regions (> 47 kb) of the genome are non-essential for replication *in vitro* [4]. In addition, large amounts of heterologous DNA (> 25 kb) can be cloned into a single recombinant [5]. The lack of stringent packaging constraints allows multiple foreign genes to be cloned into the poxvirus genome.

Poxviruses, unlike other DNA viruses, replicate in the cytoplasm of the infected cell. Consequently, enzymatic functions involved in transcription and replication must be supplied by the virus. This has several consequences regarding the use of these viruses as expression vectors. For example, eucaryotic promoters are not efficiently recognized by the poxvirus transcriptional machinery. Therefore, poxvirus promoters must be used for efficient transcription of recombinant genes. In addition, poxvirus transcripts are not spliced. Therefore, genetic material cloned into poxviruses must be in a cDNA rather than a genomic form. Finally, due to the large size and non-infectious nature of poxvirus DNA, foreign genes are cloned into poxviruses by *in vivo* recombination [1].

Vaccines

Numerous examples have been reported in which immunization with a vaccinia virus recombinant expressing an immunogenic antigen of a particular pathogen has protected target species against a subsequent challenge with the corresponding pathogen. For example, oral administration of a vaccinia virus recombinant expressing the rabies glycoprotein has protected foxes and raccoons against rabies [6, 7]. In addition, distribution of a vaccinia virus-rabies glycoprotein recombinant, RABORAL, has dramati-

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cally decreased the incidence of rabid foxes and raccoons in European and North American field trials [8, 9]. Vaccinia virus recombinants have also protected target species against vesicular stomatitis, canine distemper, rinderpest, pseudorabies and Venezuelean equine encephalitis [10–14]. Therefore, vaccinia virus recombinants are effective against a variety of infectious diseases.

Smallpox vaccination was responsible for a small number of vaccine-related complications. Not surprisingly, some complications (e.g., encephalitis and progressive vaccinia infection) were associated with the age and immunological status of the vaccinee. Different incidences of complications were also associated with different strains of vaccinia virus. For example, in the Netherlands, complications appeared in approximately 1/4000 primary vaccinees, whilst in the US, where a different strain was used, complications arose in approximately 1/250000 vaccinees [15].

In response to these safety concerns, highly attenuated vaccinia virus strains have been generated. There is evidence, however, that the genetic background of a vaccinia vector can affect the potency of a subsequent recombinant. For example, a vaccinia virus (WR strain) recombinant expressing the Epstein-Barr virus (EBV) gp340 envelope glycoprotein protected cottontop tamarins against an EBV challenge, whereas another vaccinia virus (Wyeth vaccine strain) recombinant expressing the same antigen was unable to protect tamarins against an identical challenge [16]. Therefore, there is a need for safe, but yet efficacious poxvirus vectors.

NYVAC

NYVAC is a highly attenuated poxvirus vector. It was derived from the Copenhagen strain of vaccinia virus by the precise deletion of 18 ORFs [17]. Many of these ORFs encode functions implicated in the pathogenicity and host range/replication of vaccinia virus. Consequently, NYVAC does not produce detectable ulceration or induration at the site of inoculation, has negligible pathogenicity in newborn and immunocompromized mice and, while retaining the ability to replicate efficiently in primary chick embryo fibroblasts, has a dramatically reduced replicative capacity in murine, porcine, equine and human tissue culture cells.

Despite these highly attenuated properties, NYVAC remains an efficacious vaccine vector. In a rabies challenge study [17], a NYVAC-rabies glycoprotein recombinant (NYVAC-RG) had a virtually identical 50% protective dose (PD_{50}) value as a replication-competent thymidine kinase deficient vaccinia virus-rabies glycoprotein recombinant (VV-RG) (Table 1).

Table 1. *Comparative efficacy of VV-RG, ALVAC-RG and NYVAC-RG in mice*

Recombinant	PD_{50}*
VV-RG	3·74
ALVAC-RG	3·86
NYVAC-RG	3·70

* Four to 6-week-old mice were inoculated with 2·0–8·0 $\log_{10}$ tissue culture infection dose 50% ($TCID_{50}$) of VV-RG, ALVAC-RG or NYVAC-RG. At day 14, the mice were challenged intracranially with 15 lethal dose 50% (LD_{50}) of rabies virus. At day 28, the surviving mice were counted and a PD_{50} was calculated. (Modified from ref. 17).

NYVAC recombinants have also protected target species against infectious challenges. A NYVAC recombinant expressing the preM and envelope proteins of Japanese encephalitis virus (JEV) protected pigs against JEV [18], a recombinant expressing the type A1 and type A2 haemagglutinin (HA) glycoproteins of equine influenza virus (EIV) protected horses against EIV [19] and recombinants expressing the gB or gD glycoproteins of pseudorabies virus (PRV) protected pigs against PRV [20]. These studies indicate that NYVAC retains the immunogenicity and potency of a replication-competent vaccinia virus vector, and therefore represents a safer alternative to existing vaccinia strains.

Avipoxviruses

Other poxviruses are also being developed as vaccine vectors. For example, immunization with a raccoonpox recombinant expressing the rabies glycoprotein protected raccoons against rabies [21]. In addition, fowlpox recombinants expressing the influenza virus HA and nucleocapsid proteins, the Marek's disease virus (MDV) gB glycoprotein or the Newcastle disease virus (NDV) fusion or HA glycoproteins protected chickens against influenza, MDV and NDV, respectively [22–25]. In the above examples, the raccoonpox and fowlpox vectors were used in the natural host for these viruses. The fowlpox vector, however, has also been used in animals which are not its natural host. For example, a fowlpox recombinant

expressing the rabies glycoprotein has protected mice, cats and dogs against rabies [26].

Fowlpox virus is the prototypic member of the genus *Avipoxvirus*. An attenuated strain of fowlpox has been used as a vaccine since the 1920s to control fowlpox in poultry. During this time, there have been no reports of transmission to non-avian species. Avipoxviruses do not replicate in non-avian species and can therefore be regarded as naturally attenuated for them. Although infectious particles are not produced, some viral proteins and foreign proteins under the control of appropriate promoters can be expressed in non-avian cells. These vectors are, therefore, attractive vaccine vectors in mammalian, as well as avian, species.

ALVAC. The fowlpox-rabies glycoprotein recombinant was not as potent as a replication-competent vaccinia-rabies glycoprotein recombinant (VV-RG). However, a replication-restricted canarypox-rabies glycoprotein recombinant (ALVAC-RG) was as efficacious as VV-RG. In fact, in a rabies challenge study [17], ALVAC-RG had a virtually identical PD_{50} value to VV-RG (Table 1). The reasons for the difference in potency of the two avipoxvirus recombinants are not immediately obvious.

A plaque-cloned isolate of an attenuated canarypox vaccine strain has been designated ALVAC [17]. As expected, ALVAC has a highly attenuated phenotype, and similar to NYVAC, exhibits negligible pathogenicity in newborn and immunocompromized mice [17].

Although canarypox virus does not replicate in mammalian species, an ALVAC recombinant can be as potent in mammalian target species as a comparable replication-competent vaccinia virus recombinant. For example, dogs immunized with an ALVAC recombinant expressing the measles virus HA glycoprotein generated equivalent neutralizing antibody titres and were as resistant to CDV challenge as dogs immunized with a replication-competent vaccinia virus-measles HA recombinant [11].

ALVAC recombinants have also protected other mammalian target species against infectious agents. For example, ALVAC-RG protected dogs and cats against rabies [27], an ALVAC recombinant expressing the EIV type A1 and type A2 HA glycoproteins protected horses against EIV [28] and an *ALVAC recombinant expressing the feline leukaemia virus (FeLV) gag and env proteins protected cats against FeLV [29].*

Immune responses generated by NYVAC or ALVAC recombinants

Although avipoxvirus recombinants do not replicate in non-avian species, *de novo* synthesis of the recombinant protein is necessary to induce an immune response against the recombinant protein. Animals inoculated with a fowlpox recombinant expressing the rabies glycoprotein produced antibodies against rabies. However, animals inoculated with an inactivated fowlpox-rabies glycoprotein recombinant produced antibodies against the fowlpox vector, but not against rabies. Therefore, the rabies-specific immune response elicited by this recombinant was induced by the rabies protein expressed *de novo* in the infected cell and not by the rabies protein introduced with the inoculated virus [26].

Humoral immunity. Although there are exceptions (see below), most ALVAC and NYVAC recombinants elicit a strong humoral response against the recombinant antigen [11, 18, 20, 26, 30]. In fact, NYVAC and ALVAC recombinants can elicit neutralizing antibody titres equivalent to a comparable replication-competent vaccinia virus recombinant [11, 18].

Cell-mediated immunity. Although ALVAC and NYVAC have a restricted or debilitated replicative capacity in mammalian cells, recombinants generated from these vectors can elicit cytotoxic T-lymphocytes (CTLs) in humans [31]. Data from Phase I clinical trials indicate that some individuals inoculated with an ALVAC recombinant expressing the human immunodeficiency virus type 1_{MN} ($HIV1_{MN}$) envelope glycoprotein and many individuals inoculated with the ALVAC-$HIV1_{MN}$ envelope glycoprotein recombinant and boosted with HIV1 gp160 or gp120 subunit protein generated CD8 + CTLs [31, 32]. Furthermore, the protocol of priming with an ALVAC recombinant and boosting with a subunit protein consistently allowed the demonstration of circulating CTLs in a higher percentage of individuals than protocols with the subunit preparation alone [32]. These results indicate that immunization with a non-replicating poxvirus vector, either alone or as part of a prime-boost regime, can elicit CTLs in humans.

Correlates of protection. Immunization with an ALVAC recombinant expressing the FeLV *gag* and *env proteins protected all 6/6 cats against an FeLV challenge. Immunization with an ALVAC recombinant* expressing the FeLV *gag* protein and a form

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of *env* from which the putative immunosuppressive region had been deleted protected 3/6 cats. Serological analyses indicated that none of the 12 immunized cats had detectable titres of FeLV neutralizing antibodies prior to challenge. However, whereas all the protected cats generated neutralizing antibodies 9–12 weeks after challenge, none of the cats that became infected developed neutralizing antibodies, even after challenge. Therefore, protection against FeLV was induced without detectable neutralizing antibodies at the time of challenge [29]. These results suggest that protection may be associated with a primed immune response that was quickly recalled upon challenge. Of course, the role of cell-mediated immunity may also be of critical importance.

ALVAC recombinants have also protected other species against infectious agents despite the lack of detectable neutralizing antibodies at the time of challenge. For example, dogs inoculated with an ALVAC recombinant expressing the measles virus fusion glycoprotein survived a lethal CDV challenge despite having no detectable CDV-specific neutralizing antibodies [11]. In addition, 11/12 dogs resisted a rabies challenge 36 months after being inoculated with ALVAC-RG, even though rabies-specific neutralizing antibody titres were not detectable at the time of challenge [33]. Therefore, in three separate studies, protective immunity was induced even though neutralizing antibodies were not detectable at the time of challenge. The latter study also indicates that an ALVAC recombinant can induce long-lasting immunity.

Safety. The attenuated characteristics of NYVAC and ALVAC in cell culture and laboratory animals suggest that these vectors are safe. However, the safety of each candidate vaccine must be evaluated empirically. To date, three NYVAC recombinants and seven ALVAC recombinants have been evaluated in humans. Apart from minor local reactions, there have been no serious side-effects [30, 31, 34]. Therefore, NYVAC and ALVAC appear to be safe and well tolerated in humans.

Effect of prior exposure to poxvirus vectors. Much of the world's population has been exposed to vaccinia virus. Therefore, the efficacy of poxvirus-based recombinants, and in particular, vaccinia virus-based recombinants must be evaluated in vaccinia-immune, as well as vaccinia-naive individuals. Data from a Phase I clinical trial indicated that immunization with HIVAC-1e, a replication-competent vaccinia virus recombinant expressing the $HIV1_{IIIB}$ envelope glycoprotein, elicited stronger HIV1-specific antibody and lymphoproliferative responses in vaccinia-naive individuals than vaccinia-immune individuals [35]. On the other hand, the rabies- or HIV1-specific immune responses elicited by ALVAC recombinants expressing either rabies or $HIV1_{MN}$ envelope glycoproteins were equivalent in both vaccinia-naive and vaccinia-immune individuals [30, 32]. Therefore, prior exposure to vaccinia virus does not appear to effect the ability of ALVAC recombinants to elicit a primary and/or anamnestic immune response. This question is also being addressed with NYVAC recombinants in ongoing clinical studies.

Interference from maternal immunity. One problem encountered when vaccinating very young individuals is interference from maternal antibodies. For example, vaccination of children less than 12 months old with attenuated measles vaccine does not consistently produce protective immunity and can impair successful revaccination as measured by the induction of antibody. In developed countries, where the risk of being exposed to measles virus is low, children are usually vaccinated at 15 months when maternal antibody levels have waned. In developing countries, however, where the risk of measles virus is much higher, there is a critical window of susceptibility when maternal antibody is too low to be protective, but yet too high to allow successful vaccination.

To determine whether an ALVAC recombinant could elicit a protective immune response in the presence of maternal antibodies, a rabies challenge study was done in newborn puppies. To ensure that the newborns had high levels of maternal antibodies, immune pregnant bitches were boosted with inactivated rabies vaccine 2 weeks before giving birth. Four weeks later, pups with high levels of rabies antibodies were vaccinated with ALVAC-RG. No significant increase in rabies antibodies were observed. At 3 months, when maternal antibody had waned, the pups were challenged with rabies virus. All four dogs vaccinated with a high dose of ALVAC-RG and 2/4 dogs vaccinated with a low dose of ALVAC-RG survived, whereas 0/4 unvaccinated dogs survived [33]. These results indicate that an ALVAC recombinant can elicit protective immunity in the presence of high levels of maternally-derived antibodies.

Mucosal immunity. Many pathogens infect a host via

Table 2. *Protective non-replicating poxvirus recombinants*

Pathogen	Strain	Target species
Canine distemper virus	ALVAC	Dogs
Equine influenza virus	NYVAC	Horses
Equine influenza virus	ALVAC	Horses
Feline leukemia virus	ALVAC	Cats
Japanese encephalitis virus	NYVAC	Pigs
Pseudorabies virus	NYVAC	Pigs
Rabies virus	ALVAC	Dogs
Rabies virus	ALVAC	Cats

a mucosal surface. Therefore, mucosal immunity is an important component of a protective immune response. Parenteral administration of NYVAC or ALVAC recombinants can protect target species individuals against a mucosal challenge. An ALVAC-FeLV *gag/env* recombinant protected cats against an oronasal FeLV challenge, an ALVAC-measles HA recombinant protected dogs against an intranasal CDV challenge, NYVAC-PRV gB and NYVAC-PRV gD recombinants protected pigs against an oronasal PRV challenge and an ALVAC-EIV HA recombinant and a NYVAC-EIV HA recombinant protected horses against a natural EIV infection, which is assumed to have been transmitted via the oronasal route [11, 19, 20, 29].

Use of poxviruses in immunotherapy/gene therapy

Cancer

Non-replicating poxvirus recombinants have been used prophylactically to vaccinate animals against a variety of infectious agents (Table 2). Poxvirus vectors can also be used in a variety of immunotherapeutic protocols to induce or potentiate immune responses against tumours or infectious agents. For example, many tumours express tumour-associated antigens (TAAs) that can act as immunological targets. Unfortunately, the immune response against TAAs is usually weak or non-existent. However, expression of a TAA in the context of a biological response modifier (BRM) could potentially increase the immunogenicity of that TAA. Therefore, parenteral or intratumoural inoculation with a poxvirus recombinant co-expressing BRMs (e.g., cytokines, B7-1, B7-2) and a TAA from a patient's tumour may induce an immune response capable of controlling or preventing the growth of the patient's tumour.

Systemic administration of BRMs, such as IL-2, have resulted in clinically significant tumour regression [36]. Unfortunately, toxicity associated with the systemic administration of high doses of BRMs, such as IL-2, has limited the utility of cytokine treatments. To circumvent these problems, the potential delivery of efficacious, nontoxic levels of cytokines via a viral vector has been investigated. Poxvirus vectors could therefore be used as part of a cell-based immunotherapeutic protocol in which tumour cells or tumour infiltrating lymphocytes (TILs) are infected with a poxvirus-TAA/BRM recombinant *ex vivo* and then reintroduced into the patient.

Expression of BRMs in tumour cells has increased the immunogenicity of weakly immunogenic TAAs. Mice injected with a mixture of uninfected murine colonic adenocarcinoma cells (MC38) and MC38 cells infected with a replication-competent vaccinia virus recombinant expressing the murine T-cell co-stimulatory molecules, B7-1 or B7-2, did not develop tumours, whereas mice injected with MC38 cells or a mixture of uninfected and vaccinia virus-infected MC38 cells did develop tumours. Furthermore, when the protected mice were rechallenged on the opposite flank with uninfected MC38 cells 40 days after the initial challenge, tumour formation was significantly delayed and the growth rate of the tumour substantially reduced [37].

Similar studies have also been performed with mouse bladder tumour cells (MBT-2). Mice injected with a mixture of uninfected MBT-2 cells and MBT-2 cells infected with ALVAC recombinants expressing GM-CSF, IL-12 or TNF-α did not develop tumours, whereas tumours did develop in mice injected with a mixture of uninfected and ALVAC-infected MBT-2 cells. In addition, 80% of the mice injected with a mixture of uninfected MBT-2 cells and ALVAC-IL-2-infected MBT-2 cells did not develop tumours. Furthermore, tumour-specific cytolytic activity was observed in mice injected with ALVAC-IL-2-infected MBT-2 cells (Rao et al., unpublished results). These two studies indicate that expression of a BRM in a tumour cell via a poxvirus recombinant can prime a tumour-specific immune response capable of rejecting or delaying tumour formation.

Poxvirus vectors have also been used to stimulate and expand tumour-specific CTLs/TILs *ex vivo*. Peripheral blood mononuclear cells (PBMCs) were isolated from a cancer patient with no detectable tumour-specific CTL activity. The PBMCs were infected with an ALVAC-MAGE-1 recombinant and

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used *in vitro* to stimulate TILs isolated from a tumour from the same patient. (MAGE-1 is a human TAA [38].) After amplification, MAGE-1-specific CTL activity was identified [39]. Therefore, an ALVAC-TAA recombinant was able to stimulate and expand TAA-specific CTLs from a cancer patient with no prior detectable TAA-specific cytolytic activity.

Infectious agents

ALVAC and NYVAC recombinants have also been used to stimulate and expand HIV1 envelope-specific CTLs from HIV1-seropositive individuals with undetectable or low levels of envelope-specific CTL activity. PBMCs from HIV1-seropositive individuals were infected with an ALVAC-HIV1 envelope recombinant or a NYVAC-HIV1 envelope recombinant. The infected cells were then used to stimulate *in vitro* uninfected PBMCs from the same individual. Following this procedure, the stimulated PBMCs had a high level of envelope-specific CD8+ cytolytic activity. Furthermore, the cytolytic activity of these cultures was higher than that of a culture stimulated with a replication-competent vaccinia virus-HIV1 envelope recombinant [40]. Therefore, ALVAC and NYVAC recombinants can be used to preferentially stimulate and expand specific CTL populations *ex vivo*, which could then be reintroduced into the donor to hopefully provide a therapeutic benefit.

ADENOVIRUSES

Adenoviruses are relatively large (30–40 kb) double-stranded DNA viruses. Individual members have been isolated from numerous mammalian and avian species. Clinical symptoms associated with adenovirus infection depend on the serotype, but are usually mild and rarely life-threatening. An attenuated adenovirus type 4 and type 7 vaccine has been used by the US military for the past 30 years to prevent respiratory disease in recruits. Attenuated veterinary vaccines are also used to protect against adenovirus-associated disease. The success of these vaccines has prompted the development of adenoviruses as recombinant vectors [41].

Adenoviruses have many properties advantageous for a potential vector. They are easily grown and concentrated to high titres. They can infect a broad spectrum of cells, including epithelial, liver and lung cells. Adenovirus recombinants can be easily generated using plasmids containing viral DNA [42]. In addition, adenoviruses replicate in the nucleus of the infected cell. Therefore, expression of foreign genes can utilize eucaryotic promoters, such as the adenovirus E1a promoter, the cytomegalovirus (CMV) immediate early promoter or the Rous sarcoma virus (RSV) LTR promoter [43–45]. Furthermore, adenovirus vaccines can be administered either orally, in an enteric-coated capsule, or intramuscularly.

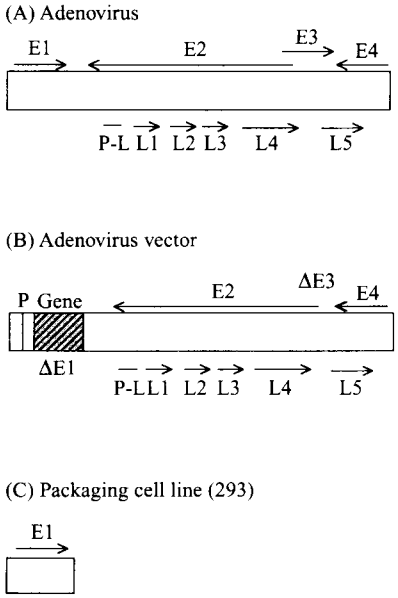

Fig. 1. Recombinant adenovirus vector system. (A) Transcription map of a typical adenovirus. Early (E) and late (L) transcripts, encoding the adenovirus non-structural and structural proteins are represented by arrows. Each transcription unit gives rise to a family of mRNAs via differential splicing events. The early genes have individual promoters. The late genes are transcribed from a single late promoter (P–L). The inverted terminal repeats (ITR) contain the regulatory sequences necessary for replication and genomic encapsidation. (B) Typical helper-free, replication-defective adenovirus vector. The foreign gene is cloned into the E1 region under the transcriptional control of an internal promoter (P). To increase cloning capacity, the E3 region has been deleted. (C) Adenovirus packaging cell line. Helper-free, replication-defective adenovirus recombinants are propagated in 293 cells, which express the E1 region constitutively. (Modified from ref. 87).

Three types of adenovirus recombinants can be generated; (1) replication-competent; (2) helper-free, replication-defective and (3) helper-dependent, replication-defective. The type of adenovirus recombinant generated depends on the insertion locus used to generate the recombinant and the adenovirus sequence retained in the subsequent virus. For example, insertion of exogenous sequence into the non-essential E3 region results in the generation of a replication-competent recombinant virus (Fig. 1A).

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On the other hand, insertion into the essential E1 region results in the generation of a helper-free, replication-defective recombinant (Fig. 1B) that can be propagated in a cell line (293) that expresses the E1 gene products (Fig. 1C). Deletion of large amounts of adenovirus DNA results in the generation of a helper-dependent, replication-defective recombinant. Propagation of helper-dependent, replication-defective recombinants requires the presence of a helper adenovirus to supply the viral structural and enzymatic functions lacking in the recombinant [41].

The amount of foreign DNA that can be packaged into an adenovirus recombinant depends on the type of recombinant desired. An adenovirus capsid can package DNA equivalent to 105% of a typical adenovirus genome. The packaging capacity, however, can be increased by deleting various regions of the adenovirus genome. For example, a replication-competent recombinant from which the non-essential E3 and E4 regions are deleted can contain 5–6 kb of foreign DNA. On the other hand, a helper-free, replication-defective adenovirus recombinant from which the non-essential E3 and E4 regions and the essential E1 region are deleted can contain 7–8 kb of foreign DNA. Of course, by deleting the whole adenovirus genome except the inverted repeats and packaging signal sequence, more than 30 kb of foreign DNA can be inserted into a helper-dependent, replication-defective recombinant [41].

Vaccines

Immunization with adenovirus recombinants can protect individuals against a subsequent infectious challenge. For example, a replication-competent adenovirus type 5 (Ad5) recombinant expressing the spike or nucleocapsid proteins of mouse hepatitis virus (MHV) protected mice against MHV [46] and a replication-competent Ad5 recombinant expressing the rabies glycoprotein protected mice against rabies [47].

However, adenovirus vectors do have potential disadvantages. For example, like other live viral vectors, they may cause disseminated disease in immunocompromized individuals [48]. Adenoviruses may also be excreted by oro-faecal and respiratory routes for months or years following infection. In fact, horizontal transmission of the adenovirus type 4 vaccine has been observed between married couples [49] and between vaccinated children and family members [50]. In addition, cotton rats inoculated intranasally with an Ad5 recombinant containing a deletion in the E3 region exhibited an increased pulmonary inflammatory response compared to mice inoculated with wild-type virus [51]. On the other hand, oral administration of chimpanzees and humans with other Ad5 recombinants containing an E3 deletion did not induce an increased inflammatory response [52, 53].

The diffusion of replication-deficient adenoviruses is very low. Therefore, in the hope of developing a safer vector, replication-defective adenoviruses have been evaluated as potential recombinant vaccine vectors. Inoculation with a replication-defective adenovirus recombinant expressing the EBV envelope glycoprotein protected cottontop tamarins against EBV [54]. In addition, immunization with a replication-defective adenovirus recombinant expressing the PRV gD envelope glycoprotein protected cotton rats against a PRV challenge. However, the protective dose of the replication-defective adenovirus-PRV gD recombinant was 1000X higher than that of a replication-competent adenovirus-PRV gD recombinant [55]. Therefore, in this model system, the potency of an adenovirus recombinant was adversely affected by the replicative capacity of the vector.

Immunotherapy/gene therapy

Classical genetic diseases

Helper-free, replication-defective adenoviruses have many properties theoretically useful for gene therapy [41]. In particular, adenovirus recombinants can efficiently transduce non-dividing cells *in vivo*. Therefore, since many potential targets for gene therapy are tissues that are either slow growing, terminally differentiated or difficult to remove and reimplant, adenoviruses are attractive vectors. On the other hand, adenovirus vectors do have some disadvantages for gene therapy. For example, since the adenovirus genome does not integrate into the chromosome of the transduced cell, the vector is eventually lost as cell division proceeds.

Cystic fibrosis (CF) is a hereditary disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Although mutations in CFTR cause a variety of abnormalities, the most severe is chronic mucus production and resulting infection at the epithelial surface of the lung. The complexity of the lung does not allow dissection and

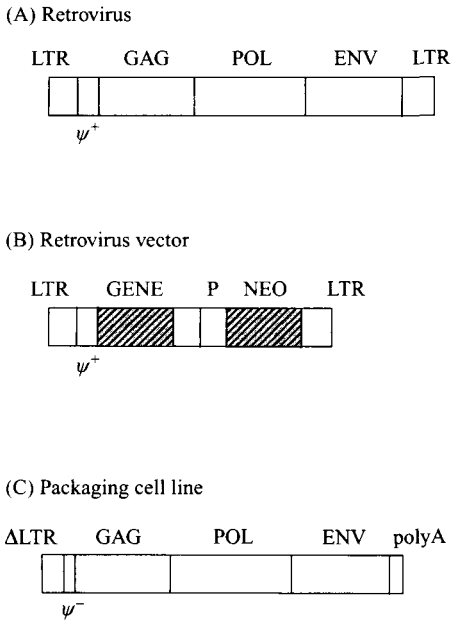

Fig. 2. Recombinant retrovirus vector system. (A) Replication-competent retrovirus genome. GAG encodes the capsid proteins, POL encodes the reverse transcriptase and integrase proteins and ENV encodes the envelope glycoprotein. The long terminal repeat (LTR) contains regulatory sequences, such as a promoter and transcription termination sequence. Ψ^+ represents the packaging signal sequence. (B) Typical replication-defective, double-expression retrovirus vector. The foreign gene is under the transcriptional control of the LTR promoter. The selectable marker gene (NEO) is under the transcriptional control of an internal promoter (P). (C) Retrovirus packaging cell line. Retrovirus recombinants are propagated in a cell line that expresses the GAG, POL and ENV gene products. Ψ^- indicates that the packaging signal sequence has been deleted. PolyA represents a polyadenylation signal sequence. (Modified from ref. 87).

reimplantation of genetically engineered lung cells. Furthermore, most of the epithelial cells on the lung surface are terminally differentiated. Therefore, reconstitution of pulmonary function via gene therapy must be accomplished with a vector that can transduce non-replicating cells *in vivo*.

Helper-free, replication-defective adenovirus recombinants expressing the CFTR gene have been inoculated directly into the airway of numerous species, including humans [43, 56, 57]. In each case, successful gene transfer was observed. However, expression of CFTR was transient and loss of expression associated with pulmonary inflammation. In a recent clinical trial [58], an adenovirus-CFTR recombinant was administered at four different doses to the nasal epithelium of 12 CF patients. The CFTR gene was detected in 4/6 patients receiving the higher doses and 1/6 patients receiving the lower doses. The percentage of transduced cells, however, was very low ($< 1\%$). Furthermore, analysis of the epithelium revealed no functional restoration of CFTR activity. In addition, although toxicity was not observed in patients receiving the lower doses, mucosal inflammation was observed in 2/3 patients receiving the highest dose. Therefore, in this study, administration of an adenovirus-CFTR recombinant to the nasal epithelium of CF patients did not restore CFTR activity and resulted in mucosal inflammation.

Haemophilia B is a hereditary disorder caused by a deficiency of blood coagulation factor IX. This disease affects approximately 1/30000 caucasian males and results in episodes of severe bleeding. Although numerous tissues have been targeted for factor IX gene therapy [59–61], the liver, being the normal site of factor IX synthesis, represents the most natural target.

Injection of a helper-free, replication-defective adenovirus-canine factor IX recombinant into the portal vein of haemophilic dogs resulted in the transduction of a significant percentage of liver cells. In fact, plasma levels of factor IX increased from 0 to 300% of normal levels and resulted in the complete amelioration of symptoms. Unfortunately, expression of factor IX was transient. Levels started to decline after 2 days and after 1–2 months had fallen below therapeutic levels [44].

The results observed in the CFTR and factor IX studies are not unique. In most instances, the use of helper-free, replication-defective adenovirus recombinants resulted in transient expression of the transduced gene and pathology of the target organ. Stable expression of recombinant adenovirus-transduced genes has only been observed in newborn mice [62], immunodeficient or immunocompromized animals [44] or immunoprivileged organs, such as the retina [63]. These results suggest that the immune system is involved in the inability to establish long-term expression of recombinant adenovirus-transduced genes.

Helper-free, replication-defective adenoviruses express viral proteins in non-293 cells when infected at high multiplicities of infection [64]. Viral-specific class I-restricted CTLs have also been shown to mediate the destruction of hepatocytes transduced with helper-free, replication-defective adenovirus recombinants [65]. Therefore, it appears that the expression of viral proteins in recombinant adenovirus-transduced cells trigger an immune response against the transduced

cells that is responsible for the transient expression and pathology observed with these vectors.

In order to minimize viral expression and prolong expression of transduced genes, second generation adenovirus vectors containing mutations in E2a and E1 have been constructed. Although deletion of these genes did not completely abolish viral expression, animals inoculated with recombinants made from these vectors exhibited less inflammation and longer expression of the transduced gene than animals inoculated with E2a+ recombinants [66, 67]. Therefore, improvements may eventually overcome the problems associated with adenovirus vectors.

Cancer

Adenovirus vectors can also be used to potentiate an immune response against tumour cells. Expression of BRMs in tumour cells via an adenovirus vector can induce a tumour-specific immune response capable of suppressing tumour growth. For example, most of the mice injected with mouse mastocytoma cells (P815) infected with a helper-free, replication-defective adenovirus recombinant expressing murine IL2 did not develop tumours, whereas mice injected with uninfected P815 cells did develop tumours. Furthermore, the protected mice did not develop tumours when rechallenged with uninfected P815 cells [68]. These results indicate that tumour cells infected with an adenovirus-BRM recombinant primed a tumour-specific immune response capable of suppressing tumour formation.

This form of immunotherapy has also been shown to be efficacious against established tumours. Up to 75% of mice injected with the adenovirus-IL-2 recombinant directly into an established P815 tumour cleared the tumour. Furthermore, the protected mice did not develop tumours when rechallenged with uninfected P815 cells [69]. Therefore, intratumoural injection of an adenovirus-BRM recombinant elicited an immune response capable of clearing an established tumour.

RETROVIRUSES

Retroviruses are a family of single-stranded RNA viruses. During the life cycle of these viruses, the normal flow of genetic information, from DNA to RNA, is reversed. Following infection, the single-stranded RNA genome of retroviruses is converted to double-stranded DNA by a retrovirus-encoded reverse transcriptase. The double-stranded DNA is then incorporated into the host cell chromosome by a retrovirus-encoded integrase. The ability to insert DNA into the genome of infected cells makes retroviruses ideal eucaryotic expression vectors [70].

The genomic organization of a simple retrovirus is shown in Fig. 2A. The *gag* gene encodes the core structural proteins, the *pol* gene encodes the reverse transcriptase and integrase proteins and the *env* gene encodes the outer membrane envelope glycoprotein. These genes are flanked by long terminal repeats (LTRs) which contain numerous regulatory sequences, including a promoter.

The generation of retrovirus recombinants can be divided into two components; the retrovirus vector (Fig. 2B) and the retrovirus packaging cell line (Fig. 2C). The retrovirus vector encodes the foreign gene product and a packaging signal sequence (ψ), but does not encode any of the enzymatic or structural proteins (i.e., *gag*, *pol* and *env*) required for the production of infectious particles. The retrovirus packaging cell line, on the other hand, encodes the enzymatic and structural proteins required for the production of infectious particles, but does not contain a packaging signal sequence. Recombinant retroviruses are generated by transfecting retrovirus packaging cells with retrovirus vector DNA [70].

Numerous retrovirus vectors have been generated. Early vectors contained a packaging signal sequence and a single foreign gene. Subsequent vectors were designed to (1) allow easy selection of transductants; (2) increase virus production and foreign gene expression and (3) decrease the potential of generating a replication-competent virus. For example, to allow easy selection of transductants, vectors that express two foreign genes (one of which is a selectable marker, such as neomycin resistance) have been generated. Vectors that express two genes can utilize either one or two promoters. A single promoter, such as the LTR promoter, can express two genes by a differential splicing mechanism. The main disadvantage of this type of vector is that splicing is not always efficient, therefore, expression of the foreign genes is not always consistent. A single promoter can also express two genes by utilizing an internal ribosome entry site (IRES). (An IRES is a sequence isolated from picornaviruses that allows cap-independent translation [71]). The most common type of double expression vector, however, utilizes two promoters (Fig. 2B).

Numerous retrovirus packaging cell lines, expressing avian, murine or primate retrovirus gene products

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have been generated. Cell lines encoding murine retrovirus gene products are the most commonly used due to the wide range of cell tropisms of different murine retroviruses. For example, for human applications, a retrovirus capable of infecting human cells could be used.

Early packaging cell lines contained a cDNA copy of a retrovirus genome with the packaging signal sequence deleted. However, since recombination between a retrovirus vector containing a packaging signal sequence and the retrovirus sequence in the packaging cells could result in the production of a replication-competent retrovirus, subsequent packaging cells contain additional modifications. For example, the PA3127 packaging cell line contains a mutation in the 5′ LTR and replaces the 3′ LTR with the simian virus SV40 polyadenylation signal sequence [72].

Vaccines

Very little work has been done to develop retroviruses as vaccine vectors. In one study, chickens inoculated with a replication-competent RSV recombinant expressing the avian influenza HA glycoprotein were protected against avian influenza [73]. These results indicate that retroviruses could potentially be used as recombinant vaccine vectors.

Immunotherapy/gene therapy

Classical genetic diseases

Adenosine deaminase (ADA) deficiency is a very rare genetic disease. Individuals with this disease lack a functional form of ADA, an enzyme involved in the purine salvage pathway. In the absence of ADA, deoxyadenosine can accumulate to levels that are toxic to certain types of cells, such as T-lymphocytes, and results in severe immunosuppression.

In 1990, ADA deficiency became the first disease to be treated by gene therapy [74]. T-cells isolated from two ADA patients were transduced with a retrovirus recombinant expressing the ADA gene. The transduced cells were expanded *in vitro* and transfused back into the patients. Four years after treatment began and 2 years after treatment was completed, $> 50\%$ of one patient's circulating T-cells and 0·1–1 % of the other patient's T-cells contained the new ADA gene [75]. In the 2 years following treatment, the number of T-cells containing the new gene remained constant, suggesting that the transduced T-cells were either long-lived or proliferating. The two individuals have also responded positively in *in vivo* and *in vitro* immunological assays (e.g. DTH skin tests, antibody production, IL-2 production and T-cell-mediated cytolysis). Although these patients are given routine injections of synthetic ADA, these results suggest that gene therapy has been beneficial.

Retrovirus vectors have also been used to deliver genes to the liver. When a retrovirus-canine factor IX recombinant was injected into the portal vein of haemophilic dogs 1–3 days after partial hepatectomies, low levels of factor IX were expressed for more than 5 months, resulting in modest improvements in blood clotting efficiencies [76].

Although encouraging, human applications would require greater expression of factor IX. In addition, the surgical invasiveness of this protocol is far from ideal. Surgical removal of part of the liver is necessary because (1) *in vivo* transduction of liver cells with a retrovirus recombinant is very inefficient and (2) *ex vivo* manipulations affect the transplantation capacity of explanted hepatocytes [77]. *In vivo* transduction is inefficient because most hepatocytes in the adult liver are quiescent and retroviruses can only integrate into the genome of actively dividing cells. However, following a partial hepatectomy, the remaining liver cells actively divide until the liver is fully regenerated. Therefore, under these conditions, *in vivo* transduction of liver cells with a retrovirus recombinant can be accomplished [78].

Efforts to overcome the restricted host-range of retrovirus vectors has led to the development of pseudotyped retrovirus vectors [79]. Pseudotyped retroviruses contain the genome of one virus and the envelope protein of another virus. Since the host range of these viruses is associated with the envelope protein, a pseudotyped retrovirus can have a much broader host-range than an unmodified retrovirus. For example, a pseudotyped retrovirus (VSV) vector has been used to transduce newborn mouse liver cells *in vivo* [80]. These results suggest that continued improvements may eventually overcome the inherent limitations associated with retrovirus vectors.

Cancer

Approximately half of the human gene therapy clinical trials have involved cancer. In general, these protocols are designed to enhance tumour-specific immune responses. The rationale is based on results from animal studies in which recombinant retrovirus-transduced cells elicit a tumour-specific immune

response capable of preventing tumour formation. For example, mice inoculated with murine neuroblastoma (C1300) cells transduced with a retrovirus recombinant expressing IFN-γ did not develop tumours, whereas mice inoculated with non-transduced C1300 cells did [81]. This protection was dependent on the level of IFN-γ produced by the transduced cells. Mice inoculated with transduced C1300 cells expressing low levels of IFN-γ developed tumours, whereas mice inoculated with transduced C1300 cells expressing high levels of IFN-γ did not. Consistent with these results, injection of a monoclonal antibody against IFN-γ abrogated the protection elicited by the transduced cells.

The immune response generated by the transduced cells was adoptive and tumour-specific. Mice inoculated with non-transduced C1300 cells 4–6 weeks after clearing the recombinant retrovirus-transduced C1300 challenge did not develop tumours, whereas mice inoculated with murine fibrosarcoma (Sa-1) cells 4–6 weeks after clearing the recombinant retrovirus-transduced C1300 challenge did develop tumours [81].

Very similar results were observed in another murine system [82]. Mice inoculated with murine fibrosarcoma (CMS-5) cells transduced with a retrovirus-IL-2 recombinant did not develop tumours, whereas tumours did develop in mice inoculated with non-transduced CMS-5 cells. Protection was dependent on the level of IL-2 produced. Mice inoculated with transduced CMS-5 cells expressing low levels of IL-2 developed tumours, whereas mice inoculated with transduced CMS-5 cells expressing high levels of IL-2 did not. Furthermore, mice injected with a mixture of non-transduced CMS-5 cells and recombinant retrovirus-transduced CMS-5 cells did not develop tumours. The protection generated in this system is also adoptive and tumour-specific. Mice inoculated with non-transduced CMS-5 cells 6 weeks after clearing the recombinant retrovirus-transduced CMS-5 challenge did not develop tumours, whereas tumours did develop in mice inoculated with two other murine fibrosarcoma cells.

In another murine system, mice inoculated with murine melanoma (B16) cells transduced with a retrovirus recombinant expressing IL-2 did not develop tumours, whereas mice inoculated with non-transduced B16 cells, or B16 cells transduced with retrovirus recombinants expressing IL-4, IL-5, IL-6, IFN-γ, TNF-α, GM-CSF, IL-IRA, ICAM or CD2 did develop tumours [83]. Furthermore, due to the progressively increasing number of cells expressing cytokines, mice receiving some of the recombinant retrovirus-transduced cells developed fatal, cytokine-related pathologies.

To avoid the potential dangers associated with inoculation of cytokine-producing tumour cells, the immunogenicity of irradiated recombinant retrovirus-transduced tumour cells was evaluated [83]. All the mice inoculated with irradiated B16 cells transduced with a retrovirus-GM-CSF recombinant and about half the mice inoculated with irradiated B16 cells transduced with retrovirus-IL-4 or retrovirus-IL-6 recombinants did not develop tumours when challenged with non-transduced B16 cells 7 days later. On the other hand, mice inoculated with irradiated B16 cells transduced with retrovirus recombinants expressing IL-2, IL-5, IFN-γ, TNF-α, IL-IRA, ICAM or CD2 did develop tumours when challenged with non-transduced B16 cells.

Inoculation with irradiated retrovirus-GM-CSF-transduced tumour cells also enhanced tumour-specific immunity in other murine systems. For example, mice inoculated with irradiated colon carcinoma (CT-26) cells, irradiated fibrosarcoma (CMS-5) cells or irradiated renal carcinoma (RENCA) cells transduced with the retrovirus-GM-CSF recombinant did not develop tumours when challenged with non-transduced tumour cells 1–3 weeks later. Furthermore, the protection was long-lasting. Most of the mice inoculated with the irradiated retrovirus-GM-CSF-transduced B16 cells did not develop tumours when challenged with non-transduced B16 cells several months later. Protection was mediated by CD4+ and CD8+ T-cells, but not by NK cells. Depletion of CD4+ or CD8+ cells abrogated protection, whereas depletion of NK cells had little or no effect.

The therapeutic application of recombinant retrovirus-transduced tumour cells may be limited by the inability to culture and transduce every type of tumour. Unlike many tumour cells, fibroblasts are easily cultured and transduced. Therefore, the potential of utilizing transduced fibroblasts to elicit a tumour-specific immune response was investigated [84]. Mice inoculated with a mixture of CT-26 cells and recombinant retrovirus-IL-2-transduced murine fibroblasts developed tumours at a lower incidence than mice inoculated with CT-26 cells alone. Furthermore, mice inoculated with a mixture of irradiated CT-26 cells and recombinant retrovirus-IL-2-transduced fibroblasts 2 weeks before a CT-26 challenge developed tumours at a lower incidence than mice inoculated with irradiated CT-26 cells alone.

Animal studies suggest that this form of immunotherapy may also be effective against preexisting tumours. Most of the mice inoculated with irradiated recombinant retrovirus-GM-CSF-transduced B16 cells 3 days after being inoculated with nontransduced B16 cells did not develop tumours [83]. In addition, about half of the mice multiply inoculated with a mixture of irradiated CT-26 cells and recombinant retrovirus-IL-2-transduced fibroblasts 3 days after being inoculated with CT-26 cells were able to clear a visible tumour. On the other hand, mice could not clear a tumour when recombinant retrovirus-IL-2-transduced fibroblasts were inoculated as close to the tumour as possible [84].

The results of these studies indicate that the production of cytokines in the microenvironment of tumour cells via a retrovirus recombinant can enhance tumour-specific immunity and potentially prevent tumour formation.

DISCUSSION

A wide variety of eucaryotic expression vectors have been developed. This review has focused on three of these vectors; poxviruses, adenoviruses and retroviruses. The biological characteristics of these viruses make them more or less suited for different applications (Table 3). For example, numerous attenuated poxvirus and adenovirus vaccines have been developed to control poxvirus and adenovirus diseases in humans and domestic animals. The success of these vaccines has prompted the development of recombinant poxvirus and adenovirus vaccine vectors. However, before these vectors can be universally accepted, certain safety issues have to be addressed. For example, like all live vaccines, poxvirus and adenovirus recombinants have the potential to elicit adverse reactions in immunocompromized individuals due to the unrestricted growth of the vector. In response to these concerns, the efficacy of replication-defective vectors has been investigated.

Two highly attenuated poxvirus vectors, NYVAC and ALVAC, have been developed. Recombinants generated from these vectors are safe and elicit humoral and cell-mediated immune responses capable of protecting target species individuals against infectious agents. In addition, protection studies indicate that replication-defective poxvirus recombinants can be as efficacious as replication-competent poxvirus recombinants. Therefore, NYVAC and ALVAC represent safer alternatives to previous poxvirus vectors.

Table 3. *Potential applications of non-replicating vectors*

Vector	Vaccine	Immunotherapy/gene therapy
Poxviruses	+	+/−
Adenoviruses	+/−	+
Retroviruses	−	+

Table 4. *Relative advantages and disadvantages of gene therapy vectors*

Vector	Advantages	Disadvantages
Adenoviruses	Can transduce non-replicating cells	Transient expression
	Can transduce cells *in vivo*	Toxicity
		No genomic integration
Retroviruses	Genomic integration	Can't transduce non-replicating cells
		Ex vivo transduction usually required

A replication-defective adenovirus vaccine vector has also been developed. However, one study indicated that a replication-defective adenovirus recombinant was not as efficacious as a replication-competent adenovirus recombinant. Therefore, replication-defective adenovirus vaccines may not be an alternative to replication-competent adenovirus vaccines.

All of the vectors discussed in this review have some application in gene therapy. However, due to the biological characteristics of these viruses, different vectors have different advantages and disadvantages (Table 4). For example, retroviruses cannot integrate into the genome of non-dividing cells. Therefore, retrovirus vectors cannot transduce terminally differentiated, non-proliferating cells. In addition, in most instances, retrovirus vectors do not efficiently transduce cells *in vivo*. Therefore, these vectors are not readily amenable to transducing complex organs, such as the lung. The inability of retrovirus vectors to be used *in vivo* requires that target cells be removed from the body; a procedure that can be unacceptably invasive. Therefore, the usefulness of retrovirus

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vectors are often limited by the cell type to be manipulated.

Adenovirus vectors can transduce non-replicating, terminally differentiated cells *in vivo*. However, due to the expression of viral proteins and subsequent immune response against adenovirus-transduced cells, these vectors are often toxic and expression of the recombinant protein transient.

Although this review has focused on poxvirus, adenovirus and retrovirus vectors, numerous other viruses, including herpesviruses, picornaviruses, alphaviruses, paramyxoviruses, adeno-associated virus (AAV), bovine papilloma virus and hepatitis delta virus have been developed as virus vectors. AAV, in particular, has several properties advantageous for a expression vector.

AAV is a defective, human parvovirus which can infect a broad spectrum of dividing and non-dividing cells. Productive replication requires co-infection with a helper virus, such as adenovirus or herpes simplex virus (HSV). In the absence of helper virus, AAV integrates into the host genome, where it persists in a latent state until rescued by an adenovirus or HSV infection. The ability to integrate into the genome of a wide variety of dividing and non-dividing cells makes AAV an attractive eucaryotic expression vector [85]. In fact, CFTR expression was observed in the epithelial cells of a rabbit lung 6 months after being inoculated with an AAV-CFTR recombinant [86]. Therefore, AAV vectors can successfully transduce non-replicating cells *in vivo* and can promote the long-term expression of potentially therapeutic proteins. Although these results are very encouraging, the packaging systems generating AAV recombinants are inefficient and cumbersome. Therefore, more work needs to be performed before AAV is a suitable vector.

All of the vectors discussed in this review have potential application in immunoprophylaxis and/or gene therapy. Hopefully, continued research will allow the potential of these vectors to be realized.

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