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Xenogenic Adenoviral Vectors

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I. Impetus and Rationale

Although human adenoviruses (HAdVs) have been extensively studied over the past four decades, it is only in the past 10 years or so that studies on animal adenoviruses have begun to approach the same level of molecular analysis. This was partly driven by the desire to characterize viruses that clearly had very different properties and host ranges compared with HAdV, but it was also recognized that natural infection of human populations would very likely induce a level of immunity that might curtail the effective use of HAdV vectors. Molecular studies of xenogenic AdVs have substantially expanded our knowledge. Understanding their biology will ultimately lead to an increased choice of gene delivery vectors, providing more options in therapeutic strategy and design.

II. Classification of Adenoviruses

Adenoviruses were classified originally on the basis of serological tests and hemagglutination ability (reviewed in [1, 2]) but the availability of genetic data has enhanced the ability to assess viral relatedness. The great majority of AdVs are classified as members of the mastadenovirus genus. This group includes all known human and many AdVs of animal origin. Bovine, porcine, canine, murine, equine, simian, and ovine viruses are all represented, some as multiple serotypes [3]. The genus aviadenovirus has also been known for many years. This group consists exclusively of viruses of avian origin, as the name suggests. Again, multiple serotypes of fowl AdV occur, with the prototype virus being the FAdV1 isolate known as CELO. A third group,
proposed as a new genus called the atadenoviruses [4–7], comprises viruses from bovine, ovine, and avian species and, tentatively, viruses from goats, deer, and possum (B. Harrach and D. Thomson and H. Lehnkuhl, pers. commun.). The OAdV7 isolate 287 has been proposed as the prototype of this group [5, 8]. Turkey hemorrhagic enteritis virus (HEV) and frog virus (FrAdV1) may constitute a fourth genus [9]. To assist in defining the potential uses of each vector it is important to understand the host range and biology of each virus. Although some information has been gleaned from genetic data, for the nonmastadenoviruses especially, few studies of the nonstructural viral gene products have been done.

III. Factors Affecting Vector Design and Utility

A. Host Range and Pathogenicity

A driving force behind the development of HAdV vectors was the knowledge that they are not associated with significant disease in healthy individuals [1]. The production of defective vectors in complementing cell lines has provided an additional margin of safety [10]. Several of the xenogenic AdVs reviewed here are being adapted for use as vaccine vectors in the homologous host. Thus, it is important that wild-type BAdV3, PAdV3, CAdV2, FAdV1, and OAdV7 cause only mild or subclinical symptoms upon experimental infection of the species from which they were isolated [11–15].

When considering viral host range it is important to distinguish between host range defined by viral replication and host range defined by the ability to transduce cells. Transduction is influenced largely by the interaction between the fiber protein and a primary cellular receptor. Some avi- and mastadenoviruses have a second fiber protein [16, 17]. The major primary receptor for HAdVs has been identified as Coxackie and adenovirus receptor (CAR) [18, 19]. This is probably also used by SAdVs (Table I) because they grow well in human cells and were propagated in human embryonic kidney 293 cells [20]. For most other xenogenic AdVs no primary receptor has been characterized, nor is it clear whether secondary receptors such as integrins [21] are involved in virus uptake. Indeed, xenogenic AdVs lack identifiable or functionally confirmed integrin-binding sequences in their penton proteins [22–27]. For fiber, the coiled coil, trimeric structure of the stalk [28] is conserved, but the distinct sequences of the cell binding domains for the avi and atadenoviruses suggest that they utilize primary receptors that are distinct from CAR. Consistent with this, although HAdV5 and OAdV7 can both infect CSL503 ovine lung cells, they do not compete with each other for entry [29]. CAdV2 is the only xenogenic mastadenovirus that has been examined with respect to cell binding and uptake. Despite the differences between the HAdV5 and CAdV2 capsids
the kinetics of uptake and trafficking of the two viruses in dog kidney cells was surprisingly similar [30]. CAdV2 shares some features of AdV2/5 tropism but also exhibits distinct characteristics. For example, CAdV2-infected Chinese hamster ovary (CHO) cells that expressed human or mouse CAR but did not bind to human dendritic cells that were efficiently infected by HAdV5. Uptake of CAdV2 in susceptible cells must be augmented principally by CAR because the Arg-Gly-Asp (RGD) motif that binds to αvβ3 integrin is absent from the CAdV2 penton. However, CAdV2 also appears capable of binding to other cell surface proteins [31]. Identifying the receptors for xenogenic adenoviruses and defining the mechanisms of virus uptake is important as it will allow target and nontarget cells to be identified, thus suggesting potential uses for each vector.
However, it is possible that amino acid variation between a natural viral receptor and its counterpart on heterologous cells may alter virus binding affinity.

B. Neutralization

HAdVs are ubiquitous in the human population. As a result of natural infection most individuals develop immunity to adenoviruses by the time they reach maturity. Antibodies against multiple serotypes are common [32] and a substantial portion have neutralizing activity [33]. Nonneutralizing antibodies can also bind to virus particles, leading to their indirect inactivation via the complement system [34]. In addition, individuals commonly develop a long-lived CD4+ T-cell response against multiple serotypes of human adenovirus [35] which may mitigate the strategy of using human adenoviruses from alternative serotypes as vectors [36, 37]. Apart from preexisting immunity, administration of a HAdV at high dose can elicit an inflammatory response [38]. The vector may also induce an immune response that can reduce the efficacy of subsequent doses, although the extent of this effect may vary with the route of administration [39, 40]. A variety of methods have been used to overcome these problems, including transient immunosuppression, blocking of antibodies with agents such as polyethylene glycol and removal of antibodies from serum by immunoapheresis [41, and references therein].

The use of xenogenic adenovirus vectors is expected to avoid neutralization due to preexisting immunity to HAdVs. To investigate this, random human sera were examined for the presence of antibodies that neutralized OAdV7 or CAdV2. Of a panel of 57 sera, most of which neutralized HAdV5 to high titer, only three also neutralized CAdV2 [42, 43]. Similarly, 13 individual and two pools of human sera that neutralized HAdV5 did not neutralize OAdV7 [44]. SAdVs were also not neutralized by antisera that neutralized HAdVs [45]. These data suggest that xenogenic adenoviruses will provide an advantage upon initial administration although it is not expected that the vector will be immunologically silent. However, whether vector is given locally or systemically may determine whether it is possible to administer more than a single dose [39, 40].

C. Genome Structure and Function

Of the xenogenic mastadenoviruses, complete nucleotide sequences have been determined for bovine (BAdV2 and 3) [24], porcine (PAdV3) [46], murine (MAdV1) [27], canine viruses (CAdV1 and 2) [26], and simian viruses [20] (Table I). For the aviadenoviruses, FAdV1 [23] was the first genome sequenced but FAdV8 [47] is now also completed. Among the atadenoviruses, ovine (OAdV7) [22], bovine (BAdV4) (B. Harrach, pers. commun.), and duck (DAdV1) [48] genomes are sequenced. The turkey (TAdV1) [49] and frog (FrAdV1) genomes [9] have also been characterized. All of these viruses...
are potential vectors for gene delivery because they can now be rationally engineered, but not all are being developed as vectors at this stage.

The viruses described above represent the extreme ranges of genome size, the largest being \( \sim 43.8 \) and \( 45 \) kb for FAdV1 and FAdV8, respectively, and the smallest being \( \sim 26.3 \) kb for TAdV1 and \( \sim 29.5 \) kb for OAdV7. The Mastadenovirus genomes range in size from \( \sim 30.9 \) (MAdV1) to \( 34.4 \) kb (BAdV3).

1. Central Core

In comparing the nucleotide sequence for prototype viruses in each genus it is apparent that there is a central core in each genome bounded by the \( pVIII \) and \( IVa2 \) genes (Fig. 1). This codes for the DNA replication, structural proteins, and accessory polypeptides required for their assembly. Most capsid proteins have homologs in each genus but proteins V and IX are unique to mastadenoviruses. Instead, OAdV7 has a gene for the structural

![Figure 1](image_url)

*Figure 1* Comparison of the genome structures of prototype viruses from the avi-, mast-, and atadenoviruses. The central core of each genome (filled rectangle) flanked by the \( IVa2 \) and \( pVIII \) genes is essentially conserved in arrangement and is truncated for simplicity. Other major open reading frames are indicated by open rectangles. Arrows indicate sites for insertion of foreign gene cassettes. The solid and broken lines indicate regions that can be provided in trans and regions that can be deleted, respectively. Note that E4 and E2 sequences have also been deleted in HAdV5 and SAdV vectors but this has not been demonstrated for other xenogenic mastadenoviruses.
protein, p32 K, that lies at the extreme left end of the genome (Fig. 1). This capsid protein complement correlates with the observation that FAdV1 and OAdV7 are more heat-stable than the mastadenoviruses [50, 51]. It will be of interest to determine whether a functionally equivalent protein exists for the aviadenoviruses. Also in the central core of HAdVs are one or two copies of VA RNA genes [52]. Except for PAdV3 [46] and SAdVs [53], these are not present in xenogenic mastadenoviruses or OAdV7 [54], but a single copy is present near the right-hand end of FAdV1 [55] and DAdV1 (Fig. 1) [23, 48].

2. Right-End Sequences

To the right of the central core the genomes vary greatly in structure and gene complement. In the mastadenoviruses the E3 region varies in size and complexity but is located between the pVIII and fiber genes (Fig. 1). HAdV2 and −5 have an E3 region of ~2.5 kb that codes for numerous polypeptides, many of which interact with components of the immune system [56]. For the xenogenic mastadenoviruses the least complex E3 region from MAdV1 appears to encode a single reading frame that may be variably spliced [57, 58]. BAdV1, −2, and −3, CAdV2, and PAdV3 and −5 have E3 regions of intermediate complexity, ranging in size from ~1.2 to 2.3 kb. These code for a variable number of putative proteins that show some homology within a species and occasionally across species [59–63]. The BAdV3 E3 codes for a 284-residue glycoprotein and a 14.7-kDa polypeptide that appears to be the homolog of the HAdV5 14.7-kDa protein. The BAdV3 gene can functionally substitute for the human gene to protect cells against tumor necrosis factor (TNF)-induced lysis [62, 64].

E3 sequences are nonessential for replication in vitro [65] and were some of the first sequences deliberately deleted in the construction of recombinant HAdVs [66]. However, it was shown that retention of E3 sequences in a HAdV5 vector dampened the immune response in a rat model, thus extending the time of transgene expression [67]. Consistent with this, a HAdV5 virus in which E3 sequences were deleted showed an enhanced inflammatory response in a Cotton rat model [68]. It remains to be determined whether these results will translate to xenogenic vectors with less complex E3 regions. However, the timing and duration of gene expression that is required is a factor to be considered in vector design.

In contrast to the mastadenoviruses, the avi- and atadenoviruses lack E3 regions between pVIII and fiber and instead have small intergenic regions of ~200 and ~400 bp, respectively, that contain signals for transcription termination and splicing of fiber RNA [69].

To the right of the fiber gene in mastadenoviruses lies the E4 region. Like HAdV2/5, a single promoter in BAdV3 and MAdV1 produces seven transcripts that encode multiple polypeptides, some of which are homologous to HAdV proteins [70, 71]. In particular, homologs of HAdV5 E4 ORF6 carry a short
amino acid motif that is highly conserved in many adenoviruses. Based on the conservation of this motif in OAdV7, where it was first recognized [22], the proposed E4 region in the atadenoviruses is penultimate to the right end of the genome (Fig. 1). Two promoters apparently control the expression of three open reading frames (ORFs), two of which contain the motif [48, 72]. No E4 region has been identified in the right-hand portion of aviadenoviruses. Indeed, the function of most reading frames in the right hand ~25% of the genome remains to be determined. In FAdV1, the products of GAM-1 and ORF22 (Fig. 1) have been identified as proteins that interact with pRb [73]. However, in comparing the related FAdV1 and FAdV8 genomes, 5 of 13 unassigned ORFs are unique to FAdV8 [74]. At the extreme right hand end of FAdV1 are 3 ORFs that can be deleted and replaced with a luciferase reporter gene cassette without affecting virus viability [51].

The extreme right ends of the avi- and atadenovirus genomes carry genes that are species specific. For example, DAdV1 has numerous ORFs of unknown function that have no counterpart in OAdV7 [22, 23, 48, 75]. Within the right-hand-end region of OAdV7 lies a series of six short reading frames (RH1 to RH6) (Fig. 1), four of which (RH1, -2, -4, and -6) are closely related to each other. This is surprising in a compact genome of only ~29.6 kb. In DAdV1 there are two ORFs that are related to each other and to those in OAdV7 [72, 76]. For OAdV7 only two transcripts from the region were detected by RT-PCR and these were spliced such that RH1 and RH6 were the only ORFs that could be translated. The apparent redundancy of these ORFs was confirmed by the fact that the reading frames RH2 to RH5 could be deleted without seriously affecting virus viability [75]. The function of these ORFs remains to be determined.

3. Left End Sequences

Left of the central core the genome structures also differ significantly (Fig. 1). For the xenogenic mastadenoviruses there are three ORFs at the left end that show homology with HAdVs [77-80]. The genome packaging signal is also present within the first ~500 nucleotides of the HAdV5 genome [81], but until recently this had not been defined for any xenogenic virus. For CAdV2, however, it was shown that the packaging region consists of a ~200-bp region that contains redundant, but not functionally equivalent sequences. The consensus sequence for HAdVs [81] is present only once and is of minor importance [82].

For the avi and atadenoviruses, some ORFs at the left end are unique to individual viruses or have homologs only within the genus. Two ORFs from the atadenoviruses show some homology with the HAdV5 E1B 19- and 55-kDa genes, suggesting that these functions are conserved. However, no homolog of the E1A gene was identified [22, 48]. An additional ORF that could encode a ~9.6-kDa protein is present in OAdV7 and BAdV4 (B. Harrach, pers.
454 Gerald W. Both

The gene for the p32 K structural protein is also present near the left end. The promoter for ORFs LH1 and LH2 is also on the opposite strand within this gene [72]. The packaging signal for atadenoviruses has not been defined but it may incorporate the ~160-bp region between the C-terminus of p32 K and the ITR. For the aviadenoviruses, ORFs with homologies to dUTPase and the REP protein of adeno-associated virus have been found [23] but there are distinct differences between FAdV1 and FAdV8 with three of eight ORFs in the left end being unique to FAdV8 [74]. It was also reported recently [83] that the cysteines and several other residues in the conserved sequence motif of E4 ORF6 are conserved in FAdV1 ORF14, which lies near the left end of the genome [23].

4. Transcription Maps

The determination of transcription maps for some xenogenic viruses has assisted vector design by complementing the data on genome structure. The major transcription units have been described for BAdV3 and PAdV3 [24, 46, 71], FAdV1 [84] and OAdV7 [72]. No transcription map has been reported for CAdV2. More detailed data is available for the E1, E3, and E4 regions of MAdV1 [58, 70, 85] and for the BAdV3 E1 [79, 86] and E3 regions [60]. For BAdV3 and PAdV3, there are minor differences in the splicing pattern within some transcription units but on a broader scale the basic units described for AdV2/5 are completely conserved. Studies of FAdV1 identified many transcripts in the genome and a major transcription unit that is controlled by the MLP. However, at the left and right ends of the genome there are 5 and 15 kb, respectively, for which the promoters and transcriptional organization is undefined [84]. In the OAdV7 genome, the left (LH1 to LH3)- and right-hand ends (RH1 to RH6), E2 and the proposed E4 region (E4.1 to E4.3), as well as the structural protein genes constitute individual transcription units. The IVa2 and p32 K ORFs also appear to be transcribed from their own promoters. The LH and E4 regions each appear to be regulated by two promoters [72]. The identification of promoter regions and transcription termination sites has identified possible sites for gene insertion that are less likely to interfere with viral functions.

D. Transforming Ability

Many AdVs are known to carry oncogenes. Members of the mastadenoviruses readily transform cells in culture [11, 87–89], although these viruses differ in their ability to induce tumor formation in animals. Among HAdVs, the group A viruses such as AdV12 are highly oncogenic, while group C (including HAdV5) and E viruses are not known to be tumourigenic (reviewed in [2, 65, 90]). BAdV3 can induce tumor formation in hamsters [91] but there are no reports of tumor induction by other animal mastadenoviruses. FAdV1
also transforms cell in vitro [92, 93] and rapidly induces tumors in newborn rodents [94, 95]. For the atadenoviruses there are conflicting reports of tumor induction in hamsters. In one study, tumor formation was reported in hamsters inoculated with BAdV8 [96]. In a second study, none of BAdV4 to 10 produced tumors [97]. More recent studies showed that OAdV7 was unable to transform cells that were transformed by HAdV5 [98]. Primary rat embryo cells were infected with HAdV5 or OAdV7 but only the former produced colonies with a transformed phenotype. Similarly, baby rat kidney cells were transformed by HAdV5 E1A/B sequences but not by the nonstructural genes of OAdV7. The apparent absence of oncogenes in the OAdV7 genome suggests that the virus interacts with the cell cycle machinery in a way that differs from the mast and aviadenoviruses, although this is yet to be defined.

The presence of oncogenes in vector genomes has important implications for vector design in that it is customary to delete these sequences for safety reasons. Continuous cell lines that express the deleted genes in trans are established to permit virus propagation. The transforming properties of the mastadenoviruses reside primarily in the E1A and E1B genes at the left end of the genome (reviewed in [2, 65, 90]). The E1A products bind to proteins of the cellular retinoblastoma (pRb) protein family [99], thereby releasing E2F transcription factors that regulate cell cycle progression into S phase [100]. The E1B 55-kDa protein binds to the tumor suppressor protein, p53, and blocks p53-mediated apoptosis [101]. The E1B 19-kDa protein is also anti-apoptotic [102]. Thus, animal adenoviruses typified by BAdV3, PAdV3, CAdV2, SAdV, and MAdV1 have E1A and E1B homologs that have similar transforming and oncogenic potential. The E4 ORF3 and ORF6 products of HAdV5 can also augment the transforming activity of the E1A and E1B genes [103-106]. However, the E4 regions of human and animal mastadenoviruses vary in sequence and complexity. Homology with HAdV5 ORF6 is always evident, especially in a cysteine-rich motif [22] that is thought to mediate ORF6/p53 interaction [83]. Furthermore, a complex between the E4 ORF6 and E1B 55-kDa proteins promotes the selective nuclear export of late viral transcripts [107] and references therein). This ORF may be therefore be conserved as it provides a core function for replication in all adenoviruses. However, other E4 ORFs in the xenogenic viruses are unique [22, 26, 108-110] and their function/transforming potential is not clear.

In FAdV1 there are no identifiable E1A/B or E4 regions in the genome [23], but recently two proteins, GAM-1 and ORF22, that interact with pRb were identified [73]. In addition, GAM-1 has been identified as an anti-apoptotic protein [111] and one that can activate the cellular heat-shock response, the latter being required for viral replication. The Hsp40 gene is a primary target [112]. GAM-1 may also functionally substitute for the E1B 19 kDa [111]. FAdV1 therefore appears to share with the mastadenoviruses an ability to disrupt complexes between pRb and the E2F transcription
factors to modulate the cell cycle, albeit via different effector proteins [99, 113]. In contrast, OAdV7, the prototype atadenovirus, lacks an identifiable E1A homolog, although it appears to carry E1B 19- and 55-kDa genes. Penultimate to the right end is a transcription unit that contains a unique ORF (E4.1) of unknown function and two ORFs (E4.2 and E4.3) which contain the conserved ORF6 cysteine-rich motif mentioned above [22, 72, 98]. These ORFs otherwise appear unrelated. Similar features are found in the DAdV1 and BAdV4 genomes [48 and B. Harrach, pers. commun.]. However, OAdV7 so far lacks oncogenic activity as the complete OAdV7 genome did not transform primary rodent cells under conditions where transformation was achieved with control HAdV5 sequences [98]. These findings invite the hypothesis that OAdV7 lacks the ability to activate the cell cycle in quiescent cells, instead taking advantage of the cycle as it progresses.

The presence or absence of transforming sequences strongly influences the design of xenogenic adenovirus vectors for gene delivery. Based on HAdV2 and −5, vectors derived from BAdV3, PAdV3, SAdV, and CAdV2 were designed such that the potentially oncogenic E1A/B homologs were deleted [20, 42, 43, 114, 115]. A similar approach could be applied to MAdV1 [116]. Such vectors are replication-defective in cells lines that do not express the deleted genes [42, 43], but in some cases, homologs from HAdV5 can substitute [114, 115]. Some vectors derived from OAdV7, avian, and PAdV3 viruses retain potential transforming genes and carry foreign DNA inserts in nonessential regions of the genome [51, 75, 117–120]. This strategy may be acceptable for vectors that are intended for gene delivery in the homologous animal or avian host but is unlikely to be acceptable for gene therapy purposes, except perhaps in the case of OAdV7, where the vector apparently lacks transforming genes.

E. Cell Lines

Successful rescue of a virus requires a cell line that can be transfected with high efficiency to initiate infection. The cells should also have abundant copies of the primary and secondary receptors to facilitate spread and the production of high titers of virus. Depending on the recombinant, the cells may or may not carry viral sequences to complement a deletion in the viral genome.

1. Primary Cell Lines

The general strategy has been to identify a cell line that is permissive for the wild-type virus and then adapt it for more specialized purposes. For propagation of BAdV3, MDBK, buffalo lung, primary kidney, and bovine cornea endothelial cells have all been tried, with MDBK cells being preferred [114, 121]. CAdV2 was grown in MDCK, dog kidney (ATCC CRL6247) or greyhound kidney [43, 121], MAdV1 in mouse 3T6 [116] and PAdV3 in swine testis cells [115]. FAdV1 recombinants were rescued in leghorn male hepatoma (LMH) cells [51]. FAdV1 can be grown in embryonic chicken kidney
cells but, for reasons of cost, is often grown in embryonated chicken eggs [23]. OAdV7 has a narrow host range and failed to grow in several ovine cell types [15]. However, it grew to high titre in CSL503 cells, a primary ovine fetal lung cell line [122] and a fetal ovine skin fibroblast line HVO-156 (C. Hofmann and P. Loser, pers. commun.).

2. Transformed Cell Lines

Primary cells are adequate for growing replication-competent recombinants. However, there was a need to produce cell lines that would complement genomic deletions and an expectation that transformed cell lines would ensure a continuous supply of cells. This encouraged attempts to develop lines equivalent to 293 cells [123]. Note that SAdVs grow in 293 cells [20]. Based on this and similar precedents [124], the E1A/B sequences of BAdV3 were used to stably transfect MDBK cells [114, 125, 126]. These grew poorly and expressed undetectable amounts of the BAdV3 E1 proteins [114] but nevertheless complemented the growth of an E1A-deleted HAdV5/lacZ recombinant [114, 125]. Attempts were also made, unsuccessfully, to transfet foetal bovine retinal cells (FBRCs) with BAdV3 E1A/B sequences. Because BAdV3 complemented HAdV5/E1A-defective replication [125], it was expected that HAdV5 E1A/B sequences would complement BAdV3/E1 deleted vectors. Transfection of FBRCs with HAdV5 E1A/B sequences in which E1A and E1B were controlled by the mouse PGK and E1B promoters, respectively, produced morphologically distinct clones, one of which was single-cell cloned and characterized as the VIDO R2 line. These cells expressed detectable levels of E1A and E1B 19-kDa, but not E1B 55-kDa protein, supported plaque formation by BAdV3 and HAdV5, and were transfected more efficiently than MDBK cells. Transfection of E1-deleted recombinant genomes into VIDO R2 cells resulted in the rescue of several viruses that carried expression cassettes [114]. For propagation of PAdV3 vectors a transformed fetal porcine retinal cell line (VIDO R1) was also produced by transfection of swine testis cells with HAdV5 E1 sequences [115]. These cells were also morphologically distinct from the parental cells. E1A and E1B 19-kDa proteins were produced, as shown by Western blots, but E1B 55-kDa protein was not detected. While PAdV3 grew well in these cells, for reasons that are not understood, an E1/E3-deleted vector and a similar virus that carried a GFP cassette in E1 grew two logs less efficiently [115]. Similarly, the E1A/B region of CAdV2 was used to transform MDBK and DK cells [42]. Again, low levels of E1A transcripts were produced and E1B transcripts were not reliably detected. Nevertheless, the cells were morphologically and phenotypically distinct from parental MDCK cells. A second series of clones was produced by transfecting DK cells with CAdV2 sequences in which E1A and E1B were controlled by the HCMV and E1B promoters, respectively. Cells produced in this way expressed detectable E1A and E1B transcripts and E1B 19-kDa protein [42] and allowed the rescue and propagation of E1A/B-deleted CAdV2 vectors [43].
Attempts were also made to produce a transformed derivative of CSL503 cells, which are permissive for OAdV7, using the left end (∼4 kb) of the OAdV7 genome [98]. The sequences used incorporated the proposed E1B homologs of OAdV7 and a 9.6-kDa ORF of unknown function. No E1A homolog was identified [72, 98]. Only two clones that grew well enough to prepare frozen stocks were obtained and these were morphologically similar to the parental cells. In contrast, transfection of CSL503 cells with HAdV5 E1A/B sequences produced morphologically distinct clones. Growth of OAdV7 in these cell lines appeared to be retarded compared with its growth in wild type CSL503 cells (Xu and Both, unpublished results).

F. Strategies for Vector Construction and Rescue

A huge amount of work carried out over some 30 years on HAdV2 and -5 has defined viral promoters, transcripts and their splice sites and genes that could be deleted or that would function in trans (reviewed in [2, 65]). The packaging capacity of the viral capsid was also shown to be ∼105% of the viral genome [127]. The strategic design of bovine, canine, porcine, simian, and murine adenovirus vectors, although based on new genetic information, has drawn extensively on historic precedents.

As precedents did not exist for the avi- and atadenoviruses it was necessary to identify intergenic regions within genomic sequences and to use mutagenesis to identify nonessential reading frames for the insertion of gene cassettes. Vector design and virus rescue was also confounded initially by the absence of transcription maps and the lack of knowledge concerning the packaging capacity of these viruses.

Construction of xenogenic adenovirus vectors first required the identification of an insertion site(s) that could stably accommodate a gene cassette without affecting virus growth. For the mastadenoviruses, vector construction strategies followed those for human Ad vectors. Genes were inserted into the nonessential E3 region of BAdV3 [126, 128] or PAdV3 [118] or between the E4 promoter and the right ITR of PAdV3 [118, 120] to generate viruses that were replication competent in noncomplementing cells. More recently, E1A/B region replacements that generated replication-deficient viruses were produced for BAdV3 [114, 121], PAdV3 [115], SAdV [20], and CAdV2 [42, 43]. It is likely that a similar strategy would be successful for MAdV1 where an infectious clone is now available [116]. For the aviaadenoviruses, a mutation strategy was used to identify nonessential regions of the genome or regions that could be complemented in trans [51]. Deletions between nucleotides 938 and 2900 were complemented by cotransfection of a plasmid that carried the left hand ∼5.5 kb of the genome. Deletion of three ORFs adjacent to the right end of the FAdV1 genome did not require transcomplementation, identifying these genes as nonessential for replication in vitro. Similarly, replication-competent FAdV8
vectors were constructed by inserting a gene cassette into sites near the right end of the genome (Fig. 1) [119]. For the atadenovirus, OAdV7, genes were initially inserted at site I (Fig. 1) in the pVIII and fiber intergenic region [44, 50, 75, 117, 129], but additional sites were subsequently identified by a mutation strategy. It was found that foreign DNA could be inserted into a unique SalI site (Site II) within ORF RH2, ~1 kb from the right end, and that ORFs in the vicinity could be deleted [75]. In addition, unique cloning sites were tolerated between the right-hand end and E4 transcription units (Site III) [72, 117].

The identification of permissible insertion sites in the genome required the construction of plasmids that enabled the rescue of infectious viruses. The first BAdV3 recombinant was constructed by recombination between a plasmid that carried BAdV3 sequences flanking the luciferase gene inserted into E3 and BAdV3 genomic DNA that had been cut with PvuI to reduce background. DNAs were transfected into MDBK cells that also expressed BAdV3 El sequences. However, this method was inefficient and produced relatively few plaques [126]. Similarly, a CAdV2 recombinant that expressed the lacZ gene was produced by recombination between the CAdV2 (Manhattan strain) genome and a plasmid that carried the expression cassette. However, this recombinant was contaminated by wild-type CAdV2 that could not be eliminated [42]. A more favorable approach was to construct a plasmid in which sequences required for the propagation of plasmid DNA in Escherichia coli were cloned into a unique restriction enzyme site that linked the ITRs of the viral genome [117]. There was one precedent for this approach [130], although others had reported that perfect palindromes longer than 30 bp were often unstable in E. coli [131] and plasmids with large palindromes based on HAdV5 were subject to rearrangement [132]. Unique restriction sites were also introduced into appropriate locations in the OAdV7 genome to allow cloning of gene cassettes. This plasmid design allowed the genome to be released intact by restriction enzyme digestion prior to its transfection into susceptible cells for virus rescue [75, 117]. Subsequently, it was discovered that such plasmids could be constructed using recombination in E. coli [133]. Infectious recombinant clones have now been constructed for BAdV3 [71, 114, 134], PAdV3 [118], CAdV2 (Toronto strain) [43], MAdV1 [116], FAdV1 [51], and OAdV7 [44]. The specific infectivity of these naked DNAs in the permissive cell line is usually low (often only a few plaques per microgram) and depends on the transfection efficiency of the cells. However, a significant advantage of this approach is that transfection of purified plasmid DNA almost invariably yields the corresponding virus without the need for extensive plaque purification that may accompany other approaches where background viruses can be generated.

Many xenogenic recombinant viruses have now been rescued. New viruses that first appeared with the formation of plaques or a cytopathic effect in the appropriate transfected cell line were amplified on fresh permissive cells to produce an infectious stock. Viruses were then characterized by restriction
enzyme, Southern blot [75, 115, 118, 126], or PCR analysis [43, 51] to confirm the integrity of the genome and expression cassette. For the vectors where an insertion strategy was pursued, it was particularly important to check the genome integrity because the packaging capacity of the new vectors was undefined. Mastadenoviruses can package 105 to ~107% of the wild-type genome [43, 120, 127], OAdV7 has a capacity of 114%, presumably because of its smaller genome and similar capsid volume [75], while the capacity of avianadenoviruses is undefined. Despite the increased packaging capacity of OAdV7, some viral genomes in which expression cassettes (ranging from 1.8 to 3.1 kb) were inserted into site I of the genome proved to be unstable upon passaging. By passage three, the genomic BamHI profile of viruses that combined the HCMV promoter with a reporter gene sometimes displayed smaller fragments [50]. In contrast, a virus that carried 4.3 kb of “stuffer” DNA was successfully rescued [75] and with the RSV promoter, two viruses with site I cassettes in opposite orientations were stable to at least passage four [44; unpublished results]. Site I instability appears to vary with sequence and possibly orientation and may reflect the need to produce adequate amounts of fiber transcript and protein. Events that lead to transgene deletion with improved fiber production may generate viruses that have a growth advantage. The stability after passage of genomes for other xenogenic recombinant vectors has not been adequately reported.

The propagation of a mixed population of CAdV2 wild-type and deleted vector [42] illustrated the potential for producing gutless vectors based on xenogenic AdVs. The principles established with HAdV5 [135, 136] will further assist this process. It will be necessary to define the packaging signal [82] and a minimum permissible genome size for a particular virus, provide a suitable a helper virus for propagation, and devise a means to purify defective particles. The benefits may be greater safety and more efficient gene delivery in a naive host and prolonged transgene gene expression.

IV. Utility of Xenogenic Vectors

Xenogenic AdV vectors can potentially be used as gene delivery vectors for a range of purposes. However, it is necessary to understand the advantages and disadvantages of vectors in particular situations so as to identify their most appropriate uses. The next section discusses the first attempts to determine the safety and utility of xenogenic vectors for vaccination or gene delivery. The following section reviews the properties and behavior of vectors in heterologous situations.

A. Veterinary Studies

Within the limits of the testing done so far, the viruses discussed in this review are of low pathogenicity in the host from which they were isolated [11–15]. Vectors designed for use in those hosts are often replication
16. Xenogenic Adenoviral Vectors

competent to facilitate vaccination by a live viral vector. In the first studies, carried out with BAdV3, the luciferase reporter was inserted directly into the E3 region where a small deletion had been introduced. Expression did not require an exogenous promoter and the vector remained replication competent in bovine cells, although its titer was reduced 10-fold [126]. In contrast to HAdV5 vectors that lacked part of the E3 region [68], this BAdV3 recombinant did not show increased pathogenicity in a Cotton rat model compared with the wild-type virus [137]. Similar replication-competent viruses that carried various forms of the bovine herpesvirus gD gene were shown to express the antigen [71] in an immunogenic form [128]. Intranasal vaccination of calves with these viruses induced gD-specific neutralizing antibodies, primed a cellular immune response and protected against viral challenge, despite the presence of preexisting serum antibodies to BAdV3 [138,139]. E1/E3-deleted replication-defective BAdV3 vectors that carried gD in the E1 region were also constructed [114]. These viruses allowed the parameters for vaccination of cattle by replicating and nonreplicating vectors to be compared. Administration of each vector at the same dose twice via the intratracheal route and once subcutaneously showed that the replication-competent vector induced superior levels of serum IgG antibodies against gD. Partial protection against challenge was obtained with the replication-competent vector. However, with the replication-defective vector challenge with BHV1 dramatically boosted the levels of serum IgG and IgA antibodies, suggesting that animals had been primed for gD-specific antibody responses [140]. Similar BAdV3 recombinants were constructed in which the bovine diarrhea virus E2 glycoprotein linked to the BHV1 gD signal peptide was expressed from the BAdV3 E3/MLP [141]. The 53-kDa protein that was expressed formed dimers and was recognized by E2 specific monoclonal antibodies. Intranasal immunization of Cotton rats with the recombinant induced E2-specific IgA and IgG responses at mucosal surfaces and in the serum. In contrast, attempts to construct vectors that expressed the bovine coronavirus hemagglutinin esterase gene from the E3 region using the strategy for the BHV1 gD gene were unsuccessful. The addition of exogenous control elements comprising an intron and the HCMV or SV40 promoter increased the level of expression but altered the kinetics. The recombinant virus also replicated less efficiently than wild-type BAdV3 [142].

Replication-competent PAdV3 vectors that express the pseudorabies gD protein or the classical swine fever virus (CSFV) gp55 protein were also constructed. The gD gene was inserted into a partially deleted E3 region without flanking sequences. In contrast to similar BAdV3 vectors, expression of gD was observed at early but not late times pi [118]. The gp55 gene linked to the PAdV3 MLP and tripartite leader sequence (TLS) was inserted at the right end between the ITR and E4 promoter. Vaccination of outbred pigs with a single dose of recombinant virus induced complete protection from lethal challenge with CSFV [120].
A FAdV8 recombinant that expressed chicken gamma interferon from the viral MLP/TLS sequences was also constructed by inserting the cassette at sites near the right end [119]. Depending on the insertion site, the recombinants displayed differing growth characteristics in chicken kidney monolayers. Insertion of the cassette adjacent to FAdV8 ORF7, about 7.2 kb from the right end, produced a recombinant with wild-type growth characteristics. In contrast to the FAdV1 viruses discussed below, deletion of the FAdV1 36-kDa homolog in FAdV8 caused a significant reduction in growth. Interferon was produced in supernatants as early as 24 h pi in proportion to the growth characteristics of each virus in vitro. Interferon levels peaked at 48 h and were maintained for at least 10 days. Chickens treated with the recombinant showed increased weight gains compared to controls and suffered reduced weight loss when challenged with a coccidial parasite [119].

An OAdV7 vector was constructed in which the 45 W antigen of *Taenia ovis* was expressed from the viral MLP/TLS elements [143], the cassette being inserted at site I (Fig. 1) [75]. This vector was used alone, or in tandem with DNA or purified 45W protein to vaccinate sheep. Prime/boost strategies where vaccination was initiated with protein or DNA and boosted with the OAdV7 vector were effective in stimulating an immune response that protected animals against challenge with the parasite [144].

The above examples illustrate that with further refinement, xenogenic vectors may have utility for vaccination and gene delivery in their respective hosts.

### B. Vector Biology

Ideally, vectors for gene transfer into human cells should be capable of transgene expression without replication or detrimental expression of viral genes. Infection of human cell lines with intact xenogenic adenoviruses established the principle that these viruses are replication defective at the inputs tested [42, 51, 76, 116, 118, 121, 126], although the molecular basis for defective replication is not understood. Studies in animal models have also allowed biodistribution profiles to be determined for some viruses.

#### 1. Transduction of Cells

Selected cell lines have been used to examine viral transduction. However, it is sometimes difficult to compare data from different laboratories because, especially in early studies, the input virus was not characterized with respect to both particle number and infectivity. BAdV3 recombinants in which a HCMV/lacZ or HCMV/GFP gene cassette was expressed from the E1 or E3 region, respectively [114, 121], were used to infect human and other cell types. The GFP recombinant replicated in cells of bovine origin and in Cotton rat lung fibroblasts, but not in cells from other species. When cells were infected with more than 5 pfu/cell of BAdV3/GFP, some GFP expression was observed at
3 days pi in 293 and HeLa but not in A549 or HepG2 cells [114]. In contrast, others found that at an m.o.i of 10 pfu/cell, at 65 h pi A549 and MRC5 cells were efficiently transduced by a BAdv3/lacZ recombinant while HeLa and 293 and primary human muscle cells were transduced with lower efficiency [121]. Since both studies used the HCMV promoter and a similar multiplicity of infection, the reason for the difference with A549 cells is unclear.

The host range of CAdv2 vectors was also investigated. Human 293, HeLa, primary myocyte, and HIB cells were infected with $10^5$ transduction units of CAdv2/RSVlacZ in the presence of wild-type CAdv2. All cell types showed β-gal expression when examined at 1 to 2 days pi [42]. In addition, replication-deficient CAdv2 vectors expressing GFP or lacZ from the HCMV and RSV promoters, respectively, were tested for their ability to transduce a range of human cell types in comparison with HAdV5/HCMV/GFP [43]. At 2 days pi HeLa, A172, and HT 1080 cells were transduced with similar efficiency by both viruses. In vivo, the CAdv2 vectors also transduced mouse airway epithelia cells with similar efficiency to a comparable HAdV5 vector. Similarly, a replication-deficient PAdv3 recombinant carrying a HCMV/GFP cassette in E1 was used to determine the ability of this vector to infect human and animal cells in vitro. At a m.o.i. of 1 pfu/cell PAdv3 apparently entered, but did not replicate in canine kidney, ovine skin fibroblasts, bovine (MDBK), and human (293, A549) cells [115].

Although an infectious clone of MAdv1 now exists, recombinant viruses have not yet been made. However, it was demonstrated by RT PCR that human 293 and primary umbilical endothelial cells were infected, the latter at low efficiency [116].

Replication-competent aviadenovirus vectors that express luciferase from the HCMV promoter [51] were constructed by inserting cassettes at the right end of FAdv1 to replace nonessential ORFs. Vectors replicated in LMH cells with kinetics similar to wild-type FAdv1. When compared to a HAdV5/luciferase recombinant for its ability to transduce human cell types, the FAdv1 recombinant showed a similar ability to express luciferase in HepG2, A549 and primary human fibroblasts [51].

Several recombinants that carried reporter genes at site I of the genome (Fig. 1) were used to investigate the host range of OAdv7 [29, 44, 50, 76, 129]. These studies showed that OAdv7 can infect, but not replicate in a variety of human cell types, including breast (MCF7, T47D2) and prostate cancer (PC3), liver carcinoma (HepG2), and retinal (911), foreskin (HFF), and lung (MRC5) fibroblasts [76]. Reporter gene expression increased proportionally with the m.o.i. Monkey (COS) and mouse prostate (RM1) cells were also infected efficiently in vitro [50 and unpublished results].

Considering the quite broad host range of OAdv7, it will be of considerable interest to identify the receptor(s) that mediates infection. In principle it is also possible to redirect the vector via an alternative receptor as was done for
HAdVs [36, 37]. It was shown [29] that the cell-binding domain of OAdV7 fiber protein could be replaced with the equivalent binding domain from HAdV5. This was the only change in the viral capsid but it profoundly altered the cell tropism of OAdV7, apparently independent of any integrin/penton RGD interaction, since this motif is absent from OAdV7 [22]. Although the hybrid virus grew less well, this result confirmed that the two viruses use distinct receptors and demonstrated that targeting of xenogenic viruses may be possible.

2. Abortive Replication in Vitro

Abortive replication of xenogenic adenoviruses probably reflects viral promoter function in human cell types. The function of early and late BAdV3 promoters in human cells was examined by RT PCR and Southern blotting [121]. In A549 and 293 cells E1A transcripts were detectable for at least 5 days. At very high m.o.i. hexon mRNA was detectable at day 3 in primary human muscle, MRC5 human lung fibroblasts, and nasal septum epithelial cells. It was also shown that CAdV2 replicated to a limited extent in some human cells, as judged by higher virus output compared with input and some expression of capsid proteins. However, this was observed only at the first passage [121]. For human cells infected with OAdV7 at m.o.i. 20 pfu/cell the situation was polarized, depending on the cell type. On the one hand, in MRC5 cells, all early promoters in the genome that were examined were active, as monitored by RT PCR amplification of selected transcripts. On the other hand, in HepG2 liver carcinoma cells, none of the early promoters had detectable activity. In most other cell types, e.g., MCF7 and T47D2 breast cancer and PC3 prostate cells, some promoters, typically E2, were active. Interestingly, in all human cell types tested, and even when the early promoters were active, transcripts from the OAdV7 major late promoter (MLP) could not be detected [72, 76]. This may be related to key events that occur in the transition from early to late protein synthesis. For HAdV2, accumulation of early gene products is not sufficient for MLP activity. DNA replication is also required for late gene expression. High-level transcription from the MLP is further dependent on a cis-acting change in the viral chromatin [145]. In addition, HAdV2 MLP activity is stimulated by trans-activating factors DBP and IVA2 [146–148]. At a gross level there is little or no DNA replication in OAdV7 infected human, compared with permissive ovine cells. However, the OAdV7 E2 promoter was active in several human cell types and large amounts of DBP transcript (and presumably, transcripts for DNA polymerase and Terminal protein) were produced [76]. Cellular factors also cooperate with viral proteins during genome replication (reviewed in [2]). The apparent absence of DNA replication may be due to the incompatibility of one or more human cell factors with binding sites on the OAdV7 ITR sequences or with other viral proteins involved in the process. There are significant differences
in putative binding sites for transcription factors between the ITRs of human and xenogenic viruses [149]. The inactivity of the OAdV7 MLP could further be due to a missing trans-activating factor, such as IVa2, whose expression in human cells has not been investigated. Such abortive replication makes it unlikely that conditionally replication-competent vectors [150, 151] based on xenogenic vectors will be developed in the near future.

3. Biodistribution and Persistence in Vivo

Few studies on the biodistribution and persistence of xenogenic AdVs in vivo have been reported, but some have been carried out with MAdV1 and OAdV7. In the homologous situation, mice were injected intraperitoneally (ip) or intranasally with $10^3$ pfu of MAdV1 and the localization of virus was monitored histologically during acute infection [152]. Endothelial cells of the brain and spinal cord showed extensive evidence of infection. Endothelial cells in lungs, kidneys, and other organs gave a positive signal, indicating a widespread involvement of the systemic circulation. Some lymphoid tissues were also positive. In experiments that examined persistence of OAdV7 it was found that $5 \times 10^3$ pfu of a recombinant OAdV7 vector injected intravenously (iv) into SCID mice produced hAAT expression that persisted for at least 60 days. However, the same vector dose in BALB/c mice was cleared by 20 days. Thus, the vector did not persist in the normal host and a substantial dose of virus ($2 \times 10^{11}$ particles) did not cause significant toxicity in normal or immunocompromised animals.

The distinct nature of the OAdV7 receptor was reflected in the biodistribution of OAdV7 following iv or ip administration of the vector to mice. OAdV7 was evenly distributed between liver, heart, spleen, and kidney [44], whereas HAdV5 vectors given iv concentrated predominantly in the liver [153]. OAdV7 given via the intraportal vein led to a greater accumulation of vector in the liver, but the vector was still found in all tissues examined [50]. In addition, when virus was injected directly into mouse skeletal muscle, cells were transduced and high levels of hAAT reporter protein were secreted in vivo [129]. By judicious adjustment of the first dose of vector it was shown that a second dose that resulted in substantial reporter gene expression could be given, raising the prospect that the vector may be suitable for prime/boost vaccination strategies. The vector was not detected in liver and spleen, indicating that it did not spread via the circulation. Expression, however, was transient and the vector DNA had essentially disappeared by day 14. Clearance occurred in the absence of detectable OAdV7 gene expression as assayed by RT PCR. As proposed for HAdV5 vectors [154] clearance may occur via presentation of antigen using an MHC class I independent mechanism.

Experiments utilizing HAdV5 and OAdV7 recombinants demonstrated a perceived advantage of xenogenic AdV, showing that OAdV7 could deliver a reporter gene in vivo in the face of preexisting antibodies against human
HAdV5 [44]. This result was encouraging from a clinical viewpoint and should be mimicked by other xenogenic AdV. It may be possible eventually to use different vectors in tandem to deliver multiple doses of the same gene [155].

C. Gene Therapy Studies

To date no gene therapy applications have been reported for xenogenic AdV. However, work is in progress in this laboratory to assess OAdV7 as a gene delivery vector for prostate cancer. The strategy is based on gene-directed enzyme prodrug therapy (GDEPT). This is a two-component cell killing system: a gene that encodes an enzyme not present in mammalian cells and a nontoxic prodrug that is converted to a toxic product by cells that produce the enzyme. Although there are several GDEPT systems [156], in this case purine nucleoside phosphorylase (PNP), an *E. coli* enzyme, and the prodrug fludarabine are being used [157]. OAdV7 vectors that express the PNP gene under the control of the constitutive RSV, or a tissue-specific prostate promoter, were constructed and tested for cell killing *in vitro* and in mouse models of prostate cancer. Viruses were injected directly into human PC3 or LN3 tumors grown subcutaneously in nude mice or into mouse RM1 tumors grown subcutaneously (sc) in immunocompetent animals. Prodrug was given systemically [158 and Voeks et al (in preparation)]. Evidence of tumor shrinkage and prolongation of mouse survival indicate that this vector and GDEPT system has potential for prostate cancer therapy.

This work has also highlighted other important issues that must be addressed for OAdV7, and for xenogenic vectors in general, if they are to be developed for clinical application. These especially include biosafety and vector growth, purification, and scaleup.

V. Biosafety

Most work with xenogenic vectors is still firmly based in the laboratory and while this is appropriate to demonstrate the utility of a vector the amount of work required for eventual exploitation of a vector in the clinic should not be underestimated.

A. Complementation and Recombination

Although the xenogenic AdV undergo abortive replication in human cells, one hypothetical situation concerning the clinical application of these vectors is their potential for interaction with opportunistic, replication-competent human adenoviruses in a patient. This may involve complementation of a replication-deficient virus or recombination between genomes to create a hybrid with
undesirable properties. *A priori,* such events seem more likely to occur between viruses that are closely related, particularly if they share a common receptor to facilitate coinfection. Evidence was sought for interaction between HAdV5 and CAdV2. However, coinfection of HeLa or A549 cells with CAdV2 (m.o.i. 10) and HAdV5 (m.o.i. 2) had no effect on the production of CAdV2 over five passages, compared with CAdV2 infection alone. DNA extracted from the cells was also digested and analyzed by Southern hybridization using a whole genome CAdV2 probe to track the DNA and look for the appearance of hybrid genomes. In coinfecting HeLa and A549 cells CAdV2 DNA disappeared after one to three passages. HAdV5 DNA became visible by passage four and its restriction enzyme profile was identical to HAdV5 alone. No CAdV2 sequences were detected in these samples by hybridization [121].

Similar experiments have been done to determine whether any productive interaction occurred between OAdV7 and HAdV5, a typical human adenovirus. No complementation of OAdV7 replication was detected in the presence of wild-type HAdV5 in MCF 7 cells, although both viruses infect these cells [76] and HAdV5 replicated with high efficiency. Similarly, when DNA from several passages of cells that were coinfected by OAdV7 and HAdV5 was analyzed by Southern blot using whole genome OAdV7 or HAdV5 probes, no hybrid genomes were detected [158a]. Considering the differences in genome structure between the two viruses (Fig. 2), the apparent lack of viable hybrid

![Figure 2](image-url)  
**Figure 2** Difference map between HAdV2/5 and OAdV7. The stippled rectangles indicate the genomes with distinct G/C content, striped boxes at each end show the ITRs of different length, and sequence and ORFs with bold type are unique. The packaging signal is shown in (ψ).
virus formation and the absence of complementation was not surprising. First, the G/C content of the two genomes is vastly different, indicating low nucleotide sequence homology. Next, the ITRs of each genome differ in length and sequence, suggesting that neither would be compatible with the DNA replication machinery of the other. Third, each virus has a distinct complement of capsid proteins, including unique proteins and distinct fibers as well as non-structural genes (Fig. 1). In addition, the packaging signals for each genome are likely to be incompatible. Thus, vectors such as OAdV7 may offer a greater margin of safety over those that are more closely related to HAdVs, such as SAdVs, with respect to potential for unwanted interactions. It is significant, therefore, that no human atadenoviruses have yet been described.

B. Oncogenes in Viral and Cellular DNA

As discussed above, replication-deficient E1-deleted vectors are rescued and propagated in continuous cell lines that were derived from primary cell lines by transformation with adenovirus E1A/B genes [42, 43, 114, 115]. While this is an advantage for cell growth and virus production it is a disadvantage for downstream processing and purification. Regulatory agencies impose strict limits on the permissible levels of contaminating DNA (10 ng/dose) in purified vector preparations [159]. A rigorous purification process is therefore required to remove potentially oncogenic DNA. Thus, an advantage of OAdV7 vectors is that they grow in a primary fetal ovine lung cell line. The trade-off is that the cells grow more slowly and have a life span of 50 to 70 doublings [122].

Oncogenes must also be removed from the vector genome. This may be more straightforward for the mastadenoviruses, where precedents exist from HAdV2/5 studies, but within this genus some viruses are more oncogenic than others [2, 65] and some ORFs exhibit unexpected transforming properties [160–162]. Progress toward oncogene identification in FAdV1 has also been made [73, 111], but others may exist. Ultimately, the regulatory authorities will require tests to be conducted on the residual oncogenicity of xenogenic vectors prior to clinical application. The apparent lack of transforming ability of OAdV7 in systems that have been used as a benchmark for such assays was therefore encouraging [98].

C. Virus/Cell Interactions

Adenoviruses undergo a lytic infection cycle in permissive cells. The mechanism behind cell lysis is not well defined in all cases but for HAdV5 it is due to the production of a “death protein” late in the infectious cycle [163]. Other mechanisms that may be involved in selective killing of tumor cells are being investigated [164]. These observations highlight the potential for interactions between a virus and a cell that may be undesirable in the context of extended gene expression or from a biosafety perspective.
Despite the inactivity of its MLP, in some cell types, typified by MRC5 lung fibroblasts, OAdV7 produced an apparent cytopathic effect (CPE) that was limited by the m.o.i. CPE was not due to viral replication because virus passaged twice on MRC5 cells failed to produce CPE in permissive CSL503 cells [76]. Thus, the effect is likely to involve an early gene product. This is currently under investigation. In this regard it is intriguing that the induction of rapid cell death following infection by certain HAdVs appears to be due to an interaction between p53 and the E1B 55-kDa product [164]. The response was abrogated by the absence of either protein due to mutation or lack of expression. Given the many genes of unknown function that exist in the expanding range of xenogenic AdVs the potential to discover other unwanted interactions exists. It may prove necessary to engineer vectors to remove deleterious genes and to grow them in complementing cell lines, but that raises complementation risks.

D. Replication Competent Viruses

A significant problem with the production of HAdV5 vectors has been the emergence of replication-competent viruses from cells that were designed to prevent their formation. Sequence overlap between the viral vector and integrated genes and subsequent recombination between them has generally been the cause [165]. Thus, PERC6 cells and matching vectors in which sequence overlaps were eliminated were specifically designed to overcome the problem [166]. An advantage offered by the xenogenic vectors is that all of them are replication-deficient in all human cell lines that have been tested. Additional work with particular vectors and cell types to understand the molecular basis for abortive replication would be very helpful in assessing the safety of new vectors.

VI. Vector Production and Purification

For vector production at the laboratory level the availability of a cell line or egg system [23, 48] for virus rescue and propagation is sufficient. Virus can be purified using methods based on cell lysis and CsCl centrifugation similar to those described for HAdV5 [10, 15, 43, 49, 137]. However, increasing success with a vector brings increasingly stringent requirements as work proceeds toward production for veterinary applications or a clinical trial. Strategic decisions taken early to facilitate subsequent steps in vector development and exploitation could save substantial time and effort later on. A key requirement for vector production is the availability of a cell line that, having been expanded and laid down as master and working cell banks (MCB/WCB), is tested and shown to be free of adventitious agents. Attention to detail in the creation and
documentation of such a cell line would pay dividends in the long term. A master virus seed stock also needs to be established. This dictates that the viral genome, including the transgene, must be stable upon serial passage such that biological activity and potency are maintained. This stock must also be free of other agents.

The issue of vector yield from the WCB should also be considered. For veterinary applications where the vector may be replication competent in the host, low yields may be less important. However, if gene therapy is being considered as an application a purified virus yield of $>10^4$ particles per cell is probably required for cost effective production of a vector.

For clinical applications in particular, a robust scheme for vector purification is required. While this might involve CsCl gradient centrifugation to produce quantities of vector for preclinical and perhaps phase I studies, such methodology is unlikely to be appropriate for producing larger amounts of vector. Methods involving chromatography may be more advantageous [167]. It is recognized that the above provides a very brief summary of issues that might be substantial for particular vector systems. However, the intention is to alert the reader contemplating the use of a new vector system to the many challenges that lie ahead in the process of chaperoning it through production and regulatory processes. The correct strategic decisions taken early can facilitate subsequent steps in vector development and exploitation.

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