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Rescue of chimeric adenoviral vectors to expand the serotype repertoire

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Abstract

The successful use of any adenoviral vectors is predicated upon the use of a serotype that is not neutralized by circulating antibodies. However, efforts to develop a diverse repertoire of serologically distinct adenovirus vectors may be hindered by the necessity to generate cell lines to allow for the successful propagation of vectors deleted of essential genes. A strategy to construct chimeric adenoviruses whereby the rescue and propagation of an E1-deleted HAdV-B-derived adenoviral vector can be achieved using existing cell lines such as HEK 293 is reported. It is further shown that this strategy may be more widely applicable.

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1. Introduction

The presence of circulating antibodies to adenovirus capsid proteins is a barrier to the use of adenovirus vectors for gene therapy and vaccines. The prototype adenovirus vectors that have been developed for gene therapy are based on human adenovirus C (HAdV-C, previously subgroup C) such as serotype 5 (HAdV-5). The prevalence of neutralizing antibodies against HAdV-C species is generally high in human populations as a result of frequent exposure to these pathogens. This fact is likely to limit the effectiveness of gene therapy vectors based on HAdV-C serotypes such as HAdV-5. One approach to overcoming this problem is to develop adenovirus vectors that rarely cause human infections (Mastrangelo et al., 1996), or to use adenoviruses from non-human sources, e.g., bovine, canine or ovine adenoviruses (Kremer et al., 2000; Mittal et al., 1995; Xu et al., 1997) because antibodies capable of neutralizing these viruses are likely to be absent in most humans. However, therapies that require subsequent gene therapy vector administration will probably require a different serotype than that used for the first administration. This will, therefore, necessitate the construction of adenovirus vectors based on several unrelated serotypes, an approach that may require the construction of the appropriate complementing cell line suitable for the manufacture of any particular serotype vector (Vogels et al., 2003).

Analysis of the nature of the protective antibodies against adenoviruses has indicated that the most important target is the major capsid protein, hexon (Gall et al., 1996; Wohlfart, 1988). Several efforts have been made to engineer the hexon so as to evade the anti-hexon antibodies by making chimeric adenoviruses harboring hexons from other serotypes (Gall et al., 1998; Roy et al., 1998; Wu et al., 2002; Youil et al., 2002). One major drawback to this method based on the above reports is that such chimeric adenoviruses can usually only be made between closely related serotypes. The speculated reason for this is that the proper assembly of the capsid particles requires optimal interactions between the hexon and the other capsid proteins such as the penton base, as well as the other structural core proteins that interact with the interior of the hexon, such as proteins IIIa, pVI, pVIII, and pIX. Furthermore, interactions with the capsid assembly mechanism such as with the 100 K scaffolding protein are likely to be important. Perturbation of any of these interactions may lead to sub-optimal capsid formation and would account for the lack of success in the generation of chimeric adenoviruses from adenoviruses that are not closely related.

The other major drawback to this approach is that it only addresses one of the three major capsid proteins. As mentioned above, the hexon has received the most attention from the point

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of view of humoral immunity because anti-hexon antibodies are very effective in neutralizing infectivity, possibly by a one-hit mechanism (Wohlfart, 1988). However, the fiber protein is known to harbor neutralization domains as well. Anti-fiber antibodies that are directed against the knob domain can block adenovirus infection (Wohlfart, 1988). Moreover, even though anti-penton base antibodies have not been thought to provide significant protection from infection, in concert with anti-fiber antibodies, they may be of importance in a gene transfer setting (Gahery-Segard et al., 1998).

In view of the above, the feasibility of constructing a chimeric adenovirus where most structural proteins, and not merely the hexon or fiber, are derived from an adenovirus of an unrelated serotype was tested. This preserves the majority of the protein–protein interactions that are involved in capsid assembly. Most of the early genes such as those encoded by the adenovirus E1 and E4 regions, that are responsible for transcriptional regulation and regulation of the host cell cycle, are retained from a different serotype that is known to result in high titer virus generation in the commonly used cell types such as HEK 293 which supplies the HAdV-5 E1 proteins in *trans*. Since coding sequences for the structural proteins are present in a contiguous stretch in the central part of the adenovirus genome, the possibility of replacing this length of DNA from one adenovirus by a homologous segment from another adenovirus was investigated. This would create a chimeric adenovirus where the majority of the capsid proteins are derived from one adenovirus species and where the flanking E1 and E4 regions are derived from another.

2. Materials and methods

2.1. Construction of plasmids containing chimeric viral genomes

Two chimeric adenoviruses were made; one that is chimeric between Ad Pan 5 (Simian adenovirus 22, ATCC VR-591) a HAdV-E adenovirus and Ad C1 (Simian adenovirus 21, ATCC VR-20) a HAdV-B adenovirus; the other that is chimeric between HAdV-5, a HAdV-C adenovirus and Ad Pan 7 (Simian adenovirus 24, ATCC VR-593) a HAdV-E adenovirus. The overall approach towards constructing chimeric viruses was first to assemble the complete E1-deleted virus DNA into a single plasmid, digest the plasmid DNA with an appropriate restriction enzyme to release the virus DNA ends, and transfet the DNA into HEK 293 cells to determine whether viable chimeric adenovirus could be rescued. Two plasmids harboring virus genomes chimeric between Ad Pan 5, and Ad C1 were initially constructed, pPan5C1short and pPan5C1long (Fig. 1A and B). The plasmid pDVP5Mlu, which contains the left end of Ad Pan 5 extended to the MluI site (15,135 bp) was cloned into this plasmid between the Smal and MluI sites. The E1 gene was functionally deleted and replaced by a DNA fragment harboring recognition sites for the extremely rare cutter restriction enzyme sites I–CeuI and PI–SceI). The 2904 bp of the right end of Ad Pan 5 was PCR amplified using the primers P5L [GCG CAC GCG TCT CTA TCG ATG AAT TCC ATT GGT GAT GGA CAT GC] and P5ITR [GCG CAT TTA AAT CAT CAT CAT CA ...

![Fig. 1. The maps of the plasmids pPan5C1short (A) and pPan5C1long (B) and pH5C7H5 (C) are shown.](image-url)
TAA TAT ACC TCA AAC] using Tgo polymerase (Roche). The PCR product was cut with *MluI* and *SwaI*, and cloned between *MluI* and EcoRV of pDVP5Mu to yield pPan5Mu + RE. A 3193-bp fragment extending from the *MluI* site (15,135) to the *ClaI* (18,328) site of Ad Pan 5 was then inserted between the same sites of pPan5Mu + RE to yield pPan5Cla + RE. The 3671 bp *ClaI* (18,529)–*EcoRI* (22,200) fragment of the chimpanzee adenovirus C1 (Simian adenovirus 21, ATCC VR-20) was cloned into pPan5Cla + RE between *ClaI* (16,111) and *EcoRI* (16,116) to yield pPan5ClaDelRI. The 10 452 bp internal *EcoRI* fragment of Ad C1 (22,200–32,651) was cloned into the *EcoRI* site of pPan5ClaDelRI to yield pPan5ClaShort. To construct pPan5ClaLong, the Ad C1 replacement was further extended by replacing the *AscI–ClaI* 10,379 bp fragment of Ad Pan 5 in pPan5ClaShort with the Ad C1 *AscI–ClaI* 10,591 bp fragment. Finally, an enhanced green fluorescent protein (eGFP) expression cassette was inserted into both pPan5ClaShort and pPan5ClaLong between the I–*CeuI* and PI–*SacI* sites to yield pPan5ClaShort-eGFP and pPan5ClaLong-eGFP, respectively.

The plasmid pH5C7H5 (Fig. 1C) harbors the (E1-deleted) virus genome that is chimeric between HAdV-5 and the chimpanzee adenovirus Ad Pan 7. To construct pH5C7H5, an existing molecular clone of an E1/ E3-deleted HAdV-5 vector was used. An internal 23,046 bp *Stul* fragment (map units 16.1–88.9) was initially deleted and the resulting HAdV-5 vector DNA fragment (that was internally deleted between the polymerase and fiber genes) was transferred to a pBR322 derivative similar to that described above, i.e., deleted between the *EcoRI* and *NdeI* sites, by inserting into a poly-linker containing *PacI* sites flanking the insert. A synthetic DNA fragment harboring restriction sites for *AscI*, *XbaI* and *EcoRI* (made by annealing together oligomers TAC TAT AGG CGC GCC TAT ATC TAG AGG GCC AAT TC and GAA TTC GCG CTC TAG ATA TAG GCG CGC CTA TAG TA) was inserted into the *Stul* site. To extend the polymerase gene of HAdV-5, this plasmid was digested with *BstZ17I* and *AscI* and a 2386 bp fragment amplified from the HAdV-5 genome (using primers TTT TTG ATG CGT TTG TTC TA CC and GTC CGG CGC GCC GCA GGG ACT TCT ACT TTC ACC), digested with *BstZ17I* and *AscI* and the resulting 2296 bp fragment was inserted. To create an *EcoRI* site at the end of the fiber–E4 junction, a 613 bp fragment was amplified from the HAdV-5 genome (using primers TTT TGCC CGG GTC TCT CTG TGT TAT GTT TCA ACC and GCT GAT TTA AGT GAG AGC ATG AGG G), digested with *EcoRI* and *BbvCI* and the resulting 525 bp fragment was inserted between the *BstZ17I* site located in the HAdV-5 E4 orf6 gene and the *EcoRI* site in the synthetic linker, to yield the plasmid pAdSendsAscRI. Next, a 3489 bp PCR fragment (generated using the primers TCG TGC TTG TGC TAT CAT GAT TGG CAT TG and GAT CGA ATT CTT TAT TCT TAT GGG ATC ATG TAT TAG AAG); the 3619 bp PCR product was digested with *EcoRI* and *XbaI*) containing the Ad Pan 7 fiber and flanking 5′-region was cloned into pAdSendsAscRI. A 994 bp *AscI–XbaI* fragment from Ad Pan 7 was then inserted to join the Ad Pan 7 and HAdV-5 polymerase gene segments. The construct was completed by sequential insertions of a 1431 bp *AscI* fragment and a 19,553 bp *XbaI* fragment to yield pH5C7H5. Finally, an enhanced green fluorescent protein (eGFP) expression cassette was inserted into pH5C7H5 between the I–*CeuI* and PI–*SacI* sites to yield pH5C7H5-eGFP.

To rescue viruses, the plasmid DNAs were digested with *SwaI* (Ad Pan 5–Ad C1 chimeras) or *PacI* (HAdV-5–Ad Pan 7 chimera) and transfected into HEK 293 cells. The chimeric adenovirus rescued from the pPan5ClaLong transfection is designated as Ad C5/C1. The chimeric adenovirus rescued from the pH5C7H5 transfection is designated Ad H5/C7.

3. Results and discussion

3.1. Rescue of chimeric viruses

Five different adenoviruses isolated from the chimpanzee, Ad C1, Ad Pan 5, Ad Pan 6, Ad Pan 7 and Ad Pan 9 (Simian adenoviruses 21–25, respectively) were fully sequenced (Roy et al., 2004a,b). These adenoviruses were then evaluated for their possible utility as gene transfer vectors as part of a repertoire of vectors that could be administered successively, i.e., antibodies against a particular vector would not significantly cross-neutralize the others (Roy et al., 2004b). It was clear that the HAdV-E (previously, subgroup E) chimpanzee adenoviruses Ad Pan 5, Ad Pan 6, Ad Pan 7 and Ad Pan 9 belonged to only two mutually exclusive neutralization serotypes whereby Ad Pan 6 belonged to one serotype and Ad Pan 5, Ad Pan 7, and Ad Pan 9 belonged to another (Roy et al., 2004b). Sequence analysis of the capsid protein sequences confirmed previous cross-neutralization data in which Ad C1 had been classified as an HAdV-B (previously, subgroup B) serotype (Roy et al., 2004a). HAdV-B adenoviruses are the only adenoviruses that do not use the coxsackie-adenovirus receptor (CAR) for binding and internalization (Roelvink et al., 1998) and can use the CD46 molecule as receptor (Gaggar et al., 2003). This has been shown to result in a transduction profile which is distinct from that seen with CAR-binding adenoviruses (Ni et al., 2005). Because antigen-presenting dendritic cells express CD46, subgroup B adenoviruses such as Ad C1 may confer useful properties to adenovirus vaccine vectors. However, attempts to cultivate Ad C1 revealed it to be fastidious in its growth characteristics (data not shown) and, therefore, possibly unsuitable for use as a gene therapy vector. Furthermore, an E1-deleted Ad C1 recombinant adenovirus could not be rescued in HEK 293 cells. The lack of success in the rescue of an E1-deleted Ad C1 recombinant adenovirus is likely to have been due to the requirement by HAdV-B adenoviruses of the expression of the cognate E1b 55 K protein in the cell line in trans (Abrahamsen et al., 1997; Vogels et al., 2003). On the other hand, because the E1-deleted HAdV-E chimpanzee adenoviruses can be adequately propagated in HEK 293 cells, the possibility of generating a chimeric adenovirus based on one of them, Ad Pan 5, while harboring the capsid proteins of Ad C1, was investigated. It may be surmised that if it were possible to replace only the hexon and the fiber, circumvention of pre-existing immunity in a gene transfer vector could be effected (Roy et al., 2005). However, in view of the previously reported difficulties associated with making hexon changes, more extensive replacements were attempted, i.e., construction of chimeras where the replacement went beyond just the hexon, to achieve
two goals. The first was to determine whether making extended replacements would allow for the rescue of viruses containing a hexon of an unrelated serotype that may not otherwise be amenable to rescue. The second goal was to test whether the growth characteristics of adenovirus vectors such as Ad Pan 5 that was found to be excellent in HEK 293 cells would also be present in the chimeric virus, particularly when the hexon (and other capsid proteins) are derived from a virus such as Ad C1 that are difficult to grow to a high yield in HEK 293 cells. An added bonus of extending the replacement to include the fiber protein would be to increase the antigenic dissimilarity beyond that afforded by a hexon change alone.

The structures of the chimeric adenovirus genomes that were constructed as plasmids are summarized in Fig. 2. The first chimeric vector that was constructed contained a genome where most of the structural protein genes between the chimpanzee adenoviruses Ad Pan 5, a HAdV-E adenovirus, were replaced by those from Ad C1, a HAdV-B adenovirus. E1-deleted Ad Pan 5 vectors that propagate to high titer in HEK 293 cells have been previously constructed, i.e., the E1 deletion of this chimpanzee adenovirus can be efficiently complemented by the human HAdV-5-derived E1 region genes that are constitutively expressed in the HEK 293 cells that are commonly used to propagate HAdV-5-based gene therapy vectors (Roy et al., 2004b). As discussed in Section 2, the viability of a chimeric construct was assessed by attempting virus rescue by assembling the complete E1-deleted virus DNA into a single plasmid, digesting the plasmid DNA with an appropriate restriction enzyme to release the virus DNA ends, and transfecting the DNA into HEK 293. Two chimeric virus plasmids were initially constructed, pPan5C1short and pPan5C1long (Fig. 1). The plasmid pPan5C1short harbors an E1-deleted Ad Pan 5 virus DNA where an internal 15,226 bp segment (bp #18,332–bp #33,557) has been replaced by a functionally analogous 14,127 bp segment (bp #18,531–bp #32,657) from Ad C1. This results in the replacement of the Ad Pan 5 proteins hexon, endoprotease, DNA-binding protein, 100 K scaffolding protein, 33 K protein, protein pVIII, and fiber, as well as the entire E3 region, with the homologous segment from Ad C1. The ClaI site at the left end of the Ad C1 fragment is at the beginning of the hexon open reading frame and the resulting chimeric open reading frame results in a translation product that is identical to the Ad C1 hexon. The EcoRI site, which constitutes the right end of the Ad C1 fragment is within the open reading frame for E4 orf 6/7. The right end was ligated to a PCR generated right end fragment from Ad Pan 5 such that the regenerated E4 orf 6/7 translation product is chimeric between Ad Pan 5 and Ad C1.

The plasmid pPan5C1long harbors an E1-deleted Ad Pan 5 virus DNA where a larger internal 25,603 bp segment (bp #7955–bp #33,557) has been replaced by a functionally analogous 24,712 bp (bp #7946–bp #32,657) segment from Ad C1. This results in the replacement of the Ad Pan 5 pre-terminal protein, 52/55 K protein, penton base, pVII, Mu, and pVI with those from Ad C1, in addition to those replaced in pPan5C1short. The AscI site at the left end of the Ad C1 fragment is at the beginning of the DNA polymerase open reading frame (residue 236 of the 1192 residue protein) and results in a chimeric protein. When the plasmids pPan5C1short-eGFP and pPan5C1long-eGFP were digested with the restriction enzyme SwaI and transfected into HEK 293 cells, a typical adenovirus induced cytopathic effect was observed when cells were transfected with pPan5C1long-eGFP, but not when cells were transfected with pPan5C1short-eGFP. Viral DNA prepared from the chimeric recombinant virus was digested with several restriction enzymes and found to have the expected pattern on electrophoresis (data not shown).

Having ascertained the extent of replacement that provided a means to construct a chimeric adenovirus, it was of interest to determine whether the method may be more generally applicable; specifically, whether a chimeric adenoviral vector based on HAdV-5 harboring capsid genes from an unrelated adenovirus would be viable. To test this, a plasmid analogous to pPan5C1long was constructed where the right and left end segments were derived from human HAdV-5 (HAdV-C) and the internal segment from the chimpanzee adenovirus Ad Pan 7 (HAdV-E). As discussed above, the AscI restriction enzyme site, which demarcates the left junction in the Ad C1-Ad Pan 5 chimeric virus lies within the adenovirus DNA polymerase open reading frame. The amino acid sequence of both Ad Pan 5 and Ad C1 DNA polymerases at this loca-
tion (residues 236–239 and 238–241, respectively) is SARR encoded by TCG GCG CGC CGT in case of Ad Pan 5 and TCG GCG CGC CGC in case of Ad C1, both of which contain the AscI recognition sequence (underlined). Both the HAdV-5 and Ad Pan 7 DNA polymerase amino acid sequences at this location are also SARR encoded by TCG GCC GTG CGC and TCG GCC CGC CGC, respectively, of which only the latter contains an AscI recognition site (underlined). Therefore, silent mutagenesis of the HAdV-5 DNA polymerase open reading frame was carried out to enable a splice identical to that carried out in pPan5C7long. The other junction was made immediately following the stop codon for the Ad Pan 7 fiber by using PCR mutagenesis to insert an AscI recognition site at that location in both Ad Pan 7 and HAdV-5 sequences. The final construct ranged from (2 to 8) 

The yields that were obtained for the chimeric recombinant adenovirus Ad C5/C1 harboring different transgene cassettes ranged from (2 to 8) × 10^{13} genome copies for a fifty 150 mm Petri dish preparation in HEK 293 cells (Table 1). These yields were somewhat lower than were obtained for equivalent preparations of recombinant E1-deleted Ad Pan 5 vectors. The yield of the 50 Petri dish culture of Ad H5/C7 eGFP was 2.5 × 10^{13}, which is similar to yields that are routinely obtained for Ad C7 eGFP.

There are several possible reasons for the viability and replication competence of the chimeric E1-deleted virus as contrasted with the absence of rescue of an E1-deleted Ad C1 recombinant in HEK 293 cells. In the growth of the Ad C5/C1 chimeric virus in HEK 293 cells, the adenoviral early region gene products of E1 and E4 are derived from HAdV-5 and Ad Pan 5, respectively. E1-deleted Ad Pan 5-derived vectors can grow to high titer in HEK 293 cells indicating that the HAdV-5-derived E1 gene products present in HEK 293 cells can adequately complement the Ad Pan 5 E1 deletion (Roy et al., 2004b). It is, thus, likely that the E1b 55 K protein from HAdV-5 that is expressed in HEK 293 cells can complex with the Ad Pan 5 E4 orf 6 protein. The E1 and E4 gene products bind, regulate and de-repress several cellular transcription complexes and coordinate their activity towards viral multiplication. Thus, it is possible that the E1 gene products supplied in trans from the HEK 293 cells and the E4 gene products from Ad Pan 5 are more optimal than are the equivalent Ad C1 gene products. This may also apply to the major late promoter whose activity is responsible for the transcription of the capsid protein genes. In the chimeric virus, the major late promoter, and the protein IVa2, which transactivates it, are derived from Ad Pan 5. However, the E2 gene products required for adenoviral DNA replication, the pre-terminal protein (pTP) and single-stranded DNA-binding protein are derived from Ad C1; the adenoviral DNA polymerase, which complexes with pTP, is chimeric in Pan5C1 but mostly Ad Pan 5-derived. The IVa2 protein has also been reported to interact with sequences at the left end of the adenovirus genome that are important for viral packaging (Zhang and Imperiale, 2000, 2003). The HAdV-5 IVa2 protein has been shown to enable the packaging of a chimeric HAdV-7 adenoviral DNA where the two inverted terminal repeats (ITRs) and the cis packaging signals were derived from HAdV-5 (Zhang et al., 2001) suggesting that IVa2 protein may confer serotype specificity in packaging. In both the Ad C5/C1 and the Ad H5/C7 chimeric constructs, the IVa2 proteins are derived from the same serotypes that contribute the ITRs and presumably the packaging signals at the left end of the genome.

The chimeric plasmid construct with the shorter replacement, pPan5C1short, encodes the Ad C1 proteins hexon and fiber as well as the intervening open reading frames for endoprotease, DNA-binding protein, 100 K scaffolding protein, 33 K protein, and protein VIII (The E3 region is also included within this region but is unlikely to impact on the viability of the chimeric virus). The absence of the rescue of viable virus from the pPan5C1short construct is interesting and informative with regards to defining future strategies for the construction of chimeric adenoviruses. When the replacement was extended to include the additional Ad C1 proteins pTP (pre-terminal protein), 52/55 K protein, penton base, pVII, Mu, and pVI, virus could be rescued. It was then possible to replicate this result using two other adenoviruses, HAdV-5 and Ad Pan 7.

In this experiment, the chimeric adenovirus construction strategy utilized the presence of AscI and Clal restriction enzyme sites present in the genes for DNA polymerase and hexon, respectively, on both Ad Pan 5 and Ad C1, i.e., an extensive investigation of other possible replacement coordinates has not been done. Thus it is possible and likely that less extensive replacements than the one present in Ad C5/C1 and Ad H5/C7 (e.g., one extending from the penton to fiber) may be viable. In this

### Table 1

| Transgene              | E3 status   | Yield (×10^{13}) | Particle/TCID<sub>50</sub> ratio |
|------------------------|-------------|------------------|-----------------------------------|
| Ebola glycoprotein     | Wild-type   | 6                | 295                               |
| Rabies glycoprotein    | Wild-type   | 3.4              | 795                               |
| SARS CoV-spike         | E3 deletion | 1.95             | 325                               |
| HIV gag                | Wild-type   | 4.7              | 246                               |
| HIV pol                | Wild-type   | 4.7              | 322                               |
| HIV gp140              | Wild-type   | 6.2              | 870                               |
| HIV gp140              | E3 deletion | 5.5              | 205                               |
| SIV gag                | E3 deletion | 2                | 150                               |
| SIV nef                | Wild-type   | 5                | 205                               |
| HIV nef-pol            | E3 deletion | 3                | 124                               |
| LacZ                   | E3 deletion | 4.4              | 70                                |
| eGFP                   | E3 deletion | 5.6              | 281                               |

The yields shown were following cesium chloride purification of the vector derived from fifty 150 mm culture dishes (293 cells) as determined by absorbance at 260 nm. For transgene cassettes where multiple preparations were made, a representative preparation is shown. The TCID<sub>50</sub> infectious titer was determined by a limiting dilution infectivity assay on HEK 293 cells where virus replication was detected using reverse transcription and PCR, probing for the viral E2a transcript. One TCID<sub>50</sub> unit corresponds to 0.7 infectious units.
Table 2
The degree of similarity between proteins of the adenoviruses that were used to make each of the two chimeric adenoviruses is shown

| Protein          | Ad Pan 5 and Ad C1 Homology (%) | Ad Pan 5 and Ad C1 Identity (%) | Ad5 and Ad Pan 7 Homology (%) | Ad5 and Ad Pan 7 Identity (%) |
|------------------|----------------------------------|---------------------------------|-------------------------------|-------------------------------|
| pIX              | 88                               | 79                              | 63                            | 43                            |
| IVa2             | 95                               | 91                              | 89                            | 83                            |
| pTP              | 93                               | 91                              | 84                            | 79                            |
| Polymerase       | 93                               | 88                              | 84                            | 77                            |
| 52/55 K          | 89                               | 84                              | 78                            | 69                            |
| IIIa             | 95                               | 93                              | 82                            | 76                            |
| Penton base      | 86                               | 83                              | 77                            | 70                            |
| pVII             | 93                               | 90                              | 79                            | 70                            |
| V                | 85                               | 78                              | 70                            | 62                            |
| pVI              | 82                               | 77                              | 72                            | 65                            |
| Hexon            | 89                               | 85                              | 84                            | 78                            |
| Endoprotease     | 91                               | 88                              | 83                            | 74                            |
| DBP              | 84                               | 76                              | 68                            | 55                            |
| 100kDa           | 82                               | 77                              | 60                            | 52                            |
| pVIII            | 95                               | 92                              | 87                            | 80                            |
| Fiber (tail only)| 59                               | 46                              | 62                            | 53                            |

The homology and identity scores were derived using the Vector NTI software (AlignX module) carrying out pairwise alignments (gap opening penalty, 10; gap extension penalty, 0.1) using the BLOSUM62 amino acid substitution matrix.

In this context, it is interesting to examine the degree of relatedness of the various adenovirus capsid proteins (hexon, penton base, and fiber), the core proteins (pVII, V) and cement proteins (pIX, IIIa, pVIII) present in the two chimeric adenoviruses (Table 2). It is clear that the overall similarity is higher between Ad Pan 5 and Ad C1 than between HAdV-5 and Ad Pan 7. The proteins involved in replication (the single-stranded DNA-binding protein, DNA polymerase, and pre-terminal protein) are also shown.

Because the pre-terminal protein and DNA polymerase form a complex, preserving both proteins from the same serotype in the construction of a chimeric construct may be a preferred configuration. In case of the chimeric adenoviruses, however, the fusion junction is within the polymerase open reading frame resulting in a chimeric protein. In the Ad C5/C1, the C-terminal 953 amino acids have been replaced by the Ad Pan 5 DNA polymerase. In this region, the homology between the Ad Pan 5 and Ad C1 DNA polymerase proteins is 95% (91% identity), i.e., a very high degree of sequence similarity exists. Similarly, in case of the Ad H5/C7 chimeric adenovirus, the C-terminal (955 amino acids) segment of the Pan 7 polymerase is 90% homologous to the HAdV-5 polymerase.

3.2. Generation of an E3-deleted Ad C1-derived chimeric vector

Adenoviral gene therapy vectors commonly have been engineered with deletions in the E3 region as a means to providing space for the insertion of larger transgene cassettes than can be accommodated by an E1 deletion alone. The E3 region in Ad C1 is longer than the E3 region in HAdV-5 (Roy et al., 2004a) as is the characteristic of subgroup B adenoviruses principally because of the presence of two additional open reading frames (Fig. 3). HAdV-5 is known to tolerate extensive deletions in the E3 region without compromising virus yields in culture showing that polyadenylation of the transcript encoding pVIII as well as the synthesis of an adequately functional L5 transcript can occur without the native elements contributing to these functions present in the E3 region. Two E3 deletions were engineered into the pPan5C1Long plasmid as shown in Fig. 3, a longer deletion (3.6 kb) extending from an NheI site encompassing all E3 open reading frames, as well as a shorter deletion (2.5 kb) extending from an NheI site to an RsrII site.
neutralizing antibodies have been directed at replacing the hexon and to a lesser extent against fiber (Roy et al., 2005).

**4. Discussion**

Previous attempts to engineer adenovirus capsids to evade neutralizing antibodies have been directed at replacing the HAdV-5 hexon with those from other serotypes. Gall et al. (1998) and Youil et al. (2002) have found that this is successful when the serotypes are closely related, but largely unsuccessful when exchanges among more distant serotypes are attempted.

It has been surmised that this is probably due to the failure of the correct protein–protein interactions required for capsid assembly and maturation, as hexon engineering to replace hyper-variable regions precisely has been shown to work in one instance (Roberts et al., 2006). Two different chimeric adenovirus vectors were constructed where one adenovirus provides E1, E2 (other than DNA-binding protein, DBP) and E4 functions, and a different unrelated adenovirus contributes the capsid protein genes, minimizing the possibility of incompatibility among the capsid protein components. The fact that HAdV-5 can be used as a recipient in such a construct opens the possibility that a wide variety of different serotypes may be used as capsid donors, potentially obviating the need to develop new complementing cell lines when the chimerizing strategy is successful. Furthermore, the HAdV-5-based helper viruses that have been developed for the construction of gutted vectors may be adapted to develop gutted adenovirus vectors packaged in capsids of serotypes other than HAdV-5, e.g., the packaging signal present at the left end of Ad H5/C7 could be altered to be flanked by loxP or ftr sequences that would be excised in the presence of cre or flp, respectively (Ng et al., 2001; Parks et al., 1996). It is possible that such an adenovirus helper would now be able to package a HAdV-5-based gutted vector construct in an Ad Pan 7 capsid.

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### Table 3

| Rabbit antiserum against | Homologous titer | Anti-C5/C1 titer |
|-------------------------|------------------|------------------|
| Ad Pan 5                | 1/655,360        | 1/40             |
| Ad Pan 6                | 1/327,680        | 1/20             |
| Ad Pan 7                | 1/163,840        | Not detected     |
| Ad Pan 9                | 1/327,680        | 1/20             |
| HAdV-5                  | 1/163,840        | 1/40             |

The pIX capsid protein in Ad C5/C1 is derived from Ad Pan 5 and 100% identical to that in Ad Pan 6; it is also 99.3% identical (141 of 142 residues) to the pIX protein in Ad Pan 7 and Ad Pan 9. However, it is only 43% identical to the pIX protein in HAdV-5.

extending from a BstZ17I site to an AgeI site as shown in Fig. 3. Upon transfection virus could only be rescued from the plasmid harboring the smaller E3 deletion.

### 3.3. Cross-neutralization among Ad C1, Ad Pan 5 and the chimeric Ad C5/C1

Since one of the principal reasons to explore adenoviruses of multiple serotypes is to overcome vector neutralization by pre-existing antibodies, it was of interest to determine whether antibodies raised against Ad Pan 5 would neutralize vectors based on the chimeric Ad C5/C1. As discussed above, in the creation of the chimeric virus one of the minor capsid proteins pIX is retained from Ad Pan 5 while the other capsid proteins, including hexon and fiber, which harbor known neutralization determinants, are derived from Ad C1. To explore the possibility that protein IX may provide neutralization determinants, high-titer rabbit anti-serum raised against Ad Pan 5 was tested for its ability to neutralize Ad C5/C1 (Table 3). Rabbit anti-sera raised against Ad Pan 6, Ad Pan 7, and Ad Pan 9 were also tested. The pIX protein is 100% identical in all 142 amino acids between Ad Pan 5 and Ad Pan 6; there is a one amino acid difference between Ad Pan 5 and Ad Pan 7 or Ad Pan 9. Therefore, any contribution to neutralization of Ad C5/C1 by anti-pIX antibodies would be expected to be present with anti-sera raised against any of the other chimpanzee adenoviruses. As seen in Table 3, the anti-Ad C5/C1 titer in all cases was negligible compared to the homologous titer. Rabbit anti-serum raised against HAdV-5 also did not have neutralizing activity against Ad C5/C1 where the sequence identity between the HAdV-5 pIX and Ad Pan 5 pIX is only 43%. Thus, it was not possible to discern cross-neutralization activity in any of the anti-sera, arguing against a role for pIX in providing neutralization determinants. This is in concordance with the previous observation that neutralization activity against adenoviruses is principally directed against hexon and to a lesser extent against fiber (Roy et al., 2005).
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