An alternative to the adenovirus inverted terminal repeat sequence increases the viral genome replication rate and provides a selective advantage in vitro

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During the development of human adenovirus 35-derived replication-incompetent (rAd35) vaccine vectors for prevention of infectious diseases, we detected mutations in the terminal 8 nt of the inverted terminal repeats (ITRs) of rAd35. The switch from the plasmid-encoded sequence 5'-CATCATCA-3' to the alternative sequence 5'-CTATCTAT-3' in the ITRs was found to be a general in vitro propagation phenomenon, as shown for several vectors carrying different transgenes or being derived from different adenovirus serotypes. In each tested case, the plasmid-encoded ITR sequence changed to exactly the same alternative ITR sequence, 5'-CTATCTAT-3'. The outgrowth of this alternative ITR version should result from a growth advantage conferred by the alternative ITR sequence. Indeed, replication kinetics studies of rAd35 harbouring either the original or alternative ITR sequence confirmed an increase in replication speed for rAd35 vectors with the alternative ITR sequence. These findings can be applied to generate recombinant adenoviral vectors harbouring the alternative ITR sequence, which will facilitate the generation of genetically homogeneous seed virus batches. Moreover, vector production may be accelerated by taking advantage of the observed improved replication kinetics associated with the alternative ITR sequence.

INTRODUCTION

Recombinant replication-incompetent human and animal adenoviruses are used extensively for their application in gene therapy and vaccination. Pre-clinical and clinical studies have shown that replication-incompetent human adenovirus 5 (rAd5)-derived vectors, although immuno- genic as vaccine vectors, are hampered by the high prevalence of pre-existing immunity to Ad5 in human populations (Buchbinder et al., 2008; McCoy et al., 2007). By contrast, the human rAd35 and rAd26 vectors are associated with both low seroprevalence and low neutralizing antibody titres and are only minimally suppressed by pre-existing Ad5 immunity (Barouch et al., 2004, 2011). This feature makes rAd35 and rAd26 vectors suitable for use as vaccine vectors. Recombinant replication-deficient adenovirus vectors can be generated and produced on helper-cell lines such as PER.C6 (Fallaux et al., 1998), HEK293 or 911 cells. These recombinant vectors harbour deletions in E1 and E3 and are often generated by plasmid transfections in helper-cell lines (Abbink et al., 2007; Havenga et al., 2006). Plaque purification ensures clonality and identity of the viral vector. For the production of research- or clinical-grade vector batches, both genomic stability and growth to high titres are crucial. Furthermore, genome sequence identity from plasmid level to larger-scale batches and sequence homogeneity in the batches are desirable.

Adenoviruses possess a dsDNA genome of about 35 kb, which has been studied in detail. All adenoviruses are characterized by inverted terminal repeats (ITRs) of about 100 bp (Dans et al., 2001; Liu et al., 2003) that are conserved among the serotypes of different adenoviral species and that harbour the origin of replication (Bernstein et al., 1986; Challberg & Rawlins, 1984; Guggenheimer et al., 1984; Harris & Hay, 1988; Hay, 1985; van Bergen et al., 1983; Wang & Pearson, 1985). Hallmarks of adenoviral DNA replication are protein-primed initiation of replication, jumping back on the template strand and strand displacement (Liu et al., 2003), all of which are inferred from studies on replication of the human subgroup C Ad5 and Ad2. These processes require the presence of both viral and cellular proteins. Replication is initiated by the formation of a stable heterodimer of two viral proteins: the precursor of the terminal protein (pTP) and the adenovirus DNA polymerase (Pol) (Enomoto et al., 1981; King et al., 1997b). The free hydroxyl group of pTP-serine580 is used as a primer, to which the first nucleotide, dCMP is covalently attached (Desiderio & Kelly, 1981; Smart &
Stillman, 1982), using nt 4–6 (3′-GTA-5′) as a template (King & van der Vliet, 1994). Following the polymerization of the first 3 nt of the newly synthesized strand 5′-CAT-3′, the pTP–CAT complex dissociates and realigns at the terminal 3′-GTA-5′ sequence of the template strand. Incorporation of 3 nt using nt 4–6 (3′-GTA-5′) as a template results in a 3 nt direct repeat. Later, the pTP–Pol complex dissociates to allow elongation, leaving the terminal protein covalently bound to the newly synthesized DNA strand. For elongation to occur, a third viral protein, the DNA-binding protein, facilitates unwinding of the dsDNA ahead of the viral polymerase (Dekker et al., 1997).

The genomes of all adenoviruses start the replication process with a small, tandem tri- or tetranucleotide repeat (Rademaker et al., 2006). Whilst most sequences display the 5′-CATCATCA-3′ sequence in the terminal 8 nt, several alternative sequences have been described (Aleström et al., 1982; Dán et al., 2001; Jacobs et al., 2004; Purkayastha et al., 2005; Rademaker et al., 2006; Shinagawa & Padmanabhan, 1980; Shinagawa et al., 1983, 1987; Tokunaga et al., 1982).

Here, we report a change in the terminal 8 nt of the ITRs of an Ad35-derived vector within the otherwise stable adenovirus genome during vector production at small and large scales in a helper-cell line. This adaptation presents a general propagation phenomenon and confers an increased vector genome replication rate in E1-complementing cell lines. The alternative ITR sequence can be exploited to generate recombinant adenovirus batches that are genomically consistent and homogeneous and display an increased replication speed during manufacturing for gene therapy or vaccine applications.

**RESULTS**

**Detection of an alternative ITR sequence during production of a replication-incompetent rAd35 vaccine vector in PER.C6 cell cultures**

An Ad35-derived replication-incompetent vaccine vector expressing *Mycobacterium tuberculosis* antigens Ag85A, Ag85B and TB10.4, as described previously (Radosavic et al., 2007), was generated in E1-complementing PER.C6 cells.

The resulting rAd35.TBS virus was plaque purified and characterized for vector integrity, identity and transgene expression at different stages of the production process. Virus DNA was isolated and completely sequenced prior to use as a seed virus for larger-scale production.

Interestingly, the seed virus genome sequence was identical to the genome encoded by the rescue plasmids with the exception of the terminal 8 nt in the left and right ITRs. The plasmid-encoded ITR sequence of the terminal 8 nt, 5′-CATCATCA-3′ (Fig. 1a), hereafter referred to as the ‘original’ ITR sequence, was no longer present at this production stage. Instead, the terminal 8 nt displayed the sequence 5′-CTATCTAT-3′ (Fig. 1b), resulting in 6 nt changes in comparison with the original ITR sequence encoded by the parental DNA plasmids. 5′-CTATCTAT-3′ is hereafter referred to as the ‘alternative’ ITR sequence.

![Fig. 1. Detection of an alternative ITR sequence during production of rAd35.TBS vaccine vector in PER.C6 cells.](http://vir.sgmjournals.org)
To investigate the inconsistency in the terminal ITR sequence further, we sequenced the ITRs at different earlier steps during the production of the vaccine vector. This analysis revealed that the original ITR sequence was still present at an earlier stage of the production process, namely at the fifth serial passage (P5) in PER.C6 cells after recombinant generation of the virus. However, a second, albeit lower signal in the sequence trace files indicated the appearance of an alternative sequence at P5 (data not shown). At P6, the sequence was mixed, probably being composed of approximately the same proportion of the original and alternative sequences. A distinct alternative sequence was detected at P7 (Fig. 1c).

**ITR heterogeneity occurs for different rAd35 vectors as well as for Ad35 WT virus upon in vitro propagation**

To address whether the observed phenomenon was a rare or common event and to examine the frequency of the switch from the original 5'-CATCATCA-3' to the alternative 5'-CTATCTAT-3' ITR sequence, we analysed four more plaques of rAd35.TBS originating from the same virus rescue. Viruses of all plaques switched to the alternative ITR sequence upon repeated passaging (data not shown).

To analyse further whether this phenomenon was restricted to rAd35.TBS or occurred for other Ad35-derived vaccine vectors, rAd35 viruses expressing different transgenes were tested. As shown in Table 1, almost all of these viruses displayed mixed or alternative ITR sequences upon propagation in PER.C6 cells. Furthermore, mixed sequences were also observed for the Ad35 WT virus propagated in PER.C6 cells, thus excluding a vector artefact. In total, 17 propagated populations of different rAd35 vectors or replication-competent Ad35 WT displayed the observed switch from 5'-CATCATCA-3' to 5'-CTATCTAT-3' in the terminal ITR sequence.

**Introduction of the alternative ITR sequence in rAd35 and rAd5 results in homogeneous virus populations**

Following up on our observation that the alternative ITR sequence frequently appeared upon propagation in cell culture, we addressed the following questions: (i) Is it possible to generate recombinant Ad vectors harbouring the alternative ITR sequence? (ii) Will this result in Ad vectors that do not convert to either the original or another ITR sequence? (iii) Do the rAd35 vectors harbouring the alternative ITR sequence display a growth advantage over the ones with the original ITR sequence?

Rescue of rAd35 and rAd5 vectors harbouring the alternative ITRs was successful, confirming that these viruses are viable in cell culture. We then constructed an rAd35.TBS harbouring either the original or alternative ITR sequence, termed rAd35.TBS.ori ITR and rAd35.TBS.alt ITR, respectively. These viruses were subjected to propagation in PER.C6 cells and the sequence of the terminal 8 nt of the ITR was monitored by specific PCR analysis of viral DNA from each viral passage number. To distinguish the original from the alternative ITR sequence, different PCR primer sets that specifically amplified either the original or the alternative ITR sequence were utilized. In the case of rAd35.TBS.ori ITR, this analysis identified a gradual decrease in the signal of the original ITR sequence between P3 and P6 and the emergence of the alternative sequence from P5 onwards (Fig. 2a). By contrast, during propagation of recombinant rAd35.TBS.alt ITR, only the alternative PCR sequence was detectable over all 10 passages (Fig. 2b).

Mixing of rAd35 vectors with alternative or original ITR sequences that were otherwise identical also led to outgrowth of vector harbouring the alternative ITR sequence, indicating a growth advantage of the presence of the alternative over the original ITR sequence (data not shown).

As we detected the switch from the original ITR sequence to the alternative ITR sequence in rAd35, a group B vector, we additionally analysed rAd5.ori ITR and rAd5.alt ITR, a group C vector harbouring either the original or alternative ITR sequence. In contrast to the results obtained with rAd35, rAd5 did not display a switch in the ITR sequence but retained the original ITRs over 10 viral passages (Fig. 2c). Additionally, rAd5.alt ITR did not display the opposite switch: rAd5 harbouring alternative ITRs was stable over 10 passages during the production of the vaccine vector. This analysis revealed that the original ITR sequence was still present at an earlier stage of the production process, namely at the fifth serial passage (P5) in PER.C6 cells after recombinant generation of the virus. However, a second, albeit lower signal in the sequence trace files indicated the appearance of an alternative sequence at P5 (data not shown). At P6, the sequence was mixed, probably being composed of approximately the same proportion of the original and alternative sequences. A distinct alternative sequence was detected at P7 (Fig. 1c).

**Table 1. ITR heterogeneity occurs for different rAd35 vectors as well as for WT virus**

Replication-incompetent rAd35 vectors harbouring different transgenes were passaged for production purposes and analysed by sequencing at viral passage number 6. The genome size and the ITR sequence are displayed. Sequences were categorized as described in Fig. 1(c).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Genome size (kb)</th>
<th>ITR</th>
</tr>
</thead>
<tbody>
<tr>
<td>rAd35.TBS*</td>
<td>32.4</td>
<td>Mixed</td>
</tr>
<tr>
<td>rAd35.Ebo.GP.Z*</td>
<td>32.4</td>
<td>Alternative</td>
</tr>
<tr>
<td>rAd35.Ebo.GP.S/G*</td>
<td>32.4</td>
<td>Mixed</td>
</tr>
<tr>
<td>rAd35.CS*</td>
<td>31.5</td>
<td>Mixed</td>
</tr>
<tr>
<td>rAd35.CS†</td>
<td>31.3</td>
<td>Original</td>
</tr>
<tr>
<td>rAd35.Luc*</td>
<td>32.0</td>
<td>Mixed</td>
</tr>
<tr>
<td>rAd35.Luc†</td>
<td>31.9</td>
<td>Mixed</td>
</tr>
<tr>
<td>rAd35.eGFP*</td>
<td>31.1</td>
<td>Mixed</td>
</tr>
<tr>
<td>rAd35.eGFP†</td>
<td>30.9</td>
<td>Mixed</td>
</tr>
<tr>
<td>rAd35.Empty*</td>
<td>30.4</td>
<td>Alternative</td>
</tr>
<tr>
<td>rAd35.Empty†</td>
<td>30.2</td>
<td>Alternative</td>
</tr>
<tr>
<td>rAd35.SIV-Gag*</td>
<td>31.9</td>
<td>Alternative</td>
</tr>
<tr>
<td>Ad35 WT</td>
<td>34.8</td>
<td>Mixed</td>
</tr>
</tbody>
</table>

*These vectors have E1 deletions according to Havenga et al. (2006) preserving the pIX promoter.
†These vectors have a partial deletion of the pIX promoter corresponding to the E1 deletion described by Vogels et al. (2003).
viral passages and did not revert to the original ITR sequence (Fig. 2d).

The alternative ITR sequence confers an increased genome replication rate to replication-incompetent rAd35 in E1-complementing cell lines

As we observed outgrowth of virus genomes with alternative ITRs, we hypothesized that viruses with alternative ITRs should have a selective advantage over those with original ITRs for rAd35. This selective advantage may be due to an increased replication rate. To test this, we used rAd35 viruses harbouring either original or alternative ITR sequences and analysed their growth kinetics. The time of onset of cytopathic effect (CPE) induced by adenoviral infection on E1-complementing cell lines is a good indication of productive infection. Therefore, we observed the CPE in infected HEK293 cells at 24, 48, 72 and 96 h post-infection (p.i.) at m.o.i. of 1000 viral particles (VPs) per cell (Fig. 3a) and 100 VPs per cell (Fig. 3b). At 24 h p.i., no CPE was observed for the m.o.i of 100 and 1000 VPs per cell. However, at 48 h p.i., advanced CPE was observed for rAd35.alt ITRs at both 100 and 1000 VPs per cell, developing into full CPE at 96 h p.i. By contrast, only limited CPE was detected for rAd35.ori ITR at these time points p.i.

To quantify the observed difference in replication kinetics, we quantified genome copies in HEK293 crude harvests (infected cells plus supernatants) at different time points p.i. by quantitative PCR (qPCR). Whilst both rAd35.ori ITR and rAd35.alt ITR grew to the same titre of approximately $10^{10}$ VP ml$^{-1}$ at the latest measured time point (90 h p.i.), rAd35.ori ITR showed delayed growth (Fig. 4). At early time points p.i., rAd35.alt ITR displayed a steeper genome amplification curve, reaching the plateau phase earlier than rAd35.ori ITR (Fig. 4a). As expected, this difference in replication kinetics was not observed between rAd5.ori ITR and rAd5.alt ITR (Fig. 4b). The genome replication advantage that was observed for rAd35.alt ITR, but not for rAd5.alt ITR, was confirmed in PER.C6 cells (Fig. 4 c, d), in which outgrowth of the alternative genome version was first observed.

Repeated passaging leads to outgrowth of the alternative ITR sequence in a variety of cell lines

To rule out that the observed switch from the original to the alternative ITRs is a phenomenon restricted to PER.C6 cells, the production E1-complementing cell line, we propagated replication-competent Ad35 WT virus in a variety of cell types: A549, HEK293, PER.C6, Hep2, HeLa and MRC5 cells. These specific cell lines were chosen to represent a variety of cell types, including cell lines derived from different tissues, of carcinoma and non-carcinoma origin, epithelial and fibroblastic cell lines and different ploidy (Table 2). The results in Table 2 show that the
switch to the alternative ITR was observed at P10 for the helper-cell lines HEK293 and PER.C6, but a switch or a mixed phenotype was also observed for the other tested cell lines, albeit at a later passage number. The observed switch of the ITR sequence is therefore considered to not be restricted to E1-complementing cells or to a particular cell type. Interestingly, the switch to the alternative ITR sequence only occurred at P15 in Hep2 cells. Furthermore, whilst viruses propagated in A549 cells showed a mixed ITR phenotype at P10, both the original and alternative ITR sequences were still present at P15, indicating a slower outgrowth of vectors harbouring the alternative ITR sequence in A549 cells.

**Extended propagation induces ITR heterogeneity in the majority of the tested adenovirus vectors derived from group B, C and D viruses**

Detection of the alternative ITR sequence in rAd35, a group B virus, but not in rAd5, a group C virus, might indicate that the switch is specific for certain species of human adenovirus vectors. To address this possibility, we passaged replication-incompetent rAd26-, rAd48-, rAd49-, rAd11-, rAd50- and rAd5-derived adenoviral vectors on PER.C6 cells. These vectors were selected from serotypes representing subgroups B, D and C.

Viral vectors were propagated until P15 after plaque purification and analysed by sequencing at P10 and P15. Two different transgenes were included for each vector serotype to exclude a transgene-specific effect.

The results of this set of experiments are shown in Table 3. We found that vectors derived from groups B, D and C switched to the alternative ITR sequence or displayed a mixed phenotype, suggesting that they would convert at a later propagation stage. In line with what we previously observed for rAd5, the original ITR sequence was maintained at P10 but started to mix at P15. By contrast, rAd48 group D vectors were the only ones to retain the original ITR sequence up to P15.

**The alternative ITR sequence is strongly represented in published human adenovirus sequences**

If the alternative ITR sequence confers a growth advantage, it may also be present in published adenovirus sequences. Therefore, an alignment of nt 1–8 of published human and
non-human ITRs was performed. Human viruses have been reported predominantly to harbour the original 5'-CATCATCA-3' sequence (Fig. 5a).

Additionally, sequences differing from 5'-CATCATCA-3' were identified and termed 'variable human adenovirus sequences'. The predominant sequence among the 'variable sequences' was the alternative sequence 5'-CTATCTAT-3' that we also identified by propagation of rAd35-derived vectors. Intriguingly, the variable sequences were found mainly in group B and D but not in group C adenoviruses.

### Table 2. The switch from original to alternative ITR during passaging is independent of host-cell type

Ad35 WT virus was rescued on A549, HEK293, PER.C6, Hep2, HeLa and MRC5 cells. For HeLa and MRC5 cells, no cytopathic effect was detected after transfection. Consequently, virus recovered from PER.C6 cells was used to infect these cell types. Viral DNA was isolated from infected cells at viral passage P10 and P15 after plaque purification and subjected to sequencing. Sequences were categorized as described in Fig. 1(c). ND, Not determined.

<table>
<thead>
<tr>
<th>Ad35 WT</th>
<th>Cell type</th>
<th>Origin</th>
<th>Ploidy</th>
<th>P10</th>
<th>P15</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293</td>
<td>Helper E1, embryonic kidney</td>
<td>Epithelial</td>
<td>Diploid</td>
<td>Alternative</td>
<td>ND</td>
</tr>
<tr>
<td>PER.C6</td>
<td>Helper E1, retina</td>
<td>Epithelial</td>
<td>Hypotriplid</td>
<td>Alternative</td>
<td>ND</td>
</tr>
<tr>
<td>A549</td>
<td>Lung carcinoma</td>
<td>Epithelial</td>
<td>Hypotriplid</td>
<td>Mixed</td>
<td>Mixed</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervix adenocarcinoma</td>
<td>Epithelial</td>
<td>Hypotriplid</td>
<td>ND</td>
<td>Alternative</td>
</tr>
<tr>
<td>Hep2</td>
<td>HeLa contaminant</td>
<td>Epithelial</td>
<td>Diploid</td>
<td>Original</td>
<td>Mixed</td>
</tr>
<tr>
<td>MRC5</td>
<td>Normal lung</td>
<td>Fibroblast</td>
<td>Diploid</td>
<td>ND</td>
<td>Mixed</td>
</tr>
</tbody>
</table>
Table 3. Extended passaging induces ITR heterogeneity or a complete shift to the alternative ITR sequence in the majority of the tested adenovirus vectors derived from group B, C and D viruses

rAd5, rAd11, rAd26, rAd48, rAd49 and rAd50 vectors harbouring different transgenes in E1 were passaged in PER.C6 cells to viral passage P10 or P15 after plaque purification. Viral DNA was recovered from infected cells, isolated by PEG 6000 isolation and subjected to sequencing. Sequences were categorized as described in Fig. 1(a). ND, Not determined.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Subgroup</th>
<th>P10</th>
<th>P15</th>
</tr>
</thead>
<tbody>
<tr>
<td>rAd26.eGFP</td>
<td>D</td>
<td>Mixed</td>
<td>Alternative</td>
</tr>
<tr>
<td>rAd26.Luc</td>
<td>D</td>
<td>Mixed</td>
<td>Alternative</td>
</tr>
<tr>
<td>rAd48.eGFP</td>
<td>D</td>
<td>Original</td>
<td>Original</td>
</tr>
<tr>
<td>rAd48.Luc</td>
<td>D</td>
<td>Original</td>
<td>Original</td>
</tr>
<tr>
<td>rAd49.eGFP</td>
<td>D</td>
<td>Alternative</td>
<td>ND</td>
</tr>
<tr>
<td>rAd49.Luc</td>
<td>D</td>
<td>Mixed</td>
<td>Alternative</td>
</tr>
<tr>
<td>rAd11.Env</td>
<td>B</td>
<td>Alternative</td>
<td>ND</td>
</tr>
<tr>
<td>rAd11.SívGag</td>
<td>B</td>
<td>Alternative</td>
<td>ND</td>
</tr>
<tr>
<td>rAd50.eGFP*</td>
<td>B</td>
<td>Alternative</td>
<td>ND</td>
</tr>
<tr>
<td>rAd50.Luc*</td>
<td>B</td>
<td>Mixed</td>
<td>Alternative</td>
</tr>
<tr>
<td>rAd5.eGFP</td>
<td>C</td>
<td>Original</td>
<td>Mixed</td>
</tr>
<tr>
<td>rAd5.Luc</td>
<td>C</td>
<td>Original</td>
<td>Mixed</td>
</tr>
</tbody>
</table>

*Rescue plasmids used for generation of recombinant Ad50 vectors contained the original ITR sequence 5′-CATCATCA-3′ instead of the published sequence (GenBank accession no. DD046417_1) as listed in Fig. 5.

The alignment of the non-human sequences (Fig. 5b) showed that 5′-CATCATCA-3′ was the most frequent sequence. Again, alternative sequences were found, e.g., the previously identified alternative sequence 5′-GATGATGT-3′, found in fowl adenoviruses. The majority of the published ITR sequences are consistent with the replication model described previously (de Jong et al., 2003; King & van der Vliet, 1994), with a small, 2, 3 or 4 nt direct repeat that is required for the jumping-back mechanism during replication initiation.

DISCUSSION

In the present study, we analysed the terminal ITR sequence in adenoviral vectors in different cell types and demonstrated that a reproducible switch from the original sequence 5′-CATCATCA-3′ to the alternative sequence 5′-CTATCTAT-3′ takes place for both replication-incompetent adenoviral vectors and replication-competent WT viruses. This suggests that outgrowth of a genome version harbouring the alternative ITR sequence is a common in vitro propagation phenomenon. We were further able to generate recombinant vectors harbouring this alternative ITR sequence. Our data revealed an increase in genome replication speed for Ad35 vectors with the alternative ITR sequence, which presumably underlies the outgrowth of the 5′-CTATCTAT-3′ mutant.

The alternative ITR sequence is not a newly discovered sequence but was published in the context of human Ad7 WT in 1980 (Shinagawa & Padmanabhan, 1980) and was also later found for human Ad4, Ad9, Ad12 and Ad16 WT (Schwarz et al., 1982; Shinagawa et al., 1983, 1987), and in bovine Ad4 WT (Dán et al., 2001). Additionally, changes in the terminal 8 nt of adenovirus strains belonging to the same serotype (Dán et al., 2001; Houng et al., 2006; Jacobs et al., 2004), and of different isolates of one strain (Schwarz et al., 1982, 1983), were reported previously.

Although the alternative ITR sequence identified in our experiments occurs in a large proportion of published human adenovirus sequences, it has never been linked to altered replication kinetics. However, larger rearrangements within the ITR sequences have been described to result in earlier CPE compared with WT virus upon repeated passaging of human Ad12 in a tumour cell line (Schwarz et al., 1982). A similar rearrangement was described for the epidemic human Ad4, in which the human Ad4 ITR was replaced by a group B like ITR in 1976 leading to outgrowth of a new version of epidemic human Ad4 in US military recruits (Houng et al., 2006). It has been hypothesized that mutations in the ITRs should be accompanied by compensatory mutations in the polymerase or pTP (King & van der Vliet, 1994). However, these compensatory mutations have not been described in context with the fowl viruses sequence 5′-GATGATGA-3′ (Rademaker et al., 2006). We also did not observe changes in the polymerase or pTP in our complete genome sequencing results of rAd35.1BS. Mechanistically, the tendency to mutate the terminal ITR sequence can be explained by the failure of proofreading in the terminal 8–12 nt of the adenovirus ITR (King et al., 1997a) due to the presence of pTP at the 5′ end of the synthesized new DNA strand. To maintain genome stability within this region, adenoviruses utilize the jumping-back mechanism, which can correct small deletions (King & van der Vliet, 1994) but might not easily correct mismatches.

It is important to note that published ITR sequences may not be a balanced representation of naturally occurring ITR sequences. In some cases, the terminal nucleotides of the ITRs have not been sequenced but are inferred to be 5′-CATCATCA-3′. Additionally, adenoviruses from diagnostic swabs are commonly grown in cell culture prior to sequencing. The passages in cell culture could also result in nucleotide changes. However, the published sequence of Ad35 stating 5′-CATCATCA-3′ as the ITR sequence results from sequencing of the complete virus sequence including the ITRs (Gao et al., 2003), which suggests that the original ITR sequence is stable in nature. Because the original sequence 5′-CATCATCA-3′ is detected in nature after a long period of virus–host co-evolution, it can be concluded that the alternative sequence 5′-CTATCTAT-3′ does not
Human sequences | Non-human sequences
---|---
Human AdV 5 | Simian AdV 48
Human AdV 2 | FJ025929
Human AdV 1 | CAT | CA
Human AdV 6 | Simian AdV 29
Human AdV 7 | FJ025916
Human AdV 19 | Simian AdV 41.1
Human AdV 32 | FJ025913
Human AdV 26 | Simian AdV 32
Human AdV 28 | FJ025909
Human AdV 36 | Simian AdV 35.1
Human AdV 46 | FJ025912
Human AdV 49 | Simian AdV 44
Human AdV 53 | FJ025899
Human AdV 56 | Simian AdV 31.1
Human AdV 3 | Human AdV 4
Human AdV 7 | AY601634
Human AdV 14 | AY803294
Human AdV 34 | AY737797
Human AdV 35 | AY128640
Human AdV 55 | FJ643876
Human AdV 4 | AY599837
Human AdV 41 | DO315364
Human AdV 3 | HQ770721
Human AdV 3 | AY599837
Human AdV 4 | AM749299
Human AdV 18 | ADRP1IT1
Human AdV 52 | DO923122
Human AdV 10 | ADRITR-1
Human AdV 19 | ADRITRRA
Human AdV 8 | AB448769
Human AdV 19 | Simian AdV 48
Human AdV 53 | AY550001
Human AdV 15 | TCA | TCA
Human AdV 7 | AY036246
Human AdV 17 | AF271992
Human AdV 19 | Simian AdV 48
Human AdV 57 | HQ003817
Human AdV 17 | HQ910407
Human AdV 19 | Simian AdV 32
Human AdV 42 | FJ619037
Human AdV 26 | EF153474
Human AdV 28 | FJ824826
Human AdV 36 | GG384080
Human AdV 46 | AY875848
Human AdV 49 | DO398929
Human AdV 53 | AB605244
Human AdV 54 | BA448770
Human AdV 56 | HM770721
Human Adv 7 | AY599836
Human AdV 31 | AM749299
Human AdV 4 | AY599837
Human AdV 41 | DO315364
Human AdV 31 | AM749299
Human AdV 18 | ADRP1IT1
Human AdV 52 | DO923122
Human AdV 10 | ADRITR-1
Human AdV 19 | ADRITRRA
Human AdV 8 | AB448769
Human AdV 19 | Simian AdV 48
Human AdV 53 | AY550001
Human AdV 15 | TCA | TCA
Human AdV 7 | AY036246
Human AdV 37 | AF271992
Human AdV 9 | AY601636
Human AdV 9 | AY036246
Human AdV 5 | AY565136
Human AdV 4 | AY601636
Human AdV 21 | AY601633
Human AdV 50 | AY737798
Human AdV 4 | AY565136
Human AdV 41 | AY191019
Human AdV 18 | GU191019
Human AdV 12 | GD000005
Consensus | CAT | CA

**Fig. 5.** Alignment of published adenovirus ITR sequences reveals the presence of the detected alternative ITR sequence in human adenovirus sequences. (a) Alignment of the repeat region of published human ITR sequences. Sequences are derived from sequencing of both the right and left ITRs. 5′−CATCATCA−3′ is categorized as ‘conserved human sequences’. Differences
have a selective advantage in vivo. The selective advantage of the alternative ITR sequence may therefore be specific for in vitro cell culture or certain cell types. The infected cell type and replication speed in the respective cell type may influence the selective pressure on the ITR sequence, resulting in the observed difference between the original Ad35 ITR sequence present in nature and the alternative ITR sequence observed upon propagation in cell culture.

Whilst the alternative sequence 5′-CTATCAT-3′ would previously have been discarded as an abnormality, our experiments clearly show that the benefits of this alternative sequence should be exploited in the production process for vectors derived from several human adenovirus serotypes for both gene therapy and vaccine applications. Remarkably, the genome heterogeneity only occurred in the terminal 8 nt of the ITRs, whereas the rest of the viral genome was completely stable over 15 viral passages. A sequence change from rescue plasmids to the upcaled virus population has two undesired results for production of clinical-grade material: first, sequence identity from plasmid level to clinical-grade material is lost; and secondly, a heterogeneous virus population is present in a large-scale batch. Insertion of the alternative ITR sequence in the rescue plasmids can circumvent these effects and result in both sequence consistency from rescue plasmids to clinical-grade material and more importantly a homogeneous virus population.

Implementation of the alternative ITR sequence further leads to a steeper genome amplification curve, which may speed up the production process and facilitate the production of complex vectors. As rAd35 vectors harbouring the alternative ITR sequence reach the plateau phase earlier than rAd35 vectors with the original ITR sequence, the timelines of the complete production process may be reduced by use of this sequence.

In conclusion, minor changes in the adenoviral ITRs can have broader implications for batch homogeneity and growth kinetics for large-scale rAdV generation in gene therapy and vaccine applications.

METHODS

**Plasmid construction.** The original ITR sequence was replaced by the alternative ITR sequence in previously described pAdapt and pBr plasmids (Havenga et al., 2006) by a fusion PCR-based cloning approach.

**Cell culture.** PER.C6 cells (Fallaux et al., 1998) were maintained in Dulbecco’s modified Eagle’s medium with 10 % FBS, supplemented with 10 mM MgCl2, A549, HEK293, Hep2, HeLa and MRC5 cells were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium with 10 % FBS.

**Adenovirus generation, infections and passaging.** If not otherwise stated, all viruses were generated in PER.C6 cells by single or double homologous recombination and produced as described previously (Havenga et al., 2006) for replication-incompetent rAds, harbouring deletions in E1 and E3 and a substitution of E4orf6 by the respective Ad5 sequence. Briefly, pAdapt, pWE and pBr plasmids (Havenga et al., 2006) were co-transfected in PER.C6 cells using Lipofectamine according to the manufacturer’s instructions (Life Technologies). Cells and medium were harvested 1 day after full CPE, freeze-thawed, centrifuged for 5 min at 1238 g and stored at −20 °C. After several amplification steps, T175 triple-layer flasks containing 70 % confluent monolayers of PER.C6 cells were infected using crude harvest material of the previous amplification step. The virus was purified using a two-step CsCl purification method. Finally, the virus was stored in aliquots at −85 °C.

To investigate the switch from the original to the alternative ITR sequence, the different viruses were passaged serially using either crude virus material after plaque purification or purified virus batches as described above. To this end, cells were infected with the respective viral vector. One day after full CPE was evident, the cells and the supernatant were harvested and frozen. The VPs were released from the cells by thawing and this crude virus material was used to infect new cells.

**Viral DNA isolation from infected cells.** DNA isolations for the ITR-specific PCR were performed as follows. VPs were released from crude virus material by repeated freeze–thaw cycles, the debris was spun down and host-cell DNA in the supernatant was removed by DNase I treatment. The VPs were disrupted by incubation with 10 % SDS and treated with proteinase K. Viral DNA was subsequently purified using a GeneClean Spin kit (MP Biochemicals) and used for PCR analysis.

For ITR sequence analysis, DNA was isolated by PEG isolation from 20 ml crude cell lysate, lysed by consecutive freeze–thaw cycles, spun down and the supernatant treated with 0.001 % (w/v) DNase I (Roche) and RNase T1 (Roche), followed by NaCl inactivation (1 M). VPs were precipitated using 10 % PEG 6000 (Sigma) on ice for 1 h, followed by a centrifugation step at 9000 g and resuspended in 1 ml SM buffer [0.1 M NaCl, 8 mM MgSO4, 50 mM Tris/HCl (pH 7.5), 0.002 % gelatin]. Viral capsid proteins were disrupted using 10 % SDS and proteinase K treatment and the DNA was extracted by phenol/chloroform precipitation. Full-length DNA was digested by EcoRI (Ad26), SphI (Ad48, Ad5), AgeI (Ad49, Ad11) or NheI (Ad50) and finally sequenced by BaseClear (Leiden, The Netherlands). For sequencing reactions, sorotype-specific primers with a reverse primer binding directly downstream of the left ITR and a forward primer binding directly upstream of the right ITR were used.

**ITR-specific PCR.** As the ITR regions are AT rich, locked nucleic acid primers were used to ensure sufficient primer binding to the template. Primers were purchased from Eurogentech. The following primers were used (lower-case letter indicate locked nucleic acid nucleotides): ori.IRR, 5′-CatacTaataATATATACC-3′; Ad5 alt IRR, 5′-CtaCTAATATATATACC-3′; Ad5 left IRR rev, 5′-CTAGTAAGTT-CCGTTGAAAAGAAG-3′; Ad5 right ITR for, 5′-GTAGTATGCGCCTAGTCACTTC-3′. PCR products were analysed on an agarose gel.
Replication kinetics by qPCR. Replication kinetics were analysed by infection of HEK293 and PER.C6 cells using 1000 VPs per cell for 3 h; the cells were subsequently washed. The presence of VPs in cells and supernatant was analysed at the indicated time points p.i. by a VP qPCR. To this end, infected cells were lysed using 0.5 % Triton X-100 (Sigma), incubated at −80 °C for 1 h and thawed.

A qPCR specific for the cytomegalovirus (CMV) promoter, present in all adenoviral vectors used, was performed using Gene Expression Master Mix (Applied Biosystems) according to manufacturer’s recommendations. Primer/probe combination sequences are: CMV for, 5′-TGGGCGGTAGGCGTGTA-3′; CMV rev, 5′-CGATCTGAGC-GTTTACATAAACG-3′; and probe, 5′-VIC-TGGGAGGTCTATAT-AAAGC-MGB-NFQ-3′, purchased from Applied Biosystems. To determine the number of VPs in individual samples, a standard curve was generated.

Sequence alignments. Adenovirus ITR sequences were obtained from BLAST searches. The alignment was created using CLC software. In cases where several sequences for one adenovirus serotype were published, they were only included if they differed from each other in the terminal 8 nt.

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