Improvements in adenovirus technology : aiming at replication specificity and vector integration

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CHAPTER 3

Relaxed Template Specificity In Fowl Adenovirus-1 DNA Replication Initiation

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Relaxed Template Specificity In Fowl Adenovirus-1 DNA Replication Initiation

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The Fowl Adenovirus 1 (FAdV-1) isolates PHELPS and OTE are highly homologous but have striking differences in the repeat region of the terminal repeat (ITR). While the repeat region in OTE conforms to the conventional human adenovirus repeat region (5'-CATCATC), PHELPS contains guanidine residues at position 1,4 and 7 (5'-GATGATG). Therefore, the FAdV-1 is currently the only known species, which contains Ads starting with guanidine residues. This implies that the FAdV-1 isolates PHELPS and OTE either have distinct template specificity at replication initiation, or alternatively, they have a relaxed specificity for replication initiation. In this study we confirmed the distinct sequence variation at the origin of DNA replication in the ITR of the FAdV-1 PHELPS and OTE isolates. Sequence analyses of the *pTP* and *Pol* genes of both PHELPS and OTE did not reveal differences that could explain the distinct template specificity. Replication assays demonstrated that linear DNA fragments flanked by either 5'-CATCATC or 5'-GATGATG termini replicated in cells upon infection with FAdV-1 OTE and FAdV-1 PHELPS. This was evident from the appearance of *Dpn*I-resistant fragments in a mini-replicon assay. From these data we conclude that the FAdV-1 has relaxed, rather than changed, its template specificity at replication initiation.

Adenoviruses (Ads) are linear double-stranded DNA viruses with genomes 34 to 48 kb in size and with terminal proteins (TP) covalently bound to the 5'ends [1-3]. At the ends an inverted terminal repeat (ITR) is located, containing the origin of replication [4-16]. The Ad genome is replicated from each end of the molecule via a strand-displacement mechanism. Most of the insight in the molecular mechanism of Ad DNA replication is derived from studies of the HAdV-5 and HAdV-2 (species HAdV-C). Preceding the initiation of replication, two viral proteins, the precursor of the terminal protein (pTP) and the Ad DNA Pol, form a stable heterodimer [17,18]. Although this complex can initiate DNA replication, the initiation is strongly stimulated by the host proteins Oct-1 [19,20] and NF-I [21]. The pre-initiation complex of pTP/Pol/Oct-1/NF-I bound to the origin initiates replication with a preference for the nucleotide at position 4 in the template strand. A specific amino acid in pTP, Ser^{580}, is used a primer for covalent binding of dCMP by

pol, generating pTP-C [22,23]. After formation of a pTP-CAT trinucleotide complex, the complex jumps back to allow base-pairing of the CAT with the first three nucleotides of the template strand [24]. After dissociation of the pTP/Pol complex the elongation can proceed requiring a third virus encoded protein, the DNA-binding protein (DBP), involved in unwinding the dsDNA ahead of the polymerase [25]. The pTP remains bound at the 5' end of the genome and protects against degradation by exonucleases. Later during infection, pTP is cleaved by Ad protease to form the Terminal Protein (TP) [26]. The jumping-back mechanism explains the short 3 (infrequently 2 or 4) base-pair direct repeat found at the termini of all Ad ITRs.

The first step of DNA replication is covalent coupling of dCMP to the pre-initiation complex. This step is well conserved. All Mastadenoviruses and Atadenoviruses characterized to date have a C residue at the 5' end of their genome, suggesting a preference of pTP to bind dCMP. This concept is supported by work of King and van der Vliet [24] who showed that mutation of G4 in the template strand blocked replication initiation of HAdV-5 in-vitro. Even in the absence of any template DNA, pTP-C/Pol complexes were formed [24]. The preference of pTP to bind dCMP was reduced in the presence of manganese ions in the replication initiation assay, suggesting that structural factors govern the template specificity.

Strikingly, whereas most of the Aviadenoviruses conform to the C rule there is an exception in the Fowl adenoviruses 1 (FAdV-1). The PHELPS isolate (gi:1314432) of the Chicken Embryo Lethal Orphan (CELO) diseases-causing Ad and isolate KUR (gi:209935) are unique among Ads in that their genomes start with the sequence 5'-GATGATG. Alignment of the KUR sequence showed that it was 99% identical to PHELPS within the first 168 bp of the right ITR. Intriguingly, another FAdV-1 isolate, OTE (gi:210033, gi:210032), of which only the sequence of the first 68 bp of the ITR has been published [27], is distinct from PHELPS and KUR in that it conforms the Ad convention and starts with the sequence 5'-CATCATC.

The genome sequence of the PHELPS isolate has been completely determined by Chiocca *et al.* [28]. The virus lacks homology with the *E1*, *E3* and *E4* regions of the Mastadenoviruses as well as the genes encoding pV and pIX. The *E2* and Late gene clusters of PHELPS are homologous to the Mastadenoviruses. Open reading frames (ORFs) homologous to HAdV-5 DBP, pTP and Pol could be identified in PHELPS (gi:1314452, gi:1314443 and gi:1314442, respectively). The 54 bp ITRs are relatively short in PHELPS compared to the 103 bp found in HAdV-5.

The unconventional occurrence of G nucleotides at position 1, 4, and 7 of some FAdV-1 isolates, as well as marked heterogeneity between different FAdV-1 isolates is intriguing. Here we confirmed the sequence difference between the FAdV-1 PHELPS and OTE and characterized their

Table 1. *Synthetic oligonucleotides used in this study.*

CELO-PE	5'-GCGTCAGTATTGGTTAGTTTTGAGG
MiniCeloL-AS	5'-GAGGGCGTCGCGAAGATCTCGTCTAAGAGGAAATACAAGAAAAAACAG
oligo-dT-20	5'-TTTTTTTTTTTTTTTTTTTT
RTCptp-rev	5'-GTGGGCTATCGTAACCTATCTGCA
RTCpol-rev	5'-CGGTATTTTGTGAACGGAGCC
CeloRTptp-for1	5'-AGGAGGAATCGACGTCGGCG
CpTP-as	5'-TAAAGCTTGCCACCATGGCGGGGACGGGGTGCATTACG
CtPT-s	5'-TGAATTCTTACAGAGGCTGACCTCGTCG
CELO-pol-s	5'-TGACGCGCTGGTAGAAGTCCCGCGAG
CELO-pol-as	5'-CCATCGCTACCAACGAGGAGATCAAAGCCAAC
CELO-pol-F4	5'-GTCTAGCCAATAGAAGCCTTCGTC
CELO-pol-R5	5'-CCTCAGACGCGTGCTCCG
BamITR-C	5'-CGCGGATCCCATCATCTATAATAACCTCAAAAACTAACGCAG
BamITR-G	5'-CGCGGATCCGATGATGTATAATAACCTCAAAAACTAACGCAG
MiniCeloL-AS	5'-GAGGGCGTCGCGAAGATCTCGTCTAAGAGGAAATACAAGAAAAAACAG
MiniCeloR-S	5'-TTAGACGAGATCTTCGCGACGCCCTCTATAGACATTATATAGAATATAACTG
cGFP-for	5'-GGGGTCATTAGTTCATAGCCCAT
cGFP-rev	5'-GCTTGATCCAAATCCAAACAGAGT

pTP and Pol genes. In addition we provide evidence that these viruses have a relaxed, rather than a changed, template specificity.

Materials and methods

Tissue culture and virus propagation. The PHELPS virus was obtained from the ATCC (VR-432, ATCC, Manassas, VA) and OTE was kindly provided by Dr. Matt Cotten (IMP, Vienna, Austria). Both PHELPS and OTE were propagated on the chicken hepatocellular carcinoma epithelial cell line, LMH (CRL-2117, ATCC). LMH cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 8% fetal calf serum (FCS), antibiotics and 3 g/l glucose in a 5% CO_2 atmosphere at 37°C. Dilutions of PHELPS or OTE were added to subconfluent LMH cells in DMEM supplemented with 2% Horse Serum (HS), antibiotics and 3 g/l glucose. Two hours post infection, the inoculum was replaced with DMEM supplemented with 8% FCS, antibiotics and 3 g/l glucose. When almost all cells showed cytopathogenic effect (CPE), approximately three days post infection, the cells were harvested in phosphate buffered saline (PBS) supplemented with 2% HS, and freeze/thawed to release virus particles. These freeze/thaw lysates were used for re-infections and mini-replicon assays.

ITR sequence determination. Virus particles from the freeze/thaw lysates were purified by CsCl density gradient as described [29]. Isolated particles were incubated with Proteinase K in 0.2% SDS, 8 mM EDTA, and viral DNA was extracted by phenol/chloroform and ethanol precipitation.
Primer extension assay: The primer CELO-PE was radiolabelled using T4 polynucleotide kinase and γ^{32}-ATP and elongated with T7 DNA polymerase in the presence of dATP, dTTP and either dCTP or dGTP. Elongated primers were size fractionated by electrophoresis on an 8% poly-acrylamide gel. For detection of the radio labelled fragments, a Kodak XAR film was used (Kodak, Vianen, The Netherlands).
Terminal transferase: A poly-A tail was added to the 3'end of the viral termini using terminal transferase (Promega, Leiden, The Netherlands). The first 800 bp of the left ITR were amplified by PCR using the primers MiniCeloL-AS and oligo-dT-20 (see Table 1). The PCR fragments were cloned in pCR2.1+ using the TA cloning kit (Invitrogen, Breda, The Netherlands) and used for sequence analysis.

Splice-site determination by rtPCR. LMH cells were infected with OTE and 18 hours post infection mRNA was isolated with the RT-PCR miniprep kit (Stratagene Europe, Amsterdam, The Netherlands). For the synthesis of cDNA, 1 µg mRNA was used in a reverse-transcriptase reaction (Promega) using the RTCptp-rev and RTCpol-rev primers to synthesize pTP and Pol cDNA respectively. For amplification of the spliced pTP and Pol fragments, the forward primer CeloRTptp-for1 was used in combination with RTCptp-rev or RTCpol-rev (see Table 1) to synthesize the pTP or Pol spliced fragments respectively in a standard PCR reaction using Taq polymerase (Perkin-Elmer Life Science, Hoofddorp, The Netherlands). The pTP and Pol PCR products were cloned in a pCR2.1 vector, and sequenced.

Isolation and sequencing of PHELPS and OTE DNA. For the isolation of pTP and Pol genes, viral DNA was isolated as described above. The pTP genes were isolated using the primers CpTP-as and CtPT-s (see table 1) in a standard PCR reaction. The 2.0 kb PCR product was digested with *Hind*III and *Eco*RI (MBI Fermentas GmbH, St. Leon-Rot, Germany) and cloned in a 5.4 kb *Hind*III/*Eco*RI fragment of pCDNA3.1+ and sequenced. Differences with the published PHELPS pTP sequenced were confirmed by direct sequencing of viral DNA.
For OTE pol, a 3.9 kb PCR fragment was amplified using the primers CELO-pol-s and CELO-pol-as and cloned in a 2.7 kb *Eco*RV digested pIC20H vector. This vector was used to sequence the OTE Pol gene.
Mini-replicon assay. The left and right genome ends of FAdV-1 OTE PCR amplified with the primers BamITR-C or BamITR-G together with MiniCeloL-AS for the left end and MiniCeloR-S for the right end. The left and right genome ends of FAdV-1 PHELPS PCR amplified with the primers BamITR-G together with MiniCeloL-AS for the left end and MiniCeloR-S for the right end (see Table 1; *Bam*HI sites are underlined in the primer sequence). By virtue of the homology in the Left ITR fragment and Right ITR fragments, both products could be linked in a second PCR reaction using either BamITR-C or BamITR-G primers and *Pfu* polymerase (Stratagene Europe). The resulting 2 kb PCR fragment was cloned in a pCR2.1+ vector using the TA-cloning kit (Invitrogen). The resulting plasmids, miniOTE-C, miniOTE-G and miniPHELPS-G were digested with NruI and ligated with a 2KB CMV-GFP cassette. The CMV-GFP cassette was synthesized with PCR from pShuttle-GFP using the primers cGFP-for and cGFP-rev and *Pfu* polymerase. The GFP cassette is used as a marker for transfection efficiency.
Subconfluent cultures of LMH cells were infected with OTE or PHELPS (m.o.i. = 1) in DMEM supplemented with 2% HS, antibiotics and 3 g/l glucose in a 5% CO_2 atmosphere at 37°C. Two hours post infection, inoculum was replaced with DMEM supplemented with 8%FCS, antibiotics and 3 g/l glucose in a 5% CO_2 atmosphere at 37°C. Six hours post infection, cells were transfected with 1 µg *Bam*HI digested miniOTE-C, miniOTE-G or miniPHELPS-G replicon plasmid, with the aid of JetPEI (Polyplus-transfection, Illkirch Cedex, France). Transfection efficiencies of 40% to 50% were achieved routinely. Viral DNA and mini-replicon DNA was isolated 54 hours post infection from LMH cells using HIRT small molecular DNA isolation procedure [30]. From the isolated DNA, 10 µg aliquots were digested

with either *Dpn*I, *Mbo*I or left undigested, and loaded on a 1% agarose gel. After electrophoresis and blotting the mini-replicon fragments were detected by Southern analysis using a radiolabelled GFP fragment as probe. For the detection of the mini-replicon backbone, a radioactive Amp-specific probe was used. It was noted that the presence of Adeno-Associated Virus in the assay severely reduced the efficiency of mini-replicon replication.

Sequnces submitted to GenBank. The coding sequences *pTP* and *Pol* genes of the FAdV-1 OTE isolate are deposited as AY421748 and AY421749, respectively. The genomic sequences of the *pTP* and *Pol* coding regions of the OTE isolate are deposited as AY421752 and AY421753. The first 773 nt of the left genome end and 819 nt of the right genome end of the OTE isolate are deposited as AY421750 and AY421751, respectively.

Results

The linear genomes of all Ads characterized to date start with a small direct repeat of 2, 3 or 4 nucleotides (Fig. 1A). To study the apparent diversity of the terminal sequences, PHELPS and OTE were propagated on LMH cells. To verify the nucleotide sequences of the PHELPS and OTE termini, the left end of their genomes were PCR amplified with DNA isolated from both PHELPS and OTE infected LMH cells as templates. The 3'-end of the bottom strand of the left-hand terminus was extended with an oligo-dA tail with the aid of terminal transferase. PCR amplification of the ITR sequences was performed using an oligo-dT primer and the FAdV-1-specific primer MiniCeloL-AS. The PCR fragments were cloned, and the plasmid clones were used for sequence analyses (accessions AY421750 and AY421751 for the left and right genome ends). The results confirmed the PHELPS and OTE terminal sequences to read 5'-GATGATG and 5'-CATCATC, respectively (Fig 1B). The homogeneity of the PHELPS and OTE terminal sequences was confirmed by primer extension assays directly on the isolated virus DNA (Fig 2). This assay determines the nucleotide at position 7 in the template strand of the ITRs. Primer extension on PHELPS DNA yielded a 34-nt product when dCTP was

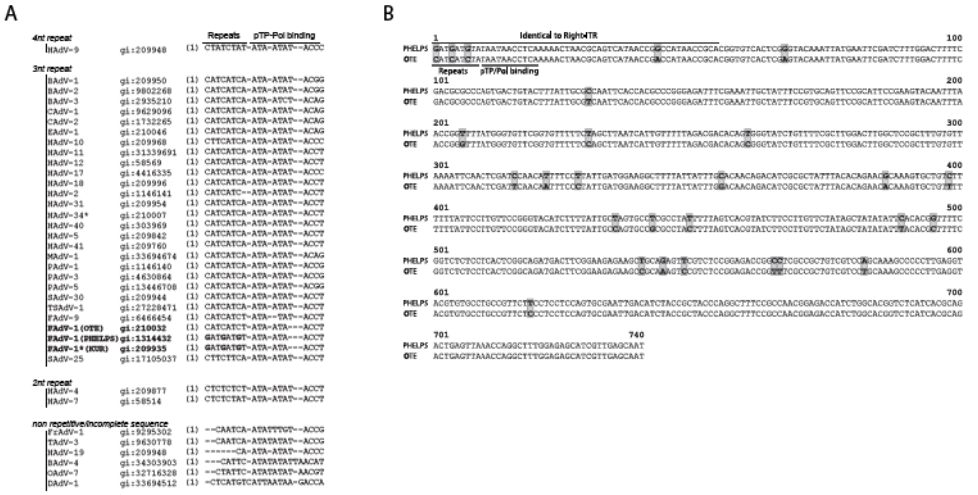

Figure 1. *Sequence conservation of the Adenovirus ITRs. (A) BLAST alignment of published Ad ITR sequences. The repeat region, pTP/Pol binding site, and the size of the repeats are annotated. The nucleotide differences in PHELPS and KUR are depicted in bold. (B=Bovine, C=Canine, D=Duck, E=Equine, F=Fowl, Fr=Frog, H=Human, M=Murine, O=Ovine, P=Porcine, S=Simian and TS=TreeSrew (Tupaia)). (*) The HAdV-34 and FAdV-1 KUR sequences are derived from the right ITR. (B) Differences between PHELPS and OTE in the first 740 nt of the Left-ITR. Two independent PCR isolates of the left ITR of OTE were sequenced and aligned against PHELPS. The repeat region and pTP/Pol-binding domain, as determined for HAdV-5, are indicated as well as the region identical to the right-ITR of PHELPS.*

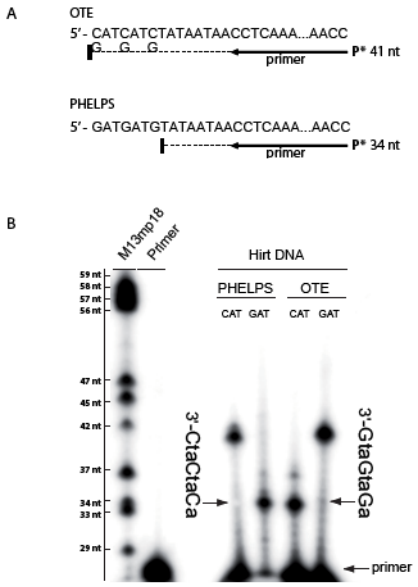

Figure 2. *Identification of nucleotide 7 in the left ITR of PHELPS and OTE. Genomic DNA was extracted from CsCl purified viral particles. A radioactive labelled probe was elongated with nucleotide mixtures depleted for dGTP or dCTP. (A) Without dCTP in the elongation mix, PHELPS would yield a product of 41 nt and OTE would yield a 34 nt product due to template dependent requirement of dCTP incorporation. (B) Elongated samples were denaturated and loaded on a sequencing gel. As a marker the ddGTP sequence lane of phage vector pM13mp18 is included.*

omitted from the elongation mix, while OTE yielded a 41-nt fragment. Primer extension in the presence of dCTP, but in the absence of dGTP generated a 41-nt fragment in PHELPS, but a 34-nt product in OTE. These data confirm the presence of guanine for PHELPS and cytosine for OTE at position 7. Furthermore, the virtual absence of the 41-nt elongation products in the lanes containing the 34-nt product confirms the homogeneity the terminal sequences in both isolates.

To study whether the difference in the initiating nucleotides is correlated with changes in the *pTP* and *Pol* sequences, the large exons containing the majority of the open reading frames for pTP and Pol from OTE DNA were cloned and sequenced. The genes were cloned by PCR from genomic OTE and PHELPS DNA and sequenced (accessions AY421752 and AY421753 for the genomic sequences and AY421748 and AY421749 for *pTP* and *Pol* cDNAs, respectively). Differences between our PHELPS sequence and the published sequence, and differences between OTE and PHELPS, were confirmed by direct sequence analyses on genomic DNA.

In PHELPS, open reading frames have been annotated for pTP (E2B pTP, AAC54905.1) and polymerase (E2B Pol, AAC54904.1). However, in HAdV-5 and HAdV-2, the translation of pTP and Pol messages are complex. The pTP and Pol proteins are translated from distinct messengers but use the same translation start codon. The majority of the coding sequences from pTP and from Pol are located in large distinct exons, but the translation initiating ATG is derived from a common upstream exon. The sequences preceding the first ATG of the large exons are essential for the activity of both proteins [31,32].

To determine the structure of the Pol and pTP mRNAs, rtPCR on RNA isolated from OTE infected LMH cells was used to characterize the presence of the upstream exon homologous to the human Ads. For cDNA synthesis we used primers RTCpol-rev for Pol and RTCptp-rev for pTP, both located downstream of the first ATG in the large exon. The forward primer (CeloRTptp-for1) was used for both OTE pTP and Pol mRNA's, and was based on sequence similarity in the shared 5' untranslated region (UTR) of HAdV-5 pTP/Pol mRNA and PHELPS genomic DNA (figure 3). The PCR products were cloned and the resulting plasmid clones were sequenced. Splice sites were predicted by aligning the cDNA fragments against the genomic sequence of PHELPS using the Sim4 algorithm [33]. Similar to HAdV-5, OTE pTP and Pol use a splice-donor site, 3 nucleotides downstream of an ATG sequence (nt 15081 in the published PHELPS sequence). The splice-acceptor site for pTP is located 159 nt upstream of the annotated ORF (nt 12155 in

published PHELPS sequence). The resulting mRNA would encode a 630 amino-acids pTP protein. This is 55 amino-acids larger than PHELPS pTP encoded by the annotated pTP ORF (AAC54905.1) and contains a conserved sequence motif [A]-[RHD]-[L]-[T]-[GN]-[Q]. The splice-acceptor site of Pol is located 610 bp upstream of the ORF (identical to nt 10476 in PHELPS) inside the pTP ORF (Figure 3). However, unlike HAdV-5, translation starting from the ATG, 6 nucleotides upstream of the splice donor, does not result in a *bona fide* Pol protein, whereas OTE and PHELPS Pol use the same splice donor, an alternative ATG must be used for the translation of Pol.

The first ATG downstream of the splice site is located at nt 10268 in both PHELPS and OTE and is in frame with the identified ORF. Translation from this start codon results in a 1255 aa polymerase protein, 134 aa larger than the annotated ORF in PHELPS (AAC54904.1). Both the pTP and Pol start codons confirm the minimal Kozak sequence (taaATGG and GaaATGG respectively). An overview of the organization is provided in figure 3.

Having established the putative ORFs made it possible to compare the sequence of the OTE pTP and Pol with the published sequence of PHELPS and other Ads (Figure 4A). We noted two differences between our sequence of PHELPS pTP and the published sequence. Our PHELPS contained $Arg^{325}Asn^{326}$ rather than Cys^{325} and Ser^{326} (numbered aa^{270} and aa^{271} in AAC54905.1). OTE and FAdV-10 are identical to our PHELPS sequence in this region. Only two differences were found between the pTP ORFs of PHELPS and OTE. PHELPS Val^{50} and Asp^{604} were changed in Leu and Glu respectively in OTE. These changes do not result in an altered amino-acids charge. In addition, the Val^{50} to Leu maps in the N-terminal part of the protein, which is unlikely to be involved in priming. Taken together these data suggest that the difference of initiating nucleotide is not caused by a functional change in terminal protein activity.

At the initiation of DNA replication Pol catalyses covalent binding of the first dCMP nucleotide to Ser^{580} of pTP. To exclude that the variation in initiating nucleotide is the result of a change in Pol function, we compared the OTE Pol sequence with the published PHELPS sequence. Eight base alterations were detected that would change the amino-acid sequence. The His^{416} to Gln and His^{455} to Gln (PHELPS; AAC54904.1 compared to OTE) alterations map in the exonuclease region of the polymerase and are therefore unlikely to be associated with the difference in initiation template-specificity. The amino acid changes Pro^{587} to His and Gly^{885} to Glu do not map in conserved regions of Pol. The changes Glu^{672} to Gly, Asn^{854} to Ser, Ser^{965} to Asn and Thr^{1203} to Ala, all map near the active site of Pol, but only the Ser^{965} to Asn is located in a conserved region. The localized sequence-similarity alignment algorithm (MACAW [34-36]) was used to find homologous-sequence blocks in the HAdV-5 and PHELPS Pol sequences. The amino-acid changes between the PHELPS and OTE sequence were compared with the HAdV-5 map and with BLAST alignments of several Ad polymerases (Fig. 4B).

The sequence comparisons of the *pTP* and *Pol* genes of PHELPS and OTE did not reveal obvious differences that would explain the difference in template specificity. Therefore it is tempting to speculate that the distinct differences at the origin of replication are not caused by a change of template specificity inherent to the pTP and Pol, but rather that the FAdV-1 Ads have a relaxed specificity. This would allow replication initiation on different templates. As a result, the 5-'GATGATG and 5'-CATCATC of PHELPS and OTE would be simply maintained by their virtue of the availability of either one of their templates sequences, and not by specificity of the pTP and/or Pol.

To test this hypothesis we constructed mini-

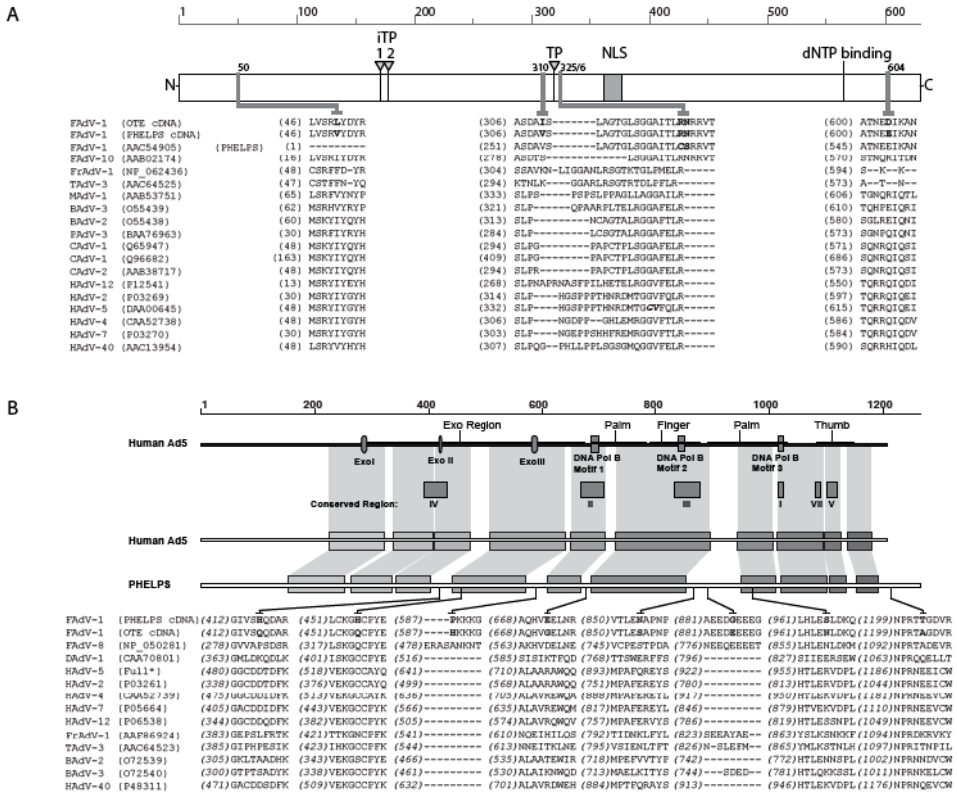

Figure 4. *Differences between PHELPS and OTE in the amino-acid sequences of pTP and Pol. Fragments encoding the pTP and Polymerase genes from PHELPS and OTE have been isolated by PCR, sequenced and compared on amino-acid levels. Amino-acid differences between PHELPS and OTE were confirmed by direct sequencing the genomic DNA. (**A**) Differences between PHELPS and OTE pTP sequences were aligned against publicly available Fowl (F), Frog (Fr), Turkey (T), Murine (M), Bovine (B), Porcine (P), Canine (C) and Human (H) pTP sequences. Protease-cleavage sites have been annotated by iTP1, iTP2 and TP as well as the amino acid bound to the 5' end of the viral genome ("dNTP binding"). The TP-cleavage site in the HAdV-5 sequence is indicated in bold/italic.*
*(**B**) PHELPS Polymerase was mapped against human HAdV-5 Polymerase by the macaw similarity align algorithm (pair wise cut-off: 33, score matrix: Blosum62). Significant similar blocks are equally shaded. Conserved domains in adenovirus Polymerase have been identified for HAdV-5 to identify similar functions in PHELPS Polymerase. Differences in OTE are aligned against know adenovirus Polymerase sequences. (*) The HAdV-5 Pol sequence is based on gi:118844 with the amino acids derived from exon 1 as described [31,32].*

replicon vectors [37] that contain a CMV-*GFP* expression cassette flanked by the 802 bp of the left terminus of the genome and the last 1154 bp of the right end of the genome of OTE. The mini-replicon is flanked by *Bam*HI restriction sites to release it from the backbone, leaving only a single additional nucleotide 3' of the normal genome end of the template strand. Three versions of the mini-replicons were generated: the miniOTE-C replicon contains the normal OTE termini, reading 5'-CATCATC, the miniOTE-G replicon harbours OTE ITR sequences, with the PHELPS sequence 5'-GATGATG at positions 1-7 and miniPHELPS-G replicon that contains the wtPHELPS ITRs. To study whether OTE could facilitate replication of the mini-replicons, LMH cells were infected with OTE. Six hours post-infection, the cells were washed and *Bam*HI-digested linear miniOTE-C and miniOTE-G replicons were introduced into the cells. Southern

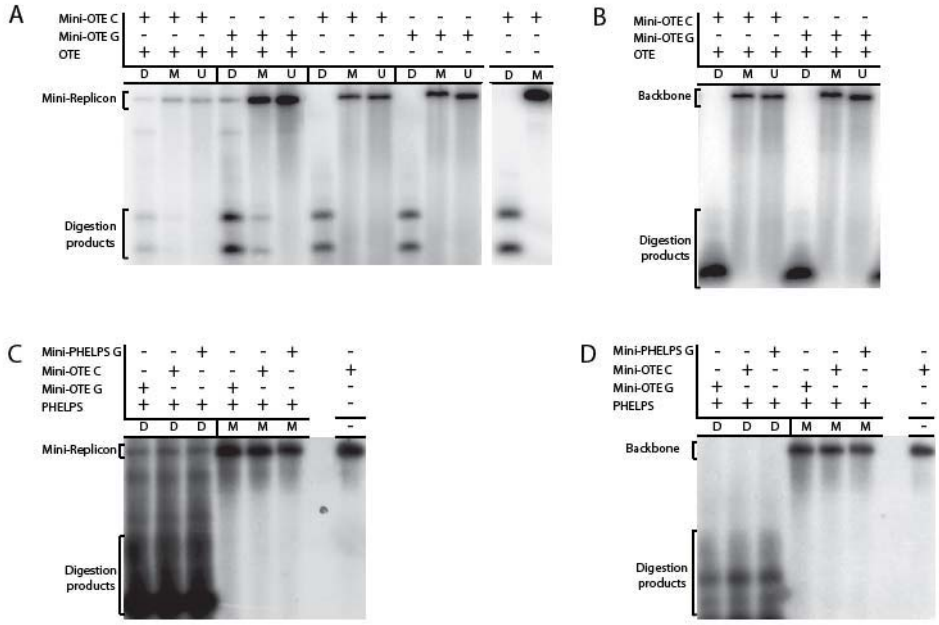

Figure 5. *Replication of mini-replicons by OTE and PHELPS. LMH cells were infected with OTE and six hours post infection, washed, and transfected with miniOTE C or miniOTE G plasmids digested with BamHI. The HIRT DNA-isolation method was used to extract low molecular weight DNA from the LMH cells 54-hours post infection. The isolated DNA was digested with DpnI (D, digests input DNA), MboI (M, digests replicated DNA) and analysed by Southern analysis (U=untreated). As a control, LMH cells were transfected with BamHI digested miniOTE-C or miniOTE-G without co-infection. (**A**) The mini-replicons were detected with a radiolabelled probe for the GFP gene. In the last two lanes, miniOTE-C plasmid digested with BamHI was loaded to indicate the size of the mini-replicon fragment. (**B**) To confirm full digestion by DpnI, a probe for the Amp gene was used to detect the miniOTE backbone. (**C**) Replication of mini-replicons with OTE ITR's with terminal C and G's (miniOTE-C and miniOTE-G), and miniPHELPS-G replicons based on PHELPS ITRs in cells infected with PHELPS. Minireplicons were detected with a radiolabelled probe for the GFP gene. The last lane contains the BamHI-digested miniOTE-C plasmid. (**D**) To confirm a full digestion by DpnI, a probe for the Amp gene was used to detect the mini-replicon plasmid backbones. In the last lane BamHI-digested miniOTE-C plasmid was loaded.*

analyses were performed on *Mbo*I-, *Dpn*I- or undigested low-molecular weight DNA extracted from LMH cells 54 hours post infection, using a GFP-specific probe to detect mini-replicon DNA (Figure 5). OTE is able to replicate both miniOTE-C- and miniOTE-G replicon constructs with equivalent efficiency as indicated by the *Dpn*I-resistant band (Figure 5A). Quantification of mini-replicon signals showed that 26% and 18% of the total undigested material was *de novo* replicated for miniOTE-G and miniOTE-C respectively. No hybridizing fragment is visible when a probe is used that detects the Amp gene that resides in the vector fragment (Fig. 5B). This clearly demonstrates that the restriction-endonuclease *Dpn*I digested the unreplicated DNA to completion. In addition, it shows that the generation of the unmethylated, *Dpn*I-resistant fragments is dependent on the presence of the FAdV-1 ITR sequences. Furthermore, the expected restriction fragments could be detected in the *Mbo*I-digested lanes (Fig. 5A), further indicating digestion of unmethylated DNA. The replication of mini-replicons was strictly dependent on the presence of OTE, since a *Dpn*I-resistant band or *Mbo*I-degradation products could not be detected in DNA isolated from non-infected cells. Similar results were obtained when PHELPS-infected LMH cells were cotransfected with miniOTE-C and miniOTE-G (Fig. 5C). To exclude that differences in mobilization are

caused by the sequence differences in the ITR sequence between PHELPS and OTE, a *Bam*HI-digested miniPHELPS-G plasmid, was transfected into PHELPS infected cell. The replication efficiencies of miniOTE-C, miniOTE-G and miniPHELPS-G were similar. When the blot was hybridized with an Amp probe to detect the backbone fragments, only degradation fragments were detected in the *Dpn*I-digested lanes, confirming the complete digested of input DNA (Fig. 5D). These data demonstrate that both OTE and PHELPS can drive replication of mini-replicons harbouring PHELPS 5'-GATGATG containing ITRs, as well as mini-replicons with the OTE 5'-CATCATC ITRs. This, together with the small differences in the sequences of the *pTP* and *Pol* genes, shows that the FAdV-1 viruses have relaxed their template specificity in the initiation of DNA replication.

Discussion

The terminal sequences of the PHELPS and OTE strains of the FAdV-1 were re-evaluated. We confirmed the striking sequence divergence between PHELPS and OTE at positions 1, 4 and 7 by sequencing of PCR amplified Ad DNA. Primer-extension assays, lacking either dGTP's or dCTP's in the elongation mixture, confirmed the presence of G or C residues at position 7 in virus DNA isolated from OTE and PHELPS infected cells. No heterogeneity was detected, excluding frequent reversion or indicating the stability of the ITR sequence in both isolates. Sequence analyses demonstrated that the *pTP* and *Pol* genes from PHELPS and OTE are strongly conserved. Sequence comparison did not reveal differences that would explain the distinct template usage. This led us to the hypothesis that the FAdV-1 isolates do not exhibit distinct template specificity, but rather the FAdV-1 has relaxed template specificity.

In Ads two mechanisms contribute to the stability of the DNA sequence at the origin of replication. Correct template replication depends on error-free replication. The proof-reading activity of the Ad Pol ensures faithful replication of the Ad genomes. However, due to the presence of pTP at the 5'end of the synthesized strand, proof reading fails in the first 8 to 12 bp of the Ad ITR [38]. To correct for deletions or mismatches Ad utilizes a jumping back mechanism. Small deletions of 1 or 2 nucleotides can be corrected in this way without compromising replication, as was shown by King and Van der Vliet [24]. However, mutations of nt 6 (A to C) or nt 3 (A to C) in the template strand resulted in a clear reduction of elongation efficiency in in-vitro assays. In contrast, mutation of nt 1 (G to A) did not affect replication efficiency. This suggests that the jumping-back mechanism can correct small deletions but can prevent mismatch incorporation at least at position 3 and 6. The relevance of the jumping-back mechanism in preventing elongation of mismatched DNA *in vivo* remains to be determined. Nevertheless, all known Ads contain a C residue at the 5'end of their ITR's, with the FAdV-1 isolates PHELPS and KUR as the sole exceptions. This suggests another mechanism to prevent mutation of the nucleotides at 1, 4 and 7. *In-vitro* replication-initiation experiments demonstrate that template DNA where nucleotide 4 is changed (C to A) did result in pTP-C formation (possibly initiated on nt. 7) but failed to generate pTP-T complexes [24]. Furthermore no elongation occurred on this template. Therefore, HAdV-5 seems to have two independent mechanisms to ensure ITR integrity. On the one hand, the template-dependent replication and jumping-back mechanism ensures generation of *bona fide* top-strands. In addition, the preference for binding dCMP of the pTP/Pol complex during replication initiation contributes to preventing mutations in the origin of replication.

This observation raised the question how the different ITRs in the otherwise closely

related strains PHELPS and OTE could have occurred. Obviously, the pTP-Pol complex derived from PHELPS must be able to bind dGMP as a substrate during initiation where this complex in OTE uses dCMP and conforms to the conventional Ads initiation sites. To test if this could explain the template specificity, we sequenced the *pTP* (both OTE and PHELPS) and *Pol* (OTE) genes, determined the splice sites for OTE pTP and Pol and compared both *OTE* and *PHELPS* sequences with the published PHELPS sequence. Like in HAdV-5, both pTP and Pol mRNAs are spliced and share the splice-donor site. The OTE pTP splice-acceptor site is located upstream of the pTP ORF as annotated for PHELPS, extending the pTP sequence with 55 amino acids. These additional amino acids have been shown to be essential for the biological activity in HAdV-5 pTP. Since the additional amino acids share a conserved motif [A]-[RHD]-[L]-[T]-[GN]-[Q] with other pTP's, the amino-terminal extended part is most likely important for the biological activity of OTE and PHELPS pTP. The splice acceptor site in OTE Pol is located 159 bp upstream of the annotated Pol ORF in PHELPS. The ATG translation-initiation codon in the upstream exon cannot be used for the translation of the Pol message, as it is employs another reading frame. Therefore, other than in HAdV-5 Pol, translation must initiate at the next start codon, located 208 bp downstream of the splice-acceptor site, resulting in an additional 134 aa compared to the Pol ORF annotated for PHELPS. The additional sequences do not code for domains conserved with other Ad Pol proteins. The splice sites for pTP and Pol as well as the alternative start codon of Pol confirm the predictions that Davison and colleagues made for PHELPS [39].

Sequence comparison of our PHELPS pTP gene with the published PHELPS sequence revealed 2 amino-acid differences. These amino acids are identical in OTE pTP. Comparison of PHELPS and OTE pTP revealed only two amino-acids changes. PHELPS Val^{50} and Asp^{604} were changed in OTE to Leu and Glu, respectively. These amino-acid differences have similar chemical properties or are located in the precursor part of the protein and therefore, most likely, do not cause substrate specificity. The striking similarities of the OTE and PHELPS pTP and Pol genes, suggest a relaxed sequence specificity rather than a distinct specificity of the pTP/Pol complex at replication initiation.

To test this hypothesis, we constructed the mini replicons miniOTE-C containing wtOTE ITRs and miniOTE-G where nt 1, 4 and 7 in the top strand of the ITR have been replaced with G's. Replication of linearized mini-replicon constructs was tested in a replication assay, that detects the absence of *dam* methylation in *de novo* replicated DNA. The results confirmed the ability of OTE and PHELPS to replicate ITRs starting with cytidine as well as guanidine residues with equal efficiencies. This replication was independent of the minor differences between PHELPS and OTE ITR sequences and specific for the mini-replicon constructs since a control plasmid lacking pTP/Pol binding domains, did not replicate.

The relaxed template specificity of the pTP/Pol complex is a pre-requisite for alterations in the FAdV-1 ITR to occur, but can only partially explain the altered ITR in PHELPS. Without the relaxed template specificity of the pTP/Pol complex, changes in the origin of replication are not possible. Therefore, the relaxation of the substrate specificity in the FAdV-1 replication machinery should have preceded the generation of the ITR sequences present in PHELPS and KUR.

The relaxed specificity of the FAdV-1 may be exploited for the generation of mobilisation-resistant adenoviral vectors for gene therapy. Vectors based on human Ads, in which the C residues in positions 1, 4 and 7 of the top strand are replaced with G residues would be resistant to mobilization by wild-type Ads [40].

Indeed, transfection of HAdV-5 vectors that harbour the sequence 5'-GATGATG at their genome ends did not result in the formation of plaques as these genomes are unable to replicate in helper cells. In contrast, wild type-ITR containing controls readily formed plaques and induced CPE (Rademaker, Van den Wollenberg, Hoeben, unpublished data), underlining the feasibility of this approach.

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