Relaxed template specificity in fowl adenovirus 1 DNA replication initiation

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INTRODUCTION

Adenoviruses (Ads) are linear, double-stranded DNA (dsDNA) viruses with genomes of 34–48 kb and with terminal proteins (TP) bound covalently to the 5′ ends (Carusi, 1977; Rekosh et al., 1977; Robinson et al., 1973). At the ends, an inverted terminal repeat (ITR) is located, containing the origin of replication (Bernstein et al., 1986; Challberg & Rawlins, 1984; Guggenheimer et al., 1984; Harris & Hay, 1988; Hay, 1985; Lally et al., 1984; Rawlins et al., 1984; Stillman & Tamanoi, 1983; Tamanoi & Stillman, 1983, 1984; van Bergen et al., 1983; Wang & Pearson, 1985; Wides et al., 1987). The Ad genome is replicated from each end of the origin of replication (Bernstein et al., 1986; Challberg & Kelly, 1981). After formation of a pTP–CAT trinucleotide complex, the complex jumps back to allow base pairing of the CAT with the first 3 nt of the template strand. A specific amino acid in pTP, Ser580, is used as a primer for covalent binding of dCMP by Pol, generating pTP–C (Desiderio & Kelly, 1981; Smart & Stillman, 1982). After dissociation of the pTP–CAT trinucleotide complex, the complex jumps back to allow base pairing of the CAT with the first 3 nt of the template strand (King & van der Vliet, 1994). After dissociation of the pTP–Pol complex, elongation can proceed, requiring a third virus-encoded protein, the DNA-binding protein (DBP), which is involved in unwinding the dsDNA ahead of the polymerase (Dekker et al., 1997). 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) (Challberg & Kelly, 1981). The jumping-back mechanism explains the short replication is derived from studies of human adenoviruses 5 and 2 (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 polymerase (Pol), form a stable heterodimer (Enomoto et al., 1981; King et al., 1997b). Although this complex can initiate DNA replication, the initiation is stimulated strongly by the host proteins Oct-1 (Hatfield & Hearing, 1993; Verrijzer et al., 1990) and NF-I (Mul et al., 1990). 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, Ser580, is used as a primer for covalent binding of dCMP by Pol, generating pTP–C (Desiderio & Kelly, 1981; Smart & Stillman, 1982). After dissociation of the pTP–CAT trinucleotide complex, the complex jumps back to allow base pairing of the CAT with the first 3 nt of the template strand (King & van der Vliet, 1994). After dissociation of the pTP–Pol complex, elongation can proceed, requiring a third virus-encoded protein, the DNA-binding protein (DBP), which is involved in unwinding the dsDNA ahead of the polymerase (Dekker et al., 1997). 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) (Challberg & Kelly, 1981). The jumping-back mechanism explains the short
3 bp (infrequently 2 or 4 bp) 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 the work of King & van der Vliet (1994), 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 (King & van der Vliet, 1994). 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 avianadenoviruses conform to the C rule, there is an exception in fowl adenovirus 1 (FAdV-1). Isolates PHELPS (GenBank accession no. U46933) of the chicken embryo lethal orphan (CELO) disease-causing Ad and KUR (GenBank accession no. M57604) are unique among Ads in that their genomes start with the sequence 5’-CATGATGATG. Alignment of the KUR sequence showed that it was 99% identical to that of isolate PHELPS within the first 168 bp of the right ITR. Intriguingly, another FAdV-1 isolate, OTE (GenBank accession nos K00939 and K00940), of which only the sequence of the first 68 bp of the ITR has been published (Shinagawa et al., 1983), is distinct from PHELPS and KUR in that it conforms to the Ad convention and starts with the sequence 5’-CATCATC.

The genome sequence of the PHELPS isolate was determined completely by Chiocca et al. (1996). 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 those of the mastadenoviruses. Open reading frames (ORFs) homologous to HAdV-5 DBP, pTP and Pol could be identified in PHELPS (GenBank accession nos AAC54914, AAC54905 and AAC54904, respectively). The 54 bp ITRs are relatively short in PHELPS and KUR in that it conforms to the Ad convention.

The unconventional occurrence of G nucleotides at positions 1, 4 and 7 of the ITRs 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 isolates PHELPS and OTE and characterized their pTP and Pol genes. In addition, we provide evidence that these viruses have a relaxed, rather than a changed, template specificity.

**METHODS**

**Tissue culture and virus propagation.** PHELPS virus was obtained from the ATCC (VR-432) and OTE was kindly provided by Dr. Matt Cotten (Institute of Molecular Pathology, 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 glucose 1⁻¹ in a 5% CO₂ atmosphere at 37°C. Dilutions of PHELPS or OTE were added to confluent LMH cells in DMEM supplemented with 2% horse serum (HS), antibiotics and 3 g glucose 1⁻¹. Two hours post-infection, the inoculum was replaced with DMEM supplemented with 8% FCS, antibiotics and 3 g glucose 1⁻¹. In all of these media, antibiotic concentrations were 100 000 IU penicillin 1⁻¹ and 100 mg streptomycin 1⁻¹. When almost all cells showed cytopathogenic effect (CPE), approximately 3 days post-infection, the cells were harvested in PBS supplemented with 2% HS and freeze–thawed to release virus particles. These freeze–thaw lysates were used for reinfections and minireplicon assays.

**ITR sequence determination.** Virus particles from the freeze–thaw lysates were purified by CsCl density-gradient centrifugation as described previously (Fallaux et al., 1996). Isolated particles were incubated with protease K in 0-2% SDS, 8 mM EDTA, and viral DNA was extracted by phenol/chloroform and ethanol precipitation.

**Primer-extension assay.** Primer CELO-PE was radiolabelled by using T4 polynucleotide kinase and [⁻³²P]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% polyacrylamide gel. For detection of the radiolabelled fragments, Kodak XAR film was used.

**Terminal transferase.** A poly(A) tail was added to the 3’ end of the viral termini by using terminal transferase (Promega). The first 800 bp of the left ITR was amplified by PCR using the primers MiniCeloL-AS and oligo-dT-20 (see Table 1). The PCR fragments were cloned in pCR2.1+ by using a TA cloning kit (Invitrogen) and used for sequence analysis.

**Splice-site determination by RT-PCR.** LMH cells were infected with OTE and 18 h post-infection, mRNA was isolated by using an RT-PCR miniprep kit (Stratagene). For the synthesis of cDNA, 1 μg mRNA was used in a reverse transcriptase reaction (Promega), using the RTPctp-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 using Taq polymerase (Perkin-Elmer). The pTP and Pol PCR products were cloned in the 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 by using the primers CTPt-as and CTPt-s (see Table 1) in a standard PCR. The 2-kb PCR product was digested with HindIII and EcoRI (MBI Fermentas), cloned in a 5-4 kb HindIII/EcoRI fragment of pCDNA3.1+ and sequenced. Differences from the published PHELPS pTP sequence were confirmed by direct sequencing of viral DNA.

For OTE Pol, a 3-9 kb PCR fragment was amplified by using the primers CELO-pol-s and CELO-pol-as and cloned in a 2-7 kb EcoRV-digested pC208H vector. This vector was used to sequence the OTE Pol gene.

**Minireplicon assay.** The left and right genome ends of FAdV-1 OTE were 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 were 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; BamHI sites are underlined in the primer sequence).
Table 1. Synthetic oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>CELO-PE</td>
<td>5'-GGTCATGATTTGGATTAGTTTTTGAGG</td>
</tr>
<tr>
<td>MiniCeloL-AS</td>
<td>5'-AGGGGCGTCGGCGAAGATCTCGTCTAAGAGGAAATACAAGAAAAACAG</td>
</tr>
<tr>
<td>oligo-dT-20</td>
<td>5'-TTTTTTTTTTTTTTTTTTTTT</td>
</tr>
<tr>
<td>RTCPtp-rev</td>
<td>5'-GTTGGCTATCGTAACTCTTCTGCA</td>
</tr>
<tr>
<td>RTCpol-rev</td>
<td>5'-GGTTATTTTTGTAAGCAGGGAGCC</td>
</tr>
<tr>
<td>CelotRTptp-for1</td>
<td>5'-AGAGGGAATCGAGTGGGCC</td>
</tr>
<tr>
<td>CgTP-as</td>
<td>5'-AAAAAGCTTGGCCACCCATGGGACGGGACGATGCTCATACG</td>
</tr>
<tr>
<td>Cpt-s</td>
<td>5'-TGAGATTCTTAGGAGGTCCTGAGTGTC</td>
</tr>
<tr>
<td>CELO-pol-s</td>
<td>5'-GCAGAGGCTGCTGATAGGTCCCGAG</td>
</tr>
<tr>
<td>CELO-pol-as</td>
<td>5'-GATGCTACAAATGGAAGCGCTAC</td>
</tr>
<tr>
<td>CELO-pol-F4</td>
<td>5'-GATCAGCCAAATAGAAGCGTGAG</td>
</tr>
<tr>
<td>CELO-pol-R5</td>
<td>5'-GATCGAGCGGTGCTGCG</td>
</tr>
<tr>
<td>BamITR-C</td>
<td>5'-GCAGGATCCCATCATCTATAATAACCTAAAACTAACGAG</td>
</tr>
<tr>
<td>BamITR-G</td>
<td>5'-GCAGGATCCCATGAGTGATCTAATACCTCAAAAAACTAACGAG</td>
</tr>
<tr>
<td>MiniCeloL-AS</td>
<td>5'-AGGGGCGTGGGCAAATCTGGCTAAGAGGAAATACAAGAAAAACAG</td>
</tr>
<tr>
<td>MiniCeloR-S</td>
<td>5'-TTAGACCGAGTCTCCTCGAGCGAGGCTCCCTATAGACATATATAGAATACTG</td>
</tr>
<tr>
<td>cGFP-for</td>
<td>5'-GGGTCTATTAGTTCTATAGGCCCAT</td>
</tr>
<tr>
<td>cGFP-rev</td>
<td>5'-GGGTCTATTAGTTCTATAGGCCCAT</td>
</tr>
</tbody>
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By virtue of the similarity in the left ITR and right ITR fragments, both products could be linked in a second PCR using either BamITR-C or BamITR-G primers and *Pfu* polymerase (Stratagene). The resulting 2 kb PCR fragment was cloned in a pCR2.1 vector using a TA cloning kit (Invitrogen). The resulting plasmids, miniOTC-C, miniOTG-G, and miniPHELPS-G, were digested with *NraI* and ligated with a 2 kb cytomegalovirus promoter–green fluorescent protein (CMV–GFP) cassette. The CMV–GFP cassette was synthesized by 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. of 1) in DMEM supplemented with 2% HS, antibiotics and 3 g glucose l^{-1} 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 glucose l^{-1} in a 5 % CO_{2} atmosphere at 37 °C. In both of these media, antibiotic concentrations were 100 000 IU penicillin l^{-1} and 100 mg streptomycin l^{-1}. Six hours post-infection, cells were transfected with 1 µg *BamH*I-digested miniOTC-C, miniOTG-G or miniPHELPS-G replicon plasmid with the aid of jetPEI (Polyplus-transfection). Transfection efficiencies of 40–50% were achieved routinely. Viral and minireplicon DNA was isolated 54 h post-infection from LMH cells by using the small molecular DNA isolation procedure of Hirt (1967). From the isolated DNA, 10 µg aliquots were either digested with *DpnI* or *MboI* or left undigested, and loaded on a 1 % agarose gel. After electrophoresis and blotting, the minireplicon fragments were detected by Southern analysis using a radiolabelled GFP fragment as probe. For detection of the minireplicon backbone, a radioactive probe specific for the amp gene was used. It was noted that the presence of adeno-associated virus in the assay severely reduced the efficiency of minireplicon replication.

**RESULTS**

The linear genomes of all Ads characterized to date start with a small, direct repeat of 2, 3 or 4 nt (Fig. 1a). To study the apparent diversity of the terminal sequences, isolates PHELPS and OTE were propagated on LMH cells. To verify the nucleotide sequences of the PHELPS and OTE termini, the left ends 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 by 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 (GenBank accession nos AY421750 and AY421751 for the left and right genome ends, respectively). 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 omitted from the elongation mix, whilst 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 of the terminal sequences in both isolates.

To study whether the difference in initiating nucleotide is correlated with changes in the pTP and Pol sequences, the large exons containing the majority of the ORFs 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 (GenBank accession nos 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, ORFs have been annotated for pTP (E2B pTP, GenBank accession no. AAC54905.1) and polymerase (E2B Pol, GenBank accession no. AAC54904.1). However, in HAdV-5 and HAdV-2, translation of the pTP and Pol messages is complex. The pTP and Pol proteins are translated from distinct messages, but use the same translation start codon. The majority of the coding sequences from pTP and 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 (Shu et al., 1987, 1988).

To determine the structure of the Pol and pTP mRNAs, RT-PCR on RNA isolated from OTE-infected LMH cells was used to characterize the presence of the upstream exon homologous to that of the human Ads. For cDNA synthesis, we used primers RTCPol-rev for Pol and RTCTptp-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 mRNAs and was based on sequence similarity in the shared 5’ untranslated region (UTR) of HAdV-5 pTP–Pol mRNA and PHELPS genomic DNA (Fig. 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 by using the Sim4 algorithm (Florea et al., 1998). Similar to HAdV-5, OTE pTP and Pol use a splice-donor site 3 nt 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 the published PHELPS sequence). The resulting mRNA would encode a 630 aa pTP protein. This is 55 aa larger than the PHELPS pTP, encoded by the annotated pTP ORF (GenBank accession no. AAC54905.1). The additional N-terminal pTP sequence contains the motif [A]-[RHD]-[L]-[T]-[GN]-[Q], conserved in other pTP species. The splice-acceptor site of Pol is located 610 bp upstream of the ORF (identical to nt 10476 in PHELPS) inside the pTP ORF (Fig. 3). However, unlike
HAdV-5, translation starting from the ATG 6 nt 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 (GenBank accession no. AAC54904.1). Both the pTP and Pol start codons conform to the minimal Kozak sequence (taaATGG and GaaATGG, respectively). An overview of the organization is provided in Fig. 3.

Having established the putative ORFs, it was possible to compare the sequence of the OTE pTP and Pol with the published sequence of PHELPS and other Ads (Fig. 4a). We noted two differences between our sequence of PHELPS pTP and the published sequence. Our PHELPS sequence contained Arg325Asn326 rather than Cys325 and Ser326 (numbered aa270 and aa271 in GenBank accession no. 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 Val50 and Asp604 were changed to Leu and Glu, respectively, in OTE. These changes do not result in an altered amino acid charge. In addition, Val50 to Leu maps in the N-terminal part of the protein, which is unlikely to be involved in priming, considering the distance to the dNTP-binding site in TP and the relatively high affinity of the N-terminal pre-part of pTP for binding to the replication enhancer Oct-1 (Botting & Hay, 1999). Taken together, these data suggest that the difference in initiating nucleotide is not caused by a functional change in TP activity.

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

**Fig. 3.** Genomic organization of the E2 region of PHELPS and splicing of E2B in OTE. LMH cells were infected with OTE and, 16 h post-infection, mRNA was extracted and used for RT-PCR to determine splice sites in Pol and pTP. The detected splice products were aligned against the available sequence of PHELPS (GenBank accession no. U46933). Splice-donor and splice-acceptor sites are annotated SD and SA, respectively, followed by the corresponding nucleotide positions based on PHELPS. ORFs are indicated by the light-grey arrows, coding sequences not part of the identified ORF are shaded dark grey and UTRs are hatched. The small arrows depict the approximate positions of the primers used in RT-PCR.
At the initiation of DNA replication, Pol catalyses covalent binding of the first dCMP nucleotide to Ser\textsuperscript{580} of pTP. To exclude the possibility 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\textsuperscript{416} to Gln and His\textsuperscript{455} to Gln (PHELPS, GenBank accession no. AAC54904.1, compared with 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\textsuperscript{587} to His and Gly\textsuperscript{854} to Glu do not map in conserved regions of Pol. The changes Glu\textsuperscript{672} to Gly, Asn\textsuperscript{854} to Ser, Ser\textsuperscript{865} to Asn and Gln and His\textsuperscript{965} to Gln (PHELPS, GenBank accession no. AAC54904.1, compared with 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\textsuperscript{587} to His and Gly\textsuperscript{854} to Glu do not map in conserved regions of Pol. The changes Glu\textsuperscript{672} to Gly, Asn\textsuperscript{854} to Ser, Ser\textsuperscript{865} to Asn and...
Thr^{1203} to Ala all map near the active site of Pol, but only the Ser^{965} to Asn change is located in a conserved region. The localized sequence-similarity alignment algorithm (MACAW; Karlin & Altschul, 1990; Lawrence et al., 1993; Schuler et al., 1991) 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. It is therefore tempting to speculate that the distinct differences at the origin of replication are not caused by a change of template specificity inherent to 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, respectively, would be maintained simply by virtue of the availability of either one of their template sequences, and not by specificity of the pTP and/or Pol.

To test this hypothesis, we constructed minireplicon vectors (Hay et al., 1984) that contain a CMV–GFP expression cassette flanked by 802 bp of the left terminus of the genome and the last 1154 bp of the right end of the genome of OTE. The minireplicon is flanked by BamHI 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 minireplicons 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 the miniPHELPS-G replicon contains the wild-type PHELPS ITRs. To study whether OTE could facilitate replication of the minireplicons, LMH cells were infected with OTE. Six hours post-infection, the cells were washed and BamHI-digested linear miniOTE-C and miniOTE-G replicons were introduced into the cells. Southern analyses were performed on Mbol-, DpnI- or undigested low-molecular-mass DNA extracted from LMH cells 54 h post-infection, using a GFP-specific probe to detect minireplicon DNA (Fig. 5). OTE is able to replicate both miniOTE-C and miniOTE-G replicon.

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**Fig. 5.** Replication of minireplicons by OTE and PHELPS. LMH cells were infected with OTE and, 6 h post-infection, they were washed and transfected with miniOTE-C or miniOTE-G plasmids digested with BamHI. The DNA-isolation method of Hirt (1967) was used to extract low-molecular-mass DNA from the LMH cells 54 h post-infection. The isolated DNA was digested with DpnI (D, digests input DNA) or 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 replicons without co-infection. (a) The minireplicons 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 minireplicon fragment. (b) To confirm full digestion by DpnI, a probe for the amp gene was used to detect the miniOTE backbone. (c) Replication of minireplicons with OTE ITRs with terminal C or G (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 full digestion by DpnI, a probe for the amp gene was used to detect the minireplicon plasmid backbones. In the last lane, BamHI-digested miniOTE-C plasmid was loaded.
constructs with equivalent efficiency, as indicated by the DpnI-resistant band (Fig. 5a). Quantification of mini-replicon signals showed that 26 and 18% of the total undigested material was replicated de novo for miniOTE-G and miniOTE-C, respectively. No hybridizing fragment is visible when a probe is used that detects the amp gene residing in the vector fragment (Fig. 5b). This demonstrates clearly that the restriction endonuclease DpnI digested the unreplicated DNA to completion. In addition, it shows that generation of the unmethylated, DpnI-resistant fragments is dependent on the presence of the FAdV-1 ITR sequences. Furthermore, the expected restriction fragments could be detected in the MboI-digested lanes (Fig. 5a), further indicating digestion of unmethylated DNA. The replication of minireplicons was strictly dependent on the presence of OTE, as a DpnI-resistant band or MboI-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 the possibility that differences in mobilization are caused by the sequence differences in the ITR sequence between PHELPS and OTE, a BamHI-digested miniPHELPS-G plasmid was transfected into PHELPS-infected cells. 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 DpnI-digested lanes, confirming the complete digestion of input DNA (Fig. 5d). These data demonstrate that both OTE and PHELPS can drive replication of minireplicons harbouring PHELPS 5’-GATGATG-containing ITRs, as well as minireplicons 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 FAdV-1 were re-evaluated. We confirmed the striking sequence divergence between isolates PHELPS and OTE at positions 1, 4 and 7 by sequencing of PCR-amplified Ad DNA. Primer-extension assays lacking either dGTP or dCTP 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 and 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 that 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, proofreading fails in the first 8–12 bp of the Ad ITR (King et al., 1997a). To correct for deletions or mismatches, Ad utilizes a jumping-back mechanism. Small deletions of 1 or 2 nt can be corrected in this way without compromising replication, as was shown by King & Van der Vliet (1994). 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 cannot prevent mismatch incorporation, at least at positions 3 and 6. The relevance of the jumping-back mechanism in preventing elongation of mismatched DNA *in vivo* remains to be determined. Despite these mechanisms, variations in the Ad ITR sequences have been reported, even within serotypes (e.g. in HAdV-4, see Fig. 1a). Nevertheless, all known Ads contain a C residue at the 5’ end of their ITRs, with the FAdV-1 isolates PHELPS and KUR as the sole exceptions. This suggests another mechanism to prevent mutation of the nucleotides at positions 1, 4 and 7. *In vitro* replication-initiation experiments demonstrate that template DNA where nt 4 is changed (C to A) did result in pTP-C formation (possibly initiated on nt 7), but failed to generate pTP-T complexes (King & van der Vliet, 1994). Furthermore, no elongation occurred on this template. Therefore, HAdV-5 seems to have two independent mechanisms to ensure ITR integrity. On the one hand, template-dependent replication and the jumping-back mechanism ensure 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 of how the different ITRs in the otherwise closely related strains PHELPS and OTE could have arisen. Obviously, the pTP–Pol complex derived from PHELPS must be able to bind dGMP as a substrate during initiation, whereas this complex in OTE uses dCMP and conforms to the conventional Ad initiation site. To test whether 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. As 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 by 55 aa. These additional amino acids have been shown to be essential for the biological activity of HAdV-5 pTP. As the additional amino acids share a conserved motif, [A]-[RHD]-[L]-[T]-[GN]-[Q], with other pTPs, the amino-terminal extended part is probably 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.
ATG translation-initiation codon in the upstream exon cannot be used for translation of the Pol message, as it employs another reading frame. Therefore, unlike 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 with the Pol ORF annotated for PHELPS. The additional sequences do not encode domains conserved in 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 et al. (2003) made for PHELPS.

Sequence comparison of our PHELPS pTP gene with the published PHELPS sequence revealed two amino acid differences. These amino acids are identical in OTE pTP. Comparison of PHELPS and OTE pTP revealed only two amino acid changes: PHELPS Val50 and Asp604 were changed 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 minireplicons miniOTC-C, containing wild-type OTE ITRs, and miniOTE-G, where nt 1, 4 and 7 in the top strand of the ITR have been replaced with Gs. Replication of linearized minireplicon 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 or guanidine residues with equal efficiencies. This replication was independent of the minor differences between PHELPS and OTE ITR sequences and specific for the minireplicon constructs, as a control plasmid lacking pTP–Pol-binding domains did not replicate (data not shown).

The relaxed template specificity of the pTP–Pol complex is a prerequisite 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 FAdV-1 may be exploited for the generation of mobilization-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 (Rademaker et al., 2002). 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 (H. J. Rademaker, D. J. M. Van den Wollenberg & R. C. Hoeben, unpublished data), underlining the feasibility of this approach.

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REFERENCES


