Viable Viruses with Deletions in the Left Inverted Terminal Repeat Define the Adenovirus Origin of DNA Replication

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(Accepted 25 October 1985)

SUMMARY

A series of human adenovirus type 2 genomes with deletions in the left inverted terminal repeat (ITR) have been constructed. Viral genomes that contained a minimum of 45 base pairs (bp) from the terminus of the genome were fully infectious and gave rise to progeny virus which maintained the deletion. In contrast, genomes containing 36 bp or less from the termini of the genome were not infectious. The boundary of a cis-acting element required for viral replication is therefore between 36 and 45 bp from the adenovirus termini and corresponds to the previously identified viral origin of replication, defined using a transfection assay to measure ori activity in vivo. The growth parameters of viruses with deletions in the left ITR were examined. These deletions had no measurable effect on plaque formation or morphology, viral DNA synthesis or early viral mRNA synthesis. Thus, it appears that DNA sequences in the left ITR, outside the replication origin, are completely dispensable for lytic viral growth in tissue culture cells.

INTRODUCTION

Human adenovirus type 2 (Ad2) contains a linear duplex DNA molecule of 35937 base pairs (bp) containing an inverted terminal repeat (ITR) of 102 bp (Arrand & Roberts, 1979) or 103 bp (Shinagawa & Padmanabhan, 1980). The 5'-OH termini of the adenovirus genome are linked via a phosphodiester bond to a β-OH of a serine residue present in the virus-coded 55000 mol. wt. terminal protein (Carusi, 1977; Rekosh et al., 1977; Desiderio & Kelly, 1981; Stillman et al., 1981). DNA synthesis initiates at the termini of the viral genome and proceeds by a strand displacement mechanism, producing a double-stranded molecule and a single-stranded molecule. DNA synthesis re-initiates at the 3' end of the displaced single strand and proceeds in the 5' to 3' direction until a completely double-stranded molecule is formed (Lechner & Kelly, 1977). The presence of ITR sequences gives the displaced single strand the potential to form a 'panhandle' structure in which the ITRs form a region of double-stranded DNA connected by a single-stranded loop (Daniell, 1976). It is not clear whether panhandle formation is an obligatory step for initiation of DNA synthesis on the displaced single strand, but it has been shown that in certain circumstances in vivo, panhandle formation does occur (Stow, 1982; Hay et al., 1984a).

To define the cis-acting DNA sequences required for adenovirus DNA replication in vivo a transfection assay has been described in which linear molecules or ‘mini-chromosomes’ containing adenovirus origins of replication replicate in the presence of adenovirus helper (Hay et al., 1984a). Using mini-chromosomes which contain two identically deleted Ad2 ITRs it has been shown that the Ad2 origin of replication (ori) extends from the terminus of the viral genome to a boundary between nucleotides 36 and 45 (Hay, 1985a). DNA sequences within the terminal 45 bp of the Ad2 genome that are important for ori function were identified by a comparison of the DNA sequence and replicative ability of mini-chromosomes containing DNA from the ITRs of other adenovirus subgroups. Palindromic DNA sequences between nucleotides 26 and 36 were thus identified (Hay, 1985b).

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It is clear that nucleotides from 46 to 103 of the Ad2 ITR are dispensable for ori function on mini-chromosomes transfected into tissue culture cells. However, these sequences may exert a more subtle influence on viral DNA metabolism that is not measured by the transfection assay. We have therefore adopted a genetic approach to analyse the function of the adenovirus type 2 ITR. Classical genetic analysis of viruses has involved random mutagenesis and selection of mutants with the desired phenotype. More recently, direct manipulation of the viral genome has allowed the construction in vitro of viral genomes with defined alterations at pre-selected sites. Given a knowledge of the sequence of the viral genome it is therefore possible to perturb specific sites in a DNA molecule and determine what effect these changes have on the biology of the virus. These methods of site-directed mutagenesis have been used with great effect to define the simian virus 40 (SV40) ori. Analysis of viable variants and deletion mutants has defined the SV40 ori as a cis-acting element of 65 bp of DNA (Cole et al., 1977; Shenk, 1978; Subramanian & Shenk, 1978; Dimao & Nathans, 1980) located in the region of the genome where bidirectional DNA replication begins (Hay & DePamphilis, 1982; Hay et al., 1984b). Application of this approach to adenoviruses is less straightforward owing to the linear nature, size and presence of proteins covalently linked to each 5' end of the genome. These problems were circumvented by the construction of the Ad5 variant d1309, which has a single XbaI site 1340 bp from the left end of the Ad5 genome (Jones & Shenk, 1979). Mutations can therefore be introduced into the cloned left end which, once reassembled into an intact viral genome in vitro, can be transfected into permissive cells and progeny virus can be isolated (Stow, 1981). We have used this approach to introduce deletions into the left ITR thus defining the Ad2 ori and establishing the role of the ITR in the life cycle of the virus.

**METHODS**

**Cells and viruses.** The 293 cell line (Graham et al., 1977) was grown in Glasgow-modified Eagle's medium containing 10% foetal calf serum. Monolayer cultures of HeLa cells were maintained in Dulbecco-modified Eagle's medium containing 7% calf serum. Ad2 and Ad5 d1309 (Jones & Shenk, 1979) were grown and titrated on these cells by the method of Williams (1970). Virus was purified on a glycerol/caesium chloride gradient (Mautner & Wilcox, 1974) followed by centrifugation to equilibrium in a second caesium chloride gradient. Virion DNA was extracted by the method of Pettersson & Sambrook (1973).

**Construction of plasmids.** Plasmid R15 (Tet') contains the Ad2 SsrI D fragment (0 to 4.9 map unit) in which the viral terminus is linked by an 18 bp GC tail to an EcoRI site (Stow, 1982). R15 was cleaved with EcoRI and XbaI, ligated to EcoRI- and XbaI-cleaved pUC13 and the products were used to transform Escherichia coli c600. Plasmids containing the viral EcoRI/XbaI fragment (pREX) were isolated from ampicillin-resistant colonies. To introduce a BamHI site adjacent to the ITR, pREX was partially cleaved with HphI, treated with T4 DNA polymerase and dNTPs and linear molecules were ligated to a BamHI linker. The ligated products were used to transform E. coli c600 and a plasmid containing a single BamHI site isolated from an ampicillin-resistant colony (pBEX). To create a plasmid in which the EcoRI site is immediately adjacent to the adenovirus terminus, pBEX was cleaved with BamHI and EcoRI and the large fragment isolated from a low melting point agarose gel. This fragment was ligated to the small fragment generated by EcoRI and BamHI cleavage of pHR1 (Hay, 1985a). pEX was thus isolated from transformed E. coli c600 and contains the terminal Ad2 XbaI fragment in which the terminal C of the adenovirus genome is part of a reconstructed EcoRI site and the ITR is bounded internally by a BamHI site (Fig. 1). The scheme used to introduce deletions into the ITR is shown in Fig. 1. A previously described series of deletions created by Bal 31 exonuclease was used as a source of deleted ITRs (Hay, 1985a). These deleted ITRs present in bacteriophage M13 (mHRnA, where n represents the number of bp from the terminus of the ITR remaining) were released from the M13 replicative form by cleavage and ligated onto the large EcoRI, BamHI fragment of pEX. The ligated products were used to transform E. coli c600 to ampicillin resistance and plasmids containing deleted ITRs isolated (pEXnA where n is the number of nucleotides of the ITR remaining).

**Construction of viral genomes.** Plasmids containing deleted ITRs (pEXnA) were cleaved with EcoRI and XbaI and treated with bacterial alkaline phosphatase (BAP). A tenfold molar excess of cleaved pEXnA DNA was ligated to the large XbaI fragment of d1309 which had been purified by sucrose gradient sedimentation (Stow, 1981). The ligated products were used to transfect two 50 mm dishes of 293 cells (Hay et al., 1984a) which were overlaid with agar (Williams, 1970) and incubated at 37 °C. Well-separated plaques were picked and used to prepare purified virus (Mautner & Wilcox, 1974).

**Structural analysis of viral genomes.** Viral DNA isolated from purified virions was cleaved with BamHI and labelled with [32P]dCMP in the presence of the Klenow fragment of DNA polymerase I. The entire reaction
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Fig. 1. Construction of viral genomes with deletions in the left ITR (shaded). Deletions (dl, represented by Δ) were present in the replicative form of mHRnΔ (Hay, 1985a). The large XbaI fragment of d1309 was isolated by sedimentation in sucrose gradients. Construction of pEX is described in the text. The HphI recognition site (in parentheses) at the boundary of the ITR is retained in this construction and a BamHI linker inserted at the cleavage site.

mixture was fractionated on a 12% polyacrylamide gel which was then dried and autoradiographed. In the hybridization analysis, purified viral DNA was cleaved with HphI and fractionated on a thin (0.3 mm) polyacrylamide gel. The DNA was denatured in situ and electroblotted in 5 mM-Tris–borate pH 8.3 on to a Pall Biodyne membrane at 400 V for 15 min in a Bio-Rad apparatus. The DNA was linked to the membrane by exposure to a short-wave u.v. source (Church & Gilbert, 1984). Hybridization, using the ITR isolated from pHRI as probe, and washing of the membrane were as described (Hay et al., 1984a).

Analysis of viral DNA synthesis. Fifty mm plates of HeLa and 293 cells were infected with 20 p.f.u./cell of purified virus. At various times after infection cells were harvested, viral DNA was selectively extracted (Hirt, 1967), denatured with alkali and spotted on to nitrocellulose filters. The membranes were baked, hybridized to nick-translated (Rigby et al., 1977) Ad2 DNA and washed as previously described for Southern blots (Hay et al., 1984a). Viral DNA synthesis was quantified by determining the 32P radioactivity present in each spot by liquid scintillation counting.

RNA preparation and analysis. Ninety mm plates of HeLa cells were infected with 25 p.f.u./cell of purified virus for 60 min at 37 °C. Medium containing 2% calf serum and 25 μg/ml arabinocytosine (araC) was then added and incubation continued for 5 h at 37 °C. Infected cells were harvested and total cytoplasmic RNA prepared (Everett, 1984). For Northern type analysis 30 μg of cytoplasmic RNA was fractionated in a 1.5% agarose gel containing
Table 1. Infectivity of deleted viral genomes*

<table>
<thead>
<tr>
<th>Plasmid DNA used in reconstruction</th>
<th>Number of plaques (duplicates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pREX</td>
<td>117, 102</td>
</tr>
<tr>
<td>pBEX</td>
<td>86, 98</td>
</tr>
<tr>
<td>pEX</td>
<td>104, 90</td>
</tr>
<tr>
<td>pEX58Δ</td>
<td>79, 88</td>
</tr>
<tr>
<td>pEX45Δ</td>
<td>95, 107</td>
</tr>
<tr>
<td>pEX36Δ</td>
<td>1, 1</td>
</tr>
<tr>
<td>pEX18Δ</td>
<td>0, 0</td>
</tr>
<tr>
<td>pEX8Δ</td>
<td>1, 0</td>
</tr>
<tr>
<td>pUC13</td>
<td>0, 1</td>
</tr>
</tbody>
</table>

* One μg of purified dl309 XbaI A fragment was ligated to 1 μg of the test plasmid which had been cleaved with EcoRI and XbaI and treated with BAP. The ligation reaction was divided in two and each aliquot was used to transfect a 50 mm dish of 293 cells. Plaques were counted 7 days post-infection.

To construct viruses with deletions in the left ITR we utilized the adenovirus type 5 variant dl309 (Jones & Shenk, 1979) which contains a single XbaI site located 3.7% from the left end of the genome. In a procedure originally described by Stow (1981) mutations are introduced into the cloned XbaI fragment which is ligated to the large genome-derived XbaI fragment. Reconstructed genomes are then transfected into tissue culture cells and their infectivity assessed. Our objective was to construct viral genomes in which part of the left ITR had been deleted. To accomplish this the EcoRI/XbaI fragment of plasmid R15 (Stow, 1982), representing 0 to 3.7% of the adenovirus genome, was first transferred to pUC13 and the resulting plasmid designated pREX. A BamHI linker was inserted at the HphI site located at the internal boundary of the left ITR. However, the resulting plasmid pBEX contains an additional 18 GC bp between the EcoRI site and the adenovirus terminus. The ITR was therefore removed from pREX and replaced with the ITR from pHRI in which the terminal C of the viral genome is part of a reconstructed EcoRI site (Hay, 1985a). Plasmid pEX therefore contains the left 0 to 3.7% of the adenovirus genome in which the ITR is flanked by EcoRI and BamHI sites.

In a previous study a series of deletions were introduced into the Ad2 ITR present in the bacteriophage vector M13 (Hay, 1985a). These bacteriophages, designated mHRnΔ (Fig. 1), were utilized as sources of deleted ITRs which could be transferred into pEX. A series of plasmids was therefore constructed in which the 3.7% Ad2 XbaI fragment contained deletions extending from the internal boundary of the ITR toward the genome terminus (pEXnΔ; Fig. 1). Viral genomes containing these deletions were reassembled by ligating the EcoRI/XbaI fragment of pEXnΔ on to the large fragment of dl309.

Infectivity of deleted viral genomes

Reassembled viral genomes with deletions in the left ITR were transfected into 293 cells and after 7 days the number of plaques was determined. The results of a single representative experiment are presented in Table 1. It should be noted that considerable variation in transfection efficiency was experienced between separate experiments (threecfold range) but results within an individual experiment were completely consistent. Regardless of transfection efficiency all experiments gave the same result: large numbers of plaques were obtained with pREX, pBEX, pEX, pEX58Δ and pEX45Δ whereas background levels of plaques were obtained with pEX36Δ, pEX18Δ, pEX8Δ and pUC13 (Table 1). These data indicate that additional
sequences present at the genome termini (pREX) and the insertion of a BamHI linker at the internal boundary of the ITR (pBEX and pEX) had no effect on infectivity of the viral genomes. Deletion of sequences from the internal boundary of the ITR up to 45 bp from the genome termini also had no effect on infectivity of the viral genomes (pEX58Δ, pEX45Δ). Comparison of plaques produced with pREX and pEX45Δ failed to demonstrate any significant variation in plaque size or morphology. The background level of plaques produced with pEX36Δ indicated that sequences located between 36 and 45 bp from the genomic termini are critical for genome infectivity. To determine the structure and properties of the deleted viruses, four well-separated plaques were picked from each plate and propagated on 293 cells.

**Structure of deleted viral genomes**

Virus from individual plaques was grown on 293 cells and DNA isolated from purified virions. All constructed viral genomes should give rise to progeny virus containing a plasmid-derived small XbaI fragment from Ad2 and a large XbaI fragment from Ad5 (d1309). It is therefore possible to distinguish between a virus constructed with plasmid-derived DNA and the parental virus (Ad5, d1309). The recognition sequence for HaeII is present at position 149 on the Ad2 genome but is absent from the corresponding position on the Ad5 genome. Viral DNAs were therefore initially screened for the presence of the HaeII cleavage site at position 149. The site was present in vREX, vBEX, vEX, vEX58Δ and vEX45Δ (data not shown). Virus isolated from the few plaques obtained when genomes were reconstructed with pEX36A, pEX18A, pEX8A and pUC13 lacked this HaeII site (data not shown) and therefore represent parental d1309. This could be due to the presence of a small amount of full-length d1309 DNA in the large XbaI fragment preparation or to incomplete separation of the small d1309 XbaI fragment from the large d1309 XbaI fragment.

To determine that the constructed viral genomes had deletions within the ITR of the predicted size, purified viral DNA was cleaved with BamHI, labelled with [32P]dCMP and analysed by PAGE (Fig. 2a). Replacement of the HphI site at the boundary of the ITR by a BamHI linker in vEX was demonstrated by the appearance of a 111 bp DNA fragment after BamHI cleavage (Fig. 2a, lane 3). Deletion of sequences within the ITR (vEX58A, vEX45A) resulted in the appearance of smaller labelled DNA fragments after BamHI cleavage (Fig. 2a, lanes 1 and 2).

The structure of both the left and right ITR can be determined by HphI cleavage and electroblotting. The HphI recognition sequence is located just inside the Ad2 ITR but the cutting site is just outside the ITR. Deletions within the left ITR therefore remove the HphI recognition sequence, which should still be present within the right ITR. vEX, vEX58Δ and vEX45Δ were all cleaved with HphI, fractionated in a polyacrylamide gel and the denatured DNA was transferred to a nylon membrane. A nick-translated 111 bp fragment containing the Ad2 ITR isolated from pHRI was used as a probe to detect sequences derived from the ITR. Cleavage of vEX with HphI resulted in the appearance of a single 106 bp hybridizing species (Fig. 2b, lane 3). Since the HphI recognition sequence occurs within the ITR, identical fragments are produced from each end of the genome. Cleavage of vEX58Δ and vEX45Δ results in the appearance of a 490 bp hybridizing species in addition to the 106 bp fragment, which is reduced in intensity with respect to vEX (Fig. 2b, lanes 1 and 2). The 490 bp bands appear as a consequence of the deletion within the ITR which removes the HphI recognition sequence and therefore creates an HphI terminal fragment of approximately 490 bp which contains the ITR. It is therefore concluded that in the absence of DNA sequence data the constructed viruses have the predicted structure with one intact ITR and a deleted ITR, although the presence of additional silent mutations cannot be ruled out.

**Growth properties of viruses containing deleted ITRs**

Comparative one-step growth experiments were carried out for vREX, vEX58Δ and vEX45Δ over a 48 h period at 37 °C. Virus titres were determined on 293 cells and HeLa cells were infected at a m.o.i. of 10 p.f.u./cell (no significant differences in particle to p.f.u. ratios were
observed). It is evident from Fig. 3 that vREX, vEX58A and vEX45A all produce virus with similar kinetics and reached an almost identical plateau level. Thus, DNA sequences located between 45 and 103 bp of the left ITR are not required for viral growth, at least in tissue culture.

**DNA replication of viruses containing deleted ITRs**

DNA replication was compared in 293 cells infected with pREX, pEX58A and pEX45A over a 48 h period at 37 °C. Infected cells were harvested at various times after infection and viral DNA was selectively extracted (Hirt, 1967; Hay et al., 1984a). Extracted DNA was spotted on to nitrocellulose and the filter probed with nick-translated Ad2 DNA. After autoradiography the spots were cut out and radioactivity was determined by liquid scintillation counting. Inspection of Fig. 4 indicates that for all three viruses the kinetics of DNA replication are indistinguishable. A similar result was also obtained on HeLa cells. Deletion of sequences within the ITR up to 45 bp from the terminus had no deleterious effect on viral DNA replication.

**Synthesis of RNA by viruses containing deleted ITRs**

Transcription of the adenovirus E1a region is controlled by upstream sequences which lie close to the ITR (Hearing & Shenk, 1983). It was therefore of interest to determine the effect that deletions within the ITR had on E1a transcription. HeLa cells were either infected with vREX,
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Fig. 3. One-step growth curves of viruses with deletions. Duplicate 50 mm plates of HeLa cells were infected with 10 p.f.u./cell of purified virus. After 1 h adsorption at 37 °C the monolayers were washed twice with Tris-buffered saline and incubated at 37 °C in medium containing 2% calf serum. At various times after infection cells were scraped into the medium, collected by sedimentation and disrupted by three cycles of freezing and thawing in 1 ml Tris-buffered saline. The yield of virus was determined by titration on HeLa cells as described by Williams (1970). O, vREX; □, vEX45Δ; △, vEX58Δ.

vEX58Δ, vEX45Δ or mock-infected. Five h after infection at 37 °C in the presence of araC total cytoplasmic RNA was isolated, and the level of E1α transcription determined by Northern analysis. RNA was fractionated in a 1.5% agarose gel containing formaldehyde, transferred to nitrocellulose and hybridized to the nick-translated 1.3 kb EcoRI–XbaI fragment from pREX. It is evident from Fig. 5 and other similar experiments that there was no significant difference in the levels of the 12S and 13S E1α-specific RNA species. Thus, it appears that sequences in the ITR outside the replication origin are not involved in the control of viral transcription.
DISCUSSION

A series of adenovirus genomes with deletions in the left ITR have been constructed. Viral genomes which contained a minimum of 45 bp from the terminus of the genome were fully infectious whereas genomes containing 36 bp or less from the termini of the genome were not infectious (Table 1). The only plaques observed on plates transfected with genomes containing only 36 bp of the ITR were due to parental d1309 virus. After 14 days, not even small plaques were observed. Structural analysis of viral genomes indicated that the ITR deletions had been maintained in the progeny virus. In contrast, transfection with mutants bearing deletions introduced at the termini of the viral genomes gives rise to progeny viruses that have repaired the deletion (Stow, 1982). In this case, however, it is envisaged that during panhandle formation the undeleted ITR serves as a template for the extension of the deleted 3' end, thus restoring the deleted sequences. Subsequently, initiation of DNA synthesis on the panhandle would generate a double-stranded molecule with completed ITRs. This scheme is inoperative when the ITR is deleted internally, since the 3' end is present at the viral terminus during panhandle formation. The boundary of a cis-acting element required for viral replication can therefore be placed between 36 and 45 bp from the adenovirus terminus. This corresponds to the previously described viral origin of replication, defined using an in vivo transfection assay for ori activity (Hay, 1985a, b). Modified viral genomes contain one deleted ITR and one complete ITR.
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Fig. 5. Synthesis of Ela-specific RNA by viruses with deletions. Ninety mm plates of HeLa cells were infected with 25 p.f.u./cell of purified virus. After 1 h adsorption at 37 °C incubation was continued at 37 °C in medium containing 2% calf serum and 25 µg/ml araC. Five h after infection total cytoplasmic RNA was prepared and Ela transcripts were determined by Northern-type analysis (details in text) using the nick-translated 0 to 3.7 map units EcoRI, XbaI fragment from pEX as probe. 12S and 13S RNAs are not resolved in this gel system. Lane 1, vREX; lane 2, vEX58A; lane 3, vEX45A; lane 4, uninfected; lane 5, 0.2 ng EcoRI, XbaI fragment from pEX.

Fig. 6. Structure of the Ad2 ITR. The boundary of ori is defined by the viable deletion vEX45A; viral genomes constructed with pEX36A are non-viable. The core region of ori contains the highly conserved sequence ATAATATACC (II) located between nucleotides 9 and 18. Also recognizable in ori is a palindromic region (— — —) which contains the binding site for NFI. In the non-essential region of the ITR is the sequence GGGXGGAG (D) that is conserved among adenoviruses and is present in the SV40 origin of replication (Stillman et al., 1982a). Also indicated is the sequence TGACGT (N) which is highly conserved among the human adenoviruses.

initiation of DNA replication on the undeleted ITR should therefore be normal and give rise to a displaced single strand. Since viral genomes containing only 36 bp of the ITR are non-infectious it follows that the DNA sequence requirement for initiation on the displaced strand is identical to that observed for the completely double-stranded molecule. Recognizable within the adenovirus origin of DNA replication (Fig. 6) is a palindromic region between nucleotides 24 and 38 and a 10 bp sequence between nucleotides 9 and 18 that is present in all human adenoviruses (Stillman et al., 1982a) and highly conserved among adenoviruses from other species (Alestrom et al., 1982). The role of these domains within the adenovirus origin of replication has been established by in vitro studies and transfection experiments in vivo.

In vitro studies have demonstrated that adenovirus DNA synthesis is initiated by a template-dependent transfer of dCMP to the viral preterminal protein catalysed by the viral DNA polymerase (Lichy et al., 1982; Stillman et al., 1982b). Using protein-free DNA molecules derived from the termini of the adenovirus genome as templates for in vitro DNA synthesis it was possible to define the minimal DNA sequence requirement for the initiation of viral DNA replication as the terminal 18 bp of the viral genome (Tamanoi & Stillman, 1983; van Bergen et al., 1983; Challberg & Rawlins, 1984; Lally et al., 1984). Additional DNA sequences between nucleotides 19 and 48 were shown to greatly enhance the efficiency of in vitro replication. This enhancement of in vitro DNA replication is dependent on the presence of a cellular protein,
nuclear factor I (NFI) (Nagata et al., 1983), and is a direct consequence of NFI binding to DNA sequences between nucleotides 19 and 48 (Guggenheimer et al., 1984; Rawlins et al., 1984; Leegwater et al., 1985). DNA sequence analysis of NFI binding sites in cellular DNA and the genomes of other viruses has shown that each binding site contains the sequence TGG(N)₆₆GCCAA, which is present in the palindromic region of the Ad2 ori (Borgmeyer et al., 1984; Siebenlist et al., 1984; Gronostajski et al., 1985; Hennighausen et al., 1985; Nowock et al., 1985). Methylation protection studies have demonstrated that NFI interacts with the guanine residues present in the consensus sequence described above (Gronostajski et al., 1985). Experiments described in this paper and transfection experiments in vitro have also pinpointed DNA sequences in this region as being important for ori function (Hay, 1985b). A point mutational analysis has further refined the DNA sequence requirements for NFI binding and stimulation of DNA replication in vitro (de Vries et al., 1985). Viral genomes containing the terminal 36 bp of the viral genome are not infectious and mini-chromosomes containing this sequence fail to replicate (Hay, 1985a). This deletion removes the two adenine residues in the NFI consensus sequence and thus stresses the importance of this site for ori function.

The isolation of viable viruses with deletions in the left end (vEX45Δ and vEX58Δ) allows investigation of the possible role of the deleted sequence. It was, however, surprising to find that deletion of these sequences had no measurable effect on plaque formation or morphology, viral DNA synthesis or viral RNA synthesis. Thus, it appears that these sequences are completely dispensable for lytic viral growth in tissue culture cells. This could be due to the presence of a copy of these sequences in the other ITR or to the presence in the left end of the genome of functionally equivalent DNA sequences. Recognizable within the non-essential region of the ITR are GC-rich sequences which are conserved among adenoviruses (Fig. 6) and are homologous to elements of the SV40 control region which contains the origin of replication and the promotors for early and late viral mRNA synthesis (Stillman et al., 1982). The role of another conserved element, TGACGT (Fig. 6), remains equally obscure. One feature common to all adenoviruses is the presence of inverted terminal repeats, which in the case of the human adenoviruses are about 100 bp. The conservation of ITRs in this size range is puzzling given that vEX45Δ grows perfectly well in tissue culture and yet has an ITR of only 45 bp. In fact, this may not be the lower level since the Ad4 ori is contained entirely within the terminal 18 bp of the genome (Hay, 1985b). If the only requirement is the presence of an identical origin of replication at each end the genome it may therefore be possible to construct viable Ad4 variants in which the ITR is reduced to 18 bp.

We thank Professor J. H. Subak-Sharpe for critical analysis of the data and for encouragement during this work and Professor W. C. Russell for critically reading the manuscript. We are particularly grateful to Nigel Stow for many valuable and enjoyable discussions on the topic of this publication.

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(Received 1 August 1985)