Adenovirus sequences required for replication *in vivo*

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ABSTRACT

We have studied the *in vivo* replication properties of plasmids carrying deletion mutations within cloned adenovirus terminal sequences. Deletion mapping located the adenovirus DNA replication origin entirely within the first 67 bp of the adenovirus inverted terminal repeat. This region could be further subdivided into two functional domains: a minimal replication origin and an adjacent auxillary region which boosted the efficiency of replication by more than 100-fold. The minimal origin occupies the first 18 to 21 bp and includes sequences conserved between all adenovirus serotypes. The adjacent auxillary region extends past nucleotide 36 but not past nucleotide 67 and contains the binding site for nuclear factor I.

INTRODUCTION

The type 2 adenovirus genome is a linear, double-stranded DNA molecule containing 35,937 base pairs (1). DNA molecules from all adenovirus serotypes have a terminal protein (2) and an inverted terminal repetition (ITR) (3, 4). ITRs differ between serotypes both in sequence and length, but all ITRs share conserved or highly homologous sequences (5). These unusual terminal structures are important for adenovirus DNA replication (for recent reviews see refs. 6-8). Stow (9) has shown that the complete removal of one ITR renders adenovirus non-viable. However, it is not obvious which, if any, sequences within the ITR are required for viability since terminal deletions penetrating, but not removing, one of the ITRs can be repaired efficiently during replication to yield wild-type progeny. Recently, Hay *et al.* (10) demonstrated that linear plasmid DNA molecules containing adenovirus terminal sequences at each end replicate autonomously when introduced into cells together with adenovirus DNA as a helper. Linear molecules with a single adenovirus terminus do not replicate under these conditions, but they can give rise to replication-proficient molecules by regenerating adenovirus termini at both ends. The regeneration of adenovirus ends requires specially constructed plasmids containing a specific geometrical arrangement of inverted repetitions

sequences (these repeats need not even be adenovirus sequences). The production of replication-proficient plasmid molecules provides a sensitive assay to determine which adenovirus terminal sequences are required in cis for replication. A clear advantage of this assay is that the replication products differ in size from the input molecules. With this assay we locate the adenovirus replication origin entirely within the first 67 nucleotides of the ITR using unidirectional deletion mutations penetrating from within adenovirus sequences toward the terminus. Moreover, this region can be divided into at least two functional domains. The first domain, termed the minimal replication origin, spans the first 18 to 21 nucleotides and includes sequences highly conserved between all adenovirus serotypes (5). The second domain increases the efficiency of the minimal origin by more than 100-fold. This auxiliary region lies immediately adjacent to the minimal origin and encompasses sequences identified as the binding site for nuclear factor I (11, 12), a cellular protein required for efficient initiation of adenovirus replication in vitro (12-15). We discuss the requirement for these adenovirus sequences for DNA replication in vivo and in vitro (12, 15-21).

MATERIALS AND METHODS

Enzymes. Restriction endonucleases, E. coli DNA polymerase I (and the Klenow fragment), T4 DNA ligase, and S1 endonuclease were from Bethesda Research Laboratories. Pronase was from Calbiochem-Boehringer Corp. The enzymes were used as recommended by the suppliers.

Cells and virus. The HeLa cell line was maintained in suspension culture in Joklik's minimal essential medium (GIBCO) containing 5% fetal calf serum. Adenovirus type 5 was grown in HeLa cells, and virus and viral DNA were purified as previously described (22). The 293 cell line (23) was maintained in monolayer culture in Dubecco's minimal essential medium (GIBCO) containing 10% fetal calf serum.

Bacteria and plasmids. E. coli JM83 (24) and E. coli JC9387 recBC sbcB (25) were grown, transformed, and selected by standard techniques (26). Selection was in the presence of ampicillin (50 µg/ml). Plasmids pUC8 (27), pMDC10 (12, 19, 21), and deletion mutants d17 through d167 (12, 19, 21) have been described. Prior to plasmid DNA isolation (28), plasmid DNA was amplified by growth in chloramphenicol (100 µg/ml). Plasmid DNA was purified by ethidium bromide-CsCl density gradient centrifugation.

Construction of plasmids containing inverted repeat sequences. Fig. 1 outlines the method to construct a plasmid containing cloned adenovirus

Figure 1. Construction of plasmid pIR10. pUC8 was cleaved with **EcoRI**, and the **EcoRI** site was blunted by treatment with **S1** endonuclease followed by repair with the Klenow fragment of DNA polymerase I. After cleavage with **BamHI**, the vector backbone was isolated and ligated to a 450 bp **BamHI-PvuII** fragment purified from pMDC10. The open boxes represent inverted repeat DNA where the arrows indicate orientation. The thick line corresponds to adenovirus DNA. pUC8 DNA is shown as the thin line. Not all restriction cleavage sites are indicated. See Fig. 3 for the detailed structure of pIR10.

terminal sequences flanked by inverted repeats. The scheme exploits the fact that re-cloning adenovirus terminal sequences and adjacent pUC9 sequences from pMDC10 into pUC8 will generate inverted repeats surrounding the adenovirus sequences. In order to prepare the appropriate vector backbone, pUC8 was cleaved with **EcoRI**, the cohesive sites were removed by **S1** endonuclease treatment, and the blunt ends were polished with the Klenow fragment of DNA polymerase I. After cleavage with **BamHI**, the linear vector backbone terminating with a **BamHI** cohesive site and a blunt end was isolated. Cleavage of pMDC10 by the combined action of **BamHI** and **PvuII** yielded many fragments due to the presence of several **PvuII** sites. A 450 bp **BamHI-PvuII** fragment carrying adenovirus sequences together with adjacent pUC9 sequences was purified from the total digest and ligated to the specially prepared vector

Figure 2. Construction of plasmid pIRd167. pIR10 was cleaved with BamHI, and the BamHI site was blunted by repair with the Klenow fragment of DNA polymerase I. After cleavage with EcoRI, the vector backbone was isolated and ligated to a 360 bp EcoRI-RsaI fragment purified from plasmid d167. The open boxes represent inverted repeat DNA where the arrows indicate the orientation. The thick line corresponds to adenovirus DNA. Plasmid vector DNA is shown as the thin line. Not all restriction sites are indicated. See Fig. 3 for the detailed structure of pIRd167.

backbone. A 3.2-kb recombinant plasmid, called pIR10 (Fig. 3), was identified in an ampicillin-resistant colony after transformation of E. coli JM83 with ligated DNA. Growth of pIR10 in E. coli JM83 generated at high frequency deleted plasmids lacking inverted repeats and adenovirus sequences. Re-cloning of pIR10 in E. coli JC9387 recBC sbcB prevented this problem (K. Wang et al., manuscript in preparation).

Fig. 2 illustrates the replacement of wild-type adenovirus terminal sequences in pIR10 with unidirectional deletion mutations penetrating from within adenovirus sequences towards the adenovirus terminus. pIR10 was cleaved with BamHI, the cohesive sites were repaired with the Klenow fragment of DNA polymerase I, and the adenovirus sequences were removed by further cleavage with EcoRI. Mutated adenovirus terminal sequences were excised from

Figure 3. Restriction endonuclease maps of pIR10 (left) and pIRd167 (right). The open boxes represent inverted repeat DNA (89 bp) where the arrows indicate the orientation. The thick line corresponds to adenovirus DNA and the thin line shows pUC8 DNA. pIR10 contains adenovirus nucleotides 1 through 355. pIRd167 contains adenovirus nucleotides 1 through 67 fused by a BamHI linker to nucleotides 358 through 639. Other deletion mutants have a structure similar to pIRd167. For example, pIRd17 contains adenovirus nucleotides 1 through 7 fused by a BamHI linker to nucleotides 358 through 639 (see Fig. 8).

plasmids d17, d112, d118, d121, d130, d136, and d167 by the combined action of EcoRI and RsaI (there are several RsaI sites in these plasmids). The appropriate EcoRI-RsaI fragment from each digest was isolated (the fragments ranged from 300 to 360 bp depending on the particular deletion mutant) and ligated to the specially prepared pIR10 backbone. As with pIR10, the initial cloning was done in E. coli JM83, but each plasmid was subsequently moved into E. coli JC9387. One of the clones, pIRd167, is shown in Fig. 3. pIR10 and pIRd167 have identical genome sizes.

Transfection. 293 cells were transfected using the DNA-calcium phosphate coprecipitation method of Graham and van der Eb (29). Each 60 mm plate was seeded with 4×10^5 cells 24 hr prior to transfection. Each plate received 3 μ g type 5 adenovirus DNA and 3 μ g plasmid DNA. Cells were shocked for 1 min with 25% glycerol 4 hr after transfection.

Extraction and analysis of DNA. Nuclei were prepared as described (30) from cells lysed with 1% NP-40 detergent in buffer containing 10 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.4. Total DNA was extracted by incubating isolated nuclei for 2 hr at 37°C in 10 mM EDTA containing 0.6% sodium dodecyl sulfate (SDS) and Pronase at a concentration of 1 mg/ml. After phenol extraction and ethanol precipitation, purified DNA was suspended in buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Approximately 1/5 of the DNA isolated from each plate was applied to each lane of a 1% agarose slab

Figure 4. Generation of a replication-proficient plasmid with two adenovirus termini from a plasmid bearing a single viral terminus. (a) *EcoRI*-linearized 3.2-kb pIR10 (see Fig. 3). The open boxes represent inverted repeat DNA where the arrows indicate the orientation. The thick line corresponds to adenovirus DNA and the thin line shows pUC8 DNA. (b) Initiation of strand displacement replication at the single adenovirus terminus. (c) Production of a daughter double-stranded molecule and a displaced parental strand at the conclusion of displacement replication. (d) Formation of a panhandle structure by base pairing between inverted repeat sequences on the displaced strand. (e) Extension of the 3'-end of the panhandle molecule using adenovirus sequences as a template. (f) Initiation of complementary replication at the adenovirus terminus of the panhandle molecule. (g) Complementary replication. (h) Production of a replication-proficient 3.6-kb daughter molecule with two adenovirus termini at the conclusion of complementary replication.

gel. After gel electrophoresis, the DNA was transferred to a Gene Screen Plus membrane (New England Nuclear) by the method of Southern (31). Hybridization was for 18 hr at 68°C in 5 x SSC, 0.5% SDS, and 1 x Denhardt's solution (26).

Figure 5. Replication of plasmids containing a single copy of adenovirus terminal sequences. Plasmids were linearized and cotransfected with helper adenovirus DNA into 293 cells. Cells were harvested 48 hr after transfection, and the total DNA analyzed by gel electrophoresis and Southern blotting. The probe was nick-translated pUC8. Lane 1: EcoRI-cut pIR10. Lane 2: Bam-cut pIR10. Lane 3: EcoRI-cut pMDC10. Lane 4: EcoRI-cut pIRM (structure shown in Fig. 7). Lane 5: EcoRI-cut pIRBH (see Fig. 7 for structure).

The probe was ^{32}P -labeled pUC8 DNA (specific activity $> 1 \times 10^8$ cpm per μg) prepared as previously described (32). After washing, the membrane was autoradiographed using Kodak SR-5 X-ray film. Autoradiograms were scanned with a Zeineh SL-504-XL soft-laser densitometer and band intensities were integrated by using an electrophoresis reporting integrator program (Biomed).

RESULTS AND DISCUSSION

Hay *et al.* (10) have shown that linear plasmid DNA containing adenovirus terminal sequences at each end replicates autonomously when introduced into cells together with adenovirus DNA as a helper. Linear molecules with a single adenovirus terminus do not replicate under these conditions, but they can give rise to replication-proficient molecules with adenovirus termini at each end if they additionally contain a specific geometrical arrangement of inverted repeat DNA. Fig. 4 diagrams the postulated sequence of events leading to the production of replication-proficient molecules. To test this scheme, 293 cells were transfected with EcoRI-linearized pIR10 DNA (Fig. 3) and helper adenovirus DNA. Fig. 5 shows the Southern blot analysis of total DNA isolated 48 hr after transfection. Two prominent bands were visualized with the nick-translated pUC8 probe (lane 1). The 3.2-kb band, identical to

Figure 6. Replication of plasmids containing deletion mutations within the single copy of adenovirus terminal sequences. Plasmids were linearized with *EcoRI* and cotransfected with helper adenovirus DNA into 293 cells. Cells were harvested 60 hr after transfection, and the total DNA analyzed by gel electrophoresis and Southern blotting. The probe was nick-translated pUC8. Input 3.2-kb plasmids generated 3.6-kb molecules as replication products. The autoradiogram is over-exposed to visualize rare 3.6-kb molecules. Lane 1: pIR10. Lane 2: pIRd167. Lane 3: pIRd136. Lane 4: pIRd130. Lane 5: pIRd121. Lane 6: pIRd118. Lane 7: pIRd112. Lane 8: pIRd17.

the input pIR10 DNA, could be detected throughout infection without significant change in intensity (not shown). The 3.6-kb band was the exact size expected for the replication-proficient product arising from pIR10. It was not visible early after transfection, but its intensity increased with time and by 60 hr equaled or exceeded the intensity of the 3.2-kb band (compare Fig. 5, lane 1 with Fig. 6, lane 1). As expected, the appearance of the 3.6-kb band depended on the presence of helper adenovirus (not shown) to supply in *trans* viral-encoded replication proteins (DNA polymerase, DNA binding protein, and pre-terminal protein). pIR10 must be linearized at the adenovirus origin since *Bam*HI-cut pIR10 (Fig. 5, lane 2) and circular pIR10 (not shown) did not give rise to the 3.6-kb band. Plasmids lacking inverted repeat DNA (pMDC10; Fig. 5, lane 3) or adenovirus terminal sequences (pIRBR,

Figure 7. Summary of sequences required for adenovirus DNA replication *in vivo*. The open boxes represent inverted repeat DNA where the arrows indicate the orientation. The thick line is adenovirus DNA and the thin line is plasmid vector DNA. B = BamHI site. R = EcoRI site. Plasmid DNA was linearized with the indicated restriction endonuclease. The extent of replication was determined from the intensity of the 3.6-kb band in Table 1. ++ = wild type (pIR10) replication. + = more than 1% but less than 50% of wild type replication. ± = less than 1% of wild type replication. - = replication not detected. pIRBR has a 377-bp EcoRI-BamHI fragment of pBR322 in place of adenovirus sequences. pIRM contains a 1080-bp NdeI fragment of M13 inserted into the NdeI site of pIR10. pIRBH contains a 346-bp HindIII-BamHI fragment of pBR322 inserted between the HindIII and BamHI sites of pIR10.

Fig. 7) also failed to generate replication-proficient molecules. These experiments clearly establish a *cis*-requirement both for adenovirus terminal sequences as well as inverted repeat sequences in the same molecule, and suggest that adenovirus replication is necessary both to produce and to amplify replication-proficient plasmid molecules. Fig. 5 also shows the results of experiments exploring changes in the architecture of plasmid molecules. pIRM contains a 1.1-kb insertion at the NdeI site which increases the distance between the inverted repeat sequences. This 4.2-kb molecule efficiently generated a new, 4.6-kb molecule with two adenovirus termini (Fig. 5, lane 4). pIRBH contains a 346-bp insertion between the BamHI and HindIII sites which increases the length between the adenovirus terminus and one copy of the inverted repeat sequences. This 3.5-kb molecule also

Table 1. Replication efficiencies of deletion mutants.

Plasmid	Intensity of 3.6-kb band ^a	
	Experiment 1	Experiment 2
pIR10	(100)	(100)
pIRd167	83	102
pIRd136	23	20
pIRd130	42	40

^aThe data are expressed as percent of the intensity of the 3.6-kb pIR10 band in each experiment. The intensity of each 3.6-kb band (in arbitrary scanning units) was normalized to the intensity of the 3.2-kb band in the same lane to correct for variable efficiencies of transfection, extraction, and blotting of plasmid molecules. The data in Experiment 1 came from a normal exposure of Figure 6 where the normalized intensity of the 3.6-kb pIR10 band was 11,500 units. The normalized intensity of the 3.6-kb pIR10 band in Experiment 2 was 27,900 units. Other deletion mutants could not be quantitated, but we estimated that the 3.6-kb band of pIRd121 could be no more than 1% of the wild-type level.

efficiently produced a new, 4.2-kb molecule with two adenovirus termini (Fig. 5, lane 5). The newly generated adenovirus terminus in this case was 700 bp long compared to 360 bp for pIR10 or pIRM.

The production of replication-proficient plasmid molecules provides a sensitive assay to determine which adenovirus terminal sequences are required in *cis* for replication. A clear advantage of this assay is that the replication products differ in size from the input molecules. Unidirectional deletion mutations penetrating from within adenovirus sequences toward the terminus were moved into plasmid molecules containing inverted repeat sequences as detailed in Fig. 2. Each of the *Eco*RI-linearized deletion mutants (Fig. 3) was tested for replication by transfecting 293 cells together with helper adenovirus DNA. Fig. 6 shows the Southern blot analysis of total DNA isolated 60 hr after transfection. Each input molecule was detected as a 3.2-kb band after visualization with the pUC8 probe. Moreover, each of the 3.2-kb bands, including the band for input pIR10, exhibited roughly equal intensities as expected. However, the mutants produced markedly different yields of replication-proficient molecules as measured by the intensity of the 3.6-kb band in each lane of Fig. 6. The mutants could be sorted into at least

Figure 8. Nucleotide sequences of deletion mutations defining the adenovirus origin. Adenovirus sequences are indicated by upper-case letters and mutated sequences by lower-case letters. Nucleotides 9 through 18 are conserved between all serotypes of human adenoviruses. Nucleotides 19 through 42 constitute the binding site for nuclear factor I.

four groups on this basis. pIRd167 (lane 2), which retains the terminal 67 nucleotides of adenovirus, replicated fully as well as wild-type (pIR10, lane 1; see Table 1). pIRd136 (lane 3) and pIRd130 (lane 4) both clearly gave rise to 3.6-kb molecules, but the yield in each case was less than 50% of the wild-type level (Table 1). pIRd121 (lane 5) and pIRd118 (lane 6) were severely limited in the production of 3.6-kb molecules. pIRd121 generated no more than 1% of the wild-type level, and pIRd118 produced an even lower level (visible on the original autoradiograph, but difficult to detect on the photographic reproduction). No 3.6-kb molecules could be detected after transfection with pIRd112 (lane 7) or pIRd17 (lane 8). These results, summarized in Fig. 7, locate the adenovirus origin within the first 67 nucleotides of the adenovirus inverted terminal repetition. Furthermore, this region can be divided into at least two functional domains as shown in Fig. 8. The first domain, termed the minimal replication origin, spans the first 18 to 21 adenovirus nucleotides and includes sequences highly conserved between all adenovirus serotypes (5). The minimal origin is absolutely required *in cis* for adenovirus replication. Deletion mutations penetrating (pIRd112) or removing (pIRd17) the conserved sequence in the minimal origin totally prevent adenovirus replication. The second domain increases the efficiency of the minimal origin by more than 100-fold. This auxiliary region lies immediately adjacent to the minimal origin and extends past nucleotide 36 but not past nucleotide 67. It encompasses sequences identified as the binding site for nuclear factor I (11, 12), a cellular protein required for efficient initiation of adenovirus replication *in vitro* (12-15). Mutants with altered binding sites for nuclear factor I do not initiate replication *in vitro* in

Figure 9. Hypothetical structure and replication properties of a recombinant plasmid produced by homologous recombination between pIRd130 and helper adenovirus. Closed box = adenovirus ITR (pIRd130 only has the first 30 bp of the ITR). Hatched box = homologous adenovirus sequences located between nucleotides 358 through 639. Box with arrow = inverted repeat sequence where the arrow indicates the orientation.

factor I-dependent assays (12, 15), but do replicate in crude extracts (16-21). The entire binding site is apparently not required for efficient use of the minimal origin *in vivo* since removal of as much as the distal half of the site (pIRd130) only reduced replication 2- to 3-fold (Table 1). Alternatively, sequences flanking deletions in pIRd130 and pIRd136 may partially restore the site for binding nuclear factor I *in vivo*, if not *in vitro* (12). This may explain why pIRd130, with less of the binding site, replicated twice as well as pIRd136 (Table 1). Nevertheless, the binding site for nuclear factor I clearly constitutes an important element of the adenovirus origin. In fact, serotypic (5) and host-specific (20) differences in the efficiency of adenovirus DNA replication may be due to variable binding of nuclear factor I to the adenovirus origin.

It is important to emphasize that we never observed homologous recombination between input plasmids and helper adenovirus molecules. Experiments with the deletion mutants provide a sensitive test for recombination. Fig. 9 shows that plasmids could acquire a wild-type ITR only by recombination within the region of homology corresponding to adenovirus nucleotides 358 through 639. Since all deletion mutants contain this region of homology, recombination cannot explain the different efficiencies of replication (Fig. 6, Table 1). Fig. 9 also shows that recombination would alter the size of the input plasmids from 3.2 kb to 3.5 kb. In fact, such hypothetical recombinant plasmids would resemble pIRBH (Fig. 5, lane 5) both in size as well as structure and, like pIRBH, give rise to 4.2-kb

replication-proficient molecules. We did not detect 3.5-kb or 4.2-kb bands after transfection with deletion mutants (Fig. 6). Moreover, we tested for recombination directly. ITR sequences were removed from all of the deletion mutants by cutting each plasmid with EcoRI and BamHI (see Fig. 3). Such "ITR-less" plasmids still contain adenovirus nucleotides 358 through 639. Replication-proficient molecules could not be rescued by recombination between helper adenovirus and any of the "ITR-less" deletion mutants (not shown).

Fig. 6 also shows that the pUC8 probe visualized higher molecular weight products which correspond in size to oligomers of the input 3.2-kb plasmids. The oligomers presumably arise from end-to-end ligation during transfection rather than by homologous recombination (see above). Only a small fraction of the input molecules apparently become joined together since oligomers were not detected after transfection with plasmids that replicate poorly (lanes 5 and 6) or not at all (lanes 7 and 8). In contrast, oligomers accumulated to observable levels after transfection with plasmids that replicate with wild-type (lanes 1 and 2) or near wild-type efficiencies (lanes 3 and 4). The polarity of joining may be restricted in order to form replication-proficient oligomers. Clues to the probable structures of oligomers come from the observation of 6.4-kb as well as 6.8-kb dimer-sized molecules (6.4-kb bands are visible in lanes 1 through 4, but the faint 6.8-kb bands in the same lanes are difficult to detect on the photographic reproduction of the autoradiogram). A replication-proficient 6.4-kb molecule could form by head-to-head joining to give a simple dimer with adenovirus termini at each end. A simple, tail-to-tail dimer would not be expected to replicate since adenovirus termini would be fused together internally. On the other hand, a head-to-tail dimer could become replication-proficient only by generating a new adenovirus terminus via the pathway outlined in Fig. 4. Such a molecule would be 6.8-kb long. Experiments to confirm the structures of the 6.4-kb and 6.8-kb dimers are in progress.

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ADDENDUM

After this manuscript was submitted, a paper was published by R. T. Hay (EMBO Journal 4, 421-426, 1985) locating the adenovirus origin entirely within the first 45 bp of the ITR. Plasmids containing 36 bp or less of the ITR were totally unable to replicate in vivo. One interpretation of these experiments is that the entire binding site for nuclear factor I is essential for in vivo replication. It is important to emphasize, however, that the deletions constructed by Hay alter not only the sequence but the size of the ITR; that is, mutant 36Δ has inverted terminal repetitions only 36 bp long. Thus, an alternative interpretation is that plasmids with ITRs smaller than 45 bp, regardless of sequence, are not able to replicate. Stow (9) has shown that an adenovirus mutant lacking all but 52 bp of one of the ITRs is still able to repair the deleted ITR efficiently. This may indicate an upper limit for the size of the adenovirus ITR. A lower limit remains to be determined.