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Template Requirements for In Vivo Replication of Adenovirus DNA

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The adenovirus (Ad) DNA origin of replication was defined through an analysis of the DNA sequences necessary for the replication of plasmid DNAs with purified viral and cellular proteins. Results from several laboratories have shown that the origin consists of two functionally distinct domains: (i) a 10-base-pair sequence present in the inverted terminal repetition (ITR) of all human serotypes and (ii) an adjacent sequence constituting the binding site for a cellular protein, nuclear factor I. To determine whether the same nucleotide sequences are necessary for origin function in vivo, we developed an assay for the replication of plasmid DNAs transfected into Ad5-infected cells. The assay is similar to that described by Hay et al. (J. Mol. Biol. 175:493–510, 1984). With this assay, plasmid DNA replication is dependent upon prior infection of cells with virus and only occurs with linear DNA molecules containing viral terminal sequences at each end. Replicated DNA is resistant to digestion with λ-exonuclease, suggesting that a protein is covalently bound at both termini. A plasmid containing only the first 67 base pairs of the Ad2 ITR replicates as well as plasmids containing the entire ITR. Deletions or point mutations which reduce the binding of nuclear factor I to DNA in vitro reduce the efficiency of plasmid replication in vivo. A point mutation within the 10-base-pair conserved sequence has a similar effect upon replication. These results suggest that the two sequence domains of the Ad origin identified by in vitro studies are in fact important for viral DNA replication in infected cells. In addition, we found that two separate point mutations which lie outside these two sequence domains, and which have little or no effect upon DNA replication in vitro, also reduce the apparent efficiency of plasmid replication in vivo. Thus, there may be elements of the Ad DNA origin of replication which have not yet been identified by in vitro studies.

The adenovirus (Ad) genome is a linear DNA molecule containing an inverted terminal repetition (ITR) of between 100 and 150 base pairs (bp) (16, 60). The replication of Ad DNA occurs by a strand displacement mechanism initiated at either end of the virus genome (1, 27, 37, 56, 57). In vitro experiments have demonstrated that initiation occurs by the covalent association of the 5′-terminal nucleotide of the nascent chain, dCMP, to the viral terminal protein (7, 12, 30, 52). This process requires the presence of the terminal protein, the viral 140-kilodalton DNA polymerase, and a host-encoded protein, nuclear factor I (29, 33, 41, 49). Elongation of the nascent chain is primed by the 3′ hydroxyl group of the protein-bound dCMP and requires additional proteins including the viral 72 kilodalton single-stranded DNA-binding protein and a host topoisomerase (14, 26, 35, 36).

Template structural and sequence requirements for Ad5 DNA replication in vitro have been determined by examining the replication of plasmids containing Ad sequences (8, 21, 28, 42, 53). A linear DNA molecule with the viral terminal sequence positioned at or near its end will support intiation in vitro (52, 55). Two domains within the viral terminal sequence have been identified. A 10-bp sequence conserved in all human Ad serotypes (bp 9 to 18 of Ad5 [50, 54]) is absolutely required for initiation to occur (8, 28, 53). This sequence may represent the binding site for one of the viral proteins involved in initiation (43). Adjacent to this conserved domain is a second domain containing a specific binding site for nuclear factor I (bp 19 to 45 of Ad5 [21, 34, 42]). The level of initiation is greatly enhanced by the presence of both nuclear factor I and its binding site, although low levels of initiation can be demonstrated in their absence (33, 42). No other specific nucleotide sequences appear to be necessary for elongation (35, 42).

Hay et al. (24) have shown that plasmids containing Ad terminal sequences can be replicated when introduced into susceptible cells along with purified viral DNA. In this paper we describe similar findings and show that the two sequence domains necessary for replication in vitro play a major role in DNA replication in vivo. In particular we show that maximal levels of replication of plasmid DNA in infected cells require an intact conserved sequence and a functional nuclear factor I binding site. In addition we show that certain mutations in sequences outside of these two domains also reduce replication in vivo. Our results confirming the role of the nuclear factor I binding site are in substantial agreement with those reported recently (23, 59).

MATERIALS AND METHODS

Materials. Restriction endonucleases *Bam*HI, *Dde*I, *Eco*RI, *Hha*I, *Hin*dIII, *Pvu*II, *Sal*I, T4 kinase, and *Hin*dIII linkers were all purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Restriction endonuclease *Mbo*I, λ-exonuclease, and T4 ligase were obtained from New England Biolabs, Inc., Beverly, Mass. *Dpn*I and calf intestine alkaline phosphatase were from Boehringer Mannheim Biochemicals, Indianapolis, Ind. *Escherichia coli* DNA polymerase, the Klenow fragment of *E. coli* DNA polymerase, and ^{32}P-labeled deoxynucleoside triphosphates were all purchased from New England Nuclear Corp., Boston, Mass. Bovine pancreatic RNase and proteinase K were obtained from Sigma Chemical Co., St. Louis, Mo. Plasmid pJM107, a pSCS101 replicon containing a 1.6-kilobase (kb) *Eco*RI fragment derived from the *E. coli* F factor (32), was a gift of Jeff F. Miller. Plasmids pMDC10, pMDC7, pMDC7-dl12, pMDC7-dl18, pMDC7-dl36, pMDC7-dl67, pMDC10-pm4,

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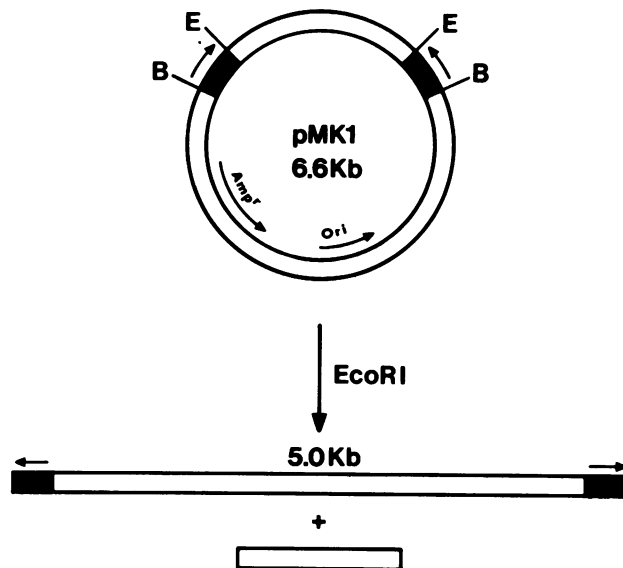

FIG. 1. Structure of plasmid pMK1 containing an inverted duplication of the Ad terminal sequence. Plasmid pMK1 contains two copies of nucleotides 1 to 358 of the left end of the Ad2 genome cloned into the *Bam*HI site of pBR322-*Eco*RI^r. These sequences are separated by a 1.6-kb *Eco*RI spacer fragment derived from the *E. coli* F factor. Digestion of pMK1 with *Eco*RI yields a 5.0-kb molecule consisting of pBR322-*Eco*RI^r with inverted copies of the Ad2 terminal sequence at its ends as well as the 1.6-kb *Eco*RI fragment.

and pMDC10-pm18 have all been described previously (8). Restriction endonuclease digestions (performed for 3 to 12 h at 37°C), fill-in reactions with the Klenow fragment of DNA polymerase I, end labeling with T4 kinase, and ligations (incubated overnight at 14°C at DNA concentrations of 20 to 30 μg/ml) were conducted as described by Maniatis et al. (31). Digestions of *Dpn*I-cut DNA with λ-exonuclease were conducted for 1 h at 37°C according to the instructions of the manufacturer. Digestions with proteinase K were performed in 50 mM Tris hydrochloride–10 mM EDTA (pH 8)–1.0% sodium dodecyl sulfate overnight at 37°C. Digestions with RNase were in 10 mM Tris hydrochloride–1 mM EDTA (pH 8) at 37°C, overnight.

Plasmid constructions. Plasmids constructed for the in vivo assay were all similar to pMK1 (Fig. 1). The backbone of each of these molecules consisted of an *Eco*RI-resistant (*Eco*RI^r) derivative of pUC9 or pBR322 that was generated by filling in the *Eco*RI site of the parent plasmid, using the Klenow fragment of *E. coli* DNA polymerase I and deoxynucleoside triphosphates. *Bam*HI-*Eco*RI or *Hin*dIII-*Eco*RI fragments containing the viral terminal sequence were cloned in the presence of an irrelevant 1.6-kb *Eco*RI spacer fragment (derived from the f7 *Eco*RI fragment of the *E. coli* F factor) into either the *Bam*HI or *Hin*dIII site of the vector. (The spacer fragment, derived from plasmid pJM107, was included in these molecules in an effort to stabilize the inverted copies of the viral terminal sequence during propagation of the plasmid in *E. coli*.)

Plasmids pMK1 and pAd both contain wild-type copies of the terminal 358 bp of the left end of Ad (Fig. 2). The deletion mutants pAd-dl12, pAd-dl18, pAd-dl36, and pAd-dl67 contain the first 12, 18, 36, or 67 bp of the Ad terminal sequence abutting bp 358 to 453 of the left end of Ad (Fig. 2). The DNA fragments (110 to 170 bp) containing the Ad terminal sequence used in the construction of these molecules were generated by digestion of plasmids pMDC7-dl12, pMDC7-dl18, pMDC7-dl36, and pMDC7-dl67 with *Pvu*II, ligation to *Hin*dIII linkers, and cleavage with *Eco*RI. The *Eco*RI-*Hin*dIII fragment was cloned into pUC9-*Eco*RI^r cut with *Hin*dIII as described above for the construction of pAd. Plasmid pAd-dl67′ contains inverted copies of the terminal 67 bp of Ad5 but lacks bp 358 to 453 of the left end. To construct pAd-dl67′, plasmid pAd-dl67 was digested with *Bam*HI to release a 1.75-kb *Bam*HI fragment consisting of the 1.6-kb spacer fragment flanked by inverted copies of the first 67 bp of Ad2. The 1.75-kb *Bam*HI fragment was ligated to pUC9-*Eco*RI^r cut with *Bam*HI, generating pAd-dl67′.

Single or double G · C to A · T transitions within or near the nuclear factor I binding domain of the Ad terminal sequence cloned in plasmids pm26,57, pm31, pm27,65, pm57, and pm63 were created by directed mutagenesis with sodium bisulfite (45). The C · G to G · C transversion at bp 35 of pm35 was generated by oligonucleotide-directed mutagenesis. (A detailed description of the construction of these mutations and their effects on viral DNA replication in vitro will be published elsewhere.) These mutant plasmids were digested with *Bam*HI and *Eco*RI to liberate a 70-bp *Eco*RI-*Bam*HI fragment which was cloned by the same method as outlined for the construction of plasmid pAd. Plasmids pAd-dl67′pm26,57, pAd-dl67′pm27,65, pAd-dl67′pm31, pAd-dl67′pm35, pAd-dl67′pm57, and pAd-dl67′pm63 were thus mutant analogs of plasmid pAd-dl67′.

Plasmids pAd-dl71pm4 and pAd-dl71pm18, containing copies of the first 71 bp of Ad2 with a C · G to T · A transition at bp 4 or 18, were constructed by use of clones pMDC10-pm4 and pMDC10-pm18 as sources of the mutant terminal regions (8). Plasmids pMDC10-pm4 and pMDC10-pm18 were digested with *Hha*I and *Eco*RI and then treated with the Klenow fragment of *E. coli* DNA polymerase I in the presence of dGTP to remove the *Hha*I overhang. The 71-bp fragments containing the Ad terminal region were isolated and ligated to *Bam*HI linkers to create *Bam*HI-*Eco*RI fragments. The approximately 74-bp *Bam*HI-*Eco*RI fragments, along with the 1.6-kb *Eco*RI fragment described

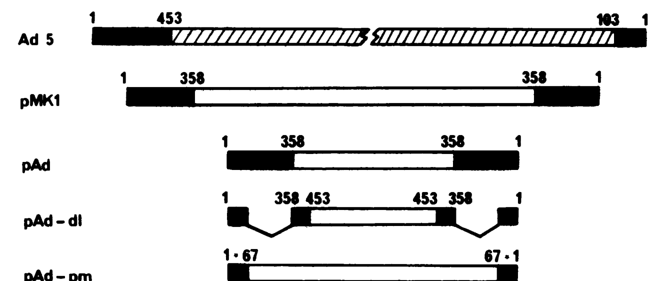

FIG. 2. Structure of *Eco*RI-linearized plasmids containing wild-type or mutant Ad2 terminal sequences. Plasmids pAd, pAd-dl (deletion mutants) and pAd-pm (point mutants) have the same general structure as plasmid pMK1 (Fig. 1). However, since the former molecules were generated by cloning into pUC9-*Eco*RI^r, *Eco*RI digestion produces a linear molecule that is 1.5-kb smaller. The deletions in the pAd-dl plasmids begin at nucleotide 358 and extend towards the terminus of the viral DNA. These molecules contain an additional 95 bp from the left end of the Ad2 genome (bp 358 to 453). Plasmid pAd-dl67′ (not shown) is a derivative of pAd-dl67 which lacks bp 358 to 453 of Ad2. Point mutant plasmids (pAd-pm) resemble pAd-dl67′ but have single or double point mutations within the 67 bp (71 bp for pAd-dl71pm4 and pAd-dl71pm18) terminal sequence.

above, were isolated and cloned into pUC9-*Eco*RI^r digested with *Bam*HI. The nucleotide sequences of the relevant regions of all DNAs used in these studies were confirmed by direct sequence analysis by a modification (2) of the method of Maxam and Gilbert.

Cells and virus. HeLa cells were maintained as monolayer cultures in minimal essential medium with 10% calf serum, penicillin-streptomycin (250 μg/ml), and L-glutamine (730 μg/ml). Adenovirus stocks were prepared as previously described (27).

In vivo DNA replication assay. HeLa cells were transferred to fresh dishes 1 to 2 days before each experiment so they would be at a density of 2.3×10^6 to 2.7×10^6 cells per 60-mm dish at the time of infection. Monolayers were infected at a multiplicity of 5 to 7 PFU per cell in 0.5 ml of minimal essential medium with 2.0% fetal bovine serum. After adsorption for 1 h at 37°C, virus was removed, and the monolayers were washed and returned to minimal essential medium with 2.0% fetal bovine serum at 37°C.

Plasmid DNA used in transfections was prepared from chloramphenicol-amplified cultures of Dam$^+$ *E. coli* 294 or JM83 by the Brij lysis method (9). DNA was digested with *Eco*RI or *Bam*HI and suspended at a concentration of 500 μg/ml in 10 mM Tris hydrochloride–1 mM EDTA (pH 8) before use in transfections. At 16 to 18 h postinfection (at which time viral replication should be proceeding at its maximal rate [18, 40]) the monolayers were each transfected with 5 μg of each plasmid DNA by the calcium phosphate method (19) followed by a 3 min shock with 15% glycerol in HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) buffered saline at 3 h after transfection (15).

At 20 h after transfection, cells were harvested from each dish, and low-molecular-weight DNA was recovered by the method of Hirt (25) modified by the inclusion of an overnight digestion of the lysate with proteinase K (100 μg/ml). The material derived from one-third of each dish was phenol extracted and ethanol precipitated. Samples were then incubated at 37°C overnight in 50 mM NaCl–10 mM Tris hydrochloride (pH 7.4)–10 mM $MgCl_2$–1 mM dithiothreitol–100 μg of RNase per ml–400 U of *Dpn*I per ml in a volume of 0.12 ml. (In some experiments one-fifth of the material was removed before *Dpn*I digestion for use as a size standard on the gel.)

*Dpn*I-digested samples were electrophoresed along with undigested samples on a 1.5% agarose gel in 40 mM Tris acetate–2 mM EDTA (pH 8.1) for 20 h at 85 V and transferred to nitrocellulose by the method of Southern (46). Baked filters were hybridized with nick translated α-^{32}P-pBR322 or α-^{32}P-pMK1 (0.45 mCi/μg) to detect plasmid and viral DNA, respectively, as described previously (31). Autoradiography was performed overnight at −70°C with a Kodak intensifying screen.

RESULTS

Characterization of plasmid replication in vivo. Hay et al. (24) have shown that plasmid molecules containing two inverted Ad termini are replicated in the presence of cotransfected Ad helper DNA. We have taken a similar approach to further investigate the structural and sequence requirements for DNA replication by Ad proteins in vivo.

Our initial experiments were performed using plasmid pMK1 which, when digested with *Eco*RI, generates a 5-kb linear molecule containing an ITR of the first 358 bp of the left end of the Ad2 genome (Fig. 1). Both pMK1 and pBR322, which was used as a negative control throughout our experiments, were digested with *Eco*RI and introduced

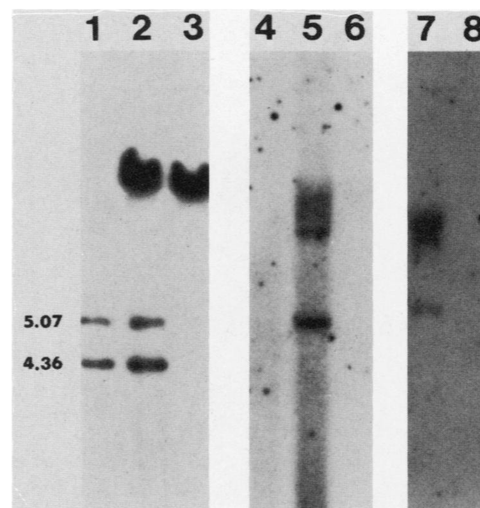

FIG. 3. Replication of plasmid DNA molecules containing an ITR composed of Ad terminal sequences in Ad-infected cells. *Eco*RI-digested pBR322 (negative control, 4.36 kb) and pMK1 (5.07 kb) were cotransfected into uninfected or Ad5-infected HeLa cells. At 20 h after transfection, cells were collected and low-molecular-weight DNA was isolated. Uncut samples and samples digested with *Dpn*I or *Dpn*I and *Mbo*I were analyzed by electrophoresis on a 1.5% agarose gel. After Southern transfer, undigested samples were probed with α-^{32}P-pMK1 to detect both viral and plasmid DNA while the *Dpn*I and the *Dpn*I and *Mbo*I digested samples were probed with α-^{32}P-pBR322 to visualize only plasmid molecules. Lanes 1 to 3, uncut DNA: 1, uninfected cells transfected with plasmid DNAs; 2, infected cells transfected with plasmid DNAs; 3, infected cells. Lanes 4 to 6, *Dpn*I-digested samples: 4, uninfected cells transfected with plasmid DNAs; 5, infected cells transfected with plasmid DNAs; 6, infected cells. Lane 7, *Dpn*I-digested DNA from infected cells transfected with plasmid DNA. Lane 8, *Dpn*I-digested DNA from lane 7 digested with *Mbo*I. The indicated mobilities of DNA molecules were determined from a reconstruction containing 50 pg of *Eco*RI-digested pMK1 (5.07 kb) and 50 pg of pUC9 (2.70 kb) run in an adjacent lane on the same gel (not shown).

into Ad5-infected or mock-infected HeLa cells by the calcium phosphate coprecipitation technique (19). Cells were harvested 20 h later, and low-molecular-weight DNA was isolated. One-fourth of it was digested with *Dpn*I (which cleaves unreplicated but not replicated DNA into several smaller fragments [38]). *Dpn*I-digested and uncut DNAs were fractionated by agarose gel electrophoresis. The DNA was transferred to nitrocellulose and hybridized with ^{32}P-labeled pBR322 to detect plasmid DNA alone or with ^{32}P-labeled pMK1 to detect both viral and plasmid DNAs. Both pBR322 and pMK1 DNA were detectable in lysates which had not been digested with *Dpn*I (Fig. 3, lanes 1 and 2). *Dpn*I-resistant DNA comigrating with the large *Eco*RI fragment of pMK1 was detectable in the lysates of infected cells (Fig. 3, lane 5). No *Dpn*I-resistant plasmid DNA was detectable in lysates of uninfected cells (Fig. 3, lane 4), and no *Dpn*I-resistant pBR322 DNA was detectable in either case. A broad smear of *Dpn*I-resistant DNA of lower electrophoretic mobility than either of the input plasmids was also detected. When Ad-infected cells were transfected with molecules of a single *Eco*RI-linearized plasmid of the same general structure as that of pMK1, the high-molecular-weight, *Dpn*I-resistant DNA resolved into a ladder of discrete bands, with adjacent bands differing in size by the length of a plasmid monomer (see Fig. 7, lanes 15 to 20). Thus, it is likely that the high-molecular-weight, *Dpn*I-

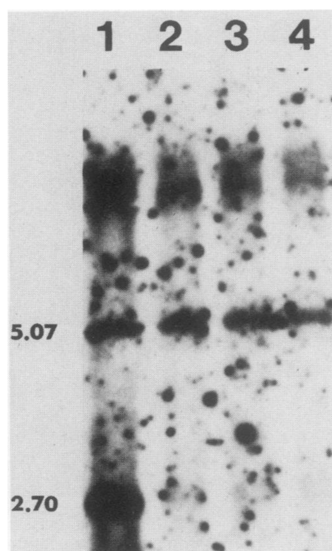

FIG. 4. Termini of *Dpn*I-resistant plasmid molecules recovered from Ad5-infected HeLa cells are protected from λ-exonuclease digestion. A 200 pg sample of *Eco*RI-digested pUC9 DNA (2.70 kb) was added to *Dpn*I-resistant low-molecular-weight DNA prepared as in lane 5 of Fig. 3. The sample was divided into four equal portions and digested with increasing amounts of λ-exonuclease. After digestion, samples were electrophoresed on a 1.5% agarose gel, transferred to nitrocellulose, and hybridized to α-^{32}P-pBR322. λ-Exonuclease: lane 1, 0 U; lane 2, 1 U; lane 3, 2.5 U; and lane 4, 5 U (all lanes at 1 h). The indicated mobilities were determined as described in the legend to Fig. 3.

resistant DNA consists of concatemers of plasmid molecules with Ad terminal sequences at each end generated either by end-to-end ligation or by homologous recombination of plasmid monomers (13, 39, 44).

The *Dpn*I-resistant DNA (Fig. 3, lane 7) was digested with *Mbo*I, which cleaves completely unmethylated but not hemimethylated or methylated DNA (17). The sensitivity of the *Dpn*I-resistant DNA to *Mbo*I (Fig. 3, lane 8) indicates that it consists of DNA molecules which have undergone multiple rounds of replication.

It is apparent from the results (Fig. 3) that only a small fraction of the input plasmid DNA served as a template for DNA replication. Therefore, we carried out several experiments to demonstrate that the *Dpn*I-resistant DNA detected in our assay was in fact the product of authentic Ad DNA synthesis. A consequence of the mechanism by which Ad DNA replication is initiated is that the ends of both nascent and mature viral DNA molecules are covalently associated with the virus-encoded terminal protein (5–7, 10, 47, 48, 58). To establish that the *Dpn*I-resistant plasmid molecules detected by our assay had this property, we digested the DNA with increasing concentrations of λ-exonuclease, a 5′ to 3′ exonuclease, the activity of which on viral DNA is blocked by the covalently attached terminal protein (3). The *Dpn*I-resistant pMK1 DNA (as well as the high-molecular-weight *Dpn*I-resistant DNA) was considerably more resistant to degradation by λ-exonuclease than *Eco*RI-digested pUC9 DNA present in the same reaction mixtures (Fig. 4). These results suggest that the termini of the *Dpn*I-resistant DNA are protected by the fragment of the terminal protein that remains covalently bound to the 5′ end of these molecules after protease digestion. Thus the *Dpn*I-resistant DNA detected in our assay has one of the unique properties expected of DNA molecules that have been replicated by an Ad-like mechanism.

Each initiation event during Ad DNA replication gives rise to a replication fork in which only one of the two strands of the duplex viral genome is copied. As a result, net synthesis of viral DNA requires the presence of an origin of replication at each end of the viral genome (51). We investigated the effect of altering the number and position of the viral terminal sequences on plasmid DNA replication (Fig. 5). Ad-infected cells were cotransfected with an equimolar mixture of three plasmids: *Eco*RI-digested pBR322, which served as a negative control; *Eco*RI-digested pMK1 or pAd, constructed in the vector pUC9 rather than pBR322 but otherwise identical to pMK1 (3.41 kb); and a derivative of pMK1 or pAd. In each case, linear plasmid DNAs containing the viral terminal sequence at both ends were replicated. In contrast, no replication was observed with *Eco*RI-digested pMDC7 (4.71 kb [Fig. 5, lane 2]) which contains the viral terminal sequence at one end only or with *Bam*HI-digested pAd (Fig. 5, lane 4). *Bam*HI digestion of pAd generates a linear DNA molecule (2.31 kb) containing an ITR in which the sequences normally located at the end of viral DNA are located approximately 350 bp from the end. Similarly, no replication was observed with uncut, circular pAd (Fig. 5, lane 3). (See Fig. 5 for a diagrammatic representation of the structure of the template molecules.) Therefore, as expected, replication of plasmid molecules in Ad-infected cells requires the presence of viral terminal sequences at both ends of a linear DNA molecule.

Sequence requirements for origin function in vivo. The Ad DNA sequences necessary for plasmid replication in vivo were analyzed with derivatives of pAd containing deletions within each copy of the terminal sequence. To insure that the interpretation of our results was not complicated by a possible requirement for a minimum length of ITR per se, the terminal sequences of all the deletions were joined to nucleotides 358 to 453 of the left end of Ad2. Thus, *Eco*RI digestion of each of the deletion plasmids generated a linear DNA molecule with an ITR of at least 100 bp. Each of the *Eco*RI-linearized deletion plasmids was cotransfected into Ad-infected cells with *Eco*RI-linearized pBR322 and pMK1 which served as internal negative and positive controls. Several samples of DNA mixtures were used in the transfections (Fig. 6, lanes 1 to 5). Plasmids containing only the first 12, 18, or 36 bp of the viral terminal sequence (Fig. 6, lane 9, 8, and 7, respectively) failed to replicate at detectable levels. In contrast, pAd-dl67 (Fig. 6, lane 6) containing the first 67 bp of the viral terminal sequence replicated at a level comparable to the control (Fig. 6, lane 10) which contained the entire ITR of viral DNA. Thus sequences located between bp 36 and 67 of the viral genome are necessary for Ad-dependent replication of plasmid molecules. Since this region of the viral genome contains the binding site for nuclear factor I, we conclude that nuclear factor I is essential for the replication of viral DNA.

The *Dpn*I-resistant DNA detected in infected cells that were cotransfected with *Eco*RI-digested pMK1 and any of the deletion plasmids included one (pAd-dl36, pAd-dl18, and pAd-dl12; Fig. 6, lanes 7 to 9) or two (pAd-dl67; Fig. 6, lane 6) new species of slower electrophoretic mobility than the input deletion plasmid DNA. The deduced sizes of these novel species are consistent with the idea that they are generated by a recombination event occurring in the 95-bp region of homology (nucleotides 358 to 453 of Ad2) between the deletion plasmid and the helper virus. Such an event would restore the wild-type terminal sequence to the mutant

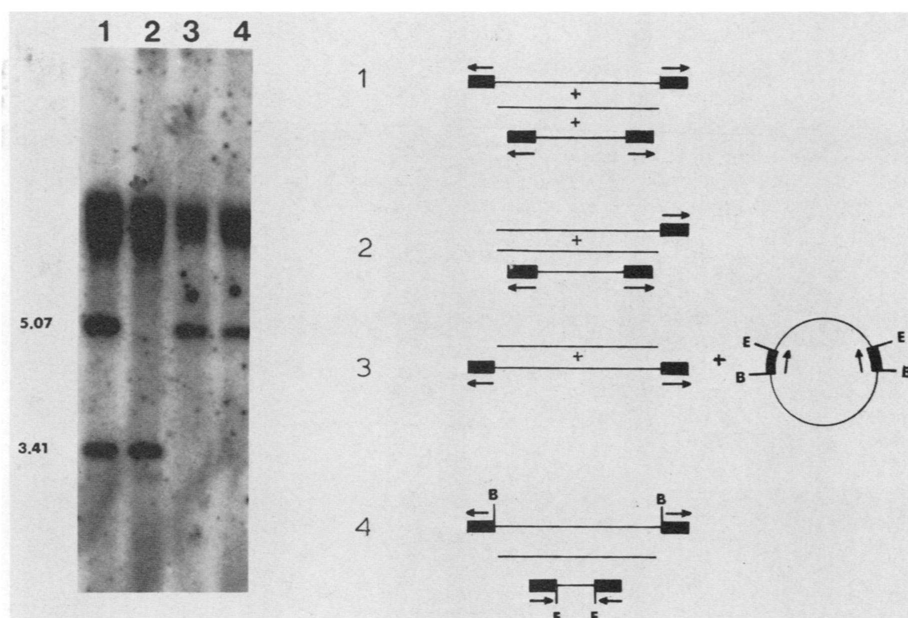

FIG. 5. Dependence of plasmid replication upon the number and position of the viral origin sequence. Low-molecular-weight DNA was prepared from Ad5-infected HeLa cells transfected with combinations of plasmids (right panel). Each transfection included *Eco*RI-digested pBR322 (4.36 kb) as a negative control and either *Eco*RI-cut pMK1 (5.07 kb) or pAd (3.41 kb) as a positive control. *Dpn*I-digested DNA was analyzed by electrophoresis on a 1.5% agarose gel, transferred to nitrocellulose, and hybridized with α-^{32}P-pBR322. Lanes: 1, *Eco*RI-digested pMK1, pBR322, and pAd; 2, *Eco*RI-digested pBR322, pAd, and pMDC7, a 4.71-kb molecule with a single copy of the terminal sequence; 3, *Eco*RI-digested pBR322 and pMK1 and uncut pAd (5.12 kb); 4, *Eco*RI-digested pBR322 and pMK1 and *Bam*HI-digested pAd, a 2.3-kb molecule flanked by Ad terminal sequences in inverted orientation with respect to that of viral DNA. The indicated mobilities were determined as described in the legend to Fig. 3. The arrowheads point in the direction of the first base pair of the Ad terminal sequence.

plasmid and extend its length by approximately 300 or 600 bp depending upon whether it occurred at one or both ends of the molecule. (In the case of replication-defective deletion mutants, only the larger recombinant would be expected to be recovered in *Dpn*I-resistant form [Fig. 5].) To provide evidence that these aberrantly sized molecules were generated by a mechanism involving recombination between the helper virus and the input deletion mutant plasmid, we introduced the mutant plasmids into infected cells in the absence of any additional plasmids. Under these conditions the *Dpn*I-resistant molecules described above were still apparent (Fig. 6, lane 11).

These results suggest that recombination between the helper virus and the plasmids occurs at a significant level and could considerably complicate the interpretation of any experiments to assess the effect of point mutations within the terminal sequence upon plasmid replication. Moreover, recombination also seems to occur between cotransfected plasmids. For example, we constructed plasmid pAd-dl67′ to eliminate the homology required for rescue of deletion mutant pAd-dl67 by Ad. Plasmid pAd-dl67′ contains an ITR composed of the first 67 bp of the Ad2 genome but lacks the additional sequences (nucleotides 358 to 453 of Ad2) described above. When this plasmid was digested with *Eco*RI and introduced into Ad5-infected cells (Fig. 6, lanes 12 and 14), the 3.32 and 3.61-kb *Dpn*I-resistant molecules observed in transfections with pAd-dl67 (Fig. 6, lanes 11 and 13) were no longer detected. However, when *Eco*RI-cut pAd-dl67′ and control plasmids were cotransfected into Ad5-infected cells, a new 3.03-kb *Dpn*I-resistant molecule was observed (Fig. 6, lane 14). A molecule of this size could have arisen from a recombination event between pMK1 and pAd-dl67′ occurring within homologous vector sequences. Thus recombination probably occurs both between transfected plasmid DNA and helper virus DNA and between cotransfected plasmids.

To analyze the Ad replication origin in more detail, we assayed the replication of several plasmids containing point mutations within the viral terminal sequence. To minimize the potential for rescue of a replication-deficient plasmid by homologous recombination with the genome of the helper virus, all of the point mutations were constructed as derivatives of the plasmid pAd-dl67′ in which the only homology to viral DNA is the inverted repeat of the first 67 bp of the viral terminal sequence. As indicated above, this plasmid contains all of the sequences necessary for full origin function, and when linearized with *Eco*RI, replicates as efficiently as a DNA molecule containing the complete viral inverted terminal repetition. Replication assays were performed with plasmids containing point mutations (Fig. 7 and 8 and summarized in Fig. 9). Plasmids pAd-dl67′pm31 (Fig. 7, lane 2) and pAd-dl67′pm63 (Fig. 7, lanes 6 and 19) replicated as efficiently as the wild-type plasmid (Fig. 7, lanes 1 and 15). The replication of plasmids pAd-dl67′pm57 (Fig. 7, lanes 4 and 17), pAd-dl67′pm27,65 (Fig. 7, lanes 7 and 20), pAd-dl71pm4 (Fig. 8, lane 3), and pAd-dl71pm18 (Fig. 8, lane 4) was reduced, and the replication of pAd-dl67′pm26,57 (Fig. 7, lanes 5 and 18) and pAd-dl67′pm35 (Fig. 7, lanes 3 and 16) was barely detectable. To quantitate the amount of each DNA transfected onto the cells, an aliquot from each DNA mixture was run on the same gel (Fig. 7, lanes 8 to 14; and Fig. 8, lanes 1 and 2).

Those mutant plasmids which were unable to replicate at the wild-type level had a more pronounced defect when cotransfected with a plasmid containing the wild-type origin (compare Fig. 7, lanes 3 and 16, pm35; Fig. 7, lanes 4 and 17, pm57; and Fig. 7, lanes 5 and 18, pm26,57). We believe that the most likely explanation for this result is that the wild-

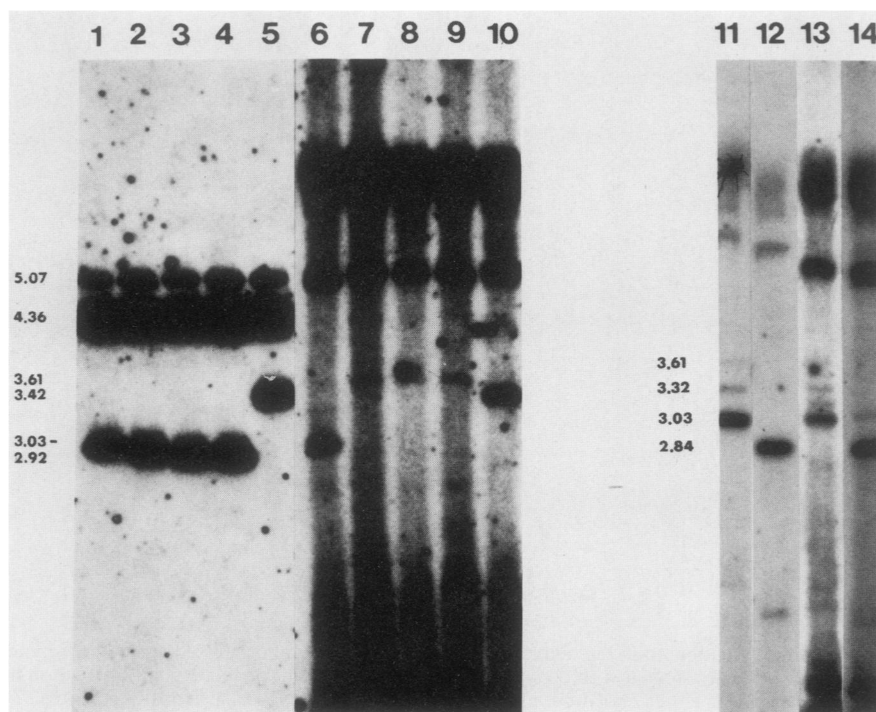

FIG. 6. Replication of deletion mutant plasmid DNA molecules. Ad5-infected HeLa cells were transfected with either a mixture of *Eco*RI-digested pBR322 (4.36 kb), pMK1 (5.07 kb), and deletion mutant plasmid (2.92 to 3.03 kb) DNA (lanes 6 to 10, 13, and 14) or with deletion mutant plasmid alone (lanes 11 and 12). Low-molecular-weight DNA was prepared as described in Fig. 3. Samples in lanes 1 to 5 contain 50 pg of each plasmid DNA prepared by removing an aliquot from each mixture of DNAs before transfection. Samples were analyzed by electrophoresis on a 1.5% agarose gel and Southern hybridization to α-^{32}P-pBR322. Lanes 1 to 5, uncut DNA: 1, pAd-dl67 (3.03 kb); 2, pAd-dl36 (2.97 kb); 3, pAd-dl18 (2.93 kb); 4, pAd-dl12 (2.92 kb); 5, pAd (3.42 kb), wild-type control. Lanes 6 to 10, *Dpn*I-digested DNA: 6, pAd-dl67; 7, pAd-dl36; 8, pAd-dl18; 9, pAd-dl12; 10, pAd. Lanes 11 and 12, *Dpn*I-digested samples containing plasmids pAd-dl67 (3.03 kb) and pAd-dl67′ (2.84 kb), respectively. Lanes 13 and 14, *Dpn*I-digested DNA (samples include control plasmids): 13, pAd-dl67; 14, pAd-dl67′. Putative recombinants between Ad and mutant DNAs are the 3.32- and 3.61-kb molecules (lanes 6, 7, 8, 9, 11, and 13). Molecules probably derived by recombination between mutant plasmid and control plasmid pMK1 include the 3.13 kb molecule (lane 13) and the 3.03-kb molecule (lane 14). Recombination of plasmid pAd-dl67 with Ad5 at one end of the molecule and with pMK1 at the other end would generate the 3.42-kb molecule in lane 13. The indicated sizes of DNA molecules were determined as described in the legend to Fig. 3 as well as from the mobilities of molecules in lanes 1 to 5.

type origin and a partially defective mutant origin compete for a factor that is limiting in infected cells.

DISCUSSION

In vitro studies using purified Ad and HeLa cell proteins to replicate plasmid DNA containing the Ad terminal sequence have defined *cis*-acting structural and sequence elements required for template activity (8, 21, 28, 42, 52, 53, 55). The template must be a linear molecule possessing the viral terminal sequence at its end. Two sequence domains within the Ad terminal region are necessary for replication. These are a 10-bp conserved sequence and an adjacent binding site for a cellular protein, nuclear factor I, which markedly stimulates initiation of Ad DNA replication (8, 21, 33, 34, 42). To confirm that the template requirements for in vitro replication are an accurate reflection of those in vivo, we assayed for the replication of linearized plasmid DNA containing Ad terminal sequences in Ad5-infected cells.

We have demonstrated that when linearized plasmid DNA containing an Ad terminal sequence at each end is transfected into HeLa cells, it is converted into *Dpn*I-resistant form by a process that depends upon prior infection of the cells with Ad. The *Dpn*I-resistant DNA shares a unique property with viral DNA, resistance to λ-exonuclease (3), suggesting that its 5′ termini are protected by the Ad terminal protein. It has been observed previously that cloned viral molecules in a circular form and viral genomes cut with a restriction enzyme so that they have only a single copy of the terminal sequence are not infectious when transfected into susceptible cells (22, 51). Our assays of the replication of plasmids containing Ad terminal sequences confirm that these sequences must be located at both ends of linear molecules for the plasmids to be replicated by Ad proteins in vivo. These results are in substantial agreement with those of Hay et al. (23, 24).

The results of several of our experiments clearly indicated that recombination occurred between transfected plasmid DNA and helper virus DNA. This complicates the analysis of plasmids with mutations in the origin region in two ways. First, studies on Ad replication in vitro have indicated that viral DNA covalently bound to an intact terminal protein is replicated more efficiently than DNA lacking the terminal protein (20). Thus it is possible that a recombination event with viral DNA resulting in the transfer of DNA containing the terminal protein to the end of the linear plasmid is a rate-limiting first step in the replication of transfected plasmids. If so, then the observed differences in the apparent replication efficiency of different mutant plasmids may reflect differences in the frequency of recombination with viral DNA rather than differences in replication per se. Although

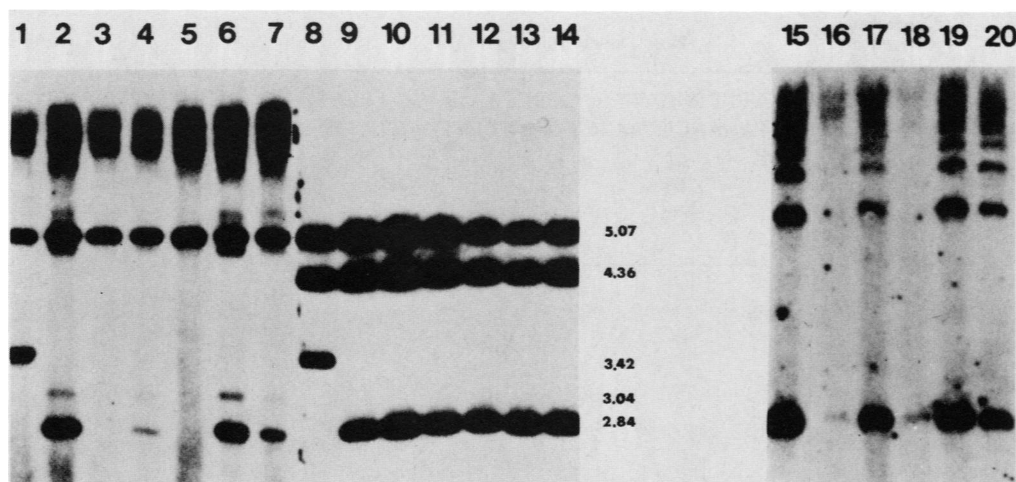

FIG. 7. Replication of plasmid molecules containing point mutations in the nuclear factor I binding region. Plasmid DNA molecules were digested with *Eco*RI and introduced into Ad5-infected cells. Replicated DNA was assayed as described in the legend to Fig. 6. Samples in lanes 1 to 7 were prepared from cells transfected with both positive (pMK1, 5.07 kb) and negative (pBR322, 4.36 kb) control plasmid DNAs and mutant DNA. Samples in lanes 8 to 14 contain 50 pg of each plasmid DNA and were prepared by removing an aliquot from each mixture of DNAs before transfection. Samples in lanes 15 to 20 were from cells transfected with mutant DNA alone. Lanes 1 to 7 and 15 to 20 contain *Dpn*I-digested DNA, and lanes 8 to 14 contain undigested DNA: 1 and 8, pAd (positive control); 2 and 9, pAd-dl67'pm31; 3, 10, and 16, pAd-dl67'pm35; 4, 11, and 17, pAd-dl67'pm57; 5, 12, and 18, pAd-dl67'26,57; 6, 13, and 19, pAd-dl67'pm63; 7, 14, and 20, pAd-dl67'pm27,65; 15, pAd-dl67' (positive control). The indicated sizes of DNA molecules were determined by comparison to the mobilities of molecules of known size in lanes 1 to 7.

we cannot completely rule out this interpretation, our results suggest that it is unlikely. First, there is no observable difference in the replication of plasmid pAd which contains an uninterrupted 375 bp of the viral terminal sequence at each end and pAd-d167' which has only 67 bp of homology with the end of the viral genome. If recombination is the limiting first step, we expect that pAd would replicate significantly better, since the target for recombination is more than fivefold longer than in the case of pAd-d167'. Second, the plasmid pAd-d167 contains two blocks of homology (of approximately equal length) with the left end of viral DNA, nucleotides 1 to 67 and nucleotides 358 to 453. Recombination with viral DNA at the second block of homology is detectable, since it results in DNA molecules approximately 300 or 600 bp longer than the input linearized plasmid (depending on whether recombination occurred at one or both ends of the DNA). We did in fact detect *Dpn*I-resistant DNA of the size expected for these recombinants (Fig. 6); the amount of these recombinants, however, is at least 10-fold less than replicated DNA of the same size as the input plasmid. Taken together our results suggest that recombination is not the limiting first step in the replication of transfected plasmids.

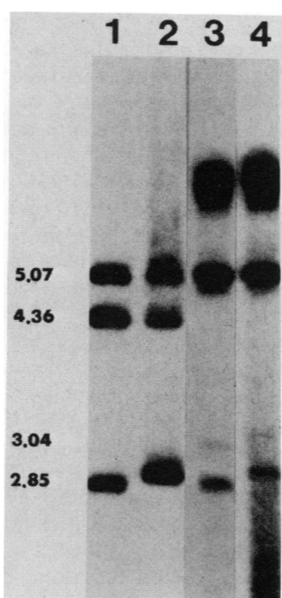

FIG. 8. Replication of plasmids containing a point mutation at bp 4 or 18 of the terminal sequence. Ad5-infected HeLa cells were transfected with 5.0 μg of *Eco*RI-digested pMK1 (5.07 kb), 5.0 μg of pBR322 (4.36 kb), and either 8.3 μg of *Eco*RI-digested pAd-dl71pm4 or 10.4 μg of *Eco*RI-digested pAd-dl71pm18. Samples were prepared and analyzed as described in the legend to Fig. 6. Lanes: 1 and 2, 50 to 100 pg of each plasmid DNA prepared by removing an aliquot from each mixture of DNAs before transfection; 3 and 4, *Dpn*I-digested DNA; 1 and 3, pAd-dl71pm4; 2 and 4, pAd-dl71pm18.

Although it appears unlikely that recombination with the helper virus DNA is an obligatory step in the replication of linear plasmid DNA, the fact that it may occur nonetheless complicates the analysis of plasmids with mutations, since recombination could result in a DNA molecule with a wild-type origin. In the case of plasmids with deletions, the wild-type recombinant is sufficiently different in size that it can be resolved from the mutant during gel electrophoresis. In the case of the point mutant plasmids, no such difference in electrophoretic mobility would be expected. We attempted to reduce the level of recombination in the case of the plasmids with point mutations by limiting the homology between viral and plasmid DNAs to 67 bp. It seems likely that recombination was in fact reduced to an acceptably low level, since several of the point mutations tested considerably reduced plasmid replication. Moreover, as indicated below, there is generally good agreement between our results and those obtained in vitro where recombination would not be expected to occur. Because of the possibility of recombination, however, the relative efficiency of plasmid

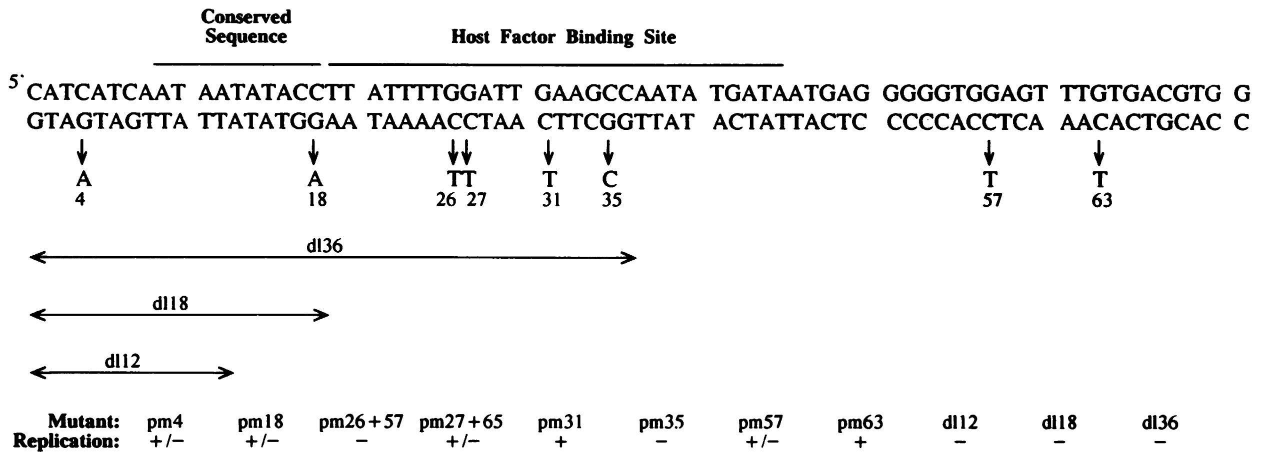

FIG. 9. The minimal Ad replication origin as defined by deletion analysis in vitro (8, 27, 40, 51) and in this study. Bp 9 to 18 are conserved among all human Ad serotypes (8, 52), and bp 19 to 45 are protected by nuclear factor I (32, 40). Point mutations and deletions whose effects were determined in vivo are indicated below the wild-type sequence. Base pairs included in the deletion mutants are indicated by arrows. The in vivo replication phenotype of each mutant is shown at the bottom.

replication in our experiments is best viewed as an upper limit.

From our analysis of the replication of plasmids with deletions and point mutations in the viral terminal sequence, we draw several conclusions. First, the binding of nuclear factor I to viral DNA is an essential step in viral DNA replication. A plasmid containing only the first 36 bp of the viral terminal sequence failed to replicate, while a plasmid with the first 67 bp replicated as efficiently as plasmids containing the entire viral ITR. The region between bp 36 and 67 is known to include sequences necessary for nuclear factor I binding (34, 42). Moreover, point mutations which reduce the binding of nuclear factor I to DNA in vitro (pm26,57, pm27,65, and pm35 [P. Rosenfeld, R. Wides, C. Hamilton, M. Challberg, and T. J. Kelly, unpublished data]) greatly reduced the efficiency of plasmid replication in vivo, and a point mutation in this region which has no effect on nuclear factor I binding (pm31) had no effect upon plasmid replication. Second, the 10-bp conserved sequence (bp 9 to 18 of Ad 2 [50, 54]) is also an essential element of viral DNA replication. A single point mutation in this region (pm18) reduced plasmid replication in vivo 5- to 10-fold. The magnitude of the effect of this mutation was not as great in our in vivo experiments as that observed in vitro (42). As discussed above, the interpretation of our experiments with point mutations is complicated by the possibility of recombination between plasmid and viral DNA, and the actual level of replication of the pm18 mutant in vivo may be less than observed. Alternatively, there may be more flexibility in the exact sequence of this region under the conditions of replication in infected cells. Finally, our experiments suggest that there are elements of the origin necessary for optimal function that have not yet been observed in vitro. In particular, a plasmid with a point mutation either at bp 4 or at bp 57 consistently replicated at levels between 1/5 and 1/10 of the cotransfected control plasmid. Neither of these two mutations has been observed to affect the efficiency of replication in vitro (8; R. Wides and T. J. Kelly, Jr., personal communication.) One obvious difference between the experiments in vivo and in vitro is that replication in vitro does not apparently involve the synthesis of DNA initiating on the single strand produced by a round of displacement synthesis (4). Thus, it is possible that there are elements of the origin which specifically affect the efficiency of initiation on the displaced single strand. Alternatively, there may be protein factors interacting with the origin which have not yet been identified in in vitro studies. The fact that the replication of pm57 was markedly reduced by the presence of a plasmid with a wild-type origin suggests that this mutation is located within the binding site for an essential factor.

It has been suggested that the initiation of replication on displaced single strands occurs at a duplex panhandle structure on circular intermediates formed by the base pairing of complementary sequences at the ends of the single strands (11). Given such a mechanism, it is possible that the length of the ITR might play a role in determining the overall efficiency of viral DNA replication. This possibility is supported by the fact that the different human Ad serotypes all have an ITR of at least 100 bp (16, 60). Yet the sequence of the various ITRs bear little resemblance to one another, except in the regions near their ends that are necessary for the initiation of DNA replication (50, 54). Here we show that a reduction in the length of the ITR to 67 bp had no effect upon the efficiency of Ad5-promoted plasmid replication. Thus it is possible that there is more flexibility in the length of the ITR than would be inferred from a comparison of the sequences of different serotypes. Alternatively, the length of the ITR might be more important in DNA molecules closer in length to that of viral DNA, or there may be some as yet unidentified additional function associated with the ITR. In summary, we have demonstrated that the two sequence domains of the Ad origin identified by in vitro studies are in fact important for viral DNA replication in infected cells. In addition, our results suggest that there are other sequences outside of these two domains which are important for optimal origin function in vivo. Further in vitro studies will be required to determine the function of these additional sequence elements.

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