MOLECULAR AND CELLULAR BIOLOGY, Feb. 1984, p. 302–309
0270-7306/84/020302-08$02.00/0

Vol. 4, No. 2

Rescue of Functional Replication Origins from Embedded Configurations in a Plasmid Carrying the Adenovirus Genome

DOUGLAS HANAHAN[1,2] AND YAKOV GLUZMAN[1]*

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724,[1] and Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138[2]

Received 6 September 1983/Accepted 21 November 1983

A variant of the adenovirus type 5 genome which lacks *Eco*RI sites has been cloned in a bacterial plasmid after the addition of *Eco*RI oligonucleotide linkers to its ends. Closed circular forms of the recombinant viral genome were not infectious upon their introduction into permissive eucaryotic cells. The linear genome released by digestion of the 39-kilobase recombinant plasmid (pXAd) with *Eco*RI produced infectious virus at about 5% of the level of wild-type controls. The viruses which arose were indistinguishable from the parental strain, and the normal termini of the viral genome had been restored. Marker rescue experiments demonstrate that provision of a DNA fragment with a normal viral end improves infectivity. When a small fragment carrying a wild-type left end (the 0 to 2.6% *Cla*I-B fragment) was ligated to *Cla*I-linearized pXAd, virus was produced with efficiencies comparable to a similar reconstitution of the two *Cla*I fragments of the wild-type genome. These viruses stably carry the left-end fragment at both ends, leaving the normal right end embedded in 950 base pairs of DNA. The embedded right origin is inactive. The consensus of the analyses reported here is that a free end is a necessary configuration for the sequences which make up the adenovirus origin of replication.

Human adenovirus type 5 (Ad5) has a double-stranded DNA genome of 36 kilobase pairs (kbp) (33). The 5′ ends of the viral DNA are covalently linked to a terminal protein through a deoxycytosine-to-serine phosphoryl bond (5, 23). The 55-kilodalton (kDa) terminal protein is a proteolytic cleavage product of an 80-kDa protein which participates in the initiation of DNA replication (2, 4, 16, 26). The terminal protein precursor forms a complex with a 140-kDa adenovirus-encoded polymerase (15, 27), after which the terminal protein precursor becomes modified through its covalent linkage to a deoxycytosine, which will subsequently become the first nucleotide of the daughter strand. During morphogenesis of the virion, the 80-kDa terminal protein precursor is specifically cleaved by an adenovirus-encoded protease to produce the 55-kDa terminal protein (3, 26).

Isolation of viral DNA commonly includes a nonspecific proteolytic digestion step (with pronase), which removes all but a few amino acids of the terminal protein from the DNA. The pronase-treated DNA is infectious upon transfection into permissive cells, which reveals that the presence of the intact 55-kDa terminal protein in the template strand is not strictly required for the initiation of adenovirus DNA replication. However, biochemical evidence indicates that the newly synthesized 80-kDa terminal protein precursor is obligatory for the initiation of synthesis of the daughter strand.

Each end of the viral genome carries an identical sequence of 103 bp (25, 35), called the inverted terminal repetition (ITR). The origin of replication has been localized to sequences lying within the ITR (reviewed in reference 4). Restriction fragments of adenovirus DNA which include one end of the viral genome have been cloned in bacterial plasmids either by GC tailing (28) or by the addition of an oligonucleotide linker to a viral end after removal of the terminal peptide. Release of such fragments from the recombinant plasmids by digestion with the appropriate restriction enzyme(s) generates a modified end that is extended by either a stretch of GC residues or one half of an oligonucleotide linker. Such cloned terminal fragments have been ligated to complementary viral DNA fragments which carry the remainder of the viral genome, including the second bona fide end. These molecules are infectious upon transfection into permissive cells, producing virus with two normal ends (28, 29). Deletions extending into the ITR of the cloned end also produce normal virus when combined with the remainder of the viral genome, provided that the deletions do not completely remove the ITR (29). These results indicate that the mutated and normal ends of the viral DNA are capable of interactions in such a manner as to allow repair of the mutant end and the consequent restoration of the wild-type virus genome. Since a normal viral end was always present in these reconstructions, it was not possible to assess the ability of the mutant end to function as a replication origin.

A number of eucaryotic viral genomes have been cloned in bacterial plasmids, and their ability to establish viral infections of permissive cells upon transfection of the cloned DNA has been examined. Simian virus 40 and polyomavirus have been cloned in a number of laboratories and have been found to be infectious if released from their plasmid vectors. Retrovirus proviruses (integrated and unintegrated forms) have been cloned and found to be infectious when the colinearity of the genome is preserved. A cloned cDNA copy of the poliovirus RNA genome is infectious, and its infectivity does not require release from the plasmid (22). Two parvoviruses, adeno-associated virus (24) and the minute virus of mice (18), have been cloned in the bacterial plasmid pBR322 and found to produce wild-type virus upon transfection of the circular plasmids into appropriate cultured cells. This demonstrates that these "integrated" configurations of the parvoviral genomes can be resolved into the normal linear forms, with the consequent restoration of their proper viral termini. We have cloned the genome of Ad5 into a bacterial plasmid to examine its infectivity characteristics.

* Corresponding author.

MATERIALS AND METHODS

Molecular cloning of Ad5. Fifty micrograms of virion DNA from the *Eco*RI-resistant (*Eco*RI^r) virus Ad5 was incubated for 3 h at 37°C in 0.3 N NaOH, conditions which have been shown to cleave the serine-to-deoxycytosine phosphoryl bond between the terminal protein and the adenovirus DNA, leaving the DNA with a 5′ phosphate group (1). The solution was neutralized with the addition of 100 mM Tris (pH 8) and 0.3 N HCl, and the NaCl concentration was adjusted to 1 M. The DNA was reannealed by incubating the solution at 68°C for 12 h, followed by slow cooling to 20°C, and finally by precipitation under ethanol.

The reannealed DNA was suspended, and 5 μg was ligated to 100 ng of *Eco*RI oligonucleotide linkers (octamers) by using T4 DNA ligase. After phenol extraction and ethanol precipitation, the DNA was digested with the *Eco*RI. The adenovirus DNA was then ligated to 0.5 μg of the bacterial plasmid pXf3 (10), which was cleaved once with *Eco*RI.

The ligated DNA was used to transform *Escherichia coli* DH1 (10) by a transformation procedure which efficiently transfers large plasmids (10). The transformed cells were plated on a nitrocellulose filter at a density of 20,000 per 100-mm filter. The colonies which arose were replicated and prepared for hybridization as described (11). The colonies were probed with ^{32}P-radiolabeled Ad5 DNA, and one colony was identified. This clone (pXAd) was amplified and subjected to the evaluation described below.

Plasmid DNA was prepared from *E. coli* DH1(pXAd) on a number of occasions by various protocols for cell growth and DNA extraction (17). Yields of supercoiled DNA ranged from 25 to 300 μg per liter of culture, with an average of 100 μg per liter. No method proved significantly different, and therefore, none is described. pXAD was purified by equilibrium centrifugation in a cesium chloride-ethidium bromide density gradient.

Cell culture, viruses, and viral DNAs. Human 293 (8) and HeLa cells were propagated in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum or 10% calf serum. Ad5 and the *Eco*RI^r variant of Ad5 (14) were propagated on HeLa cells. Viral DNA was extracted from CsCl-purified virions as described by Petterson and Sambrook (21). Intracellular viral DNA was isolated by modified Hirt procedure as described previously (7).

DNA analysis. Restriction endonucleases were purchased from New England Biolabs and were used as recommended by the vendor. T4 polymerase was from Bethesda Research Laboratories. Restriction fragments were end labeled by using DNA polymerase I large fragment (New England Biolabs) in the presence of α-^{32}P-labeled deoxynucleoside triphosphates at 20°C for 30 min. Adenovirus restriction fragments used in marker rescue experiments were fractionated on low-melting-point agarose, the appropriate ethidium bromide-stained bands were removed, and the DNAs were separated from the agarose by repeated extractions with 68°C phenol, followed by a $CHCl_3$ extraction and ethanol precipitation. The 3′ ends of adenovirus DNAs were radiolabeled by incubating the duplex DNA with T4 polymerase, first in the presence of dCTP, followed by the addition of the three remaining deoxynucleoside triphosphates. The T4 polymerase, in the absence of triphosphates, processes in a 3′ to 5′ direction, employing an exonucleolytic activity (17). Given deoxynucleotide triphosphates, it elongates DNA in a 5′ to 3′ direction under the specifications of the template strand. In the reaction described here, T4 polymerase plus dCTP removes 3′ sequences until the first dGTP (the seventh nucleotide for wild-type adenovirus DNA) is encountered, at which time the enzyme stalls, alternatively removing and replacing dCTP. The viral DNAs were so treated for 15 min at 20°C. Then, [^{32}P]dTTP, [^{32}P]dATP, and [^{32}P]dGTP were added, and the reaction was incubated for 15 min to replace the missing nucleotides at the 3′ end with radiolabeled ones.

RESULTS

Molecular cloning of Ad5. A molecular clone of an *Eco*RI^r variant of human Ad5 was established in *E. coli* as a recombinant with the plasmid pXf3. The terminal protein was removed from the 5′ ends of the viral DNA by treatment with sodium hydroxide. The single strands were reannealed, and *Eco*RI oligonucleotide linkers were ligated onto the ends. After digestion with *Eco*RI, the DNA was ligated to *Eco*RI-cleaved pXf3 and used to transform *E. coli* DH1. The colonies that arose were screened with radiolabeled Ad5 DNA, a single hybridizing colony was picked and purified, and the plasmid it contained was analyzed.

The plasmid pXAd is 39 kilobases (kb) in length, the expected size for the combination of the 36-kb viral gene with the 3.2-kb plasmid. Restriction mapping revealed that the recombinant carried sequences consistent with those of the viral DNA, and no gross rearrangements or instabilities (such as large deletions) were observed. Figure 1 shows a map of the orientation of the *Eco*RI-linked *Eco*RI^r Ad5 DNA in pXf3 and also compares representative restriction digests of Ad5, *Eco*RI^r Ad5, and pXAd DNAs. The sequences of this 36-kb human virus are stable as a recombinant plasmid in *E. coli* by the criterion of restriction mapping.

In the course of evaluating the cloned viral DNA, it was observed that the *Cla*I site located at 2.6% on the 0 to 100% genetic map (35) could not be digested with the restriction endonuclease *Cla*I. The *Cla*I site in Ad5 DNA is coincident with two *Mbo*I sites (GATCGATC), which are recognition sites for the *dam* methylase of *E. coli* (12). The methylation of adenine renders the *Cla*I site resistant to *Cla*I digestion, and thus, only the *Cla*I site in pXf3 can be cleaved when pXAd is prepared in the *dam*$^+$ strain DH1. *Cla*I digestion thus linearizes the recombinant plasmid, a quality which will prove useful below.

Infectivity of pXAd. The ability of the cloned viral DNA to reestablish itself as virus was examined by transfecting the DNA into permissive cells. Human 293 cells carry integrated copies of Ad5 DNA encompassing E1A and E1B genes, which are efficiently expressed (8) and can complement adenovirus mutants in this region. Nevertheless, the major reason for choosing these cells is their high transfection efficiency with adenovirus DNA, a quality which is necessary for the quantitative experiments reported here. The results of a series of transfections are presented in Table 1 and Fig. 2. The closed circular plasmid was unable to produce virus. However, if the viral genome was released from the plasmid by digestion with *Eco*RI, subsequent transfection produced plaques at about 5% of the efficiency of pronase-treated viral DNA. If the plasmid was linearized with *Cla*I, plaques were also produced, although at much lower levels (about 0.1 to 0.5% of the levels of controls). The *Cla*I-digested form can be viewed as a viral genome with two tails, one 3.2 kb in length, the other 30 bp long. Other variations in the treatment of the cloned DNA would not produce virus: *Eco*RI cleaved and then cyclized; *Eco*RI cleaved and then concatenated; or partially digested with *Bam*HI (data not shown).

The growth characteristics of viruses that arose from transfection of *Eco*RI-cleaved plasmid pXAd were assessed.

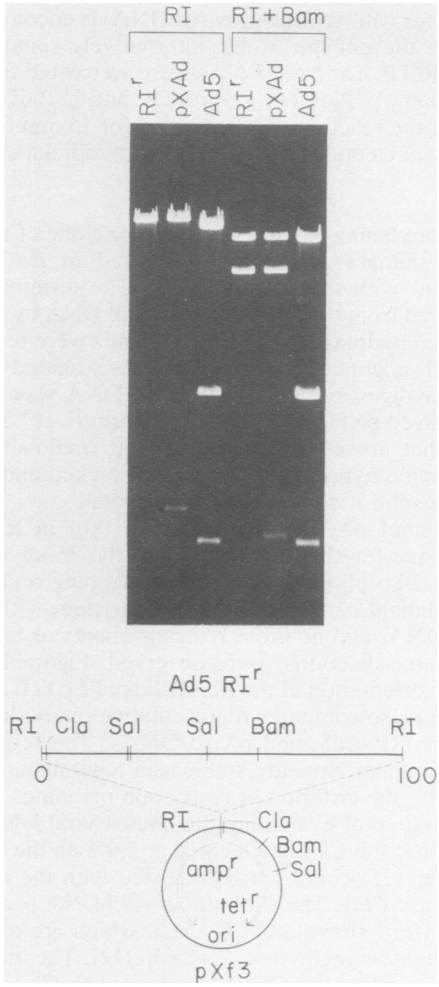

FIG. 1. Structure of plasmid pXAd. The restriction endonuclease digestion patterns of Ad5, *Eco*RI^r Ad5, and pXAd were compared by electrophoresis through a 0.7% agarose gel in the presence of ethidium bromide, using the endonucleases *Eco*RI and *Eco*RI plus *Bam*HI. The orientation of the *Eco*RI^r Ad5 genome in the plasmid vector pXf3 is depicted below the gel.

The viruses infected HeLa and CV1 cells in a manner indistinguishable from wild-type adenovirus. They grew at similar rates and produced DNA in CV1 cells and virus in HeLa or 293 cells at wild-type levels. Viral DNAs were isolated either from infected cells by the modified Hirt extraction procedure or from virus particles purified by equilibrium centrifugation. The analysis of the DNAs revealed that all viral DNAs arising from pXAd transfections were resistant to digestion with *Eco*RI. Virus arising from the transfections of cloned DNA is denoted XAd, the plasmid DNA is denoted pXAd, and normal virus is denoted Ad.

Nature of the ends of XAd virus. When plasmid pXAd was cleaved with *Eco*RI, the viral genome which was released carried extra nucleotides on its ends, derived from the oligonucleotide linkers that were ligated onto its bona fide ends (Fig. 3). Analysis of five XAd viruses that arose after transfection of *Eco*RI-digested pXAd indicates that these extra nucleotides are not maintained in the virus. The results for two of these are shown in Fig. 3. The virus DNAs were radiolabeled at their 3′ ends by using T4 DNA polymerase. *Eco*RI-cleaved pXAd was radiolabeled by end filling, using DNA polymerase I (large fragment). The labeled DNAs were then digested with *Hha*I, which cleaves 81 bp from the normal adenovirus ends, within the 103-bp ITR. The DNAs were analyzed on a polyacrylamide gel with a DNA sequence ladder as a length standard. Both XAd viruses had 81-bp terminal *Hha* fragments identical to that of wild-type Ad5 DNA, whereas the end-labeled pXAd had 87-bp *Hha* fragments (Fig. 3). Thus, the establishment of viral infection has included the restoration of ends of the correct length, and probably the original sequence as well. Since the terminal protein is covalently attached to the DNA strand through a serine-to-deoxycytosine phosphoryl bond, it is of note that an extra deoxycytosine, available from the *Eco*RI oligonucleotide linker, was not used as the 5′ terminal deoxycytosine in any of these viruses.

TABLE 1. Specific infectivity and market rescue of pXAd DNA[a]

DNA	Plaques per plate	
	Expt 1	Expt 2
pXAd (circular)		
Alone	0, 0	0, 0
Plus Ad5 *Eco*RI-A	ND[b]	9, 4
Plus Ad5 *Eco*RI-B	ND	1, 3
*Cla*I-digested pXAd		
Alone	1, 0	7, 0
Plus Ad5 *Eco*RI-A	52, 33	7, 97
Plus Ad5 *Eco*RI-B	24, 49	5, 57
Plus Ad5 *Sal*I-B	0, 13	12, 23
Plus Ad5 *Sal*I-C	0, 1	ND
pXAd/*Eco*RI	56, 28	31, 70
pXAd/*Eco*RI self-ligation	0, 1	ND
Ad5 (0.1 μg)	73, 101	46, 49

[a] One microgram of plasmid pXAd DNA was transfected into a monolayer of 293 cells in a 6-cm petri dish according to the calcium phosphate coprecipitation procedure. Each calcium phosphate coprecipitate was divided into two halves, and each half was applied to one plate. The variance observed between duplicate plates is probably a consequence of clumping of the coprecipitate, which would result in unequal distribution of DNA to the two plates. Four hours later, the cell monolayer was boosted with 15% glycerol (28). After 2 h, the cells were overlaid with 0.65% agar. Plaques were counted 6 to 10 days later. Marker rescue transfections were done with 1 μg of pXAd DNA and a 3 to 5 molar excess of different wild-type DNA fragments which were purified by agarose gel electrophoresis.

[b] ND, Not determined.

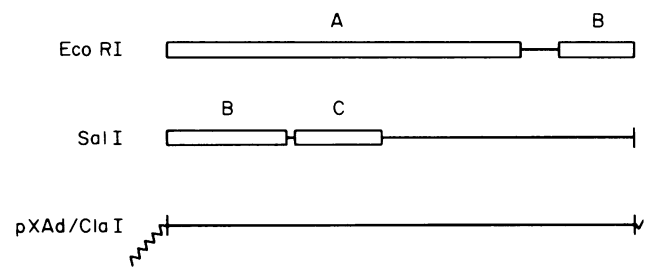

FIG. 2. Restriction fragments of wild-type Ad5 DNA (open bars) used in the marker rescue experiments presented in Table 1. pXAd/*Cla*I represents the structure of plasmid pXAd digested with *Cla*I; wavy line represents the pXf3 sequence.

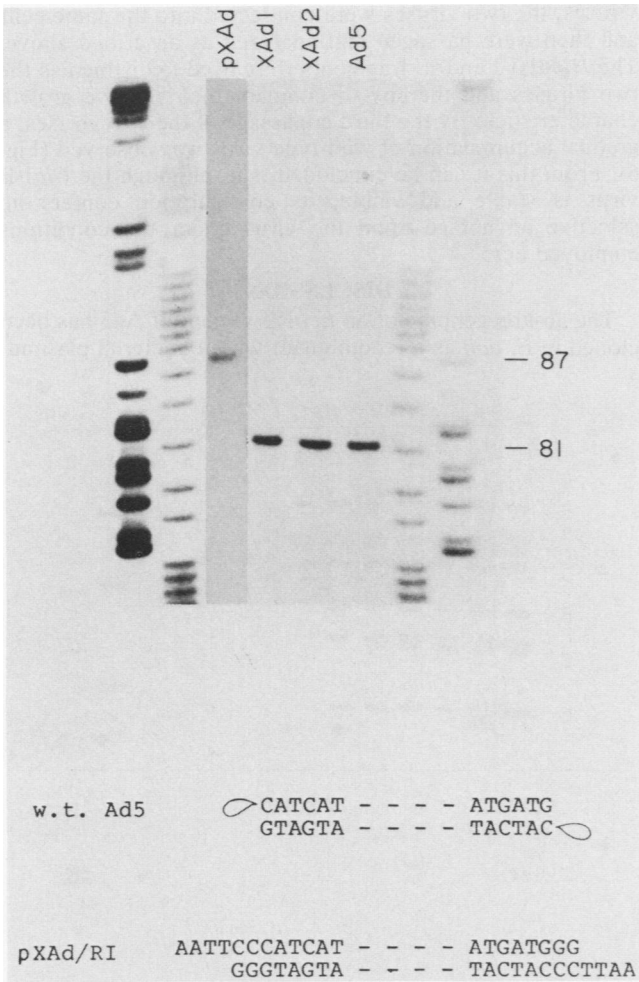

FIG. 3. Analysis of the ends of XAd viral DNAs. The DNAs of two independently isolated XAd viruses and wild-type Ad5 were radiolabeled at their 3′ ends by using T4 polymerase. *Eco*RI-cleaved pXAd was 3′ end labeled by using the large fragment of DNA polymerase I to fill in the *Eco*RI overhang. All DNAs were digested with *Hha*I and were fractionated on an 8% polyacrylamide gel with DNA sequence ladders as length standards. A comparison of the structure and sequence of the ends of Ad5 and of the corresponding ends of *Eco*RI-cleaved pXAD is shown in the diagram below the gels. The residual oligopeptide of the terminal protein is represented as the loop linked to the 5′ dCMP of Ad5.

Marker rescue of pXAd. To evaluate possible explanations for the low specific infectivity of the cloned viral DNA, marker rescue experiments were conducted by using agarose gel-purified restriction fragments derived from wild-type viral DNA. Two types of marker rescues were attempted. In one type, purified Ad5 fragments were mixed with plasmid pXAd; in the other, the Ad5 fragments were ligated to pXAd.

The mixing experiments employed the *Eco*RI-A and -B fragments of Ad5, which include the left and right ends of the viral genome, respectively (Table 1 and Fig. 2). Marker rescue by using each of these fragments with *Eco*RI-cleaved pXAd did not appreciably improve the infectivity, possibly because the high basal level of *Eco*RI-cleaved pAXd alone obscured a low level of marker rescue. However, the *Eco*RI-A and -B fragments did improve the transfection efficiency of both closed circular pXAd and of *Cla*I-cleaved pXAd. In each case, the basal efficiency was undetectable or low (respectively), and improvements by marker rescue were demonstrated (Table 1 and Fig. 2). The *Sal*I-B and -C fragments were also used in one marker rescue experiment with *Cla*I-cleaved pXAd. Here the B fragment, which includes the left end, improved transfection, but the internal C fragment did not. The results indicate that DNA fragments which include either wild-type viral terminus improve infectivity.

Marker rescue by mixing of fragments requires recombination between them, which is a relatively improbable event in transfection assays. Therefore, additional marker rescue experiments were conducted in which the cloned viral genome and the rescuing viral DNA fragment were ligated together and then mixed with carrier DNA and transfected.

In these marker rescue experiments, *Cla*I-digested pXAd was employed (Table 2 and Fig. 4). The linearized viral genome has a 30-bp pXf3 tail on the right end and a 3.2-kb pXf3 tail on the left. The *Cla*I-B fragment of Ad5 was used as the rescuing fragment: it consists of the left end (2.6%) of Ad5 (918 bp). Thus, there is little genetic information other than the end itself, in contrast to the marker rescues described above, in which 15 to 75% of the genome was included. However, it is noteworthy that this fragment does contain a sequence which is necessary for correct packaging of the viral DNA into virions (9, 13).

When the ligation products of Ad5 *Cla*-B to *Cla*I-cleaved pXAd were transfected into 293 cells, the specific infectivity increased significantly over the levels observed with the plasmid alone. The *Cla*-B marker rescue produced about 150 plaques per μg (Table 2 and Fig. 4), whereas *Cla*I-cleaved pXAd alone produced 1 to 5 plaques per μg (Table 1 and 2) and *Eco*RI-cleaved pXAd produced about 50 plaques per μg (Table 1 and Fig. 2). Furthermore, the efficiency was comparable to that of a reconstruction in which the isolated Ad5 *Cla*I-A and -B fragments are ligated together to restore the viral genome. Thus, the *Cla*I-B fragment confers wild-type

TABLE 2. Marker rescue of plasmid pXAd DNA by ligation to left end wild-type Ad5 fragment[a]

DNA	Plaques per plate
pXAd cleaved by *Cla*I	1, 0
pXAd cleaved by *Cla*I plus Ad5 *Cla*I-B	>200, 124
Ad5 *Cla*I-A	10, 15
Ad5 (*Cla*I-A plus *Cla*I-B)	>200, 161
Ad5 (0.1 μg)	73, 101

[a] One microgram of either *Cla*I-digested pXAd or gel-purified Ad5 *Cla*I-A fragment were ligated to a fourfold molar excess of Ad5 *Cla*I-B fragment and used to transfect the monolayer of 293 cells as described in Table 1, footnote *a*.

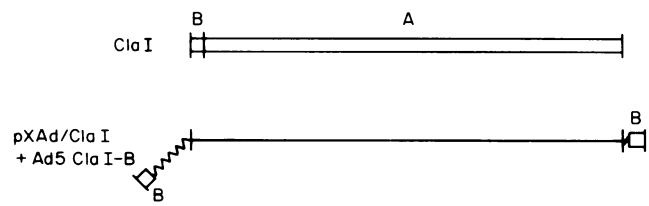

FIG. 4. Final products of ligation of *Cla*I-digested pXAd (pXAd/*Cla*I) to the Ad5 *Cla*I-B fragment (see Table 2).

infectivity upon the cloned virus, and it can be concluded that the low infectivity is in some way related to the nature of the ends; an *Eco*RI end (with its extra nucleotides) is not equivalent to the bona fide end with a short peptide linked to it. In addition, the *Cla*I end of *Cla*I-cleaved pXAD, with its 30-bp tail, is clearly inferior to both of these.

Analysis of *Cla*I-B-rescued virus. The transfection of ligations between *Cla*I-digested pXAd and the Ad5 *Cla*I-B fragment produced virus at levels comparable to those of reconstructions of the wild-type viral genome. Since *Cla*I-digested pXAd has pXf3 tails on each end, an analysis of the virus which arose was conducted. DNAs extracted from 10 independently isolated viruses were identical, but different from *Eco*RI^r Ad5 and XAd DNAs. Figure 5 shows analysis of the DNA of one of these viruses (XAdC), which was amplified and banded in a CsCl gradient. Comparison of the *Hin*dIII restriction patterns of Ad5, XAd, and XAdC reveals that the *Hin*dIII-I fragment (the normal right end) was absent from XAdC and that a new fragment (*Hin*dIII-I′), approximately 900 bp larger, had appeared in its place. Digestions of XAd and XAdC with *Hin*dIII plus *Eco*RI and *Hin*dIII plus *Cla*I demonstrate that the *Hin*dIII-I′ fragment carries closely linked *Eco*RI and *Cla*I recognition sites. The *Hin*dIII-I′ fragment was cleaved by *Eco*RI into two smaller fragments. One comigrated with the *Hin*dIII-I fragment of XAd, which is the normal right end. The second migrated slightly behind the rescuing *Cla*I-B. *Hin*dIII-I′ was also cleaved by *Cla*I into two fragments. One comigrated with the authentic *Cla*I-B left-end fragment. The second fragment was slightly larger than the normal *Hin*dIII-I band. The interpretation of these results is that the rescuing *Cla*I-B fragment was maintained on the right end of the virus after its ligation to the *Cla*I site 30 bp upstream from the right-end ITR. Thus, the right end of XAdC carries a short stretch of pXf3 sequences followed by 2.6% of the viral genome originating from the left end. This modified right end is stable, and it persists despite the presence of a direct repeat of its ITR sequence (the normal right end) 950 bp downstream. The 3.2-kb tail on the left end of *Cla*I-digested pXAd was removed, and the bona fide left end was restored. The structure of XAdC is outlined in Fig. 5 to show the structure of the virus genome as deduced from this analysis.

Growth characteristics of *Cla*I-B-rescued virus. One of the *Cla*I-B viruses (XAdC) was passaged serially to evaluate both its stability and its growth rate relative to XAd. Figure 6 presents this analysis. Each virus (XAdC and XAd) was used to infect permissive cultured cells (HeLa) at similar multiplicities of infection. Virus and viral DNA were extracted from duplicate infections. The virus was similarly passaged twice more, and then viral DNA was collected. The input and third-passage DNAs were evaluated by digestion with *Hin*dIII (Fig. 6). XAdC maintained the *Hin*dIII-I′ fragment, and no normal *Hin*dIII-I could be detected. Both XAdC and XAd produced similar amounts of DNA in parallel infections and extractions. Thus, the modified right end of XAdC is stable, and the virus is propagated at levels similar to XAd itself. Therefore, the presence on the right end of 2.6% of the viral genome originating from the left end is not unfavorable to virus proliferation in the cultured cells employed in these analyses.

The extra sequences in XAdC viral DNA were not removed by recombination or other interactions between the embedded and terminal ITRs, which raises the possibility that the modified structure of the right end is of some benefit to the virus and hence is selectively maintained. To assess subtle differences in the multiplication of XAd and XAdC viruses, the two viruses were coinfected into the same cells and then were passaged and analyzed as described above. The *Hin*dIII-I and -I′ fragments were used to distinguish the two viruses and thereby to compare their relative growth characteristics. By the third copassage of the two viruses, a gradual accumulation of wild-type virus was observed (Fig. 6). From this it can be concluded that, although the *Cla*I-B virus is stable and viable, its configuration confers no selective advantage upon the virus under the conditions employed here.

DISCUSSION

The 36-kbp genome of an *Eco*RI^r variant of Ad5 has been cloned in *E. coli* as a recombinant with a bacterial plasmid.

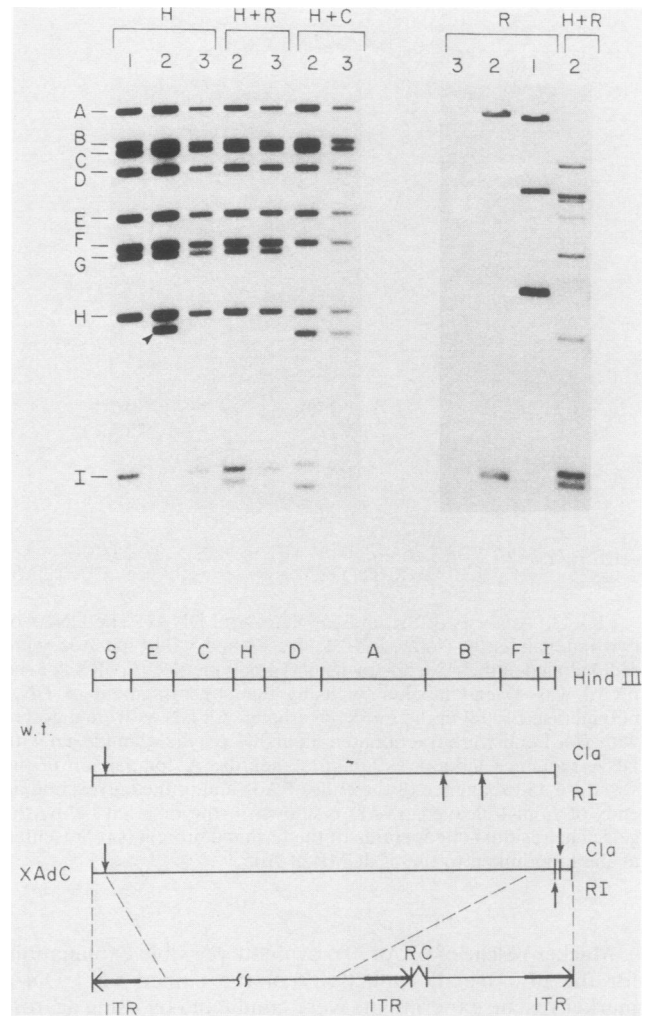

FIG. 5. Structure of the XAdC virus derived from marker rescue with Ad5 *Cla*I-B. DNAs from wild-type Ad5 (tracks 1), XAd virus (tracks 3), and XAdC virus (tracks 2) were digested with the noted combinations of *Hin*dIII (H), *Eco*RI (R), and *Cla*I (C), radiolabeled by using the large fragment of DNA polymerase I, and then analyzed by electrophoresis on a 1% agarose gel followed by autoradiography. Arrow marks the position of the *Hin*dIII-I′ band in the *Hin*dIII digestion of XAdC (track H-2). Since XAd has no *Eco*RI sites, it is not labeled in the analysis of *Eco*RI alone, and hence, no band appears, even though the DNA is present by ethidium bromide straining. The restriction map of XAdC is shown below the gels to describe the orientation of the sequences maintained on the right end, which consist of a short segment of pXf3 sequences (*Eco*RI to *Cla*I), followed by the Ad5 *Cla*I-B fragment.

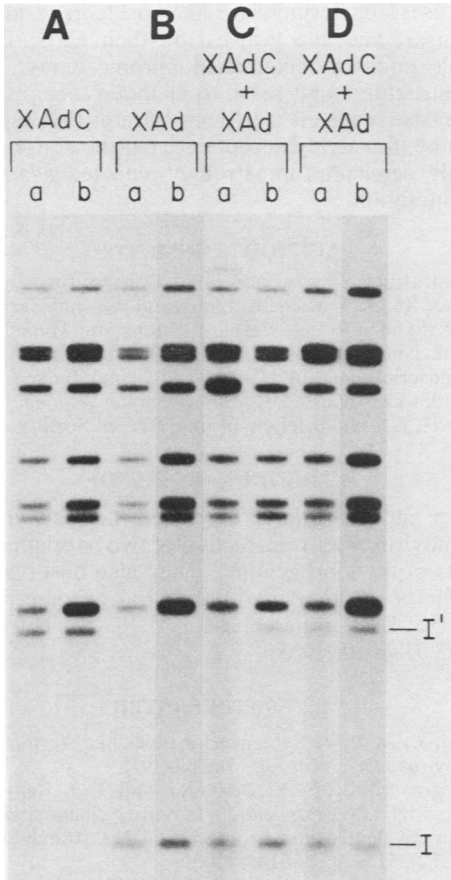

FIG. 6. Stability of duplicated left end present on the right end of XAdC. Virus stocks of XAdC and XAd were used to infect HeLa cells both separately and together, using the *Hin*dIII-I and -I' difference to distinguish XAd from XAdC. The DNAs of these viruses were analyzed before the first passage (tracks b) and after three passages by viral infection (tracks a). End labeling of the *Hin*dIII digestions were followed by electrophoresis and autoradiography. Overexposure did not reveal the *Hin*dIII-I fragment in XAdC alone, which would be indicative of resolution of XAdC into XAd (see Fig. 5). In the mixing experiments, XAd and XAdC virus were coinfected at ratios of 2:1 (C) and 1:1 (D).

The ability of the cloned viral DNA to produce virus upon transfection of permissive human cell lines was evaluated. The results indicate that (i) the closed circular plasmid is not infectious; (ii) the closed circular or concatenated viral genome produced by first releasing the viral DNA from the plasmid with *Eco*RI and then treating the DNA with T4 ligase is not infectious either; (iii) the linear DNA produced by digestion of pXAd with *Eco*RI is infectious at about 5% of the levels obtained with viral DNA; and (iv) the linear DNA produced by digestion of pXAd with *Cla*I is also infectious to about 0.1 to 0.5% of control levels. Thus, a linear form of the DNA is necessary for productive transfection.

The virus which arose from transfections of *Eco*RI-digested pXAd multiplied at rates similar to those wild-type virus, and biochemical analysis revealed that the normal virus end had been restored, with the consequent loss of the terminal sequences derived from the *Eco*RI oligonucleotide linker added during the molecular cloning. Marker rescue experiments using purified fragments of wild-type Ad5 DNA (isolated from virions) demonstrated that providing a normal virus end improved the transfection efficiency of both closed circular pXAd and *Cla*I-digested pXAD. In particular, ligation of the Ad5 *Cla*I-B fragment onto *Cla*I-digested pXAd produced plaques at levels comparable to a reconstruction using the Ad5 *Cla*-A and -B fragments. This indicates that the addition of a normal viral terminus can improve the infectivity of the cloned viral genome to levels comparable with those of wild-type DNAs.

Free ends are a component of the adenovirus origin of replication. The consensus which develops from these observations is that an essential feature of the adenovirus origin of replication is the presence of its normal terminal sequences as a free end. Some definition of a free end can be obtained by comparing the transfections of *Eco*RI-cleaved and *Cla*I-cleaved pXAd. *Eco*RI-cleaved pXAd leaves six extra nucleotides on the 5' end, whereas *Cla*I-cleaved pXAd carries 30 additional nucleotides on the right end and 3.2 kb on the left end. The respective transfection efficiencies are 5 and 0.1% of those obtained with viral DNA, which carries no extra nucleotides and includes a few amino acids linked to its 5' ends. Thus, progressively embedding the terminal ITR in DNA produces consequent reductions in the ability of the DNA to produce virus upon transfection into permissive cells.

Previous work with cloned viral ends has always employed a second wild-type end in the transfections. Thus, the appearance of a corrected end can be explained by an interaction between the mutant and normal ends (28, 29). In the experiments reported here, transfection with two mutant ends nevertheless produced wild-type virus. Therefore, the mechanism of repair in this situation may differ from that proposed previously in studies in which one mutant and one wild-type end were present.

An embedded origin is inactive. Strong support for the necessity of presenting the sequences of the origin as a free end comes from the analysis of the viruses produced by the ligation of Ad5 *Cla*I-B to *Cla*I-digested pXAd. The XAdC viruses all contain two copies of the normal adenovirus left end (the *Cla*I-B fragment). One is stably present on the right end of the viral genome. The normal right end is embedded in 950 bp of DNA, with its ITR aligned as a direct repeat of the ITR at the new right end. These viruses are stable on passage and multiply at a level close to that of wild-type virus. These results have several implications. First, the embedded origin is inactive for the initiation of replication since no wild-type virus appears, which would be a consequence of initiation at this origin. Second, the embedded origin cannot serve to terminate replication since specific termination at the embedded origin would again produce wild-type virus, which was not observed. Finally, the interactions which demonstrably occur to repair mutant ends evidently do not act on this embedded origin, perhaps because of the greater homology between the two left ends (918 bp) than between two ITRs (103 bp) alone (see Fig. 5). Furthermore, recombination between the two directly repeated ITRs on the right end would produce normal virus. This suppression of repair and recombination between the actual ends and the embedded ITR is particularly striking in view of the efficient removal of the 4 kb of DNA which extends off the normal left end after the ligation of Ad5 *Cla*I-B to *Cla*I-digested pXAd. However, preservation of such an extended left end would produce a viral genome of 40 to 41 kb, which should be inefficiently packaged and thus not expected to persist. There is clearly a bias in which the normal left end is always restored, but the left-end sequences on the right end are not removed to restore the

normal right end. Defective adenoviruses have been isolated and shown to stably persist as large palindromes which contain adenoviral information originating only from the left end (6, 7). This indicates that the configuration of left-end sequences on both the left and right ends is favorable under certain conditions.

In sum, these results and those discussed above argue that the adenovirus origin of replication is inactive if embedded in other DNA. This is in contrast to the parvoviruses, another class of linear-DNA viruses, which are capable of producing wild-type virus upon transfection of closed circular recombinant DNAs. Thus, the parvovirus origin of replication can function in the recombinant, with the consequent resolution of the normal ends from their embedded state, and does not need the conformation of a free end, whereas the related adenovirus origin demonstrably requires one.

Comparison of in vivo and in vitro results. Transfections of the closed circular recombinant plasmid (pXAd) were never observed to produce infectious virus. Several groups have reported that cloned adenovirus origins do not correctly initiate replication in vitro when the origin is embedded in a closed circular plasmid (31, 34; M. D. Challberg and D. R. Rawlins, Proc. Natl. Acad. Sci. U.S.A., in press). In contrast, Pearson et al. (19) reported that a closed circular plasmid carrying an adenovirus origin can produce newly synthesized DNA strands in vitro which originate near that origin. This suggests that the embedded origin is still capable of some form of interaction with components of the replication apparatus. Our results indicate that if such an interaction occurs in vivo, it will not efficiently produce infectious virus.

The viral DNA produced by digestion of the recombinant plasmid is different from normal virus DNA in two ways. Extra nucleotides are added onto the normal virus end, and the DNA carries a 5′ phosphate instead of the few amino acids that remain after removal of the terminal protein with pronase. The high infectivity of pronase-treated viral DNA demonstrates that the terminal protein on the template is not necessary for the initiation of DNA replication in vivo. Using a fractionated in vitro system, Tamanoi and Stillman (31, 32) have shown that an *Eco*RI end is reduced two- to threefold in the efficiency of initiation relative to an end carrying the intact terminal protein. Thus, the presence of a few nucleotides on the normal end may not compromise its status as a free end. The in vivo results described in this report show a much greater reduction in transfection efficiency than does the in vitro assay for initiation. One explanation for this disparity is the transfection assay. It has been amply demonstrated that the introduction of DNA into cultured cells as a calcium phosphate-DNA coprecipitate produces rapid concatenation of the exogenous sequences (20). This would be expected to occur in the transfections described here, and concatenation of the adenovirus genome will embed the ends and render them inactive. The terminal protein is maintained on the ends of the viral DNA throughout the life of the virus after its participation in the initiation cycle. It is possible that one role of the terminal protein is to maintain the sequences of the origins as free ends by protecting the DNA from access to repair functions in the cell that might either degrade or embed the ends, thereby inactivating the origins of replication and incapacitating the virus. It is of note that virtually all DNA viruses have evolved strategies or structures to protect their ends and thus maintain their episomal status. In particular, simple DNA ends are rarely encountered. The viral genomes either are circular (e.g., papovaviruses) or carry proteins (e.g., adenoviruses, bacteriophage ϕ29) or hairpin loops (e.g., parvoviruses, vaccinia viruses) on their ends. Telomeres, the stable ends of mammalian chromosomes, have also evolved structures that serve to maintain them as free ends (30). The case reported here demonstrates that simple linear ends can be transiently acceptable, but the consequences of such ends persisting are strongly selected against during viral proliferation.

ACKNOWLEDGMENTS

We thank Bruce Stillman and Fuyu Tamanoi for helpful discussions; Bruce Stillman, Michele Manos, and Winship Herr for critical reading of the manuscript; Barbara Ahrens and Mark DeLuca for excellent technical assistance; Patti Barkley for patient preparation of the manuscript; and Mike Ockler and Debbie Balke for artwork.

This work was supported by grants from the National Institutes of Health. D.H. is a junior fellow of the Harvard Society of Fellows.

ADDENDUM IN PROOF

Berkner and Sharp have described the recovery of wild-type adenovirus after transfection of two overlapping cloned fragments of the viral genome. They also observed restoration of the bona fide ends of the viral genome during this process (K. L. Berkner and P. A. Sharp, Nucleic Acids Res. **11**:6003–6020, 1983).

LITERATURE CITED

1. **Carusi, E. A.** 1977. Evidence for blocked 5′ termini in human adenovirus DNA. Virology **76**:380–394.
2. **Challberg, M. D., S. V. Desiderio, and T. J. Kelly, Jr.** 1980. Adenovirus DNA replication in vitro: characterization of a protein covalently linked to nascent DNA strands. Proc. Natl. Acad. Sci. U.S.A. **77**:5105–5109.
3. **Challberg, M. D., and T. J. Kelly, Jr.** 1981. Processing of the adenovirus terminal protein. J. Virol. **38**:272–277.
4. **Challberg, M. D., and T. J. Kelly.** 1982. Eukaryotic DNA replication: viral and plasmid model systems. Annu. Rev. Biochem. **51**:901–934.
5. **Desiderio, S. V., and T. J. Kelly.** 1981. Structure of the linkage between adenovirus DNA and the 55,000 molecular weight terminal protein. J. Mol. Biol. **145**:319–337.
6. **Deuring, R., G. Klotz, and W. Doerfler.** 1981. An unusual symmetric recombinant between adenovirus type 12 DNA and human cell DNA. Proc. Natl. Acad. Sci. U.S.A. **78**:3142–3146.
7. **Gluzman, Y., and K. Van Doren.** 1983. Palindromic adenovirus type 5-simian virus 40 hybrid. J. Virol. **45**:91–103.
8. **Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
9. **Hammarskjold, M. L., and G. Winberg.** 1980. Encapsidation of adenovirus 16 DNA is directed by a small DNA sequence at the left end of the genome. Cell **20**:787–795.
10. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
11. **Hanahan, D., and M. Meselson.** 1983. Plasmid screening at high colony density. Methods Enzymol. **100**:333–342.
12. **Hattman, S., J. E. Brooks, and M. Masurekar.** 1978. Sequence specificity of the P1 modification methylase (M Eco P1) and the DNA methylase (M Eco dam) controlled by the Escherichia coli dam gene. J. Mol. Biol. **126**:367–380.
13. **Hearing, P., and T. Shenk.** 1983. The adenovirus type 5 E1A transcriptional control region contains the duplicated enhancer element. Cell **33**:695–708.
14. **Jones, N., and T. Shenk.** 1978. Isolation of deletion and substitution mutants of adenovirus type 5. Cell **13**:181–188.
15. **Lichy, J. H., J. Field, M. S. Horwitz, and J. Hurwitz.** 1982. Separation of the adenovirus terminal protein precursor from its associated DNA polymerase: role of both proteins in the initiation of adenovirus DNA replication. Proc. Natl. Acad. Sci. U.S.A. **79**:5225–5229.

16. **Lichy, J. H., M. S. Horwitz, and J. Hurwitz.** 1981. Formation of a covalent complex between the 80,000-dalton adenovirus terminal protein and 5'-dCMP *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **78**:2678–2682.
17. **Maniatis, T., E. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Merchlinsky, M. J., P. J. Tattersall, J. J. Leary, S. F. Cotmore, E. M. Gardiner, and D. C. Ward.** 1983. Construction of an infectious molecular clone of the autonomous parvovirus minute virus of mice. J. Virol. **47**:227–232.
19. **Pearson, G. D., K.-C. Chow, R. E. Enns, K. G. Ahern, J. L. Corden, and J. A. Harpst.** 1983. In vitro replication directed by a cloned adenovirus origin. Gene **23**:293–305.
20. **Perucho, M., D. Hanahan, and M. Wigler.** 1980. Genetic and physical linkage of exogenous sequences in transformed cells. Cell **21**:309–317.
21. **Pettersson, U., and J. Sambrook.** 1973. The amount of viral DNA in the genome of cells transformed by adenovirus type 2. J. Mol. Biol. **73**:125–130.
22. **Racaniello, V. R., and D. Baltimore.** 1981. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science **214**:916–919.
23. **Robinson, A. J., H. B. Younghusband, and A. J. D. Bellet.** 1973. A circular DNA-protein complex from adenoviruses. Virology **56**:54–69.
24. **Samulski, R. J., A. Srivastava, K. I. Berns, and N. Muzyczka.** 1983. Rescue of adeno-associated virus from recombinant plasmids: gene correction within the terminal repeats of AAV. Cell **33**:135–143.
25. **Steenbergh, P. H., and J. S. Sussenbach.** 1979. The nucleotide sequence of the right-hand terminus of adenovirus type 5 DNA: implications for the mechanism of DNA replication. Gene **6**:307–318.
26. **Stillman, B. W., J. B. Lewis, L. T. Chow, M. B. Mathews, and J. E. Smart.** 1981. Identification of the gene and mRNA for the adenovirus terminal protein precursor. Cell **23**:497–508.
27. **Stillman, B. W., F. Tamanoi, and M. B. Mathews.** 1982. Purification of an adenovirus-coded DNA polymerase that is required for initiation of DNA replication. Cell **31**:613–623.
28. **Stow, N. D.** 1981. Cloning of a DNA fragment from the left-hand terminus of the adenovirus type 2 genome and its use in site-directed mutagenesis. J. Virol. **37**:171–180.
29. **Stow, N. D.** 1982. The infectivity of adenovirus genomes lacking DNA sequences from their left-hand termini. Nucleic Acids Res. **10**:5105–5119.
30. **Szostak, J. W., and E. H. Blackburn.** 1982. Cloning yeast telomeres on linear plasmid vectors. Cell **29**:245–255.
31. **Tamanoi, F., and B. W. Stillman.** 1982. Function of adenovirus terminal protein in the initiation of DNA replication. Proc. Natl. Acad. Sci. U.S.A. **79**:2221–2225.
32. **Tamanoi, F., and B. W. Stillman.** 1983. Initiation of adenovirus DNA replication in vitro requires a specific DNA sequence. Proc. Natl. Acad. Sci. U.S.A. **80**:6446–6450.
33. **Tooze, J. (ed.).** 1982. DNA tumor viruses: molecular biology of tumor viruses, part 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Van Bergen, B. G. M., P. A. van der Ley, W. van Driel, A. D. M. van Mansfeld, and P. C. van der Vliet.** 1983. Replication of origin containing adenovirus DNA fragments that do not carry the terminal protein. Nucleic Acids Res. **11**:1975–1989.
35. **Van Ormondt, H., J. Maat, A. De Waard, and A. J. van der Eb.** 1978. The nucleotide sequence of the transforming HpaI-E fragment of adenovirus type 5 DNA. Gene **4**:309–328.