

Cloning of a DNA Fragment from the Left-Hand Terminus of the Adenovirus Type 2 Genome and Its Use in Site-Directed Mutagenesis

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The *HpaI* E fragment (0-4.5 map units) of adenovirus type 2 (Ad2) DNA was cloned in the plasmid vector pBR322. Excision of the viral insert with *PstI* and *XbaI* generated a fragment which comigrated with Ad2 *XbaI*-E (0-3.8 map units), and this fragment was ligated to the 3.8-100 fragment generated by *XbaI* cleavage of the DNA of the Ad5 mutant, *dl309* (N. Jones and T. Shenk, Cell 17:683-689, 1979). Transfection with the ligation products resulted in the production of progeny virus which was able to replicate on both HeLa and line 293 cells, demonstrating the biological activity of the sequences rescued from the plasmid. Small deletions were introduced around the *SmaI* site (map position 2.8) within the cloned viral insert, and the altered DNA sequences were reintroduced into progeny virus as described above. The mutant viruses grew well on line 293 cells but plaqued with greatly reduced efficiency on HeLa cells, exhibiting a host range phenotype similar to previously described mutants with lesions located within this region of the genome. When plasmid-derived left-end fragments containing pBR322 DNA sequences to the left of map position 0 were ligated to the 3.8-100 fragment of *dl309* DNA, the infectivity of the ligation products was not reduced. However, all progeny viruses examined yielded normal-size restriction enzyme fragments from their left-hand ends, indicating that the bulk of the pBR322 DNA sequences are removed either prior to or as a consequence of the replication of the transfecting DNA molecules.

The left-hand 11% of the genomes of adenovirus serotypes 2 and 5 (Ad2 and Ad5) contains genes which play important roles in both cellular transformation and the early stages of lytic virus infection (reviewed in reference 35). This early region (E1) contains two separate transcription units, E1a (map coordinates 1.4-4.4) and E1b (4.5-11.2) (38). Several overlapping spliced mRNA's are specified by both E1a and E1b (3, 7, 21, 32), and a number of polypeptides encoded by these regions have been identified (14, 16, 18, 22, 28, 36).

The presence of overlapping genes considerably complicates the investigation of the roles of individual E1 polypeptide products during the lytic cycle and in transformation. Recently, host range (*hr*) mutants of Ad5 have been isolated (17, 19) and are proving useful in the analysis of these processes (2, 20, 22). These mutants, which exhibit defects in transformation (11, 19), are unable to replicate in HeLa cells, but grow normally on line 293 cells, a line of human embryo kidney cells transformed by sheared Ad5 DNA (12). Mutant virus growth appears to be comple-

mented (in at least some instances) by E1 products expressed by the line 293 cells.

Two classes of Ad5 *hr* mutants have been described. Harrison et al. (17) obtained *hr* mutants (which probably contain point mutations) after treating Ad5 virions with nitrous acid. The mutations represented two complementation groups which mapped within regions E1a and E1b, respectively (9; R. Galos and J. Williams, personal communication). Jones and Shenk (19) first isolated an Ad5 mutant, *dl309*, which contained a single *XbaI* site at map position 3.8 but retained the ability to replicate on both HeLa and line 293 cells. Mutants were then selected for resistance of their DNAs to cleavage with *XbaI*. These mutants contained deletions or substitutions around the *XbaI* site and exhibited a host range phenotype.

This paper describes a method for isolating additional adenovirus *hr* mutants which contain lesions within region E1a. The Ad2 *HpaI* E fragment, which lies between map coordinates 0 and 4.5, was initially cloned in the plasmid vector pBR322 (4). This DNA fragment has a DNA sequence virtually identical to that of the corresponding Ad5 fragment (11; T. R. Gingeras, M. Kelly and R. J. Roberts, personal communi-

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cation), and the cleavage sites of nearly all restriction enzymes, including *Xba*I, are located in precisely equivalent positions. Mutations are introduced within the cloned *Hpa*I E fragment, and the altered DNA sequences are then transferred to progeny virus by excising a fragment corresponding to Ad2 *Xba*I-E (map coordinates 0-3.8) from the plasmid, ligating it to the large *Xba*I fragment of *dl*309 DNA (3.8-100), and transfecting cells with the ligated DNAs.

MATERIALS AND METHODS

Cells, viruses, and DNAs. HeLa cells grown in suspension were used for the propagation of wild-type Ad2 and the Ad5 mutant *dl*309 (obtained from T. Shenk). Monolayer cultures of HeLa and line 293 cells (provided by J. Williams) were maintained in Dulbecco-modified Eagle medium supplemented with 10% calf serum, 100 μ g of streptomycin per ml, and 100 μ g of penicillin per ml. Virus titrations were performed on HeLa or line 293 cell monolayers in 50-mm-diameter plastic petri dishes (17). *hr* mutants were grown on line 293 cell monolayers. Ad2, *dl*309, and *hr* mutant viruses were purified, and their DNAs were extracted as previously described (24).

DNA transfections. Monolayers of line 293 cells were infected with DNA by the calcium phosphate precipitation method (13) followed by a "boost" using 20% glycerol at 4-h postinfection (9, 33). Infected plates were overlaid with 0.7% agar, and single, well-isolated plaques were picked for analysis.

Restriction endonuclease digestions and gel electrophoresis. Restriction enzymes were obtained from Bethesda Research Laboratories or New England Biolabs, and the conditions for digestion of DNA were as recommended by the supplier. DNA fragments were separated by electrophoresis through 1% agarose or 7% acrylamide gels; the fragments were stained in buffer containing 0.5 μ g of ethidium bromide per ml and visualized during illumination with UV light (31).

Preparation of the large *Xba*I fragment of *dl*309 DNA. A 75- to 100- μ g amount of *dl*309 DNA was cleaved to completion with *Xba*I and the digested DNA was loaded on a preformed 5 to 20% gradient of sucrose in 1 M NaCl-20 mM Tris-hydrochloride (pH 7.8)-10 mM EDTA. Centrifugation was at 35,000 rpm for 6 h at 20°C in an SW41 rotor. Fractions were collected from the bottom of the tube, and those containing the large fragment were identified by subjecting small portions of each to electrophoresis through a 1% agarose gel. The fractions were pooled, diluted threefold with water, and ethanol precipitated. The DNA pellet was resuspended in 10 mM Tris-hydrochloride (pH 7.4)-1 mM EDTA at a concentration of about 100 μ g/ml.

Cloning the Ad2 *Hpa*I-E terminal fragment. The unseparated *Hpa*I fragments of Ad2 DNA, or pBR322 DNA (4) which had been linearized with *Pst*I, were tailed with approximately 20 deoxycytosine (dC) or deoxyguanosine (dG) residues, respectively, with terminal transferase under the conditions described by Roychoudhury et al. (29). The tailed fragments were chromatographed through a G75 Sephadex column,

ethanol precipitated, and resuspended in water. Tailed viral and plasmid DNAs were allowed to anneal, each at a concentration of 25 μ g/ml, in 0.1 M NaCl-0.5 mM EDTA for 2 h at 42°.

Escherichia coli χ 1776 was transformed with the hybrid molecules by using conditions developed by D. Hanahan (manuscript in preparation). Tetracycline-resistant colonies were screened for the presence of the *Hpa*I E fragment by in situ hybridization (15) to the Ad2 *Sma*I J fragment (0-2.8 map units) labeled in vitro with ³²P nick translation (26).

Mutagenesis of plasmid HE4 at the *Sma*I site. Plasmid HE4, which contains a cloned copy of the *Hpa*I E fragment, was cleaved to completion at position 2.8 in the viral sequences with *Xma*I (an isoschizomer of *Sma*I which generates a 5' overhang of four nucleotides [8]). A 5- μ g amount of the linearized DNA was digested with 5 U of S1 nuclease in a 100- μ l reaction mix for 30 min at 37°C. The S1-treated DNA was incubated in the presence of T4 DNA ligase for 16 h at 14°C and the products used to transform *E. coli* χ 1776 (10). Plasmids were extracted from 2-ml bacterial cultures started from single independent transformed colonies and screened for their susceptibility or resistance to *Sma*I digestion as described by Gluzman et al. (10).

Growth of plasmids. Cultures (200 ml) of bacteria were grown in broth containing 15 μ g of tetracycline per ml, and the plasmids were amplified in the presence of 170 μ g of chloramphenicol per ml added for approximately 12 h at an optical density of 0.6 at 575 nm. Plasmids were extracted from the pelleted bacteria, and supercoiled DNA was purified by cesium chloride-ethidium bromide density gradient centrifugation as described by Tanaka and Weisblum (34).

Reassembly of intact viral genomes. Plasmids containing wild-type or mutated *Hpa*I-E sequences were cleaved with enzymes *Pst*I and *Xba*I, and the resulting double digest fragments were ligated to the large *Xba*I fragment of *dl*309. Typical ligation reactions contained 1 μ g of the *dl*309 *Xba*I large fragment (*dl*309A) and 4 μ g of digested plasmid in a reaction volume of 40 μ l. The DNAs were incubated with 100 U of T4 DNA ligase (New England Biolabs, using their recommended buffer) for 16 h at 14°C. The total ligation reaction was used in the transfection of two replicate 50-mm petri dishes of line 293 cells.

Containment procedures. Bacteria (*E. coli* χ 1776) containing hybrid plasmids were grown under P2 conditions, and all experiments involving viruses containing sequences derived from recombinant plasmids were performed in a P2 laboratory, as approved by the Office of Recombinant DNA Activities of the National Institutes of Health.

RESULTS

Cloning of the *Hpa*I E fragment of Ad2 DNA. The 5' terminus of each strand of the adenovirus genome is covalently linked to a protein molecule (5, 25, 27, 30), a few amino acids of which remain attached even after treatment of the viral DNA with proteases. These residues, which block the action of polynucleotide kinase

and λ exonuclease on the 5' termini (6), would also be expected to prevent the direct ligation of these ends to other DNA molecules. To enable the molecular cloning of terminal DNA fragments, use was therefore made of their accessible 3' ends. The unseparated *HpaI* fragments of Ad2 DNA were tailed with dC residues by using terminal transferase and annealed to pBR322 tailed with dG residues at the single *PstI* site. This procedure results in the regeneration of *PstI* sites (as described later and illustrated in Fig. 5a) and therefore permits excision of the viral insert from the plasmid. After transfection of 3×10^8 *E. coli* χ 1776 with an annealed mixture containing 250-ng-tailed pBR322 and 250-ng-tailed *HpaI* fragments of Ad2, over 100 colonies which hybridized to ^{32}P -labeled Ad2 *SmaI* J fragment (0–2.8 map units) were obtained (see Materials and Methods). Two such colonies were purified, and the plasmids (HE3 and HE4) were prepared from them and analyzed in detail. *PstI* digestion of both HE3 and HE4 yielded two fragments, one of which comigrated with linear pBR322, whereas the other exhibited a slightly lower mobility than the Ad2 *HpaI* E fragment (Fig. 1a). There are no cleavage sites for *PstI* within the *HpaI* E fragment, and the slightly reduced mobility of the cloned viral fragments is probably because of the presence of the dG-dC tails. Digestion of HE3 and HE4 with *XbaI*, which cleaves at a single site in the viral insert, and *EcoRI*, which has a single site within the pBR322 sequences, demonstrates that the viral fragments are inserted in opposite orientations in these plasmids (Fig. 1b and d). A fragment which comigrated with the terminal *XbaI* E fragment was generated by double digestion with *XbaI* and *PstI* (Fig. 1c).

Cloned sequences retain their biological activity. The products of *XbaI* plus *PstI* digestion of HE3 or HE4 were ligated to the large fragment generated by *XbaI* cleavage of *dl309* DNA (*dl309* A), purified as described in Materials and Methods. One of the products of the ligation would be expected to correspond to an intact adenovirus genome composed of fragments with map coordinates 0–3.8 and 3.8–100 derived from the plasmid and *dl309* DNAs, respectively. The resulting ligation products were used to transfect line 293 cells. Plaques were obtained after infection with the ligated fragments, but not after single infection with the cleaved HE3 or HE4 DNAs or with the *dl309*A fragment. The number of plaques was similar to that obtained when equivalent amounts of the two *XbaI* fragments of *dl309* DNA were ligated together and used to transfect line 293 cells (Table 1).

When progeny virus from cells infected with

the ligated *dl309* A and HE3 or HE4 DNA fragments was assayed on line 293 and HeLa cells, approximately equal virus titers on the two cell lines were obtained for each of the 12 plaques tested (e.g., 3/1 and 3/2, Table 3). The E1a functions specified by the cloned *HpaI* E fragment and required for viral replication on HeLa cells (17, 19) are therefore expressed when this DNA fragment is reconstituted into an intact viral genome.

The *XbaI* site was regenerated in the DNAs of all rescued virus examined, and digestion with other restriction enzymes indicated that normalized fragments were produced from the left-hand end of these DNAs (e.g., compare *XbaI* and *SmaI* digests of 3/1 and *dl309* DNAs in Fig. 4).

Mutagenesis of plasmid HE4. The above experiments indicated that fragments corresponding to Ad2 *XbaI* E could be excised from plasmids HE3 and HE4 and transferred to non-defective progeny virus. This suggested that it might be possible to introduce mutations into the cloned viral sequences and use a similar technique to generate viruses carrying these lesions. The use of line 293 cells for the initial DNA transfections would permit the isolation of mutants with lesions in essential (in addition to nonessential) DNA sequences, provided that the E1a products expressed by the line 293 cells could complement the resulting defects.

To test this system, small deletions were introduced into plasmid HE4 around the single *SmaI* site (2.8 map units on the Ad2 genome). The plasmid was cleaved with *XmaI* (an isoschizomer of *SmaI* which generates a 5' overhang of four nucleotides upon cleavage; reference 8). The resulting linear molecules were treated sequentially with S1 nuclease and DNA ligase, and the ligation products were used to transform *E. coli* χ 1776. Twenty tetracycline-resistant colonies were picked, small-scale plasmid extractions were performed, and the products were incubated with *SmaI*. Six plasmids resistant to enzyme digestion were obtained (see Materials and Methods for details).

Figure 2 shows the products of digestion of the wild-type plasmid (HE4) and two *SmaI*-resistant plasmids, Sx1 and Sx15, with the enzymes *EcoRI* and *SmaI*. All three plasmids were linearized by *EcoRI* treatment alone, but only HE4 yielded two fragments when digested with both enzymes, confirming the loss of the *SmaI* site from the two mutant plasmids.

HE4 was digested with the multicut enzyme *Sau96I* alone, and also with *Sau96I* and *SmaI* to identify the *Sau96I* fragment containing the *SmaI* site. Comparison of the *Sau96I* profiles of HE4, Sx1, and Sx15 indicated that small dele-

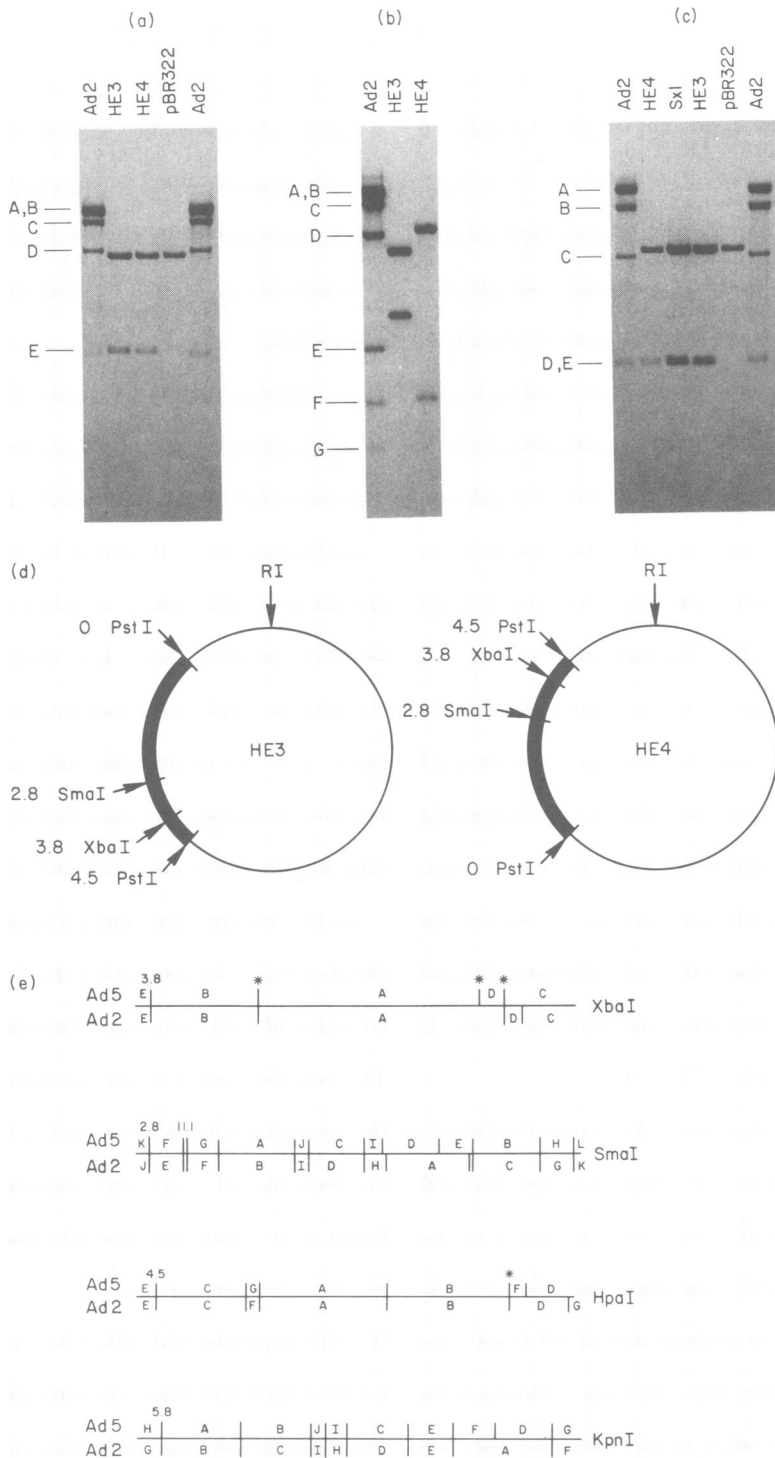


FIG. 1. Analysis of plasmids HE3 and HE4. (a) pBR322, HE3, and HE4 DNAs digested with PstI and subjected to electrophoresis through a 1% agarose gel with Ad2 HpaI markers (HpaI fragments F and G are too faint to be clearly seen in this photograph). (b) HE3 and HE4 DNAs digested with EcoRI and XbaI and run with Ad2 XbaI markers. (c) pBR322, HE3, HE4, and Sx1 DNAs digested with PstI and XbaI and run with Ad2 XbaI markers. Plasmid Sx1 is an SmaI-resistant derivative of plasmid HE4 (see mutagenesis of plasmid HE4). (d) Structure of HE3 and HE4 showing the position of the cleavage sites for SmaI, XbaI, PstI, and EcoRI and the orientation of the viral inserts. (e) Physical maps for Ad2 and Ad5 DNAs for the enzymes XbaI, HpaI, SmaI, and KpnI (adapted from reference 35). dl309 DNA has the same maps as Ad5 except that one HpaI site and three XbaI sites have been lost (marked *, reference 19).

TABLE 1. Transfer of cloned Ad2 left-end DNA sequences to progeny virus^a

DNA I (0-3.8 map units)	DNA II (3.8-100 map units)	Plaques/plate
	<i>dl309 A</i>	0, 0
HE3	<i>dl309 A</i>	37, 28
HE4	<i>dl309 A</i>	22, 22
<i>dl309 B</i>	<i>dl309 A</i>	26, 27
HE3		0, 0
HE4		0, 0
<i>dl309 B</i>		0, 0
		0, 0

^a A 1- μ g amount of *dl309 A* fragment was ligated to either 4 μ g of HE3 or HE4 DNA cleaved with *Xba*I and *Pst*I, or to 0.9 μ g of the small *Xba*I fragment of *dl309* DNA (*dl309 B*). In each case, the amount of the 0-3.8 fragment present was equivalent to 23 μ g of intact Ad2 DNA. Each ligation reaction was used to transfect two 50-mm dishes of line 293 cells. Single infection controls were set up by using the above-mentioned amounts of unligated DNA. Plaques were counted 8 days postinfection.



FIG. 2. Resistance of *Sx1* and *Sx15* DNAs to cleavage with *Sma*I. HE4, *Sx1*, and *Sx15* DNAs were cleaved with *Sma*I and *Eco*RI (tracks labeled a), or with *Eco*RI alone (labeled b), and the fragments were separated by electrophoresis through a 1% agarose gel.

tions of about 7 and 14 nucleotides, respectively, had occurred within this 173-base-pair fragment (T. Gingeras, et al., personal communication) of *Sx1* and *Sx15* (Fig. 3).

Construction of virus lacking the *Sma*I site at 2.8 map units. Mutant plasmids *Sx1* and *Sx15* were cut with *Xba*I and *Pst*I, the fragments were ligated to the *dl309 A* fragment, and line 293 cells were transfected as previously

described for the wild-type plasmid HE4. Plaques were again produced in monolayers infected with the ligation products (Table 2). Twenty-four independent plaques were picked from plates which received the mutant plasmid DNAs, the virus from these plaques was passaged on line 293 cells, and small-scale preparations of viral DNA were made. These were cleaved with *Sma*I, and in each case the cleavage site at 2.8 map units was missing. As an example,

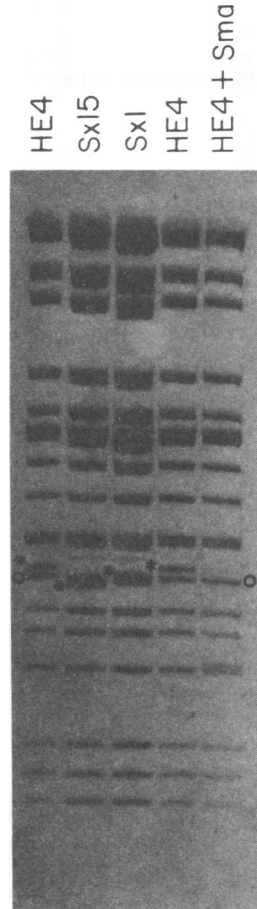


FIG. 3. Plasmids *Sx1* and *Sx15* contain small deletions around the *Sma*I site. HE4, *Sx1*, and *Sx15* DNAs were digested with *Sau*96I, and HE4 DNA was also digested with *Sau*96I and *Sma*I (HE4 + *Sma*). The fragments were separated through a 7% acrylamide gel. * indicates the *Sau*96I fragment of HE4 (173 base pairs), which contains the *Sma*I site, and the position of the corresponding fragments generated from *Sx1* and *Sx15* DNAs. ° indicates a *Sau*96I fragment of 164 base pairs generated from within the viral insert of each of the plasmids (sizes of the *Sau*96I fragments were derived from the DNA sequence data of T. R. Gingeras et al., personal communication).

TABLE 2. Transfer of mutated left-end Ad2 DNA sequences to progeny virus^a

DNA I (0-3.8 map units)	DNA II (3.8-100 map units)	Plaques/plate
	<i>dl309</i> A	0, 0
HE4	<i>dl309</i> A	22, 25
Sx1	<i>dl309</i> A	53, 40
Sx15	<i>dl309</i> A	24, 29
HE4		0, 0
Sx1		0, 0
Sx15		0, 0

^a Conditions were as described in footnote a of Table 1, except that the 0-3.8 fragments were from the wild-type plasmid HE4 and *Sma*I-resistant plasmids Sx1 and Sx15 derived from it.

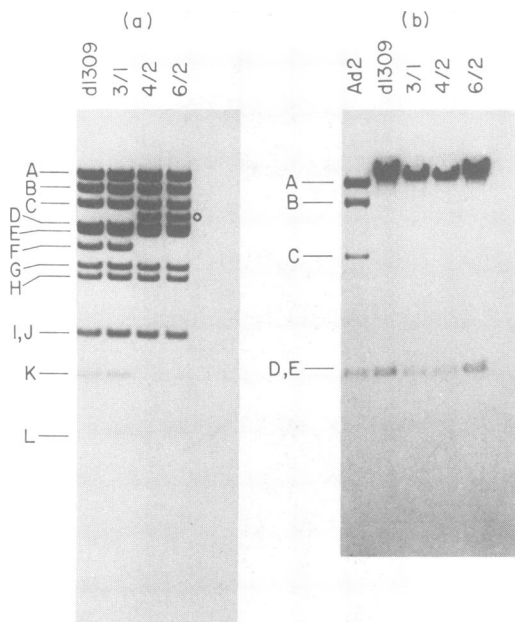


FIG. 4. Analysis of viruses lacking the *Sma*I site at 2.8 map units. (a) *Sma*I digest patterns for the DNAs of *dl309* and rescued viruses 3/1, 4/2, and 6/2, which contain sequences derived from plasmids HE4, Sx1, and Sx15, respectively. o, New fragment generated from 4/2 and 6/2 DNAs. (b) *Xba*I digest patterns for Ad2, *dl309*, 3/1, 4/2, and 6/2 DNAs.

Fig. 4 shows the *Xba*I and *Sma*I profiles for three viruses (3/1, 4/2, and 6/2) containing sequences derived from plasmids HE4, Sx1, and Sx15, respectively. The digest patterns for *dl309* and 3/1 DNAs are indistinguishable, but 4/2 and 6/2 DNAs have lost the *Sma*I K and F fragments and yield a new band of higher molecular weight, corresponding to about 11 map units. This not only indicates the loss of the *Sma*I cleavage site at 2.8 map units, but also confirms that the left-hand end sequences of these viruses are derived from the plasmid DNA.

All three viruses yielded two *Xba*I fragments indistinguishable from those generated from *dl309* DNA, demonstrating the regeneration of the *Xba*I site upon ligation and consistent with the presence of only small deletions around the *Sma*I site of the mutant plasmids.

Viruses which have lost the *Sma*I site at 2.8 map units exhibit a host-range phenotype. The virus from independent plaques picked from plates infected with *dl309* A and the fragments derived from plasmids HE4, Sx1, or Sx15 was passaged on line 293 cells, and the yield of progeny was titrated on line 293 and HeLa cells. Table 3 shows the titers of *dl309* and two known *hr* mutants of Ad5 (*dl312* [19] and *hr* 1 [17]) on these two cell lines. *dl309* and rescued virus containing sequences from plasmid HE4 (3/1 and 3/2) plaqued with approximately equal efficiency on the two cell lines, whereas *hr* 1, *dl312*, and viruses rescued using plasmids Sx1 (4/1 and 4/2) and Sx15 (6/1 and 6/2) all showed a greatly reduced efficiency of plaquing on HeLa cells. The *Sma*I site mutants constructed with in vitro-mutagenized plasmid, therefore, exhibit a host-range phenotype similar to that previously described for *hr* 1 and *dl312*. It should also be noted that the plaques produced by all the *hr* mutants (i.e., *dl312*, *hr* 1, 4/1, 4/2, 6/1, and 6/2) on HeLa cells were very much smaller and less distinct than those produced by the wild-type viruses (*dl309*, 3/1, and 3/2).

Structure of the left-hand ends of rescued virus. The DNA sequences present at the left-

TABLE 3. Titration of rescued viruses on HeLa and line 293 cells^a

Virus	Plasmid used in virus construction	Titer on HeLa cells (PFU/ml)	Titer on line 293 cells (PFU/ml)
<i>dl309</i>		2.0×10^9	3.0×10^9
<i>dl312</i>		4.2×10^4	6.2×10^8
<i>hr</i> 1		8.7×10^4	8.5×10^7
3/1	HE4	7.9×10^8	7.5×10^8
3/2	HE4	1.6×10^8	3.6×10^8
4/1	Sx1	1.5×10^5	3.7×10^8
4/2	Sx1	1.8×10^5	1.2×10^8
6/1	Sx15	2.4×10^5	5.7×10^7
6/2	Sx15	2.7×10^6	5.1×10^8

^a Individual plaques were picked from line 293 cell monolayers infected with the DNA ligation products indicated in Table 2. Virus from these plaques contained left-end sequences derived from wild-type plasmid HE4 (3/1 and 3/2) and its *Sma*I-resistant derivatives Sx1 (4/1 and 4/2) and Sx15 (6/1 and 6/2). The virus was passaged once on line 293 cells, and the resulting stocks were titrated on HeLa and line 293 cells in parallel with a known wild-type virus *dl309* (19) and two known *hr* mutants *dl312* (19), and *hr* 1 (17).

hand termini of viruses rescued by using the plasmid-derived Ad2 *Xba*I E fragment are of particular interest. The nucleotide sequences at these termini, and at the junctions between the pBR322 and Ad2 DNAs in the plasmids HE3 and HE4, have not yet been determined. However, the facts that the *Hpa*I E fragment was cloned by dG-dC tailing and the resulting plasmids (HE3, HE4) contain a *Pst*I site corresponding to 0 map units on the viral genome indicate

that the left-hand end of the viral fragment (0-3.8) cleaved from the plasmids is not identical to the corresponding end of the authentic Ad2 *Xba*I E fragment. Thus, the DNA sequences present at the left-hand terminus of genomes reconstructed by ligation with plasmid-derived DNA fragments differ from those found on DNA extracted from virions (Fig. 5a). Transfection with these molecules nevertheless results in the generation of progeny virus.

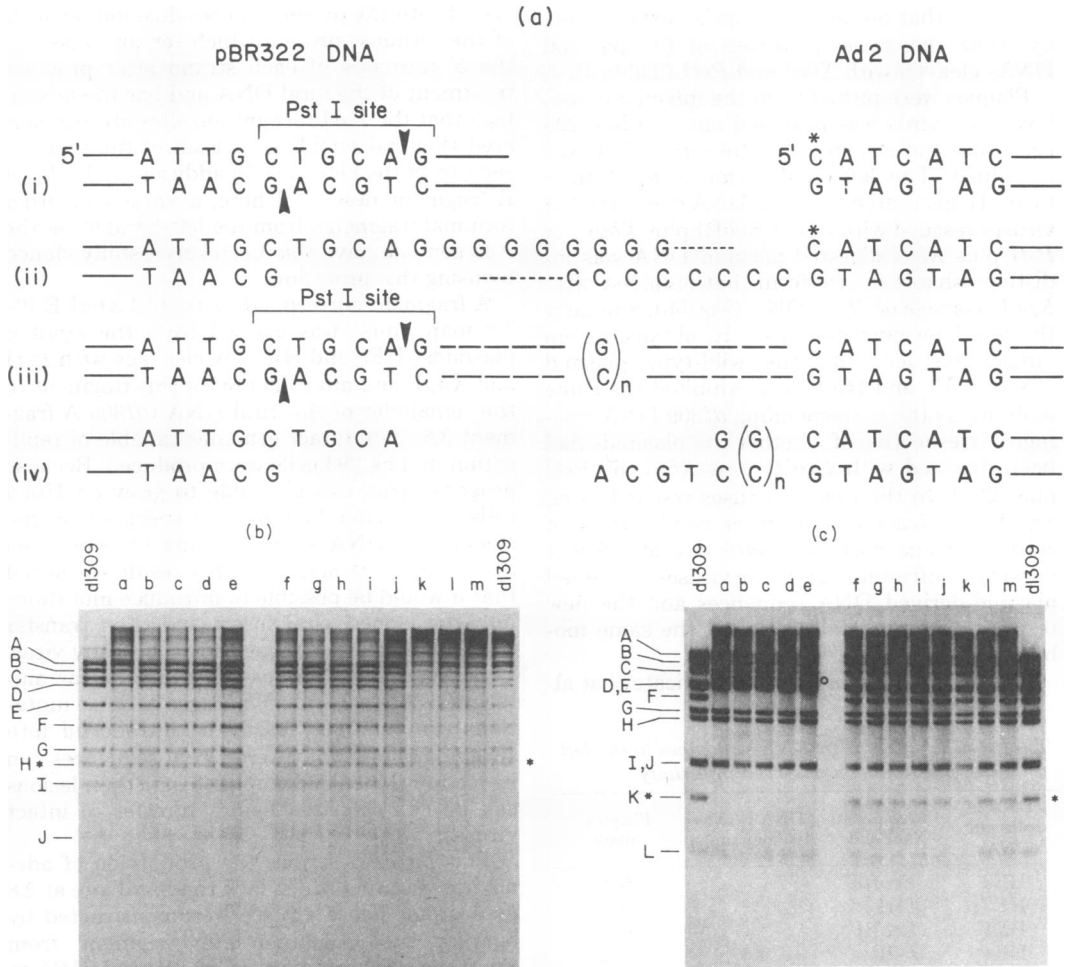


FIG. 5. Structure of the left-hand end of the cloned *Hpa*I E fragment and of rescued viruses. (a) Diagram of the cloning procedure showing (i) sequence around the *Pst*I site in pBR322 DNA and at the left-hand terminus of Ad2 DNA. * indicates the 5' dC residue to which the terminal protein is linked (see text). (ii) Sequences present after tailing of *Pst*I cut pBR322 with dG residues and the left-hand terminus of Ad2 with dC residues. (iii) Hypothetical sequence at the junction between pBR322 and viral DNA in hybrid plasmid (e.g., HE4) showing a homopolymer stretch of n residues. (iv) Sequence at the left-hand terminus of the viral DNA after excision of the insert with *Pst*I. (b) *Kpn*I digest patterns of dl309 DNA, and DNAs of independently isolated viruses rescued using *Eco*RI-*Xba*I-cleaved Sx1 (a,b,c); *Pst*I-*Xba*I-cleaved Sx1 (d,e); *Eco*RI-*Xba*I-cleaved HE3 (f,g); *Pst*I-*Xba*I-cleaved HE3 (h,i); *Eco*RI-*Xba*I-cleaved HE4 (j,k); and *Pst*I-*Xba*I-cleaved HE4 (l,m). *, Left-hand terminal fragment H of dl309 DNA. (c) *Sma*I digests of above viral DNAs. *, left-hand terminal fragment, K, of dl309 DNA. °, left-hand terminal fragment of viruses lacking the cleavage site at 2.8 map units.

To test the effect of additional DNA sequences lying to the left of position 0 on the efficiency of virus rescue, plasmids HE3, HE4, or Sx1 were cleaved with *Xba*I and *Eco*RI, and the products ligated to the *dl*309 A fragment. In this instance the ligation products would be expected to contain approximately 3,600 (in the case of HE4 and Sx1) or 750 (HE3) base pairs of pBR322 DNA attached to the viral DNA to the left of position 0 (see Fig. 1).

Transfection with these molecules resulted in the production of plaques with an efficiency similar to that obtained in parallel experiments by using the same quantities of the plasmid DNAs cleaved with *Xba*I and *Pst*I (Table 4).

Plaques were picked from the infected monolayer, the virus was passaged once on line 293 cells, and the structure of the viral DNA was examined. The left-hand terminal *Kpn*I fragment, H, generated from the DNA of each of the viruses rescued with either *Eco*RI plus *Xba*I-, or *Pst*I plus *Xba*I-digested plasmid DNA was indistinguishable in size from that obtained after *Kpn*I cleavage of *dl*309 DNA (Fig. 5b). Similarly the *Sma*I terminal fragment, K, obtained from viruses rescued with the wild-type plasmid DNAs, HE3 and HE4, also exhibited the same mobility as the corresponding *dl*309 DNA fragment, irrespective of whether the plasmids had been digested with *Eco*RI plus *Xba*I, or *Pst*I plus *Xba*I. In the case of viruses rescued using Sx1 DNA cleaved with either combination of restriction enzymes, the *Sma*I site at 2.8 was missing, confirming that these viruses contained plasmid-derived DNA sequences and the new terminal fragments all exhibited the same mobility (Fig. 5c).

These experiments therefore indicate that al-

though the initial transfecting molecules must contain additional pBR322 DNA sequences linked to the viral genomes at position 0, the bulk of these sequences are not present in progeny virus DNA.

DISCUSSION

Hybrid clones containing the Ad2 *Hpa*I E fragment (0-4.5 map units) inserted into the *Pst*I site of the plasmid vector pBR322 were constructed by using the dG-dC tailing procedure. This method circumvents problems associated with the presence of residual amino acids of the terminal protein which remain linked to the 5' terminus of each strand after protease treatment of the viral DNA and has the advantage that the *Pst*I recognition sites are regenerated (Fig. 5a) enabling excision of the viral inserts from the plasmids. In addition to the *Hpa*I E fragment described here, a variety of other terminal fragments from the left-hand 9% of the Ad2 genome have also been successfully cloned by using this procedure.

A fragment corresponding to Ad2 *Xba*I-E (0-3.8 map units) was excised from the *Hpa*I-E plasmids (HE3 and HE4) by cleavage with *Pst*I and *Xba*I, and after ligation of this fragment to the remainder of the viral DNA (*dl*309 A fragment 3.8-100), intact genomes capable of replication on line 293 cells were produced. Because progeny virus was also able to grow on HeLa cells, the region 1a functions specified by the cloned viral DNA sequences must have retained their biological activity. This result suggested that it would be possible to introduce mutations into the cloned viral insert and then transfer these altered DNA sequences to progeny virus. Using plasmid DNAs as the substrate for mutagenesis has several advantages because mutations can relatively easily be introduced into specific regions, the altered viral sequences can be readily cloned and amplified, and their lesions can be characterized before transfer to intact viruses.

This paper describes the production of adenovirus mutants which lack the *Sma*I site at 2.8 map units. These viruses were constructed by ligating the 0-3.8-map-unit fragment from *Sma*I-resistant derivatives of plasmid HE4 to the *dl*309 A fragment. *Sma*I cleaves Ad2 and Ad5 DNAs 13 times; therefore, the probability of generating this type of mutant by the procedure of Jones and Shenk (19), which depends on restriction enzyme cleavage of full-length viral DNA followed by religation of the fragments, is exceedingly low. In addition virus from each of the plaques tested contained left-end sequences derived from the plasmid DNA, making it unnecessary to screen large numbers of plaques to obtain the desired mutant.

TABLE 4. Effect of pBR DNA sequences to the left of position 0 on observed infectivity^a

DNA I (plasmid DNA)	Cleaved with <i>Xba</i> I and:	DNA II (3.8-100 map units)	Plaques/plate
HE3	<i>Eco</i> RI		0, 0
HE3	<i>Pst</i> I		0, 0
HE4	<i>Eco</i> RI		0, 0
HE4	<i>Pst</i> I		0, 0
Sx1	<i>Eco</i> RI		0, 0
Sx1	<i>Pst</i> I		0, 0
		<i>dl</i> 309 A	0, 0
HE3	<i>Eco</i> RI	<i>dl</i> 309 A	38, 40
HE3	<i>Pst</i> I	<i>dl</i> 309 A	34, 27
HE4	<i>Eco</i> RI	<i>dl</i> 309 A	12, 21
HE4	<i>Pst</i> I	<i>dl</i> 309 A	26, 21
Sx1	<i>Eco</i> RI	<i>dl</i> 309 A	66, 51
Sx1	<i>Pst</i> I	<i>dl</i> 309 A	38, 34

^a Conditions as for Table 1 except the plasmid DNAs were cleaved with *Xba*I and either *Pst*I or *Eco*RI as indicated before ligation.

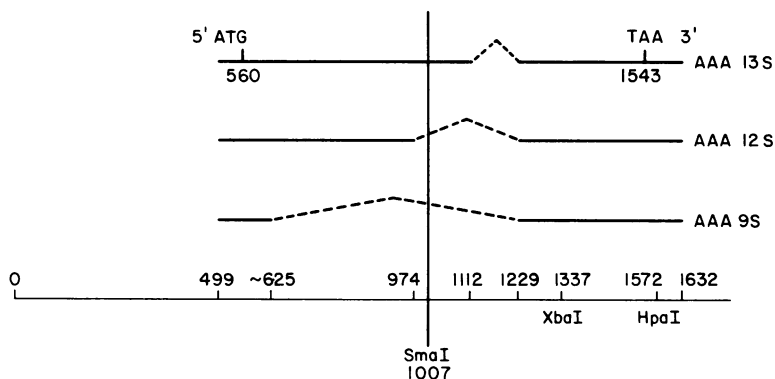


FIG. 6. Location of *Sma*I site in relation to the E1a mRNA's. The positions of the initiation and termination codons, the 3' ends of the RNAs and the splice points for the 13S and 12S mRNA's are taken from the sequence data of Perricaudet et al. (23). The structure of the 9S mRNA is from Chow et al. (7), and the position of the 5' end of the E1a mRNA's from Baker and Ziff (1). T. R. Gingeras, M. Kelly, and R. J. Roberts (personal communication) provided the Ad2 genomic DNA sequence and the locations of the restriction endonuclease cleavage sites.

The mutant plasmids Sx1 and Sx15 contained small deletions (approximately 7 and 14 nucleotides, respectively) at the *Sma*I site, introduced into the hybrid plasmid HE4. Inspection of the nucleotide sequence for the Ad2 genome (T. R. Gingeras et al., personal communication), and comparison with the mRNA sequences for region E1a (23), show that sequences corresponding to the *Sma*I site at position 2.8 are present in the largest E1a message (13S). However, this site is within the introns for the genes specifying the smaller 12S and 9S mRNA's (see reference 7 and Fig. 6). Thus, provided that the 12S and 9S mRNA's are synthesized, processed, and translated normally, the new lesions will affect only one of the products of these overlapping genes. The virus mutants obtained exhibited a host-range phenotype similar to that previously described for other E1a mutants (17, 19), and a series of viruses containing different lesions around the *Sma*I site is being characterized in detail to determine the nature of the defect.

The approach described here, in which mutations are initially introduced into cloned viral sequences and then transferred to progeny virus, depends for its success on the availability of permissive cell lines which can complement defects resulting from the mutagenesis. In the experiments described here, the ability of line 293 cells to complement defects in E1a (9, 17, 19) facilitated the isolation of mutants with small deletions located at 2.8 map units. The method should hopefully prove of more general use in the genetic analysis of other regions of the adenovirus genome as cell lines expressing genes from these regions become available (e.g., constructed by co-transformation as described by Grodzicker and Klessig [14a]).

One point of particular interest in this work concerns the nature of the sequences present at the left end of the viruses rescued by using plasmid-derived fragments. It was shown (Fig. 5 and Table 4) that even when long sequences of pBR322 DNA remain attached to the left of position 0 on the transfecting DNA molecules, the DNA is infectious and the left-most fragment generated from the rescued virus is of normal size. Whether the additional pBR322 sequences are excised before replication of the ligated molecules or are lost as a consequence of the replication of these molecules is not yet known. If the latter situation is correct, it suggests that new viral DNA strands can be initiated at a specific sequence (corresponding to the terminal sequence) that can be recognized even when located internally within a viral DNA molecule. Alternatively, for DNA strands initiated at the right end of the viral DNA, a sequence-specific mechanism might determine the position at which the synthesis of the strand is terminated (corresponding to the normal left-hand terminus). It should be possible to introduce mutations around the origin of viral DNA replication into cloned terminal fragments and use them to provide insights concerning the mode of adenovirus DNA replication *in vivo* or *in vitro*.

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