Proc. Nat. Acad. Sci. USA
Vol. 68, No. 5, pp. 1037–1041, May 1971

Simian Virus 40 Integration Site in an Adenovirus 7–Simian Virus 40 Hybrid DNA Molecule

THOMAS J. KELLY, JR. AND JAMES A. ROSE

Laboratory of Viral Diseases and Laboratory of the Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Communicated by Robert J. Huebner, March 3, 1971

ABSTRACT **The E46$^+$ strain of Adenovirus 7 is a mixed-virus population containing defective Adenovirus 7–SV40 hybrid particles and helper, nonhybrid Adenovirus 7 particles. We have applied electron microscopic mapping techniques to obtain a physical map of the genome of the hybrid particles present in E46$^+$PL1, a substrain of E46$^+$ derived from a single two-hit plaque. DNA molecules extracted from purified E46$^+$PL1 virions were found to be linear duplexes, with a mean length of 10.9 μm. When these molecules were denatured and renatured, a unique heteroduplex was formed that presumably derived one of its strands from an Adenovirus 7–SV40 hybrid molecule and the other from a nonhybrid Adenovirus 7 molecule. This heteroduplex was double-stranded, except for a short region near one end where the two strands were not paired. On the basis of measurements of the lengths of the single- and double-stranded regions in the heteroduplex, the structure of the Adenovirus 7–SV40 hybrid genome can be reconstructed as follows: The hybrid genome contains 16% less Adenovirus 7 DNA than the nonhybrid Adenovirus 7 genome. This deletion consists of the segment of DNA that maps between 0.05 and 0.21 molecular lengths in the nonhybrid Adenovirus 7 DNA molecule. The deleted DNA has been partially replaced by an amount of heterologous DNA equivalent to 75% of the complete SV40 genome. A model for the generation of the hybrid genome is presented.**

The E46$^+$ strain of adenovirus 7 (Ad. 7) (1) contains two types of virus particles: (*a*) hybrid particles that contain both Ad. 7 and SV40 DNA in the same Ad. 7 capsid, and (*b*) nonhybrid Ad. 7 particles that contain only Ad. 7 DNA (2–4). Hybrid particles differ from nonhybrid particles in several biological properties: they are defective and require coinfection with nonhybrid Ad. 7 for their replication (3, 4); they are able to induce SV40 T and transplantation antigens (but not SV40 capsid antigen or virus) (5–7); and they have a greatly increased oncogenic potential (8). To date it has not been possible to purify the hybrid particles away from the excess nonhybrid helper particles in the E46$^+$ population (3, 9).

Electron microscopic techniques have been developed for mapping the position and size of bacteriophage deletion and substitution mutations (10, 11). These techniques were applied to obtain a physical map of the genome of the hybrid particles present in E46$^+$PL1, a substrain of E46$^+$ derived from a single (two-hit) plaque*. There is evidence that the Ad. 7 and SV40 components of the hybrid-particle genome are covalently linked in a single DNA molecule (12). Thus, denaturation and renaturation of E46$^+$PL1 DNA should yield some molecules composed of one hybrid and one nonhybrid strand (heteroduplexes). Such heteroduplexes would be double-stranded molecules, except where the two strands are not homologous. Lack of homology between the two strands could arise from at least two sources: the presence of SV40 DNA in the hybrid strand and/or the deletion of a segment of Ad. 7 DNA from the hybrid strand. In either case, the nonhomologous regions would be expected to give rise to single-stranded loops, whose precise location and size could be determined. In this report, we show that a unique heteroduplex is formed after renaturation of denatured E46$^+$PL1 DNA. On the basis of measurements of the lengths of the single- and double-stranded regions in this heteroduplex, we propose a model for the structure of the E46$^+$PL1 hybrid-particle genome.

Abbreviations: SV40, Simian Virus 40; Ad. 7, Adenovirus 7.

* The E46$^+$PL1 substrain was obtained from a plaque induced by plating the E46$^+$ strain on monkey cells. Plaque induction required simultaneous infection with both a hybrid and a nonhybrid particle (3), and both types of particles were recovered from plaques.

MATERIALS AND METHODS

Viruses. The E46$^+$PL1 strain of Ad. 7 originated as a plaque isolate from the E46AG8 passage of the E46 pool of the LL strain of Ad. 7 (3). It contains a mixture of hybrid (Ad. 7–SV40) and nonhybrid (Ad. 7) virions. The E46$^-$ strain (nonhybrid Ad. 7) was originally isolated from the E46 pool by limiting-dilution passage in human embryonic kidney cells (HEK) (3). Pools of both virus strains were obtained from W. P. Rowe. E46$^+$PL1 was passed in African green monkey kidney cells (AGMK) and E46$^-$ in HEK cells. Stocks of these strains were free of Adenovirus-associated virus by complement-fixation tests (13).

Virus Production and Purification. E46$^+$PL1 was produced in primary cultures of AGMK cells infected at a multiplicity of about 10 tissue culture infectious doses$_{50}$ per cell. Cells were harvested on the 4th day after infection, at which time 90% of the cells showed cytopathic lesions. E46$^-$ was produced in KB cells in a suspension culture (15). Both E46$^+$PL1 and E46$^-$ were purified as before (14, 15), except for omission of the genetron extraction step.

Purification of Viral DNA. Bands of purified E46$^+$PL1 or E46$^-$ virus obtained from CsCl density gradients were dialyzed into SSC (0.15 M NaCl–0.015 M sodium citrate), then incubated with pronase (16) (1 mg/ml) in the presence of 0.3% sodium dodecyl sulfate at 37°C for 2 hr. The mixture was extracted by gentle rocking with an equal volume of redistilled phenol equilibrated with water. The organic phase

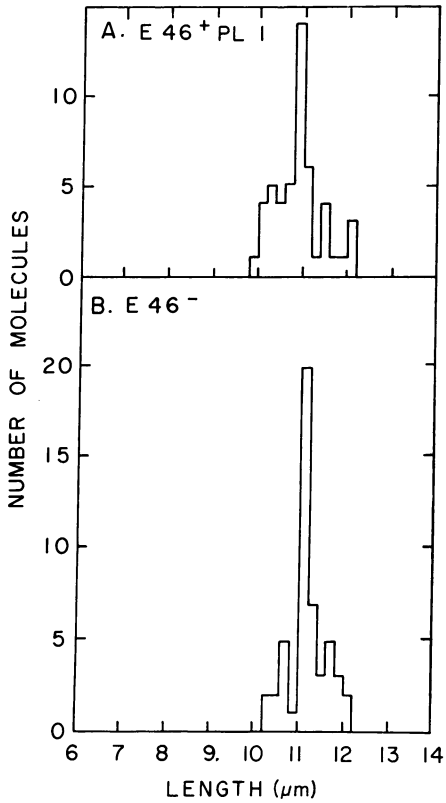


FIG. 1. Length distribution of E46+PL1 and E46− DNA molecules mounted for electron microscopy by the aqueous procedure. The lengths of all unambiguous molecules were determined. The mean length of 49 E46+PL1 molecules was 10.9 ± 0.5 μm and of 50 E46− molecules was 11.2 ± 0.4 μm.

was removed and the aqueous phase was extracted with phenol two more times. The aqueous phase was then dialyzed into Tris–EDTA buffer (0.01 M Tris, pH 8.5–0.02 M EDTA). DNA concentration was measured by absorbance at 260 nm.

Denaturation and renaturation of DNA was performed as described by Davis, Simon, and Davidson (11). The SV40 DNA used in this work was a gift of Dr. M. Martin.

Electron Microscopy. The conditions for mounting DNA for electron microscopy were those described by Davis, Simon, and Davidson (11). For the aqueous technique, the spreading solution contained 0.5–1.0 μg/ml DNA, 0.1 mg/ml cytochrome *c*, and 0.5 M NH_4 acetate; the hypophase was 0.25 M NH_4 acetate. For the formamide technique, the spreading solution contained 0.5–1.0 μg/ml DNA, 0.1m g/ml of cytochrome *c*, 0.1 M Tris (pH 8.5), and 0.01 M EDTA in 60% formamide (V/V); the hypophase was 10% formamide (V/V)–0.01 M Tris, pH 8.5. Electron micrographs were taken on Kodak Electron Image Plates with a Siemens Elmiskop 101, at magnifications from 3000 to 9000, with a 20-μm objective aperture and 40 kV accelerating voltage. Magnification was calibrated with a grating replica (E. F. Fullum: 0.883-μm spacing). Contour lengths were measured from tracings of enlarged molecular images with a Keuffel and Esser 620300 map measurer.

RESULTS

Electron Microscopy of Native E46+PL1 and E46− DNA Molecules. Fig. 1*A* shows the length distribution of the native DNA molecules extracted from E46+PL1 virus particles. All the molecules in this preparation were linear. Under the conditions used in preparing the electron microscopic grids, (aqueous technique) (11), single-stranded DNA characteristically appears as a collapsed "bush" (see below). No intramolecular bushes were observed. The mean length of the molecules in the E46+PL1 population was 10.9 ± 0.5 μm. The E46− (pure nonhybrid Ad. 7) population also contained linear duplex DNA molecules (Fig. 1*B*). The mean length of these molecules (11.2 ± 0.4 μm) was not significantly different from that of the E46+PL1 molecules.

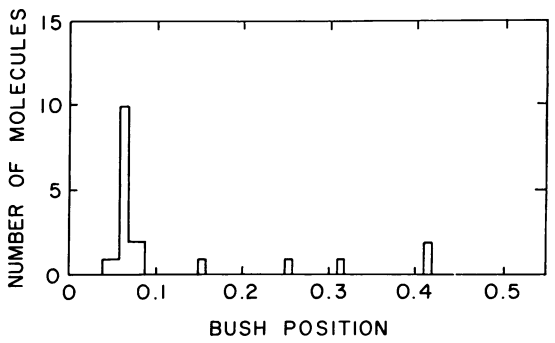
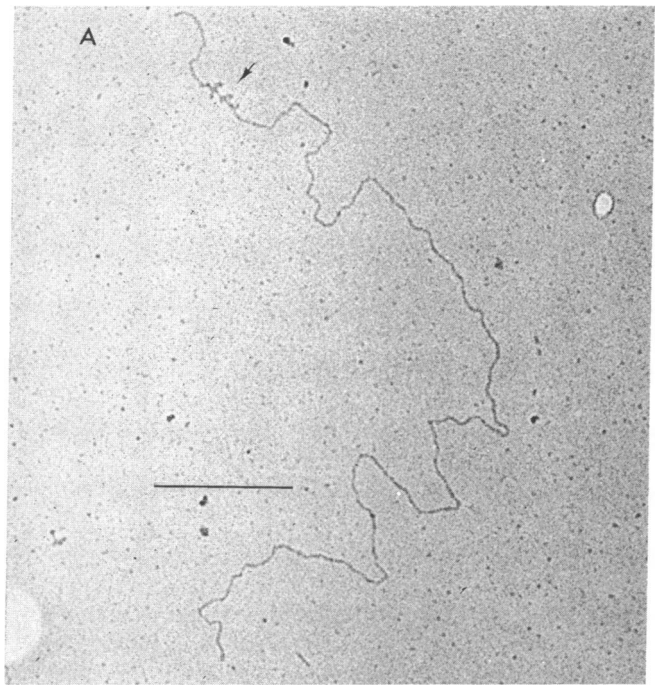
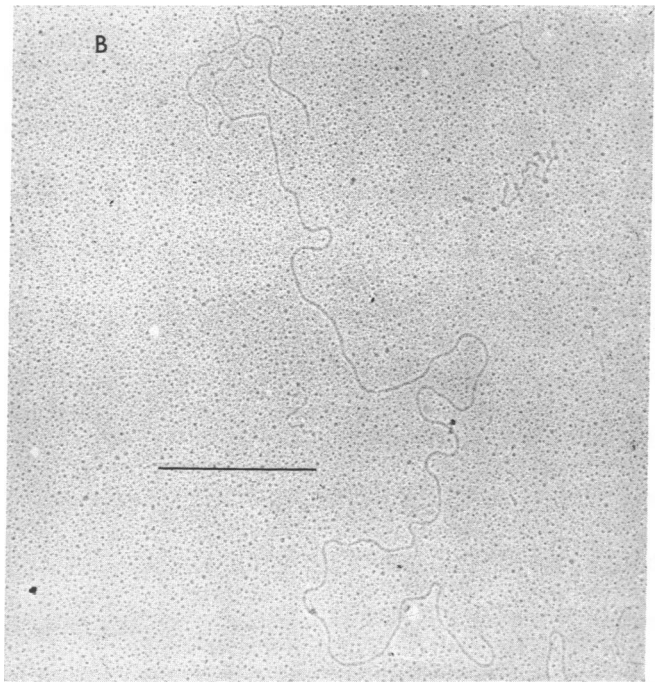


FIG. 2. Histogram of bush positions in denatured and renatured E46+PL1 DNA molecules. The preparation of E46+PL1 DNA of Fig. 1 was denatured and renatured, then mounted for electron microscopy by the aqueous procedure. The bush position was determined for all unambiguous molecules with internal bushes. The abscissa is the distance of the center of a bush from one end of the molecule measured as a fraction of the total double-stranded length of the molecule.

Electron Microscopy of Denatured and Renatured E46+PL1 DNA. A preparation of E46+PL1 DNA was denatured in alkali and then renatured at room temperature (11). With the aqueous technique, heteroduplexes composed of one Ad. 7 DNA strand and one Ad. 7–SV40 hybrid DNA strand should contain one or more uniquely located single-stranded bushes. However, preparations of this type also generally contain molecules with bushes that are not due to the existence of nonhomologous regions in the two DNA strands, but to the presence of single-strand breaks (17). In particular, about half of the molecules in the E46+PL1 preparation (and in control preparations of E46−) had bushes at one or both ends. The double-stranded portions of these molecules were very heterogeneous in length and were shorter than molecules without terminal bushes. It is likely that these molecules result from the renaturation of two DNA strands, one or both of which is broken. (See ref. 17 for a discussion of this point.) For molecules without terminal bushes, 16 of 49 molecules photographed (33%) had a single internal bush uniquely located at a fractional map position of about 0.06 (Fig. 2). Interestingly, this bush did not originate from a single point (as might be expected in the case of a heteroduplex in which one strand contains a simple insertion of a length of nonhomologous DNA), but was spread over a small region and was composed of several "bushlets" (Fig. 3*A*). Previous studies (17) have suggested that this particular bush morphology is characteristic of a heteroduplex in which one strand is wild type and the other contains a genetic substitution, i.e., there is a deletion of a portion of the strand, with partial or complete replacement with nonhomologous DNA.



A. Preparation mounted for electron miscroscopy by the aqueous procedure. The molecule shown has a single internal bush, at a fractional map position of about 0.06 (see Fig 2).



B. Preparation mounted for electron microscopy by formamide technique. The molecule shown has two unpaired single strands close to one end.

FIG. 3. Heteroduplex molecules prepared by denaturation and renaturation of E46$^+$PL1 DNA molecules (11). The bars on each plate represent 1 micrometer.

In the present case, the existence of a deletion is confirmed by the fact that the molecules with bushes were about 17% shorter than molecules without bushes (Fig. 4). It should be noted that five molecules contained internal bushes that were apparently located at random (Fig. 2). These are probably a result of nonspecific aggregation or of renaturation of broken strands. When a preparation of E46$^-$ DNA was denatured and renatured, eight randomly located internal bushes were observed among the 62 molecules photographed. The mean length of the molecules in this control preparation (10.7 μm) was not significantly different from that of the molecules without bushes in the E46$^+$PL1 DNA preparation (10.8 μm).

Denatured and renatured E46$^+$PL1 DNA was also mounted for electron microscopy by the formamide technique (11). With this technique, single strands are extended and measurable, and are generally distinguishable from duplex strands by being thinner and more kinky. As expected, only one type of heteroduplex was seen on these grids. A typical example is shown in Fig. 3*B*. At a point close to one end of the heteroduplex, there was an unpaired region with one of the two single strands significantly longer than the other. Table 1 summarizes length measurements of 22 of these heteroduplexes, together with a similar number of homoduplexes (uniformly double-stranded molecules), unrenatured single strands, and single-stranded SV40 circles (added to the preparation at the time of mounting for electron microscopy). The lengths of the unpaired strands were about 11 and 16% of the length of the Ad. 7 homoduplexes. The duplex portions of the heteroduplex measured about 5 and 80% of the length of Ad. 7 homoduplexes. A control preparation of E46$^-$, treated identically to the E46$^+$PL1 preparation, contained no molecules with unpaired regions or single-stranded loops. The mean length of renatured E46$^-$ molecules (11.6 μm) was similar to that of the homoduplexes in the E46$^+$PL1 preparation.

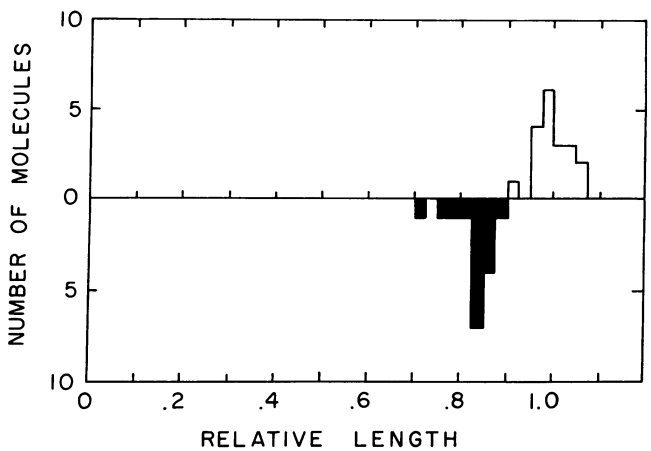


FIG. 4. Histogram of relative lengths of molecules with and without bushes in a preparation of denatured and renatured E46$^+$PL1 DNA molecules. The inverted histogram represents the molecules with bushes mapping between 0.04 and 0.09 in the histogram of Fig. 2 (mean length: 8.9 μm). The upright histogram represents a random sample of the molecules without bushes on the same grid (mean length: 10.7 μm). Since the heteroduplex frequency in this preparation was about 33%, it can be computed that about 95% of the molecules without bushes were Ad. 7·d. A7 homoduplexes. The data are plotted relative to the mean length of the Ad. 7·Ad. 7 homoduplexes (taken as 1.0).

TABLE 1. *Lengths of molecules in a preparation of denatured and renatured E46+PL1 DNA**

Molecule	Length (μm)	Standard deviation (μm)
Ad. 7·Ad. 7–SV40 heteroduplexes		
Short duplex segment	0.56	0.03
Long duplex segment	9.0	0.6
Short single-stranded segment	1.25	0.07
Long single-stranded segment	1.86	0.11
Homoduplexes (Ad. 7)	11.3	0.4
Unrenatured single strands (Ad. 7)	11.3	0.5
SV40 single-stranded circles†	1.66	0.15

* The length of single-stranded DNA was corrected to equivalent double-stranded length by a normalization factor equal to the ratio of the mean length of the homoduplexes (11.3 μm) to the mean length of the unrenatured single-strands (11.4 μm). For purposes of this standardization procedure, it was assumed that all of the unrenatured single strands were Ad. 7 and all of the homoduplexes were of the Ad. 7·Ad. 7 type. Since the E46+-PL1 population contains a considerable excess of nonhybrid molecules over hybrid molecules, the error resulting from this assumption was negligible. If random assortment of strands during renaturation is assumed, it may be calculated from the observed frequency of heteroduplexes (33%) that about 95% of the homoduplexes in this preparation derived both strands from Ad. 7. Similarly, about 80% of the unrenatured single-strands were Ad. 7. twenty-two of each type of molecule in the table were measured (n).

† Added to the preparation at the time of mounting for electron microscopy.

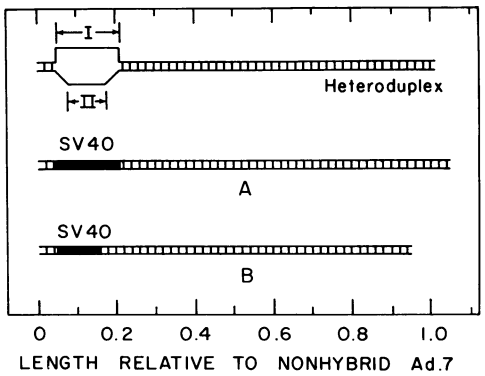


FIG. 5. Models for the structure of the Ad.7–SV40 hybrid DNA molecule. The Ad.7·Ad.7–SV40 heteroduplex contains two unpaired strands. Two models for the structure of the Ad. 7–SV40 hybrid DNA molecule are possible. Structure *A* corresponds to assigning the longer unpaired strand (I) to Ad.7–SV40 and the shorter (II) to Ad.7. This structure contains 11% less Ad.7 DNA than nonhybrid Ad.7. The deleted segment of Ad.7 DNA has been replaced with a somewhat longer segment of heterologous DNA. The size of the heterologous segment is equivalent to 1.12 SV40 genomes. Structure *B* follows from the opposite strand assignment. It contains 16% less Ad.7 DNA than nonhybrid Ad.7. The deleted DNA has been replaced by a heterologous segment, equivalent to 75% of a complete SV40 genome. Structure *A* is about 5% longer than a nonhybrid Ad.7 DNA molecule and structure *B* about 5% shorter.

DISCUSSION

The results presented in this paper indicate that when DNA prepared from E46+PL1 virions is denatured and renatured, a unique type of heteroduplex is formed. Although not directly demonstrated, it seems likely that this heteroduplex is composed of one Ad. 7 strand and one Ad. 7–SV40 hybrid strand. Other hypotheses, such as that the heteroduplex is formed by strands from two genetic variants of Ad. 7, are less likely for two reasons. First, the E46+PL1 virus used in these experiments was ultimately derived from a single plaque and second, the E46− (nonhybrid) population did not form these discrete heteroduplexes after denaturation and renaturation. Given this interpretation for the composition of the heteroduplex, there remains the problem of determining which of the two unpaired strands belongs to Ad. 7 and which belongs to Ad. 7–SV40. There are two possible ways of assigning the unpaired strands corresponding to two possible structures for the Ad. 7–SV40 hybrid genome (Fig. 5). These two structures are distinguishable by virtue of the fact that structure *A* contains about 5% more DNA than the nonhybrid Ad. 7 DNA molecule and structure *B* contains about 5% less DNA. It can be calculated that Ad. 7 capsids containing structure *A* would have a buoyant density in CsCl about 2 mg/ml higher than that of nonhybrid Ad. 7 virions, whereas Ad. 7 capsids containing structure *B* would have a buoyant density about 2 mg/ml lower†. The hybrid virions in the E46+PL1 population are 1–2 mg/ml less dense than nonhybrid Ad. 7 virions (9). It appears likely, therefore, that structure *B* is correct, i.e., the longer unpaired strand should be assigned to Ad. 7 and the shorter unpaired strand to Ad. 7–SV40. This view is supported by the fact that if the total double-stranded length of the heteroduplex is added to the length of the longer unpaired strand, the resulting sum (11.4 μm) is very close to the total length of an Ad. 7 homoduplex (11.3 μm). On the basis of this strand assignment and the data of Table 1, it may be deduced that the hybrid genome contains 16% less Ad. 7 DNA than the nonhybrid Ad. 7 genome. This deletion consists of the segment of DNA mapping between 0.05 and 0.21 molecular lengths in the nonhybrid Ad. 7 DNA molecule. The deleted DNA has been partially replaced by an amount of heterologous DNA equivalent to 75% of the complete SV40 genome.

The genetic functions specified by the segment of adenovirus DNA deleted from the hybrid genome are not known. However, this deletion, probably accounts for the defectiveness of hybrid particles (3, 4).

The unique location of the unpaired strands in the Ad. 7: Ad. 7–SV40 heteroduplex indicates that the nonhybrid Ad. 7 DNA molecules constitute a nonpermuted population (21). Using denaturation mapping techniques, Doerfler and Kleinschmidt have reached a similar conclusion regarding Ad. 2 (22).

The E46+ virus population was derived from a vaccine strain of Ad. 7 that had been propagated in monkey cells. The hybrid particles appeared after attempts to eliminate contaminating SV40 virus by passage with SV40 antiserum (1). In retrospect, it seems clear that this procedure applied a strong selective pressure in favor of hybrid genomes that had incorporated the portion of the SV40 genome that is

† Density changes ($\Delta\rho$) were calculated from: $\Delta\rho = 0.036\gamma/(1 + 0.098\ \gamma)$, where γ is the fractional change in the size of the genome. This equation follows from Eq. (1) of ref. 20 when $\rho_{DNA} = 1.711$, $\rho_{PROT} = 1.30$, and $\rho_0 = 1.34$.

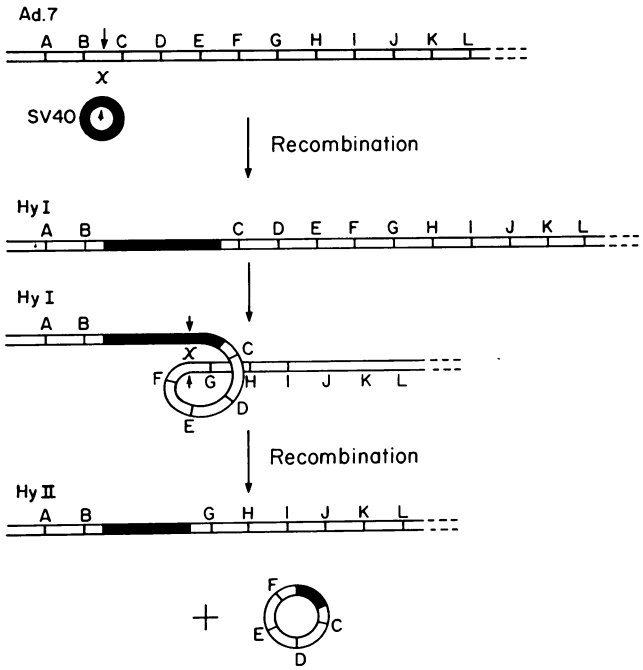


FIG. 6. The origin of the Ad.7–SV40 hybrid DNA molecule —a possible model. The model proposes that the Ad.7–SV40 hybrid is generated as a result of two independent recombination events involving two different regions of limited homology (*arrows*) between Ad.7 and SV40 DNA molecules. The first recombination event results in the insertion of the complete SV40 genome into the linear continuity of the Ad.7 genome. This event is similar to the postulated mechanism for λ prophage insertion (19). The second recombination event is intramolecular, and results in the deletion of portions of both the Ad.7 and the SV40 DNA to form a molecule whose structure is formally analogous to that proposed for the Ad.7–SV40 hybrid in the E46$^+$PL1 population on the basis of the heteroduplex data. (It is postulated that the circular fragment that is also produced is nonviable.) This is similar to the proposed mechanism for production of λdg DNA molecules (19). An alternative model may be considered in which the second recombination event is intermolecular, involving HyI and a second Ad.7 molecule. This mechanism produces two products: one is identical to HyII and the other is an Ad.7–SV40 hybrid molecule that contains a duplication of part of the Ad.7 DNA.)

responsible for enhancing the growth of Ad. 7 in monkey cells (18). It is interesting to speculate on the nature of the molecular events that originally produced the hybrid genome. The structure of the hybrid genome described here suggests that two independent recombination events were involved. A possible two-step mechanism is shown in Fig. 6. In the first step, a recombination event between a linear Ad. 7 DNA molecule and a circular SV40 DNA molecule results in the insertion of the entire SV40 genome into the continuity of the Ad. 7 genome. The resulting "hybrid" genome might be expected to be either unstable (due to the possibility of reversal of the reaction that produced it) or nonviable (due to the difficulty of encapsidating its enlarged genome). A second (intramolecular) recombination event removes these difficulties by simultaneously deleting portions of both the Ad. 7 and the SV40 DNA. The resulting molecule has a structure analogous to that deduced for the Ad. 7–SV40 hybrid in the E46$^+$PL1 population on the basis of the heteroduplex data. This mechanism implies the existence of two regions of limited homology between Ad. 7 and SV40. It is possible that other regions of homology might exist, and therefore, that SV40 DNA is potentially capable of integrating into other regions of the Ad. 7 genome.

We would like to thank Drs. Andrew M. Lewis, Jr., and W. P. Rowe for many helpful discussions and Dr. S. Polmar for preparing some of the reagents used in this work.

1. Huebner, R. J., R. M. Chanock, B. A. Rubin, and M. J. Casey, *Proc. Nat. Acad. Sci. USA*, **52**, 1333 (1964).
2. Rowe, W. P., and W. E. Pugh, *Proc. Nat. Acad. Sci. USA*, **55**, 1126 (1966).
3. Rowe, W. P., and S. G. Baum, *J. Exp. Med.*, **122**, 955 (1965).
4. Boeyé, A., J. L. Melnick, and F. Rapp, *Virology*, **28**, 56 (1966).
5. Rowe, W. P., and S. G. Baum, *Proc. Nat. Acad. Sci. USA*, **52**, 1340 (1964).
6. Rapp, F., J. Melnick, J. Butel, and T. Kitahara, *Proc. Nat. Acad. Sci. USA*, **52**, 1348 (1964).
7. Rapp, F., S. S. Tevethia, and J. Melnick, *J. Nat. Cancer Inst.*, **36**, 703 (1966).
8. Duff, R., and F. Rapp, *J. Virol.*, **5**, 568 (1970).
9. Baum, S. G., W. H. Wiese, and W. P. Rowe, *J. Virol.*, **5**, 353 (1970).
10. Westmoreland, B. C., W. Szybalski, and H. Ris, *Science*, **163**, 1343 (1969).
11. Davis, R., M. Simon, and N. Davidson, *Methods in Enzymology*, ed. L. Grossman and K. Moldave (Academic Press, New York, 1970), in press.
12. Baum, S. G., P. R. Reich, C. J. Hybner, W. P. Rowe, and S. M. Weissman, *Proc. Nat. Acad. Sci. USA*, **56**, 1509 (1966).
13. Hoggan, M. D., N. R. Blacklow, and W. P. Rowe, *Proc. Nat. Acad. Sci. USA*, **55**, 1467 (1966).
14. Rose, J. A., P. R. Reich, and S. M. Weissman, *Virology*, **27**, 571 (1965).
15. Green, M., and M. Piña, *Virology*, **20**, 199 (1963).
16. Thomas, C. A., K. I. Berns, T. J. Kelly, Jr., *Procedures in Nucleic Acid Research*, ed. G. L. Cantoni and D. R. Davies (Harper & Rowe, New York), 1966, p. 535.
17. Davis, R. W., and N. Davidson, *Proc. Nat. Acad. Sci. USA*, **60**, 243 (1968).
18. Rabson, A. S., G. T. O'Conor, I. K. Berezesky, and F. S. Paul, *Proc. Soc. Exp. Biol. Med.*, **116**, 187 (1964).
19. Campbell, A., *Advan. Genet.*, **11**, 161 (1962).
20. Weigle, J., M. Meselson, and K. Paigen, *J. Mol. Biol.*, **1**, 379 (1959).
21. Thomas, C. A., Jr., *J. Gen. Physiol.* (Suppl.), **49**, 143 (1966).
22. Doerfler, W., and A. K. Kleinschmidt, *J. Mol. Biol.*, **50**, 579 (1970).