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Addition of Extra DNA Sequences to Simian Virus 40 DNA In Vivo

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The possible addition of extra sequences to simian virus 40 (SV40) DNA was analyzed by electron microscopy in two different cell systems, productively infected monkey cells and activated heterokaryons of monkey and transformed mouse 3T3 cells. We found that the closed circular DNA fraction, extracted from monkey cells at 70 h after infection with nondefective SV40 at a multiplicity of infection of 6 PFU/cell, contained oversized molecules (1.1 to 2.0 fractional lengths of SV40 DNA) constituting about 8% of the molecules having lengths equal to or shorter than SV40 dimer DNA. The oversized molecules had the entire SV40 sequences. The added DNA was heterogeneous in length. The sites of addition were not specific with reference to the *Eco*RI site. These results suggest that recombination between monkey and SV40 DNAs or partial duplication of SV40 DNA occurs at many sites on the SV40 chromosome. The integrated SV40 DNA is excised and replicates in activated heterokaryons. In this system, besides SV40 DNA we found heterogeneous undersized and oversized molecules containing SV40 sequences in the closed circular DNA population. Additions differing in size appeared to be overlapping and to have occurred at a preferential site on the SV40 chromosome. These results support the hypothesis that host DNA can be added to SV40 DNA at the site of integration at the time of excision.

Since simian virus 40 (SV40) DNA in transformed cells has been shown to be linked covalently to cell DNA (11), the integration of SV40 DNA into cell DNA, which occurs in both permissive and nonpermissive systems (4, 5), appears to be one of the essential early events for stable transformation of infected cells. The simplest model for the integration would be that the linear insertion of the entire circular SV40 DNA into cell DNA results from the recombination of the two DNAs by a single crossover (1). From this model it is predicted that the SV40 provirus is excised from cell DNA by the reverse process, or circularization of viral DNA at the site of integration. This model is consistent with the fact that the SV40 provirus is induced to initiate production of infectious progeny virus when the transformed cells are fused with SV40-susceptible monkey cells (8, 16). It is also predicted that recombinant molecules containing both virus and cell DNA sequences are occasionally generated, possibly by an abnormal circularization of the provirus at the time of excision.

SV40 DNA from defective virions produced and accumulated during serial undiluted passages in monkey cells (14, 17) has been shown to contain host DNA sequences (9). The types of defective DNA that may contain the cell DNA sequences are substitution molecules (12) and insertion molecules having a deletion at a different site (19). Whether both or only one of the two types are recombinant molecules is unclear. Defective DNA molecules isolated from virions do not necessarily represent those initially formed because selection of viral DNA may occur during encapsidation. Thus, it is possible that an abnormal circularization of provirus results in the formation of a variety of substitution and addition molecules, but only those having appropriate lengths can be encapsidated into mature virions and replicate during the subsequent virus propagation.

In the present study we examined by electron microscopy the heterogeneity in size and base sequence of free (nonencapsidated) intracellular closed circular DNA molecules produced in two systems, productively infected monkey cells (in which SV40 DNA may be integrated and excised) and heterokaryons produced by the fusion of SV40-transformed and permissive cells (in which SV40 provirus is excised and replicated). We found that extra DNA sequences can be added to SV40 DNA in the two systems.

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MATERIALS AND METHODS

Virus. The small-plaque-type SV40, clone Sd (plaque purified from a stock of strain 777), was used. The standard nondefective virus stocks for infection or purification of virions had been prepared by propagation at a low input multiplicity of infection (MOI; 0.001 PFU/cell) in monkey cells. For the preparation of standard SV40 DNA, DNA was extracted from virions purified from infected cells by the method described previously (18).

Cell lines. The TC7 clone of the CV1 line of African green monkey kidney cells (a gift from J. A. Robb) was used for the propagation of virus and for cell fusion with SV40-transformed mouse 3T3 cells. The SV40-transformed mouse 3T3 cell lines, TR-3T3-L and TR-3T3-M, were used for the induction of SV40 provirus by cell fusion. TR-3T3-L cells contain the entire SV40 genome (small-plaque type of strain 777) and yield infectious virus after fusion with monkey cells (13). TR-3T3-M cells have a defective SV40 genome and generate defective SV40 DNA molecules having T-antigen-inducing capacity after fusion with monkey cells (15, 21). Circular DNA induced from these two mouse cell lines has been partly characterized (21), as well as the DNA from the defective virions rescued from TR-3T3-M by triple cell fusion (19, 20).

Virus infection. Confluent monolayer cultures of TC7 were infected with the standard (nondefective) SV40 at an MOI of 6 PFU/cell. After an adsorption period of 90 min, cultivation at 36°C was continued for 70 h before DNA extraction.

Cell fusion. The method used for cell fusion was that described by Uchida and Watanabe (13). The egg-adapted strain Z of HVJ (Sendai virus) was a gift from Y. Hosaka, the Wistar Institute. DNA was extracted from heterokaryon cultures at 70 h after plating.

DNA extraction from virions. Viral DNA was extracted from virions that had been purified by two-cycle banding in CsCl density gradients. The virions suspended in 0.01 M EDTA solution (pH 7.6) were digested with Pronase (0.1 mg/ml) at 36°C 4 h. The digested virion preparation was extracted with phenol and then with chloroform-isoamyl alcohol (24:1, vol/vol). DNA forms I and II were separated by equilibrium density centrifugation (7). Ethidium bromide was removed by extraction with isoamyl alcohol or isopropanol (7). Pancreatic DNase I was used to introduce random nicks in DNA I (11). Nicked DNA was separated from DNA I by isopycnic centrifugation (7).

DNA extraction from infected cultures or heterokaryon cultures. Closed circular DNA was extracted selectively by the method of Hirt (6) followed by dye-buoyant density centrifugation (7). The cells were lysed with a solution containing 0.01 M EDTA, pH 7.6, and 1% sodium lauryl sulfate (1 ml/100-mm-dish culture). An NaCl (5 M) solution was added to the pooled lysate to give a final concentration of 1 M. After gentle mixing, the centrifuge tubes containing lysed cells were kept in a refrigerator overnight. The tubes were centrifuged in a JA20 rotor of a Beckman J21B centrifuge at 10,000 rpm and 5°C for 30 min. The Hirt supernatant fraction was deproteinized with phenol extraction followed by chloroform-isoamyl alcohol (24:1) extraction. DNA was precipitated with ethanol (70%) at −20°C. The concentrated DNA was banded twice in dye-CsCl gradients, and the closed circular DNA fraction was isolated. In the case of DNA from infected TC7 cells, closed circular DNA (0.3 ml) was layered on top of 3 ml of a neutral CsCl solution (ρ = 1.35) and centrifuged in a Beckman SW50.1 rotor at 35,000 rpm and 10°C for 4 h. Appropriate fractions from the gradient were combined for further studies.

Digestion with *Eco*RI restriction endonuclease. The reaction mixture (0.2 ml) containing 0.1 M Tris-hydrochloride (pH 7.6), 0.01 M $MgCl_2$, 10 to 50 μg of closed circular DNA, and 500 U of R·*Eco*RI (Miles Laboratories, Inc., Elkhart, Ind.) was incubated at 36°C for 30 min. Digestion was stopped by the addition of EDTA to give a final concentration of 0.02 M. The mixture was extracted with chloroform-isoamyl alcohol (24:1) twice, and DNA was banded in dye-CsCl gradients.

Electron microscopy. The Kleinschmidt aqueous procedure, using formamide, was essentially that described by Davis et al. (3). The solution (20 μl), which contained DNA (1 to 4 μg/ml), Tris-hydrochloride (0.5 M, pH 7.6), formamide (50%), and cytochrome *c* (0.1 mg/ml), was spread on distilled water in a Teflon dish (diameter, 5.0 cm). The DNA-protein monolayer was picked up on copper grids covered with Parlodion film. The grids were immersed in 95% ethanol, stained with uranyl acetate (5×10^{-5} M in 90% ethanol), immersed in isopentane, and shadowed with platinum-palladium (80%/20%) while being rotated. For heteroduplex formation, randomly nicked or *Eco*RI-digested DNA was denatured in 0.3 M NaOH for 10 min at room temperature. One volume of denatured DNA was neutralized with 3 volumes of a solution containing 0.67 M Tris-hydrochloride, pH 7.6, and 67% formamide. The neutralized DNA was incubated at room temperature for 45 min, at which time about 50% renaturation occurs. Then, cytochrome *c* was added, and DNA-protein was spread on water and picked up on grids. Specimen grids were examined in an Elmiskop 1A at an accelerating voltage of 80 kV and photographed on Kodak Electron Image Plates (6.5 × 9 cm) at a magnification of ×6,400 or ×12,200. Negatives were projected with a Nikon 6C profile projector on tracing paper at a magnification of ×20 or ×10. Lengths of DNA were measured on tracings with a map measure (Keuffel and Esser Co., Switzerland) or a Model 75 Linear Measuring Probe equipped with a digital readout instrument (Los Angeles Scientific Instrument Co. Inc., Los Angeles, Calif.). For the determination of the length distribution of nondenatured ring DNA, molecules originating from a limited area smaller than a single mesh square were used unless stated otherwise. For the measurement of heteroduplexes, homoduplex ring DNA or *Eco*RI-generated linear DNA molecules of SV40 within the same field were used as the internal reference whenever possible. In cases in which these molecules were not available, magnification was calibrated with a grating replica. The lengths were expressed as fractional lengths of nondefective SV40 DNA.

RESULTS

DNA from productively infected monkey cells. When superhelical circular DNA was extracted from monkey cells at 70 h after infection with nondefective SV40 at high multiplicities (4 to 50 PFU/cell), a new class of circular DNA molecules, which were longer than monomeric SV40 DNA but shorter than dimeric DNA, was occasionally found. These oversized molecules were not found in DNA preparations from purified virions. In an attempt to enrich the sample with the population of these oversized molecules, closed circular DNA from infected monkey cells (MOI = 6) was sedimented through a neutral CsCl gradient (Fig. 1). After centrifugation, appropriate fractions were combined to prepare pools I and II, which contained the 22 to 27S leading fractions and the 21S peak fractions, respectively. Figure 2 shows the length distributions of the two samples measured on nicked circular molecules by electron microscopy. The presence of oversized ring DNA molecules (longer than 1.1 fractional lengths of SV40 DNA) was evident in the two samples. More oversized molecules were found in pool I than in pool II. The presence of undersized molecules (mostly deletion molecules) was more evident in pool II than in pool I. The proportions of each component in pools I and II can be calculated directly from Fig. 2. Since the relative amounts of pools I and II are known from Fig. 1, the proportion of each component in the two samples combined can be calculated accordingly. The oversized and undersized molecules were found to constitute 8 and 6%, respectively, of the ring molecules having sedimentation velocities of 21 to 27S.

We examined pools I and II by the heteroduplex method to determine whether the oversized molecules had SV40 DNA sequences. Randomly nicked DNA molecules were denatured and renatured for heteroduplex formation. The great majority of the molecules were homoduplex rings, but occasionally heteroduplexes having a single-stranded loop or segments were found. Over 100 heteroduplexes were photographed without selection as they were observed during scanning. Their representative types are shown in Fig. 3. There were molecules with a substitution loop (Fig. 3m and n) or those having two loops each, but the majority of the heteroduplexes had a single deletion or an addition loop each. Length measurement (using homoduplex ring molecules as the internal reference) revealed that there were two classes of heteroduplexes in the latter group. The first class consisted of those having an addition loop. Since the double-stranded ring portions of the molecules in Fig. 3a to h had a unit length of nondefective SV40 DNA, their single-stranded segments were regarded as addition loops. The second class was composed of those having a deletion loop. Since the sum of a single-stranded loop and a double-stranded ring had one unit length of SV40 DNA in the case of the molecules shown in Fig. 3i to l, their single-stranded loops were considered to be deletion loops. The presence of heteroduplex molecules each with an addition loop is evidence that the oversized ring molecules contain entire SV40 DNA sequences and extra DNA sequences. Most of the substitution molecules had a length similar to or slightly shorter than that of nondefective SV40 DNA (Fig. 3m and n); 1 out of 226 heteroduplexes had a substitution sequence longer than the deleted viral sequence (Fig. 3o). Table 1 shows the proportions of each type of heteroduplex. Molecules with an addi-

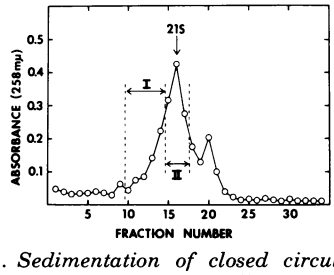


FIG. 1. *Sedimentation of closed circular DNA from SV40-infected monkey cells. DNA was extracted selectively by the Hirt method and ethidium bromide-CsCl density gradient centrifugation from TC7 cells at 70 h after infection with nondefective SV40 at an MOI of 6 PFU/cell and sedimented through a neutral CsCl gradient (mean density, 1.35 g/cm³) at 35,000 rpm for 4 h in a Beckman SW50.1 rotor. Drops were collected from the bottom of the tube for fractionation. Appropriate fractions were combined to give pools I (22 to 27S) and II (21S).*

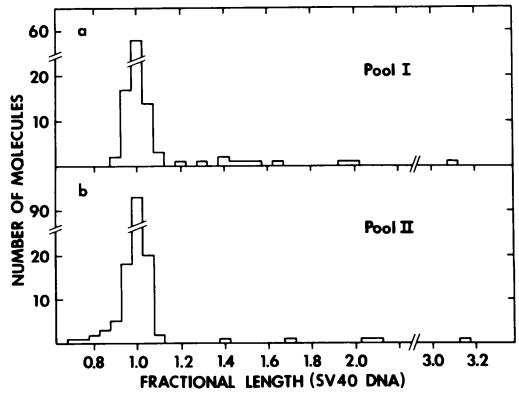


FIG. 2. *Length distributions of circular DNA molecules extracted from SV40-infected monkey cells. (a) Pool I of Fig. 1; (b) pool II of Fig. 1. Lengths have been expressed as fractional lengths of nondefective SV40 DNA.*

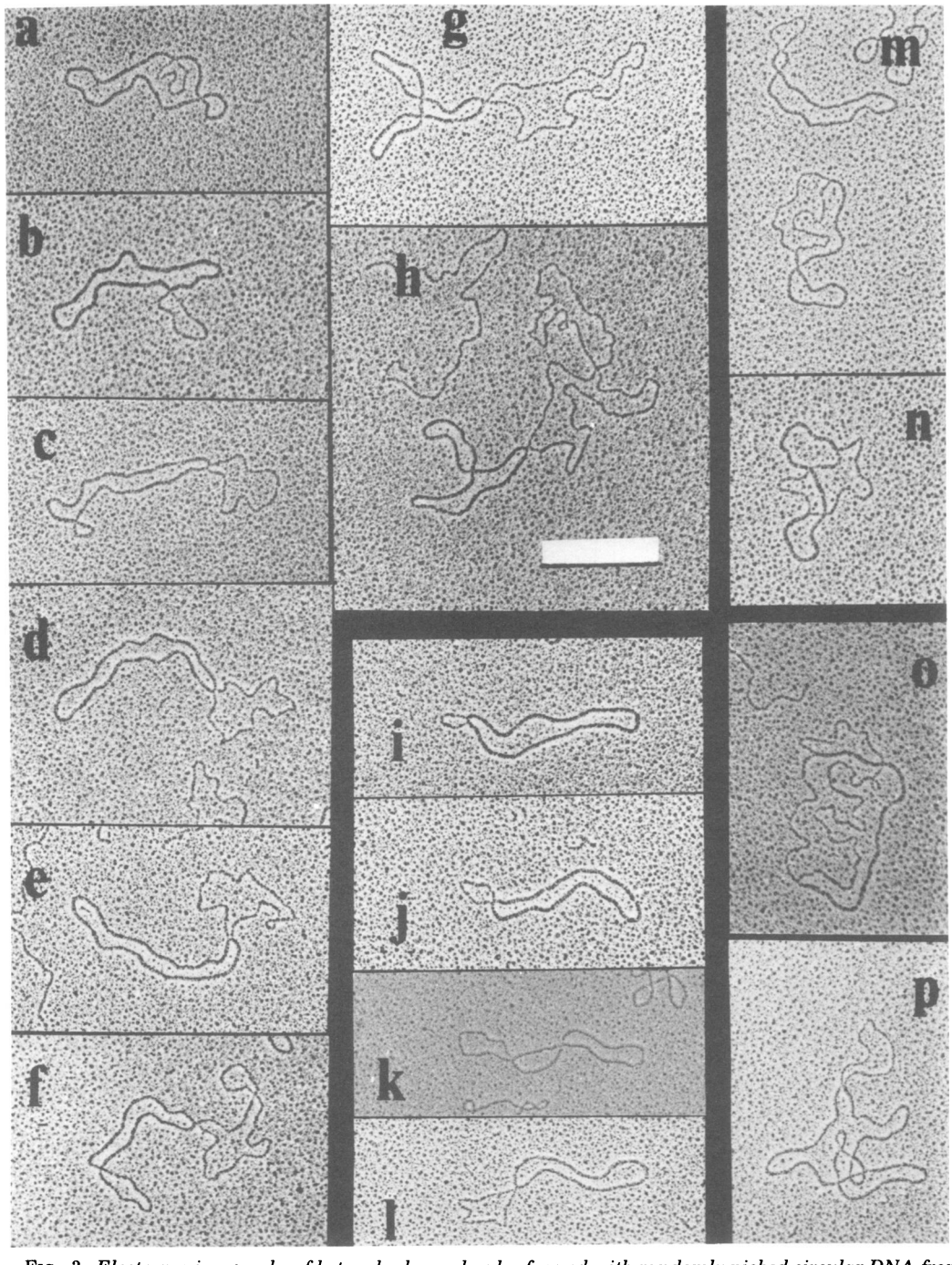


FIG. 3. *Electron micrographs of heteroduplex molecules formed with randomly nicked circular DNA from SV40-infected monkey cells. (a to h) Heteroduplexes with an addition loop. Additions for (a) to (h) are 0.26, 0.35, 0.53, 0.59, 0.79, 1.00, 1.11, and 2.09 fractional lengths of SV40 DNA, respectively. (i to l) Heteroduplexes with a deletion loop. Deletions for (i) to (l) are 13, 19, 31, and 34%, respectively. (m) Molecule with a deletion loop and molecule with a substitution loop. (n) Heteroduplex with a substitution loop. (o) Heteroduplex with a substitution loop. The substituting strand is longer than the substituted (deleted) segment of viral strand. (p) Heteroduplex with a loop of 0.45 and a double-stranded portion of 1.64 fractional lengths. Bar represents 0.5 μm.*

TABLE 1. *Classification of heteroduplexes formed with randomly nicked circular DNA from SV40-infected monkey cells*

Heteroduplexes with:	No. of molecules in pool: I[a]	No. of molecules in pool: II[b]	Pools I and II combined[c] (%)
One addition loop	64 (54)[d]	31 (29)	38
One deletion loop	21 (18)	46 (43)	33
One substitution loop	20 (17)	23 (21)	20
Two loops	13 (11)	8 (7)	9

[a] 22 to 27S fractions of Fig. 1.
[b] 21S fractions of Fig. 1.
[c] For the calculation of pools I and II combined, the percentage of each sample was corrected for the relative amounts of two samples (I and II) of Fig. 1.
[d] Numbers in parentheses indicate percentages.

tion loop (38% for pools I and II combined) occurred among heteroduplexes approximately at the same frequency as for those with a deletion loop (33% for pools I and II combined). Figure 4a and b shows the length distribution of addition loops, indicating that the added DNA is very heterogeneous in amount.

To determine the site of addition with reference to the *Eco*RI cleavage site, DNA pools I and II were digested with *Eco*RI under conditions in which all nondefective SV40 DNA I molecules are converted to linear molecules (in practice, about 1% of the molecules in a standard DNA sample were not cleaved by the enzyme). The digested DNA was denatured and renatured for heteroduplex formation. The *Eco*RI-resistant fraction of pools I and II was less than 2% of the total molecules. The types and frequencies of the heteroduplexes found with *Eco*RI linear DNA were essentially the same as those of randomly nicked molecules (Fig. 5). The linear heteroduplexes with a deletion or an addition loop constituted the majority of the total heteroduplexes. By length measurement (linear homoduplexes were used as the internal references) it was possible to discriminate addition from deletion. Addition loops were heterogeneous in size, as shown in Fig. 4c and d. A comparison of Fig. 4a and d shows a tendency of the longer DNA molecules to be more susceptible to *Eco*RI cleavage than the shorter ones. It should be noted that some addition loops had a unit length of SV40 even after *Eco*RI digestion. Figure 6 shows the sites of addition with respect to the *Eco*RI site. As can be seen, additions apparently have occurred at many sites on the SV40 chromosome. Although there is no clear evidence for a specific site for addition, there is a suggestion that the majority (75%) of additions took place in a region between 0.25 and 0.75 unit on the physical map of SV40 DNA (between 0.25 and 0.5 fractional length in Fig. 6).

We further examined 65 linear heteroduplex molecules of SV40 DNA unit length that had a deletion loop (Fig. 5f and g) to determine whether there is a preferential site for deletion. Deletions appeared to be heterogeneous in size and to have occurred at random sites on the SV40 chromosome (Fig. 7). A total of 17 linear heteroduplexes with a substitution loop were also examined. Although the sites for substitution in nine molecules were at random, eight appeared to have a common substitution site that covers a section of SV40 DNA between 0.159 ± 0.005 (standard deviation [SD]) and 0.385 ± 0.017 (SD) from the *Eco*RI site. The substituting DNA segments of these eight (for deleted segment of viral DNA, 0.456 ± 0.016 [SD]) were somewhat heterogeneous and had a mean length of 0.217 ± 0.038 (SD). Besides these substitution molecules sensitive to *Eco*RI cleavage, there were some resistant molecules whose substituting segments cover the *Eco*RI site.

From these results obtained during a single cycle of SV40 replication in permissive TC7 cells, we conclude that variable amounts of extra DNA can be added at many sites on SV40 DNA, that the frequency of occurrence of addition and deletion molecules is similar, and that there is no preferential site for deletion on the SV40 chromosome, although additions seem to occur more frequently at the region further away from the *Eco*RI site.

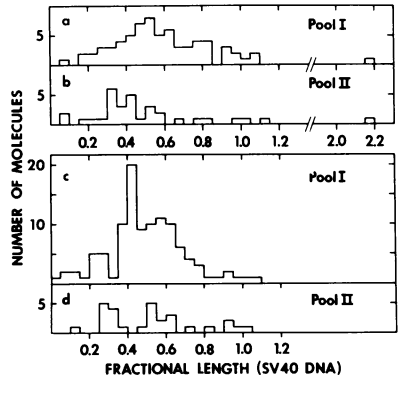


FIG. 4. *Length distributions of addition loops. (a and b) Addition loops were measured on the heteroduplexes formed with randomly nicked circular DNA molecules from SV40-infected monkey cells (Fig. 3a to h) and have been expressed as fractional lengths of SV40 DNA. Pools I and II are from the gradient shown in Fig. 1. (c and d) Addition loops are those of the heteroduplexes formed with EcoRI-generated linear DNA from SV40-infected monkey cells shown in Fig. 5a to c.*

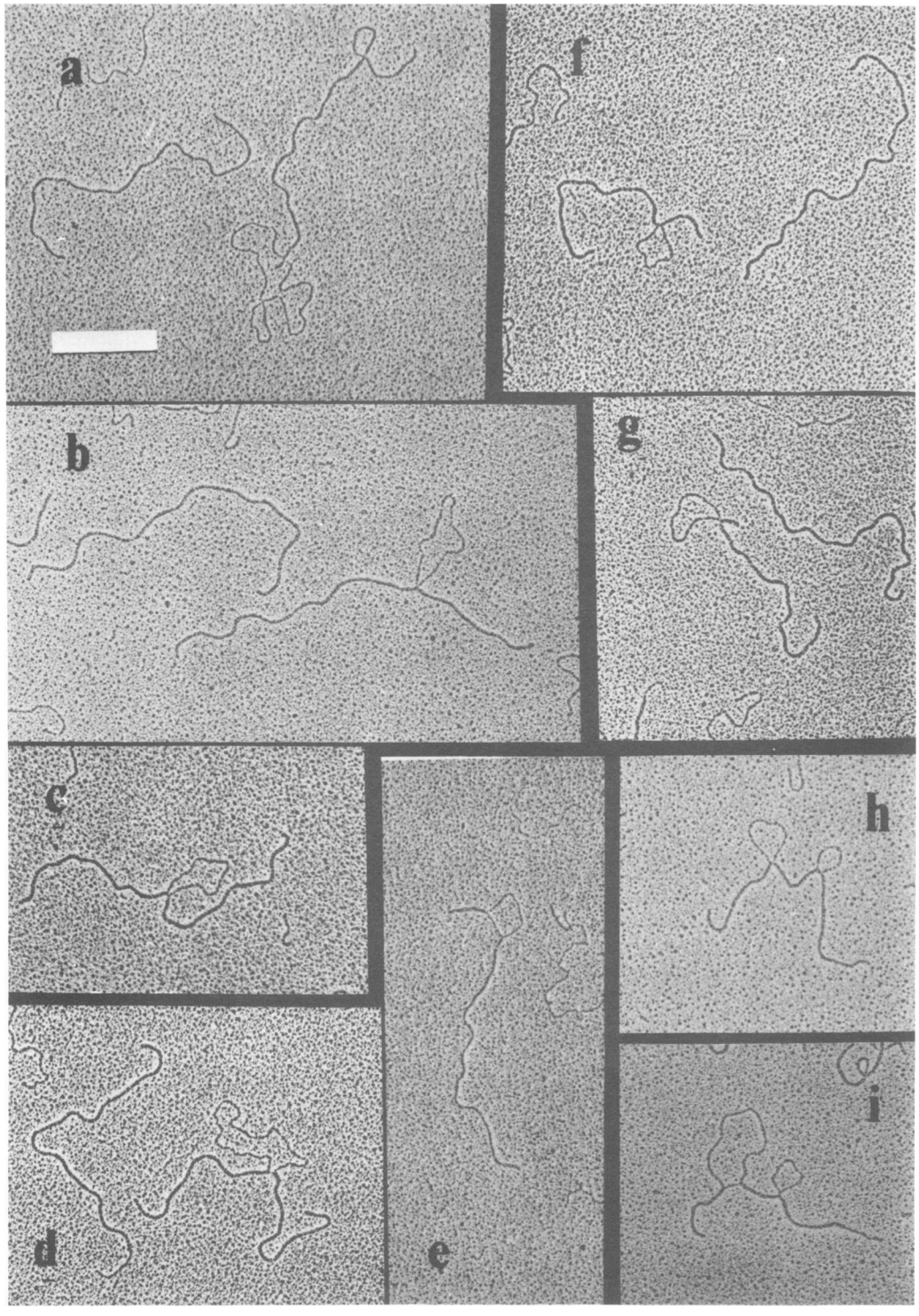


FIG. 5. *Electron micrographs of heteroduplexes formed with EcoRI-generated linear DNA molecules from SV40-infected monkey cells. (a) Linear SV40 homoduplex and heteroduplex with an addition loop of 1.052 fractional lengths. (b) Homoduplex and heteroduplex with an addition loop of 0.510. (c) Heteroduplex with an*

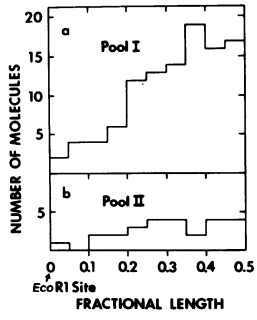


FIG. 6. *Sites of the addition with reference to the EcoRI cleavage site in productive SV40 infection. The number of molecules has been plotted against the distance from the nearer end of the heteroduplex to the addition loop. Pools I (a) and II (b) DNA are from the gradient shown in Fig. 1. The length distributions of addition loops are shown in Fig. 4c and d.*

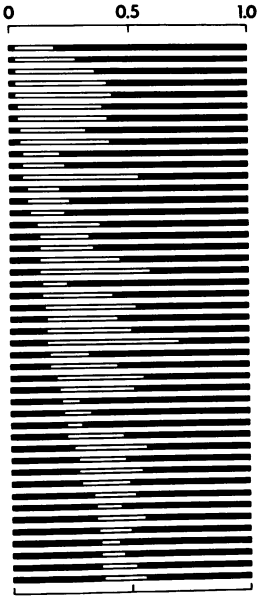


FIG. 7. *Sites of the deletion in EcoRI-generated linear DNA from SV40-infected monkey cells. A total of 46 heteroduplexes with a deletion loop were selected randomly from 65 molecules from pools I and II of Fig. 1. The molecules have been arranged so that the shorter distances from the EcoRI site are to the left. The deletion is indicated by an unfilled rectangle.*

DNA from activated heterokaryons of monkey and transformed mouse cells. When SV40-transformed mouse cells are fused with monkey cells, there is an appearance of infectious progeny; in this system the excision of SV40 DNA is believed to be followed by its autonomous replication, leading to the production of mature virions. Circular DNA from heterokaryon cultures has been partially characterized in respect to its biological activity (15, 21) and length distribution (21). Heterogeneous circular DNA molecules ranging in size from 1.0 to 3.0 μm (undersized and oversized ring molecules) were characteristic of these heterokaryon cultures. In the present study we used the same cell lines to show that the oversized molecules are viral DNA containing additional sequences.

Figure 8 shows the length distribution patterns of circular DNA obtained from heterokaryons of two mouse cell lines, each fused with monkey cells. The pattern for TR-3T3-L that carries the nondefective SV40 genome (Fig. 8a)

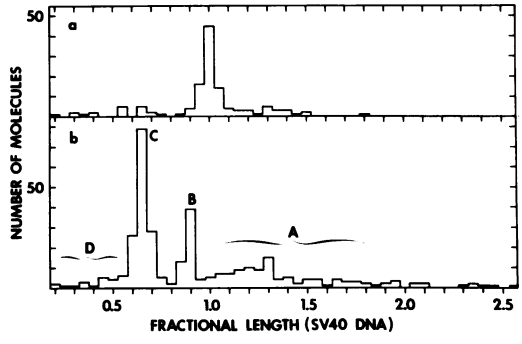
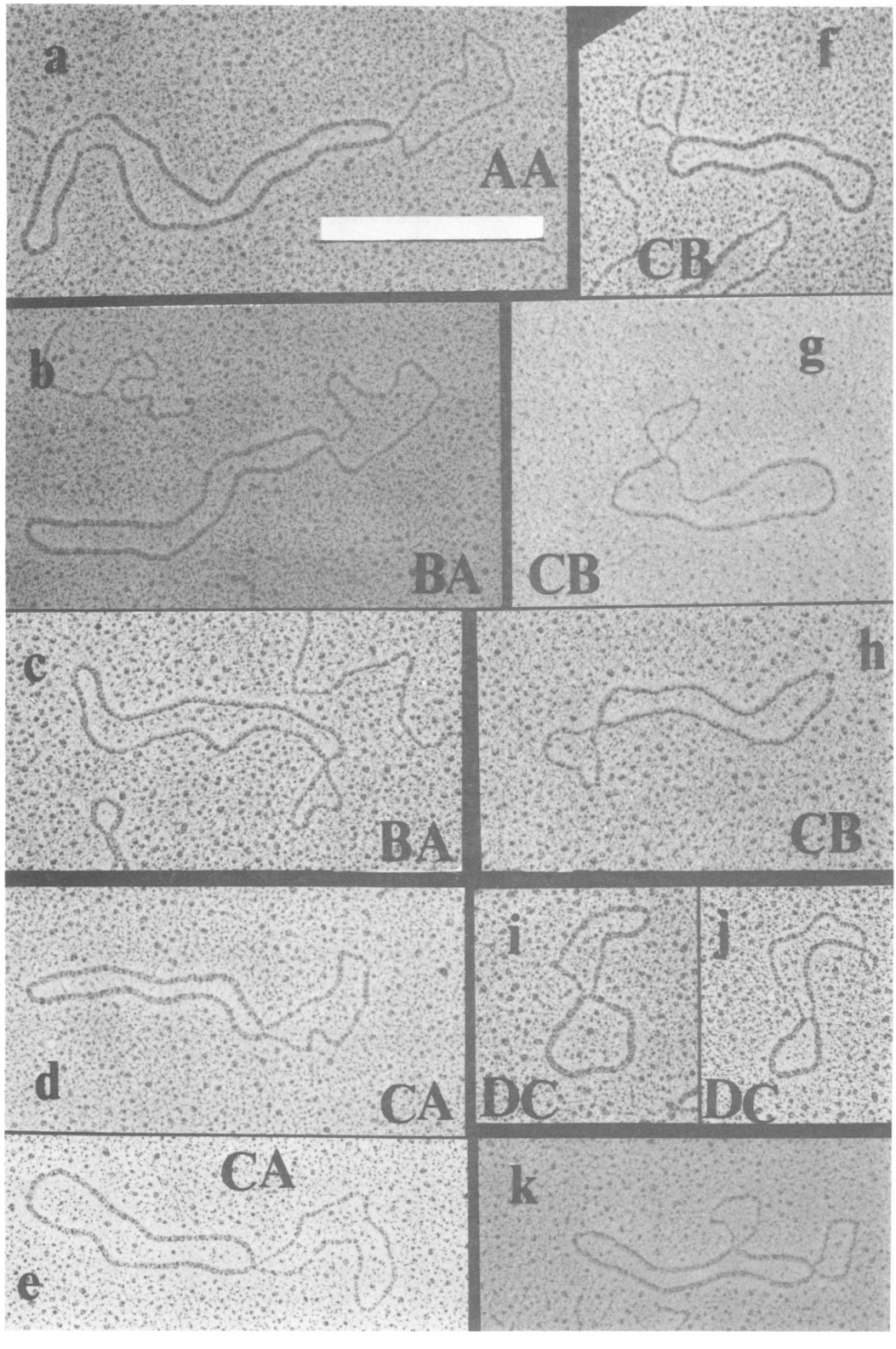


FIG. 8. *Length distributions of circular DNA from heterokaryon cultures of monkey and SV40-transformed mouse cells. Closed circular DNA was extracted selectively from cells at 70 h after plating after cell fusion by UV-irradiated Sendai virus. Measurement was done on nicked molecules and expressed as fractional lengths of nondefective SV40. (a) Monkey cells plus mouse 3T3 cells transformed by nondefective SV40 (TR-3T3-L cell line). Since the DNA concentration was low, molecules from various parts of the specimen grid were measured. (b) Monkey cells plus mouse 3T3 cells transformed by defective SV40 (TR-3T3-M cell line). Heterogeneous circular molecules have been arbitrarily grouped as classes A, B, C, and D on the basis of size.*

addition of 0.421. (d) Homoduplex and heteroduplex with a substitution loop. The substituting DNA strand (0.75) is much longer than the substituted small segment (0.01) of viral DNA. (e) Heteroduplex with a substitution loop. The substituted segment of the viral strand is longer than the substituting strand. (f and g) Linear homoduplex and heteroduplex with a deletion loop. Deletions are 30 and 38% for (f) and (g), respectively. (h) Heteroduplex with an addition and a deletion loop. (i) Heteroduplex with a substitution and an addition loop. Bar represents 0.5 μm.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was the same as that reported previously (21). This circular DNA fraction contained small amounts of oversized and undersized molecules in addition to those having the full length of SV40 DNA. These molecules could not be characterized further because of the lower DNA yield. Upon fusion with monkey cells, another cell line, TR-3T3-M, yielded an amount of DNA, whose length distribution pattern is shown in Fig. 8b, sufficient for subsequent studies. The DNA molecules in this preparation were classified arbitrarily into four groups: classes A, B, C, and D (Fig. 8b). The defective mutant DNA rescued from TR-3T3-M by triple cell fusion (TR-3T3-M containing defective SV40, TR-3T3-Y2 containing a large-plaque-type, nondefective SV40, and permissive monkey cells) had a length of class B molecules (19).

A mixture of classes A, B, C, and D molecules was denatured and renatured for heteroduplex formation. Of 120 heteroduplexes photographed, 111 molecules had a single deletion or addition loop (Fig. 9a to j), 2 molecules had a substitution loop, and 7 molecules had two loops each. On the basis of length measurement, the heteroduplexes with two loops were further characterized as three molecules with an addition and a deletion loop, one molecule with two addition loops (Fig. 9k), and three molecules with a substitution and an addition loop.

The heteroduplexes with a single addition or deletion loop were classified on the basis of length measurement (Fig. 10). All possible major combinations of the hybrid between different classes were recorded: the heteroduplexes between class A molecules, between classes B and A, between classes C and A, between classes D and A, between classes C and B, between classes D and B, and between classes D and C. The proportions of various hybrid types were approximately those expected from the proportions of four classes of DNA (Fig. 8b, classes A, B, C, and D). These results indicate that the majority of the molecules from the different classes (A, B, C, and D) are all related to one another and that their sequences are overlapping.

Since it has been shown that the defective SV40 carried by the TR-3T3-M cell line has a deletion of 35% (19), including the *Eco*RI cleavage site (20), we added nondefective *Eco*RI-generated linear SV40 DNA to the DNA preparation from heterokaryons at a ratio of 2:1 to characterize the four classes (A, B, C, and D) of DNA with respect to the complete SV40 DNA. With these heteroduplexes (Fig. 11 and 12) the original deletion (including the *Eco*RI site) was visualized as two single-stranded tails, and other additions or new deletions were visualized as a single-stranded loop. On the basis of length measurements of single- and double-stranded parts of heteroduplexes, they were classified into groups expected for hybrids between nondefective *Eco*RI linear DNA and different classes of DNA from heterokaryons. There were some molecules indicative of dimeric forms (Fig. 11a and b), which belong to

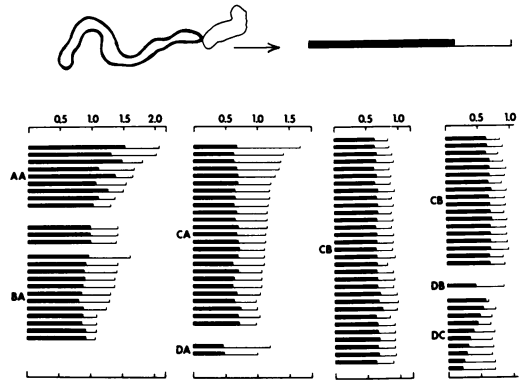

FIG. 10. *Classification of heteroduplexes formed with circular DNA from activated heterokaryons of monkey and SV40-transformed mouse cells. The proportion of those with a single loop of the type shown here and in Fig. 9a to j was 111 out of 120 heteroduplexes examined. The double- and single-stranded segments in fractional lengths have been depicted here as filled rectangles and lines, respectively. The heteroduplexes were classified on the basis of the length of the double-stranded segment and the sum of the double- and single-stranded segments into the hybrid groups shown. For instance, BA indicates hybrids between classes B and A of Fig. 8b, since the double-stranded segment and the sum of double- and single-stranded segments were close to the lengths of classes B and A molecules, respectively.*

FIG. 9. *Electron micrographs of heteroduplexes formed with circular DNA from activated heterokaryons of monkey and SV40-transformed mouse cells. Nicked circular DNA, whose length distribution is shown in Fig. 8b, was denatured and renatured for heteroduplex formation. On the basis of length measurement on single- and double-stranded portions, the heteroduplexes were interpreted to be hybrids between DNAs of various classes (A to D) of Fig. 8b. (a) Hybrid between class A molecules. (b and c) Hybrid between classes B and A molecules. (d and e) Hybrid between classes C and A molecules. (f to h) Hybrid between classes C and B molecules. (i and j) Hybrid between classes D and C molecules. (k) Heteroduplex with two addition loops. Its double-stranded portion is as long as a class C molecule. Bar represents 0.5 μm.*

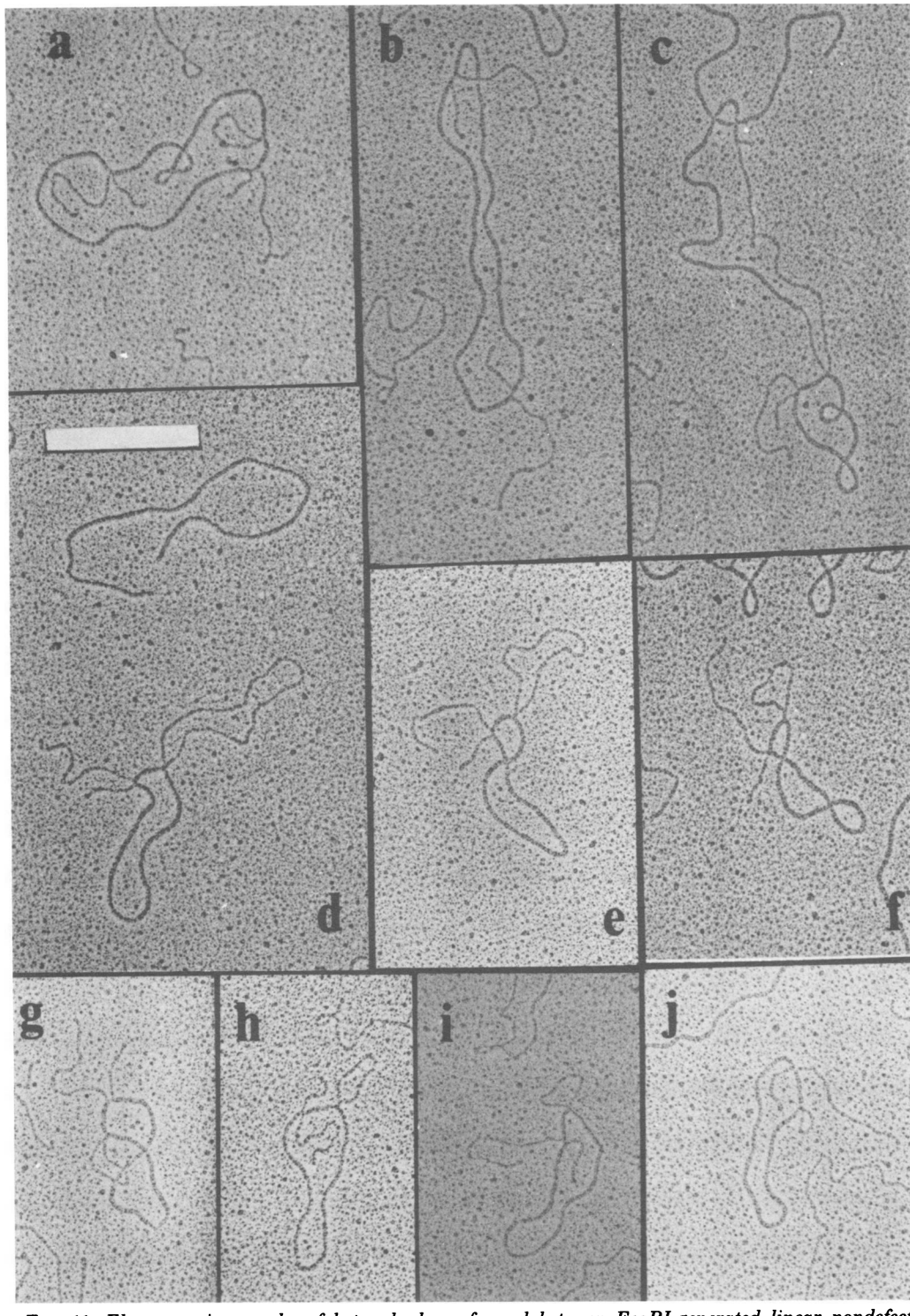

FIG. 11. *Electron micrographs of heteroduplexes formed between EcoRI-generated linear nondefective SV40 DNA and nicked circular DNA generated in SV40 provirus activation (classes A and B of Fig. 8b). Two single-stranded tails indicate the site of the deletion. From unpublished data, the shorter tail is believed to cover the region between 0 and 0.10 (clockwise) map unit and the longer tail is believed to cover the region*

class A molecules. Other molecules of class A formed heteroduplexes, each having two single-stranded tails (for the deletion site) and a single-stranded loop of variable length (for the addition) (Fig. 11c to f). The hybrids of class B molecules had two tails (for the deletion) and an addition loop having a fairly uniform length (Fig. 11g to i and 12a). The heteroduplexes between nondefective linear and class C molecules had two single-stranded tails for the deletion of 0.34 fractional length (Fig. 12d to g). The molecules shown in Fig. 12h to k were interpreted to be hybrids between *Eco*RI linear DNA and class D DNA having double deletions, the original deletion being indicated by two single-stranded tails and the new deletion being indicated by a single-stranded loop. Except for class D heteroduplexes (Fig. 12h to k) and class A dimer heteroduplexes (Fig. 11a and b), the double-stranded portions of all heteroduplexes shown in Fig. 11 and 12 had a fractional length of 0.66, and their single-stranded tails had a fractional length (for the deletion) of 0.34. These results clearly indicate that class C, class B, and at least part of class A molecules share a common deletion mutant genome of SV40 and that classes B and A molecules have additions. Length measurements also revealed that class D molecules were deletion molecules originating from class C.

For the determination of the addition site with reference to the deletion site, the heteroduplex molecules having two single-stranded tails (for the deletion including the *Eco*RI site) and one addition loop (Fig. 11c to j and 12a to c), which belong mostly to classes B and A heteroduplexes, were selected and examined. Figure 13a shows that the great majority of addition molecules had extra DNA sequences at a fixed site (0.065 fractional length from the deletion site), although there were also some additions at different sites (Fig. 12b and c). Figure 14 shows the length distribution of addition loops in heteroduplexes having a fixed distance (0.065 unit of SV40 DNA) between the deletion and the addition. As can be seen, the majority of loops had a length of about 0.2 fractional length, which apparently had originated from class B molecules. A few heteroduplex molecules had a loop shorter (Fig. 11j and 14) and some had a loop longer (Fig. 14) than those of the majority of class B. They were heterogeneous in size and, except for the shorter ones, must have originated from class A molecules (Fig. 11c to f). From Fig. 13 and 14 we conclude that additions for classes B and A molecules appear to have occurred at the same preferential site on the SV40 chromosome. These additions appear to be mostly overlapping because the heteroduplexes formed with the mixture of classes A, B, C, and D were mostly those with an addition loop (Fig. 9a to h and 10), and only 1 out of 120 heteroduplexes had two addition loops, which would be indicative of additions at two different sites (Fig. 9k).

DISCUSSION

Chromosome aberrations of SV40, such as deletions and substitutions (9, 12, 17), have been described with DNA isolated from virions obtained after several serial high-multiplicity passages. In the present study we examined the changes of SV40 DNA that occur during a single cycle of virus production. We found, besides deletion and substitution molecules, oversized molecules (entire SV40 sequences plus inserted extra DNA sequences) in monkey cells infected with nondefective SV40 at an MOI of 6 PFU/cell. The added DNA sequences may be of either cellular or viral origin or both, since it is known that SV40 DNA from mature virions can contain host cell DNA sequences (9) and that partial duplication of SV40 DNA can occur during virus propagation (10). At present it is not possible to determine the origin of these extra DNA sequences by nucleic acid hybridization techniques because DNA samples from cells were not completely free from linear DNA fragments.

Oversized addition molecules have not been found in DNA from purified virions, irrespective of the type of passage. Apparently, there is a certain limitation of size for DNA molecules to be encapsidated into mature virions. DNA molecules from virions, if they had an inserted DNA segment, had a deletion at a different site (19). Since changes of SV40 DNA reported so far appear to be the results of selection at var-

between 0.76 and 0 (clockwise) map unit on the EcoRI physical map of SV40 DNA. The single-stranded loops are interpreted to represent the addition, since the sum of the lengths of the double-stranded portion and two single-stranded tails comes to one fractional length. (a) Class A heteroduplex (dimer of classes B and C molecules). (b) Class A molecule (dimer of class C molecules). (c) Class A molecule with a deletion (two tails) and a large addition loop. The addition loop is partially double stranded. (d to f) Class A heteroduplex with a deletion (two single-stranded tails) and an addition loop with variable length. A linear homoduplex of nondefective DNA used as the internal reference is shown in (d). (g to i) Class B heteroduplex with a deletion (two tails) and a fairly homogeneous addition loop. (j) Heteroduplex in which the addition loop is shorter than that of the class B heteroduplex. Bar represents 0.5 μm.

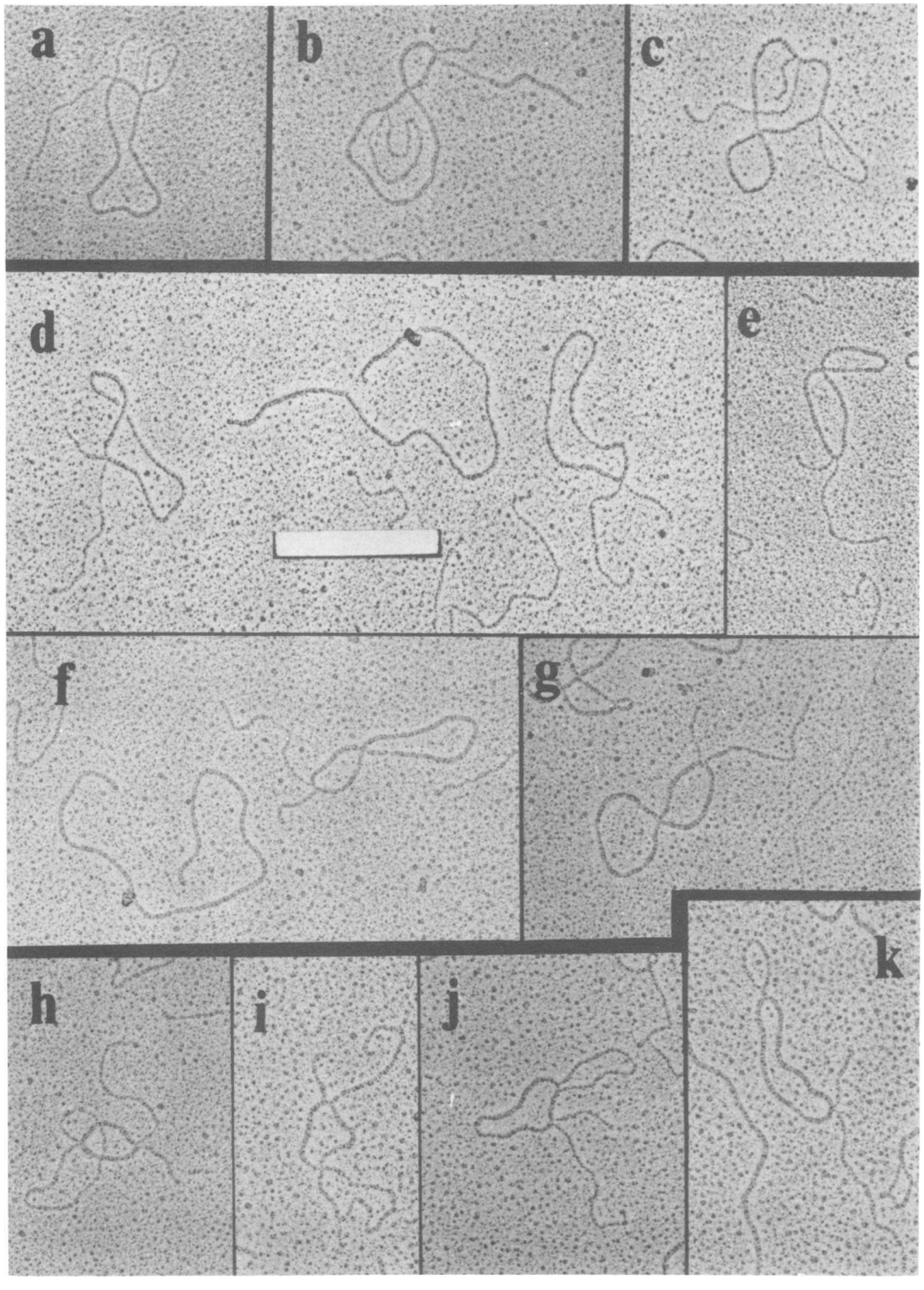

FIG. 12. *Electron micrographs of heteroduplexes formed between EcoRI-generated linear nondefective SV40 DNA and nicked circular DNA generated in SV40 provirus activation (classes B, C, and D of Fig. 8b). Heteroduplexes were interpreted in the same way as for Fig. 11. (a to c) Class B heteroduplex. The distance between the deletion (two tails) and the addition (a loop) in heteroduplexes of (b) and (c) is significantly longer than that in the majority of class B heteroduplexes shown in (a) and Fig. 11g to i. (d to g) Class C heteroduplex with two single-stranded tails for a deletion. Two linear homoduplexes of nondefective SV40 DNA are seen in (d) and (f). (h to k) Class D heteroduplexes with double deletions. The two single-stranded tails represent the original deletion. The single-stranded loop represents the second because the sum of the single-stranded loop and the double-stranded portion gives a length of class C molecules.*

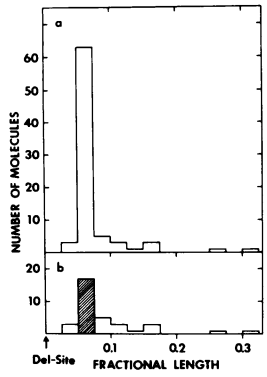

FIG. 13. *Sites of the addition with reference to the deletion site in circular DNA generated in SV40 provirus activation. The number of molecules having a deletion (two tails) and an addition (a loop with variable length) has been plotted against the shorter distance between the deletion and the addition. Some representative heteroduplexes used for this analysis (classes B and A heteroduplexes) are shown in Fig. 11c to j and Fig. 12a to c. (a) All molecules with a deletion and an addition have been included here. The population constituting the peak (0.05 to 0.075 fractional length) contains classes A and B heteroduplexes. The rest of the molecules are mostly class B heteroduplexes (the sum of the lengths of the double-stranded portion and the single-stranded loop comes to class B length). (b) Shadowed area contains only class A heteroduplexes (Fig. 11c to f).*

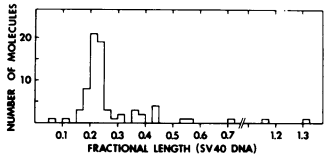

FIG. 14. *Length distribution of addition loops of the heteroduplexes having a fixed distance (0.065 fractional length) between the deletion and the addition. The heteroduplexes constituting the peak in Fig. 13a were selected. The number of molecules has been plotted against the fractional length of the addition loop. The population constituting the majority, with the peak at about 0.2 fractional length (20% addition), appears to be class B heteroduplexes (Fig. 11g to i). The molecules with an addition loop longer than that of the majority have been interpreted to be class A heteroduplexes (Fig. 11c to f), which are included in the shadowed area of Fig. 13b.*

ious stages (replication and encapsidation of DNA) during serial passages, they do not necessarily represent those occurring during a single cycle of DNA replication. Our results showed that addition, deletion, and substitution occur at similar frequencies during a single cycle of infection of monkey cells with nondefective SV40 at a high MOI (Table 1). Our results for deletion sites (Fig. 7) are essentially the same as those reported for DNA from virions (10, 20), whereas the findings for substitution sites differ from those reported for DNA from virions (2). Whether there are some preferential sites for substitution is still unclear.

As to the mechanism, we favor the integration-excision hypothesis because it accounts well not only for the generation of addition molecules but also for the generation of substitution and deletion. Addition and substitution molecules could be made through abnormal circularization of the SV40 provirus linearly integrated into cell DNA. Deletion molecules could result from the excision of a small segment from free nondefective SV40 DNA by the same mechanism as for the provirus excision. However, this model has to be tentative until the added DNA sequences have been proved to originate from cell DNA.

The second cell system we used in this study was heterokaryon cultures of monkey and SV40-transformed mouse cells. This system presumably involves two steps, the excision of linearly integrated SV40 provirus by circularization from host cell DNA and the replication of circular SV40 DNA in multinucleated cells. We, therefore, expected that the addition would occur at a unique site on the SV40 chromosome with each transformed cell line, the site at which the viral DNA had been integrated into the cell DNA. In this work we have shown that the heterogeneous circular molecules described previously (21) include SV40 deletion and addition molecules probably originating from activated heterokaryons. The fact that additions have overlapping sequences and have occurred at a preferential site on the SV40 chromosome supports the idea that extra DNA is added to SV40 at the time of provirus excision and the hypothesis that the chromosome aberrations of SV40 are generated through integration-excision.

Four classes (Fig. 8, classes A, B, C, and D) of circular DNA containing SV40 DNA sequences were generated by cell fusion from the TR-3T3-M cell line (21 and present study). From previous studies (15, 19–21), the defective SV40 carried by this cell line was interpreted to be a deletion-insertion mutant having a length of class B DNA. We have recently determined the structure of this defective mutant (details will be described elsewhere). Using DNA from virions that had been rescued by the triple cell fusion technique (19) and DNA directly obtained from heterokaryon cultures (DNA used in this study), we mapped the sites of deletion and insertion with reference to *Eco*RI and *Hpa*II cleavage sites. The results showed that class C DNA has a deletion of 0.34 fractional

length between 0.76 and 0.10 (clockwise on the physical map, assuming the *Eco*RI site as 0 point) and that class B DNA has the same deletion, including the *Eco*RI site and an addition (insertion) of 0.21 fractional length at 0.70. It appears reasonable to infer that additions of variable amounts of DNA for class A molecules have occurred also at 0.70 unit from the *Eco*RI site.

From previous and present results, many possible models could be constructed to account for the generation of classes A, B, and C molecules. Figure 15 shows one of the most simplified models. We assume that a deletion mutant DNA has been integrated into mouse DNA at 0.70 (or at some point within the inserted sequences) on the physical map of SV40 DNA. At excision, circularization between a and b in Fig. 15 and between a and c results in the generation of classes C and B molecules, respectively. The sequence between b and c in Fig. 15 could be monkey DNA inserted into SV40 DNA during productive infection before transformation of mouse cells, because the length of viral DNA from virions used for transformation had a length of class B molecules (19). However, it is also possible that this sequence is mouse DNA and that the sites a, b, and c are small sections of a common nucleotide sequence that facilitates recombination. Any circularization between points outside the sequence between a and c generates heterogeneous class A molecules that have overlapping additions and the addition site common to both classes A and B molecules. Circularization between points within and outside the integrated viral DNA would yield substitution molecules.

If the added sequences in class B molecules had originated from monkey DNA, they were not generated through abnormal circularization at the time of excision. Class B molecules could simply result from normal excision. Therefore, we excluded molecules that had a fixed distance between the addition and the deletion and were considered to be class B heteroduplexes (having addition loops of a uniform length) from Fig. 13a and obtained Fig. 13b, which includes class A addition molecules with a fixed distance between deletion and insertion and class B molecules with variable distances between the two. Still, it is clear that there is a preferential site on the SV40 chromosome for the addition that may have occurred at excision.

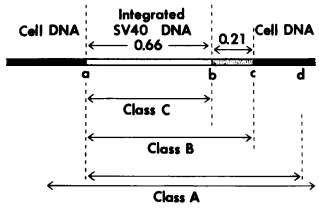

FIG. 15. *A possible model for the addition of host cell DNA to SV40 DNA at excision. The sequence between (a) and (b) is SV40 deletion mutant DNA integrated into mouse DNA in cell line TR-3T3-M. The sequence between (b) and (c) is the addition found in class B molecules of Fig. 8b. Circularization between (a) and (b) and between (a) and (c) generates classes C and B molecules of Fig. 8b. Abnormal circularization between (a) and (d) or between any points outside the viral DNA yields class A molecules of Fig. 8b.*

Other changes of SV40 DNA, however, must have occurred after excision during its subsequent replication in heterokaryons. For example, the second deletion of class D molecules (a single-stranded loop in Fig. 12h to k) could have occurred during the replication of class C DNA after provirus excision, because the sites of the second deletion with respect to the first deletion (two single-stranded tails) were not specific and were not compatible with the hypothesis that deletion molecules are made at excision from integrated provirus shown in Fig. 15. It is also possible that some class C molecules result from a specific deletion occurring in class B molecules during DNA replication. Some of the class B molecules that have an addition at a site different from that of the majority (Fig. 12b and c) may have occurred through reintegration and excision of class C DNA during its replication.

In conclusion, in permissive cells during one cycle of virus replication and in heterokaryons, in which the provirus is induced, we found a new class of oversized DNA molecules that have the entire SV40 DNA sequences plus inserted extra DNA sequences. The amounts of added DNA were found to be variable and heterogeneous in both cases. Whereas the addition sites were not specific on the SV40 chromosome in productive infection, there was a preferential site for addition in the provirus induction by heterokaryon formation. These results support the hypothesis that cell DNA can be added to SV40 DNA when linearly integrated viral DNA is excised by circularization, although the nature of added sequences remains to be characterized.

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LITERATURE CITED

1. Campbell, A. M. 1962. Episomes. Adv. Genet. 11:101-145.
2. Chow, L. T., H. W. Boyer, E. G. Tischer, and H. M. Goodman. 1974. Electron microscopic mapping of the attachment sites on SV40 DNA during lytic infection. Cold Spring Harbor Symp. Quant. Biol. 39:109-117.
3. Davis, R. W., M. Simon, and N. Davidson. 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. 21:413-428.
4. Hirai, K., and V. Defendi. 1972. Integration of simian virus 40 deoxyribonucleic acid into the deoxyribonucleic acid of permissive monkey kidney cells. J. Virol. 9:705-707.
5. Hirai, K., J. Lehman, and V. Defendi. 1971. Integration of simian virus 40 deoxyribonucleic acid into the deoxyribonucleic acid of primary infected Chinese hamster cells. J. Virol. 8:708-715.
6. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26:365-369.
7. Hudson, B., W. B. Upholt, J. Devinny, and J. Vinograd. 1969. The use of an ethidium analogue in the dye-buoyant density procedure for the isolation of closed circular DNA: the variation of the superhelix density of mitochondrial DNA. Proc. Natl. Acad. Sci. U.S.A. 62:813-820.
8. Koprowski, H., F. C. Jensen, and Z. Steplewski. 1967. Activation of production of infectious tumor virus SV40 in heterokaryon cultures. Proc. Natl. Acad. Sci. U.S.A. 58:127-133.
9. Lavi, S., and E. Winocour. 1972. Acquisition of sequences homologous to host deoxyribonucleic acid by closed circular simian virus 40 deoxyribonucleic acid. J. Virol. 9:309-316.
10. Mertz, J. E., J. Carbon, M. Herzberg, R. W. Davis, and P. Berg. 1974. Isolation and characterization of individual clones of simian virus 40 mutants containing deletions, duplications and insertions in their DNA. Cold Spring Harbor Symp. Quant. Biol. 39:69-84.
11. Sambrook, J., H. Westphal, P. R. Srinivasan, and R. Dulbecco. 1968. The integrated state of viral DNA in SV40-transformed cells. Proc. Natl. Acad. Sci. U.S.A. 60:1288-1295.
12. Tai, H. T., C. A. Smith, P. A. Sharp, and J. Vinograd. 1972. Sequence heterogeneity in closed simian virus 40 deoxyribonucleic acid. J. Virol. 9:317-325.
13. Uchida, S., and S. Watanabe. 1969. Transformation of mouse 3T3 cells by T antigen-forming defective SV40 virions (T particles). Virology 39:721-728.
14. Uchida, S., K. Yoshiike, S. Watanabe, and A. Furuno. 1968. Antigen-forming defective viruses of simian virus 40. Virology 34:1-8.
15. Watanabe, S. 1975. Virus DNA synthesizing ability of T antigen-forming defective SV40 produced by successive undiluted passages. J. Gen. Virol. 26:49-57.
16. Watkins, J. F., and R. Dulbecco. 1967. Production of SV40 virus in heterokaryons of transformed and susceptible cells. Proc. Natl. Acad. Sci. U.S.A. 58:1396-1403.
17. Yoshiike, K. 1968. Studies on DNA from low-density particles of SV40. I. Heterogeneous defective virions produced by successive undiluted passages. Virology 34:391-401.
18. Yoshiike, K. 1968. Studies on DNA from low-density particles of SV40. II. Noninfectious virions associated with a large-plaque variant. Virology 34:402-409.
19. Yoshiike, K., A. Furuno, and S. Uchida. 1974. Rescue of defective SV40 from a transformed mouse 3T3 cell line: selection of a specific defective. Virology 60:342-352.
20. Yoshiike, K., A. Furuno, S. Watanabe, S. Uchida, K. Matsubara, and K. Takagi. 1974. Characterization of defective simian virus 40 DNA: comparison between large-plaque and small-plaque types. Cold Spring Harbor Symp. Quant. Biol. 39:85-93.
21. Yoshiike, K., S. Watanabe, K. Suzuki, and S. Uchida. 1975. Circular DNA from heterokaryons of SV40-transformed mouse and African green monkey cells. Jpn. J. Microbiol. 19:237-240.