Spontaneous Rearrangement of Integrated Simian Virus 40 DNA in Nine Transformed Rodent Cell Lines

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Frequencies of spontaneous DNA rearrangement within or near integrated simian virus 40 (SV40) DNA were measured in four transformed mouse and rat cell lines of independent origin and in five clones of the SV40-transformed mouse line SVT2. Rearrangements were detected as polymorphisms of restriction enzyme fragment length in subclones of the lines. At least 17% of the subclones of each line had detectable rearrangements. The rate of rearrangement was calculated to be at least 5×10^{-3} events per cell per division. No rearrangements were detected in sequences of an immunoglobulin gene, part of the coding region of the mouse protein p53, and five proto-oncogenes. The possible role of recombination between duplicated segments of integrated SV40 DNA in generating rearrangements was studied in the five SVT2 clones, which differed in the number of duplications within a single SV40 DNA segment. The SVT2 clone that had no duplications, M3, became rearranged further at least as frequently as did closely related lines with one, two, or three duplications. Another line in this group that had one small duplication, X1, had a much higher frequency of rearrangement than did the others; integrated SV40 DNA of X1 became mostly rearranged within 100 cell divisions. The examples of M3 and X1 suggested that the high rate of rearrangement characteristic of integrated SV40 DNA was influenced more by the presence of particular sequences within or near integrated SV40 DNA than by the number or extent of duplicated sequences.

For unknown reasons, integrated simian virus 40 (SV40) DNA in transformed nonpermissive mouse and rat cells is frequently unstable. Free SV40 DNA does not replicate detectably in nonpermissive cells, yet integrated SV40 DNA undergoes rearrangements, seen as polymorphisms of restriction enzyme fragment length in Southern blots of cellular DNA probed with SV40 DNA (6, 7, 9, 15, 17, 18, 33, 43, 44, 47, 48, 51, 52, 58). Frequencies of rearrangement have varied from not detectable (10, 58, 41) to detectable in subclones selected for changes in transformation (17, 33, 43) to detectable in unselected subclones (6, 18, 51, 52, 58) or in uncloned mass cultures (15, 51). Most rearrangements have been observed in recently transformed cells (6, 18, 33, 51, 52), which raises the possibility that integrated SV40 DNA becomes more stable with time after integration.

Nonpermissive infection of mouse and rat cells by SV40 can be distinguished from semipermissive infection of rat or hamster cells by polyomavirus, in which free viral DNA replicates at a low rate (54, 69). In polyomavirus-transformed rat cells, spontaneous rearrangements of integrated viral DNA, including deletions (4) and duplications (19), have also been observed. Rearrangements in polyomavirus-transformed cells may include excision of viral DNA similar to the excision of SV40 sequences from nonpermissive SV40-transformed mouse cells fused with permissive monkey cells (11).

To explain rearrangements of integrated SV40 DNA in the transformed nonpermissive mouse line SVT2, Sager et al. (58) proposed that rearrangements were results of nearby insertions and excisions of transposable DNA elements, which incidentally involved integrated SV40 DNA. The sites of transposition were proposed to be exceptional, which explained the variability in frequencies of rearrangement found between different SV40-transformed cell lines and between subclones of SVT2 (58). The possibility of trans-

posable elements prompted a search for extrachromosomal SV40 DNA which was not found in SVT2 despite considerable effort (58) but was found in another SV40-transformed mouse line that had been more recently infected (34). No other evidence for spontaneous excision of integrated SV40 DNA has yet been reported in SV40-transformed mouse or rat cells; therefore, the role of extrachromosomal DNA in the rearrangement of integrated SV40 DNA is still unclear.

Another possible mechanism for rearrangement of integrated SV40 DNA is homologous recombination between duplicated segments of integrated SV40 DNA. Duplications of viral DNA are widespread in cells transformed by polyomavirus (8) and by SV40 (6, 9, 10, 18, 35, 58). Recombination between duplicated segments might lead to deletions or to further amplification. In polyomavirus-transformed rat cells, duplications were required both for excision of extrachromosomal DNA containing viral sequences and for additional amplification of integrated viral DNA (20). Further evidence for recombination between duplicated segments of integrated viral DNA is provided by changes in the sizes of SV40 T antigen in transformed mouse and rat cells. The SV40 super T antigens of 100 to 145 kilodaltons (kDa), larger than the wild-type size of 94 kDa, resulted from transcription of DNA containing partial tandem duplications in the T-antigen-coding sequence (14, 16, 28, 36, 39, 47, 48, 63); for example, SVT2 has a 1.8-kilobase (kb) tandem direct duplication in the coding sequence (58) and produces a 100-kDa super T antigen (28). The evidence for recombination between duplicated segments in SVT2 is the appearance of 94-kDa wild-type large T antigen in extended cell culture, correlated with spontaneous deletion of the 1.8-kb duplicated segment in the coding sequence (15). The same phenomenon, disappearance of a super T antigen correlated with deletion of a duplicated segment, was also observed in an SV40-transformed rat line (49). Despite the widespread duplications in integrated viral sequences in SV40-transformed cells and demonstrated deletions of duplications, it is

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not yet known whether recombination between duplications is the major mechanism of rearrangement of integrated SV40 DNA.

In this study, we tested two predictions suggested by the considerations above: first, that SVT2 was exceptional among transformed lines cultured for many generations in having a high frequency of rearrangement, and second, that duplications within integrated SV40 DNA are required for a high frequency of further rearrangement. In a limited study, we also explored the possibility that SV40 transformation was correlated with frequent rearrangements in other genes. Neither of the predictions was confirmed. In a survey of four well-established mouse and rat lines not related to SVT2, we found consistently high frequencies of rearrangement of integrated SV40 DNA, detectable without selection. We also reexamined SVT2, comparing it with four of its subclones which had different arrangements of SV40 DNA but which all had the same mouse flanking sequences. Surprisingly, the high frequency of rearrangement did not depend on the number of preexisting duplications, since a subclone with no duplications had a frequency of rearrangement as high as or higher than did three closely related lines with duplications. Another of the SVT2 subclones, X1, which had a single tandem duplication smaller than that of SVT2, had the highest frequency of SV40 DNA rearrangement yet observed. We concluded that the high frequency of spontaneous rearrangement is indeed characteristic of integrated SV40 DNA and that the high frequency does not depend on duplications and may be affected by small sequence changes within or very near the integrated SV40 DNA.

MATERIALS AND METHODS

Cell lines. Five previously isolated SV40-transformed rodent cell lines were studied: the BALB/c mouse lines SVT2 (1), 3T3/SV40 clone 4 (53), and SVA31 E7 (isolated by Y. Ito and obtained from L. Crawford; see reference 37); Swiss mouse line SV3T3 clone 9 (isolated by M. Vogt and obtained from F. O'Neill via W. Eckhart and J. Sambrook; see reference 67); and Fischer rat line 1_1 (25). For clarity, we have given three of the lines shorter names; 3T3/SV40 clone 4 is called B4, SVA31 E7 is called E7, and SV3T3 clone 9 is called clone 9. Our SVT2 (27) had the same restriction map as did that of Sager et al. (58). Four clones isolated directly or indirectly from SVT2 were also studied; D3 was a nontransformed (revertant) line isolated from SVT2 (27), M3 and M4 were clones of D3 selected in agar for spontaneous retransformation, and X1 was an unselected clone of SVT2 found by screening for rearrangements.

Culture conditions. Cells were grown as monolayers on polystyrene tissue culture petri dishes in the Dulbecco modification of Eagle minimal essential medium containing 1 mM sodium pyruvate, which is required for growth of SVT2 and its subclones. The medium was supplemented with 5% calf serum except in the cases of D3, M3, and M4, which were cultured in 10% calf serum. All lines were cloned in monolayer culture by seeding 50 or 100 cells per 100-mm petri plate and picking well-isolated colonies after 8 to 14 days. Monolayer cloning efficiencies were higher than 10%. X1, M3, and M4 were isolated initially in medium containing 0.35% agar, which selects for transformation, and were subcloned in agar and in monolayer culture. Cell cultures were fed daily at densities of more than 5×10^6 cells per 100-mm petri plate and were harvested at 1×10^7 to 2×10^7 cells per 100-mm petri plate. Because of frequent spontaneous rearrangements of integrated SV40 DNA, extended cell

culture was avoided except in one experiment, described below. Cell lines were stored frozen in liquid nitrogen as soon as possible after cloning. All lines were screened for mycoplasma infections; one was infected and was cured by recloning in antibiotics (31).

Isolation of high-molecular-weight cellular DNA. Monolayer cultures were rinsed twice with ice-cold phosphate-buffered saline and then lysed at 23°C on the petri plates in a solution containing (in 3 ml/10^7 cells) 1% sodium dodecyl sulfate (SDS), 50 mM trisodium EDTA, 50 mM Tris hydrochloride (pH 8), and 200 µg of self-digested pronase per ml (grade B; Calbiochem-Behring, La Jolla, Calif.; self-digested by incubation of a solution of pronase [2 mg/ml] in 0.5 M trisodium EDTA [pH 9] for 2 h at 37°C). The lysed cells were scraped into a tube, incubated for 30 min at 45°C, and extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (50:50:1). Nucleic acids in the aqueous phase were precipitated at 23°C by adding 2 volumes of 95% ethanol at 23°C. The precipitated nucleic acids were dissolved in 10 mM Tris hydrochloride (pH 7.5)–0.1 mM EDTA and then adjusted to 40 mM NaCl–50 mM Tris hydrochloride (pH 7.5)–10 µg of boiled RNase A per ml. The mixture was incubated for 60 min at 37°C, adjusted to 100 µg of self-digested pronase per ml–25 mM trisodium EDTA–0.5% SDS, and incubated for 30 min at 45°C. The DNA was extracted once again with phenol-chloroform-isoamyl alcohol, precipitated with ethanol, and redissolved in 10 mM Tris hydrochloride (pH 7.5)–0.1 mM EDTA. DNA concentrations were determined fluorometrically after depurination and conjugation to diaminobenzoic acid (30).

Southern blots. Cellular DNA was digested at 50 ng/µl with several combinations of restriction endonuclease at 2 to 4 U/µg of DNA for 15 to 18 h at 37°C (or 2 h at 65°C for *Taq*I) in the buffer recommended by the enzyme supplier. Digested DNA was treated with pronase plus SDS, extracted with phenol-chloroform-isoamyl alcohol, and precipitated with ethanol as was done for preparation of the high-molecular-weight DNA described above. The precipitated digested DNA was dissolved at 250 ng/µl in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–20% sucrose–0.05% bromophenol blue and applied at 5 µl (1.25 µg of DNA) per lane to a 0.45 or 0.7% agarose horizontal slab gel (13 by 19 by 0.3 cm) in Tris borate-EDTA buffer (50). Samples were allowed to rest in the sample wells for 2 h before the power was turned on to allow some diffusion of small molecules, giving a more uniform initial electric field and sharper bands of the high-molecular-weight fragments (29). Electrophoresis was overnight for 500 to 700 V · h. Denaturation, neutralization, transfer of DNA to a nitrocellulose membrane, washing, and baking were performed by standard procedures (50). A novel background-reducing reagent, the synthetic heparin substitute polyanetholesulfonic acid (Calbiochem), was used in the prehybridization and hybridization steps in place of Denhardt mixture (22) and heterologous DNA according the example of Singh and Jones (62), who used heparin. Prehybridization (30 min) and hybridization (20 h) were at 68°C in 5× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM sodium phosphate (pH 6.7), 5 mM sodium pyrophosphate, 0.1% SDS, and 200 µg of polyanetholesulfonic acid per ml. The probes were SV40 DNA or bacterial plasmid DNA labeled with [^{32}P]dATP or -dCTP by nick translation (55) and denatured in 0.2 N NaOH–0.1% SDS–salmon sperm DNA (1 mg/ml) for 10 min at 23°C. The plasmids used were pMycEcl carrying part of c-*myc* (21), pSSV-11 carrying part of v-*sis* (56), pEJ carrying part of c-Ha-*ras* (61), pHiHi carrying part of c-Ki-*ras* (24), pAB1-sub9 carrying v-*abl* (65),

TABLE 1. Frequencies of rearrangement of integrated SV40 DNA in transformed cells other than SVT2

Cell line	Cloning 1		Cloning 2	
	Rearranged clones/total	Frequency	Rearranged clones/total	Frequency
B4	22/22	1.0	6/24	0.25
Clone 9	8/8	1.0	5/19	0.3
E7	8/8	1.0	13/21	0.6
1_1	2/7	0.3	4/14	0.3

pCk carrying part of a mouse immunoglobulin kappa light chain (46), and p53 clone 9, a cDNA clone of the mouse protein p53 (5). After hybridization, blots were washed with agitation twice for 5 min at room temperature and then three times for 20 min each at 60°C; all washes were in 0.1× SSC plus 0.1% SDS. Damp-dry blots were heat sealed between sheets of 1-ml polyethylene and exposed for 1 to 14 days at −70°C to XAR X-ray film (Eastman Kodak Co., Rochester, N.Y.) with one intensifying screen.

RESULTS

A high frequency of spontaneous rearrangement in integrated SV40 DNA was found in four lines other than SVT2. To extend the study of spontaneous rearrangements to well-established lines other than SVT2, we examined B4, E7, clone 9, and 1_1. Each had been isolated independently after infection with SV40 and had been characterized for transformed properties but not for spontaneous rearrangements in integrated SV40 DNA. B4 and E7 were derived from BALB/c mouse embryo cells or cell lines, as was SVT2; clone 9 was derived from an outbred Swiss mouse embryo line, and 1_1 was derived from a Fischer rat embryo line. The frequency of spontaneous rearrangements was measured by the method of Sager et al. (58); the frequency of polymorphisms of restriction enzyme fragment length was measured in unselected subclones, probing with SV40 DNA. Results are summarized in Table 1, and representative blots are shown in Fig. 1 and 2.

Every subclone of mouse lines B4, E7, and clone 9 had a different pattern of restriction fragments containing SV40 sequences after the first cloning (Table 1 and Fig. 1). The Fischer rat line 1_1 had fewer polymorphisms, possibly because of more recent previous cloning; among seven subclones examined, five had the same set of fragments and two had different sets (Table 1).

The multiple polymorphisms suggested high frequencies of rearrangement, but because the lines had been cultured for many generations before our cloning, all four lines were cloned again to search for continuing rearrangements. As parent cultures for the second sets of subclones, we chose from each first set the clone with smallest number of EcoRI fragments. Polymorphisms were found again in all four of the second sets of subclones (Table 1). The frequency of rearrangement was similar to or greater than the frequency of 0.2 observed in SVT2 by Sager et al. (58). Rearrangements appeared to be independent, occurring one or more times during growth of individual clones, because the altered fragment patterns were different from each other and because clones with rearrangements were often mixed, with parental fragments and novel fragments found in the same clone of cells. (The subject of mixed clones is pursued
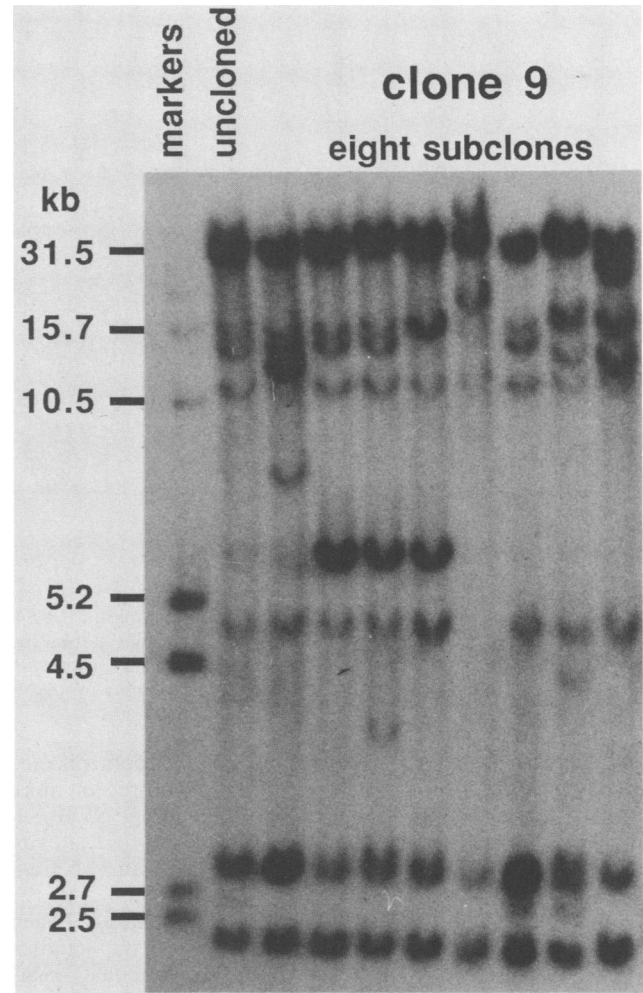

FIG. 1. Polymorphisms in subclones (first cloning) of the SV40-transformed mouse line clone 9. Eight subclones of clone 9 were isolated in monolayer culture. DNA was isolated from the subclones, digested with EcoRI, and examined on a Southern blot probed with SV40 DNA. All eight subclones had patterns different from those of both the parent culture and each other.

further in the Discussion.) We concluded that rearrangements were continuing to occur in all of the lines examined and that the high frequency of rearrangement found in SVT2 was typical of integrated SV40 DNA in transformed mouse and rat cells.

Duplications in integrated SV40 DNA had no measurable effect on the frequency of further rearrangement. The frequency of rearrangement of integrated SV40 DNA might depend on exchanges between duplicated sequences of integrated SV40 DNA, as required in polyomavirus-transformed cells (20) and as proposed for the deletion or amplification of drug resistance genes (60). Intrachromosomal gene conversion is another possible event dependent on duplications (40). To test the effect of duplications on the frequency of rearrangement by our methods, we needed cell lines that differed only in duplications within integrated SV40 DNA, including a line with no duplications.

None of the cell lines described above were suitable for the test, since they had diverse patterns of integration and all had SV40 DNA duplications. B4, E7, clone 9, and 1_1 plus their subclones must have all had duplications in SV40
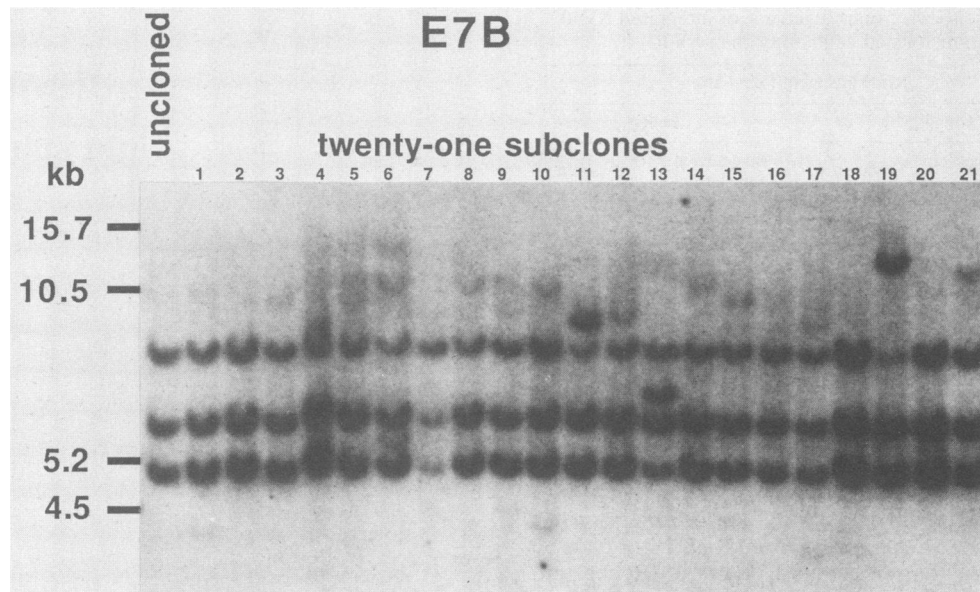
FIG. 2. Polymorphisms in subclones (second cloning) of the SV40-transformed line E7. E7 was cloned and analyzed as described in the legend to Fig. 1, producing eight clones which were all different (not shown). The clone with the smallest number of *Eco*RI fragments, E7B, was cloned again in monolayer culture, and DNA from the second set of subclones was analyzed. Shown are the results of a Southern blot of *Eco*RI digests of DNA from E7B and its 21 subclones probed with SV40 DNA.

DNA, because each had three or more *Eco*RI fragments (see Fig. 1 and Fig. 2 for clone 9 and E7B) and three or more *Bam*HI fragments (data not shown) containing SV40 sequences. Since there are single *Eco*RI and *Bam*HI sites in SV40 viral DNA, there should be no more than two fragments if the integrated DNA lacks duplications.

We were able to make a study of the relationship between the frequency of rearrangement and preexisting duplications by comparing SVT2 with three of its spontaneously rearranged subclones, D3, M3, and M4. The four lines in question, whose properties are summarized in Table 2, had a single segment of integrated SV40 DNA bounded by the same mouse sequences (Fig. 3). SVT2 had one 1.8-kb tandem direct duplication in the T-antigen-coding sequence (58; Fig. 3). D3, derived from SVT2, had an additional tandem direct duplication, giving D3 four segments of repeated SV40 DNA (Fig. 3). Lines M3 and M4, both derived from D3, had deletions within the integrated SV40 DNA of D3 (Fig. 3). The arrangement in M4 apparently resulted from a crossover between the first and second duplicated segments of D3, and the arrangement in M3 apparently resulted from a crossover between the first and fourth duplicated segments of D3. In summary, M3 had no duplications, SVT2

had one duplication, M4 had two duplications, and D3 had three duplications.

There was no correlation between the number of duplications and the frequency of rearrangement of integrated SV40 DNA (Table 3). M3, which had no duplications, had a frequency of rearrangement similar to or higher than those of SVT2, M4, and D3. The lack of correlation and the unexpectedly high frequency found in X1 (discussed below) suggested instead that an unknown effect other than homologous recombination was the major factor determining the frequency of rearrangement. Frequent deletions were not ruled out, but the other effect apparently contributed more to the frequency.

Rearrangements in subclones of M3 (Fig. 4) were of special interest because of the unique lack of preexisting duplications. To see whether the rearrangements in M3 were different from rearrangements in the other members of the SVT2 family, restriction maps of integrated SV40 DNA and flanking mouse DNA were determined in four rearranged M3 subclones. The rearrangements were all different and all included tandem direct duplications (data not shown), similar to rearrangements in SVT2 and other SV40-transformed cells. We concluded that preexisting duplications in inte-

TABLE 2. Characteristics of SVT2 and subclones

Cell line	Origin	Growth in agar	Antigen size (kDa)		Ploidy
			Large T-super T[a]	Small t	
SVT2	SV40 infection of embryo cells[b]	Yes	100	None	Diploid
X1[c]	Unselected clone of SVT2	Yes	94	20	Diploid
D3	Selected from SVT2[d]	No	100, 108	None	Hypotetraploid
M3	Agar-selected from D3	Yes	94	20	Hypotetraploid
M4	Agar-selected from D3	Yes	94, 100	20	Hypotetraploid

[a] Detected and quantified as described elsewhere (28). Amounts of labeled large T-super T per cell after a 2-h labeling period were similar (data not shown).
[b] Described elsewhere (1).
[c] Subclone X1.15 had properties the same as those of its parent, X1.
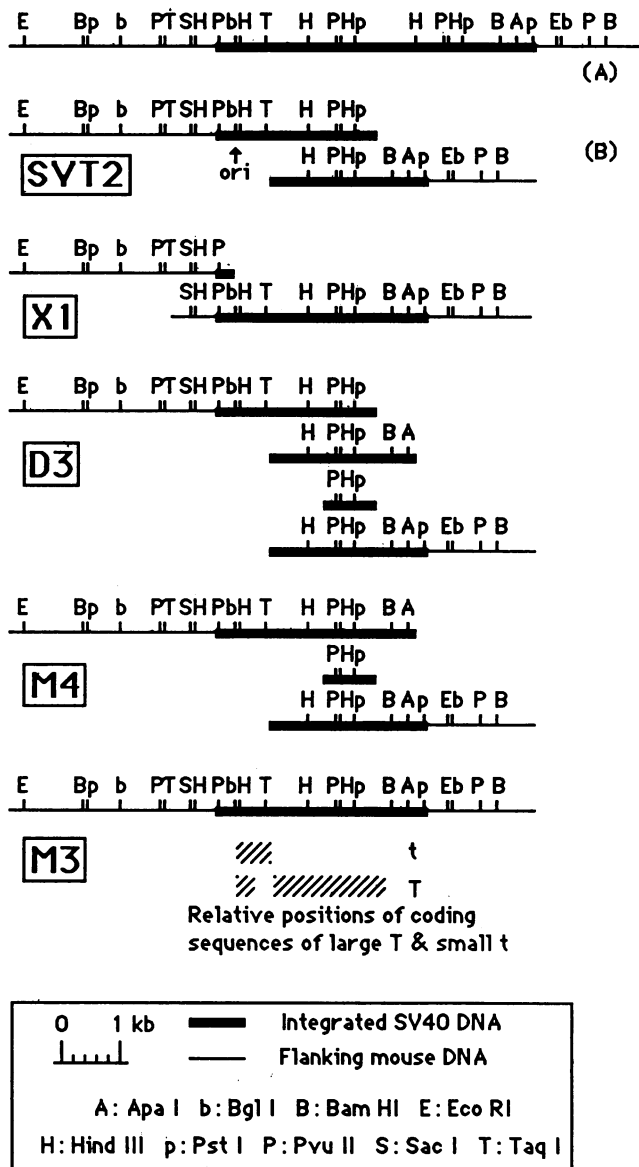[d] Described elsewhere (27).

FIG. 3. Restriction maps of SVT2 and its subclones. The maps were made from the results of single and multiple digests of the nine restriction enzymes indicated in the figure plus HaeIII, whose sites are not shown. (A) Restriction sites in SVT2 shown in the usual notation as a continuous segment of integrated SV40 DNA (━━━) and flanking mouse DNA (──────). The 1.8-kb tandem direct duplication in SVT2 was not readily apparent in panel A, so an alternate notation is given in panel B, in which the map shown in panel A is presented in discontinuous overlapping segments to show the duplication. The ends of the segments in panel B are meant to denote the ends of the duplication, not breaks or discontinuities; DNA sequences in all cases were continuous from left to right and from top to bottom. In the maps of the other lines derived from SVT2, we have used the notation of panel B to emphasize other tandem duplications and to show positions of the ends of duplications and deletions. The four segments of SV40 sequences in D3 can be interpreted as a single further tandem direct duplication of SV40 DNA in SVT2. The apparent additional duplication joined sequences near the right end of the second segment of SV40 DNA in the SVT2 map to a second copy of SVT2 sequences starting in the middle of the first segment. M4 and M3 had rearrangements compared with their parent, D3; M4 had a deletion produced by an apparent exchange between the top two segments of duplicated

TABLE 3. Frequencies of rearrangement in SVT2 and subclones

Parent culture	No. of duplications	Cloning method	Frequency of rearrangement
SVT2	1	Monolayer	4/20[a] (0.2)
X1	1	Agar	7/16 (0.4)
X1.15[b]	1	Agar	8/18 (0.4)
X1.15	1	Monolayer	10/10[c] (1.0)
D3	3	Monolayer	2/12 (0.2)
M3	0	Monolayer	11/41[d] (0.3)
M4	2	Monolayer	3/17 (0.2)

[a] Data from Sager et al. (58).
[b] X1.15 was an agar subclone of X1 with no detectable rearrangements.
[c] Shown in Fig. 6.
[d] Nineteen subclones are shown in Fig. 4.

grated SV40 sequences were not required to produce more tandem duplications, the major type of spontaneous rearrangement found in SV40-transformed mouse cells.

Line X1 had a distinctly higher frequency of rearrangement than did SVT2. An even higher frequency of rearrangement in X1 was demonstrated by rearrangements in all monolayer subclones (Table 3; see also Fig. 6) and by rearrangements in a continuously passaged uncloned culture (Fig. 5). At weekly passage 5 of uncloned cultures, SVT2 and X1 each had a single EcoRI fragment. At weekly passage 9, SVT2 still had a single EcoRI fragment with no obvious change in mobility, but X1 had relatively little of its original fragment and instead had a collection of larger fragments. The average change to larger EcoRI fragments in the mass culture of X1 at passage 9 (Fig. 5) was in agreement with the larger average size of EcoRI fragments of individual unselected X1 clones (data not shown). In passages 10 and 11 of X1 in mass culture, the patterns of restriction fragments changed still more, including EcoRI fragments smaller than the single fragment seen in earlier passages (data not shown). The reason for the higher frequency of rearrangement in X1 is not yet known, but it appears that sequence changes within or near the integrated SV40 of SVT2 increased the frequency of rearrangement to higher levels.

No rearrangements were found in seven cellular sequences. The instability of integrated SV40 DNA might reflect a high rate of rearrangements throughout the genome, such as that found in Bloom's syndrome human cells (2, 66) or in "amplificator" mutant hamster cells (23, 26); perhaps the instability seen in integrated SV40 DNA was a reporter of more general instability in SV40-transformed mouse cells. To address this possibility, Southern blots of clones showing high frequencies of rearrangements in SV40 sequences were probed or reprobed with sequences from seven mouse genes (see Materials and Methods). In several efforts, rearrangements were observed only in integrated SV40 DNA, not in the alternate sequences. One of the alternate sequences, the light-chain immunoglobulin sequence, would not be expected to be expressed in fibroblasts and so presumably would be free to diverge through rearrangement. In the

SV40 DNA in D3. M3 had a larger deletion produced by an apparent exchange between the first and last of the duplicated segments in D3. X1 had rearrangements resulting in the deletion of the 1.8-kb tandem duplication of SVT2 plus generation of another tandem duplication which contained the left mouse-SV40 junction. T' e presumptive origin of SV40 DNA replication in integrated SV. DNA is at ori in the SVT2 map, near b, the BgII site.
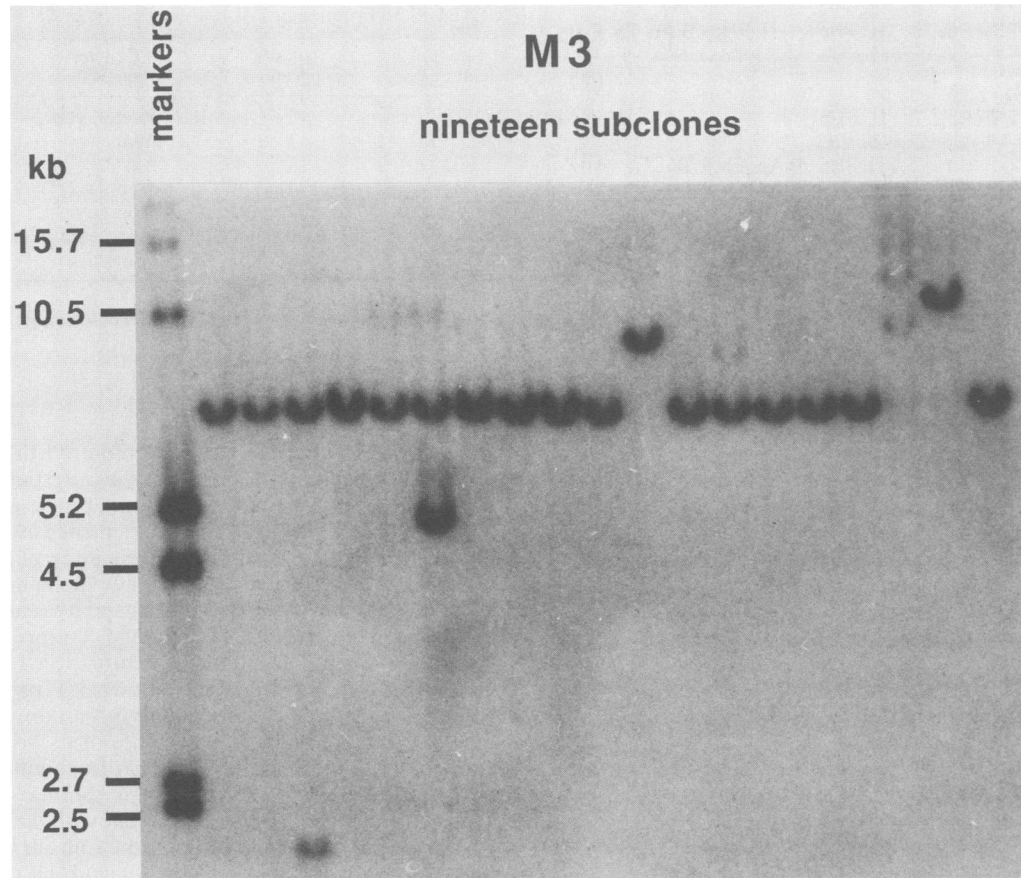
FIG. 4. Polymorphisms in unselected subclones of M3. Nineteen subclones were isolated from M3 in monolayer culture and examined as described in the legend to Fig. 1. The subclones shown represent all clones isolated in one experiment. The clone in the lane next to the markers has the same pattern as was found with EcoRI digests of the parental M3 cells.

direct comparison shown in Fig. 6, multiple rearrangements were found in integrated SV40 DNA but none were found in the immunoglobulin sequence. The lack of polymorphisms in alternate sequences also showed that partial digestion with restriction enzymes was not an artifactual source of polymorphisms in blots probed with SV40 DNA. We concluded that a high frequency of rearrangement either is characteristic of integrated SV40 DNA or is characteristic of mouse sequences immediately adjacent to integrated SV40 DNA or else is confined to other selected segments of the mouse genome which we missed in our small sample of alternate probes.

Test of sensitivity of detection of minor restriction fragments. To compute an approximate rate of rearrangement (see Discussion), we measured the sensitivity of detecting small proportions of polymorphisms under our experimental conditions by making dilutions of a preparation containing one single-copy fragment with SV40 sequences. We could detect the fragment in about 40 ng of digested cellular DNA but not in 20 ng (Fig. 7), which means that we could detect novel fragments only if the fragments were present at 1/32 or more of the amount of the standard fragment.

DISCUSSION

All nine lines had a continuing high frequency of spontaneous rearrangements in integrated SV40 DNA. The fraction of unselected subclones with detectable rearrangements was
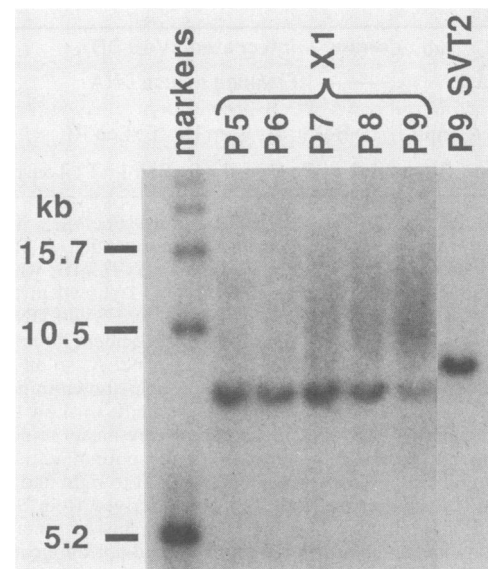

FIG. 5. Rearrangements found in X1 cells in extended culture. X1.15 (an agar subclone of X1) and SVT2 were grown continuously in monolayer culture with weekly passaging (trypsinization and reseeding). DNA was extracted from cultures at each weekly passage, digested with EcoRI, and analyzed on a Southern blot probed with SV40 DNA. X1 is shown at passages 5 through 9, and SVT2 is shown at passage 9.
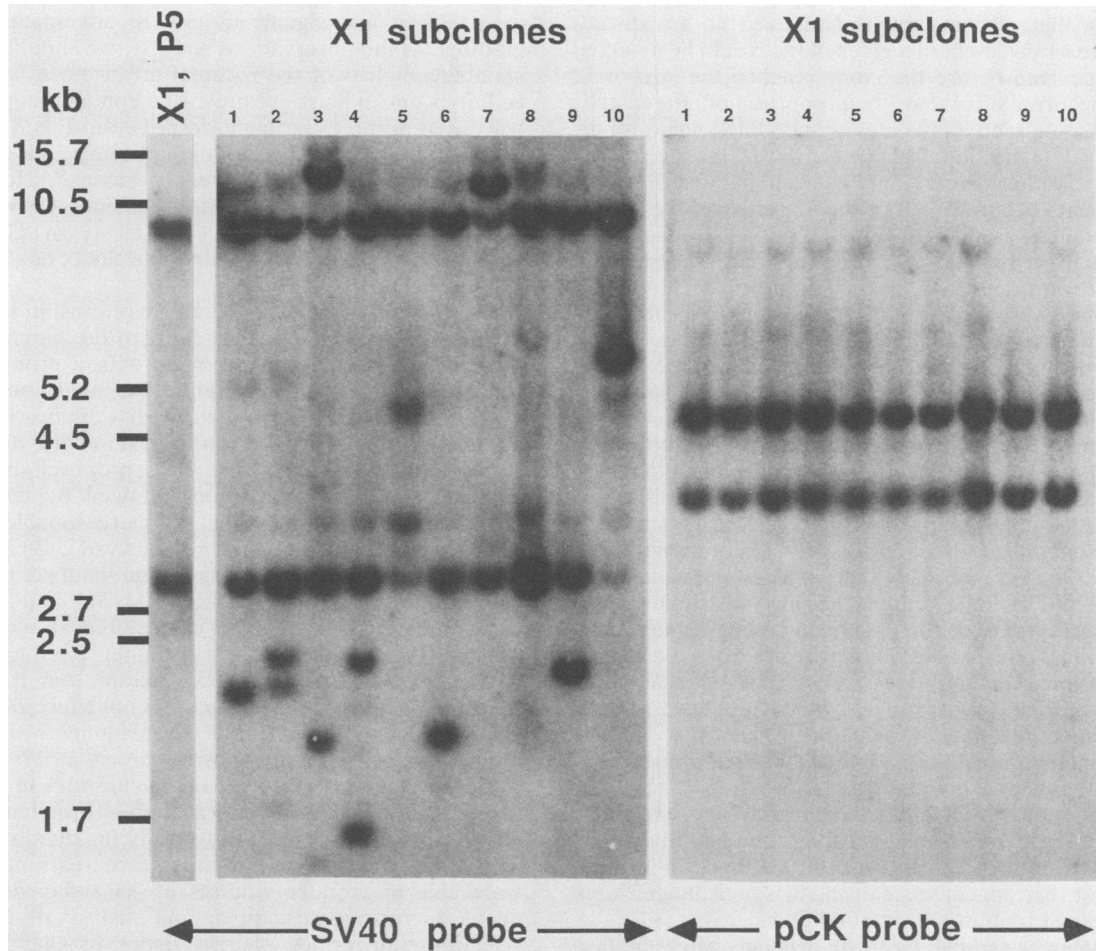
FIG. 6. X1 subclones analyzed with two probes. Ten subclones were isolated from X1.15 (an agar subclone of X1) in monolayer culture. DNA from the parental DNA and from each of the 10 subclones was digested with TaqI and analyzed for polymorphisms in integrated SV40 DNA and in a mouse immunoglobulin sequence (pCk).

between 17 and 100% in the five independently isolated lines and in the four subclones of SVT2. The frequencies of rearrangement in the independently isolated lines were similar to or higher than that of SVT2, which had been thought previously to have an unusually high frequency of rearrange-
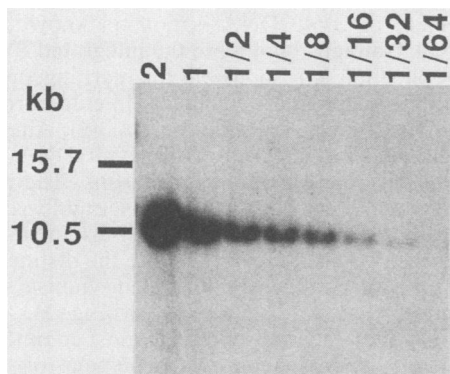

FIG. 7. Dilution series to test sensitivity of Southern blots. DNA from the M4 subclone M4F10 was digested with EcoRI and applied to a Southern blot in serial twofold dilutions from 2.5 μg to 20 ng. The standard amount applied to other blots was 1.25 μg (lane 2).

ment (58). Previous observations (6, 18, 51, 52) had suggested that integrated SV40 DNA was unstable in recently infected lines and that SVT2 was a special case for another reason (58). Results presented here with four other well-established lines (B4, clone 9, E7, and 1₁) suggest instead that SVT2 is not a special case, that the high frequency is characteristic of integrated SV40 DNA, and that the frequency remains high indefinitely.

Rates of SV40 DNA rearrangement were at least 5×10^{-3} events per cell per division. We made a rough estimate of the lower limit of the rate of rearrangement from the frequencies of observed polymorphisms and the measured sensitivity of detecting them. For the calculation, we assumed that rearrangements generally conferred no growth advantage in monolayer culture, as suggested by cloning efficiencies greater than 10% and by the slow appearance of detectable rearrangements in uncloned cultures. In our most rapidly rearranging lines, X1 and its subclone X1.15, rearrangements took seven weekly passages to be detectable (Fig. 5), in addition to the 2 or 3 weeks required for initial growth of the clone. In the less frequently rearranging line SVT2, rearrangements in a mass culture were not observed at passage 9 (Fig. 5), although rearrangements in a mass culture of SVT2 were observed eventually, after 31 passages (15).

Assuming that rearrangements conferred no growth advantage, a rearrangement in a given clone would be detected only if it occurred before the clone reached the size of 32 cells. If it occurred after that, the proportion of altered cells in the population would give a correspondingly low proportion of novel restriction fragments, lower than could be detected by Southern blots (Fig. 7). Assuming that observed rearrangements occurred before the 32-cell size, the rate of rearrangement, r, is related to the observed frequency of rearrangement, f, by $r \geq f/31$, since there are 31 individual cell divisions in the growth of a single cell to a clone of 32 cells. The lowest frequency of rearrangement in the nine cell lines studied here was 2/12 in D3 (Table 3). By using $r \geq f/31$, the approximate rate of rearrangement in D3 was $\geq 5 \times 10^{-3}$ events per cell per division. The other lines had higher rates.

The estimate gives a minimum rate for at least three reasons. Small rearrangements might give electrophoretic mobility differences too small to detect, blot conditions are often not as favorable as those reflected in Fig. 7, and rearrangements might give cells a selective disadvantage in culture, for example by reducing expression or activity of T antigen. We suspect that many rearrangements of integrated SV40 DNA did affect T antigen because restriction maps showed interruptions of the T-antigen-coding sequence by tandem duplications (58; Fig. 3). An example of a rearranged line with an apparent alteration of SV40 gene expression was the nontransformed revertant D3, which produced a novel 108-kDa super-T antigen (Table 2). In previous studies, rearrangements in integrated SV40 DNA sometimes caused the complete loss of T-antigen expression (44, 64); however, all members of the SVT2 family examined to date expressed one or more forms of T antigen (Table 2 and additional data not shown). Continued expression of T antigen in all lines suggests that rearrangements causing loss of T antigen were selected against by our culture conditions.

Mixed clones suggested frequent deletions after duplications. Several subclones with rearrangements appeared to be mixed, with both novel fragments and parental fragments. Some mixed clones presented an apparent contradiction to our assumption of the absence of growth advantage for cells with rearrangements. The contradiction is illustrated in the following hypothetical example. Consider a single rearrangement which occurred early in the growth of a clone, giving a mixed clone with a novel fragment and a parental fragment. The earliest that the rearrangement might occur would be in the first division, so that one half of the cells would have the rearrangement. If there were no selection for either arrangement, the amounts of the novel fragment and the corresponding parental fragment would be equal. More likely, the amount of the novel fragment would be less than that of the parental fragment because a rearrangement would be more likely to have occurred later in the growth of the clone than in the first division. With the assumptions above, a novel fragment in a mixed clone should never be more prominent than the parental fragment; otherwise, it would appear that cells with the novel fragment had a selective growth advantage within the clone, contrary to our assumption. Nevertheless, we frequently found mixed subclones with more prominent novel fragments accompanied by reduced parental fragments. The sixth subclone of M3 (Fig. 3) is not an example of this effect because the parental fragment was not reduced even though there was a prominent novel fragment. Examples of clones with reduced parental fragments are E7B subclones 11, 13, and 19 (Fig. 2) and X1 subclones 3, 5, 7, and 10 (Fig. 6). Four of the ten monolayer clones of X1 had reduced parental fragments. If cells with novel frag-

ments often had significant growth advantages in mixed cultures, as the observations suggest, we should have noted spontaneous loss of the parental arrangement within a few cell divisions in mass cultures. The continuous passaging of cells used in the experiment described in Fig. 5 was similar to the first few divisions of cloning because a low density of cells was seeded in each weekly passage. However, the proposed rapid loss of parental fragments was not found in continuous passage; the most rapid loss, in X1, took nine passages plus the initial growth of the clone, or about 100 cell divisions (Fig. 5).

The dilemma can be resolved by proposing that duplications are often unstable and revert to the parental arrangement by deletion. A mixed clone with a prominent novel fragment and a reduced parental fragment could originate from a rearranged founder cell, with a duplication followed by deletion of the duplication in some of the progeny cells during growth of the clone. The proposal assumes two rearrangements, a duplication followed by deletion. The proposal of frequent deletions seems reasonable since deletions have been documented previously (15, 49) and since we found three different deletions in our small set of clones, in the rearrangements that produced M3, M4, and X1. Furthermore, in each of the seven cases cited above, the novel fragment was larger than the fainter parental fragment. However, regardless of the explanation, our calculation of the minimal rate of rearrangement is not affected because the novel fragments found in the D3 subclones were present in minor amounts (data not shown).

SV40-transformed lines are not too unstable to make homogeneous cultures for biochemical studies. Frequent rearrangements might make it difficult to study the unknown property of X1 responsible for its high rate of rearrangement. We were able to produce cultures of X1 sufficiently homogeneous for restriction mapping and analysis of T antigen by selecting transformed characteristics in agar culture and exploiting the founder effect. The procedure was to clone X1 in agar and then test several clones for rearrangements with Southern blots while storing recently cloned live cells in liquid nitrogen. When an unrearranged homogeneous clone was found, the clone was thawed and grown just long enough to perform the study. Selection in agar culture apparently helped to keep the original arrangement, because more than half of X1 agar subclones were not rearranged (Table 3) whereas X1 subclones isolated by less selective monolayer culture were all rearranged (Table 3 and Fig. 6).

Possible molecular mechanism of spontaneous rearrangement of integrated SV40 DNA. The molecular mechanism of rearrangement of SV40 DNA is not yet known, but it is possible that T-antigen binding to the integrated SV40 origin of DNA replication is responsible for rearrangements, as it could be in producing rearrangements in extrachromosomal SV40 sequences during transfection (16, 49). Alternatively, the cellular sites of integration of SV40 DNA might be intrinsically unstable for unknown reasons. The restriction map of integrated SV40 DNA in our most rapidly rearranging line, X1, unfortunately does not allow us to discriminate between these two possibilities because the distinctive small duplication in X1 contains both flanking mouse sequences and sequences close to the SV40 origin (Fig. 3).

Since tandem duplications were the most common form of rearrangement, spontaneous rearrangement of SV40 sequences might use one or more of the mechanisms currently proposed for the initiation of gene amplification. The current proposals are overreplication or rereplication of DNA sequences due to multiple initiations in a single cell cycle (11,

59, 60), an increased general instability of the genome in certain cells (26), and random excision of episomes from chromosomes followed by extrachromosomal replication without segregation and then reintegration (13). Overreplication of integrated viral DNA has been observed in SV40-transformed hamster cells treated with carcinogens (38) and in polyomavirus-transformed hamster cells treated with mitomycin C (3), but the use of DNA-damaging drugs has made the results hard to interpret. Evidence for overreplication of other sequences (42, 57, 68) has been claimed to be artifactual (32) or explained by excision and replication of acentric fragments (12). Excision and extrachromosomal replication of SV40 sequences have been found after fusion with permissive monkey cells (10, 67) and in SV40-transformed hamster cells infected with herpes simplex virus (45). The semipermissive cells and the use of DNA-damaging drugs complicate interpretations of these results. Most cases of gene amplification are rare so that initial events must be deduced indirectly after many cell divisions. By contrast, the much higher frequency of spontaneous rearrangement of integrated SV40 DNA should make it possible to find direct evidence for initial events.

ACKNOWLEDGMENTS

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