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Evolutionary Variants of Simian Virus 40 Which Are Impaired in Early Lytic Functions but Transform Nonpermissive Cells

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From an undiluted passaged virus stock, two size classes of defective simian virus 40 (SV40) DNA were isolated from which two evolutionary variants were cloned. By means of restriction enzyme and heteroduplex analysis, physical maps of the mutants have been constructed. Both mutants contained the region of SV40 DNA coding for the early proteins plus some adjacent sequences (the region from 0.120 to 0.685 map unit, clockwise, on the standard SV40 DNA map). Furthermore, each mutant contained, in the form of two inverted repeats, four times the sequences from the region 0.625 to 0.685 map unit, clockwise. Some biological properties of the mutant DNA were examined, and we found that the mutant DNA (i) has, as compared with SV40 DNA, an impaired ability to induce T antigen in permissive and nonpermissive cells; (ii) does not complement a thermosensitive *A* mutant of SV40; (iii) replicates very inefficiently without a helper; and (iv), as an apparent contradiction, transforms nonpermissive baby rat kidney cells as well as SV40 DNA. A hypothetical mechanism for the expression of the mutant DNA that might explain the observed biological properties is presented.

The determination of the total nucleotide sequence of simian virus 40 (SV40) DNA has permitted the precise localization of the known genes (12, 27). The initiation codon for the two known early proteins, the t and T antigens, is located at position 0.648 map unit on the standard SV40 map, and the coding sequence proceeds counterclockwise from this point. Proceeding clockwise from 0.648 map unit, the next protein (VP2)-coding sequence starts at 0.767 map unit. As pointed out earlier (12, 27), the DNA sequence between 0.648 and 0.767 map unit contains not only the origin for DNA replication, which is at or near 0.663 map unit, but also the information for initiation of early transcription. The 5′ end of the early mRNA has been mapped near 0.66 map unit (10). The exact position where primary transcription starts and the location of the sequences necessary for a faithful action of the cellular RNA polymerase is not yet known. The binding site for the RNA polymerase need not be directly at or adjacent to the transcription initiation site but may possibly be separated from it by DNA sequences having a regulatory function.

In this report we describe the isolation, physical mapping, and biological properties of evolutionary variants of SV40 that have retained the entire early coding region plus some immediately adjacent sequences in their genomes. According to the physical analysis, the boundaries of this region are at 0.685 and 0.120 map unit, so the mutants contain a stretch of about 130 base pairs preceding the presumed initiation site for the early transcription at 0.66 map unit. As is shown in this paper, the mutants are, however, defective in the expression of early functions in the permissive cell system. Our results suggest that the defect is overcome when the viral genome has become integrated into cellular DNA where cellular sequences can be used to initiate a transcription of the integrated viral genome. Based on the results, several deductions about the SV40 sequences necessary for early transcription and about the transcription of integrated viral DNA can be made.

MATERIALS AND METHODS

Cells and virus. Cultures of BSC-1 cells, primary baby rat kidney (BRK) cells and transformed BRK cells were initiated in Eagle basal medium with Hanks salts and supplemented with 10% calf serum and antibiotics (100 U of penicillin per ml and 100 μg of streptomycin per ml). Cultures were refed with Eagle basal medium with Earle salts and 5% calf serum. Strain VA45-54 was used as the wild-type (WT) SV40 virus and has been used for the previously described (33) serial undiluted passaged virus stocks. The *ts* mutants *A*28 and *B*4 were kindly provided by P. Tegtmeyer.

Purification of defective SV40 DNA. Defective SV40 DNA, isolated from an undiluted serial passage virus stock, was separated into several size classes by

agarose gel electrophoresis (33), and the different size classes were isolated from the agarose gel. Each size class of defective DNA was then propagated in the presence of WT SV40 DNA as helper. After pretreatment with DEAE-dextran (23), BSC-1 cells growing in 3-cm petri dishes were coinfected with 0.2 μg of WT SV40 DNA and 0.1 μg of defective DNA. When about 75% of the cells showed cytopathic effect, 0.4 ml of the frozen and thawed lysate (2.5 ml) was used to infect BSC-1 cells in Roux bottles with a surface area of 150 cm^2 to obtain larger amounts of defective DNA. The isolated viral DNA was subsequently treated with the *Eco*RI restriction endonuclease. Most of the defective DNA in each size class proved to be resistant to cleavage by *Eco*RI and could be separated from the cleaved WT SV40 DNA by equilibrium centrifugation in a cesium chloride-ethidium bromide gradient (26).

Cloning of variants. From the *Eco*RI-resistant DNA of two size classes of defective DNA (91 and 78% of unit length) two evolutionary variants, ev2200 and ev2201, were cloned by the infectious-center method previously described (13). By using the DEAE-dextran method, we coinfected 10^6 semiconfluent BSC-1 cells in 5-cm petri dishes with 12.5 ng of SV40 DNA (infectivity, 2×10^6 PFU/μg) and 12.5 ng of *Eco*RI-resistant defective DNA. At 2 h postinfection (p.i.) the cells were trypsinized, replated in serial 10-fold dilutions with 10^6 uninfected BSC-1 cells, and overlaid with agar on the next day. Small plaques were picked on day 18 p.i. Plaques were suspended in 0.5 ml of phosphate-buffered saline (PBS), and after freeze-thawing, 0.2 ml of the suspension was added to BSC-1 cells in 3-cm petri dishes. The dishes were kept at an angle of about 30° for 1 h to enhance locally the multiplicity of infection. When about 75% of the cells showed cytopathic effect, the cultures were frozen and thawed, and from 1 ml of the lysate (2.5 ml), viral DNA was isolated (16) and analyzed by agarose gel electrophoresis. In six of eight examined virus lysates, viral mutant DNA was present with a size identical to the defective DNA used to infect the cells. To obtain larger amounts of virus for the isolation of DNA of the evolutionary variants, 0.4-ml amounts of the appropriate lysates were used for the infection of BSC-1 cells in 150-cm^2 Roux bottles. The WT SV40 DNA was separated from the mutant DNA by *Eco*RI digestion and equilibrium centrifugation in CsCl gradients (26).

T-antigen induction and immunofluorescence. Semiconfluent BSC-1 cells growing in a Leighton tube containing a cover slip were pretreated for 30 min with a DEAE-dextran solution (23). The optimal DEAE-dextran concentration of 500 to 1,000 μg/ml could not be used because of the cells detached from the cover slips; with a concentration of 100 μg/ml, the cells remained attached. After pretreatment, 50 μl of a solution of DNA in PBS was applied to the cover slip that had been removed from the tube, and the cover slip was incubated for 15 min at room temperature. The cover slip was then extensively washed with PBS and placed again in the Leighton tube with fresh growth medium. To study T-antigen induction in the nonpermissive system, 60 to 80% confluent BRK cells were infected with DNA by the calcium phosphate technique (2). The infection was carried out in Leighton tubes, and at 48 h p.i., the cover slips were washed with PBS. The cells were fixed with a mixture of cold (−20°C) methanol and acetone (1:1, vol/vol) and tested for the presence of SV40-specific T antigen by the indirect immunofluorescence technique (25) by using sera from hamsters with SV40 virus-induced tumors and fluorescein isothiocyanate-conjugated swine anti-hamster serum. The test for T antigen in transformed cells growing on cover slips was done in the same way.

Transformation. Primary cultures of kidneys from 5- to 6-day-old Wistar rats growing in 5-cm plastic petri dishes were infected with DNA by the calcium phosphate technique essentially as described previously (2), and the transformation was assayed by focus formation.

Replication assay. Semiconfluent BSC-1 cells growing in 25-cm^2 plastic bottles (Falcon Plastics) were infected with DNA by using DEAE-dextran at 500 μg/ml in PBS. Cells grown at 40.5°C were labeled from 24 to 48 h p.i. with 2 ml of serum-free medium containing 10 μCi of [*methyl*-^{3}H]thymidine (24 Ci/mmol) per ml. Cells grown at 33°C were labeled from 48 to 72 h p.i. Viral DNA was extracted by the Hirt procedure. (16). The lysis buffer (0.35 ml per bottle) contained a known quantity of ^{14}C-labeled SV40 DNA that was previously digested with endonuclease *Hin*dII+III. This allowed a comparison for the recovery of viral DNA. The Hirt supernatant was extracted twice with phenol and extracted once with chloroform-isoamyl alcohol (24:1, vol/vol), and the DNA was concentrated by ethanol precipitation. The precipitate was dissolved in 50 μl of 10 mM Tris (pH 7.6)–5% (wt/vol) sucrose. Samples (5 μl) were removed for a determination of the recovery of ^{14}C-labeled DNA. Then, samples containing nearly equal quantities of ^{14}C-labeled DNA were subjected to horizontal agarose (1.5%) slab gel (20 by 20 by 0.5 cm) electrophoresis. The DNA was transferred to a nitrocellulose sheet by the blotting procedure described by Southern (34). Before the transfer, the gel was soaked in twice the volume of the gel of 1 M formic acid (adjusted to pH 3.0 with NaOH) for 30 min. Hereafter the gel was soaked two times for 45 min each time in twice the volume of the gel of 0.2 M NaOH–0.6 M NaCl and one time for 45 min in 0.6 M NaCl–1 M Tris (pH 7.4) (4). The acid treatment followed by the alkaline treatment causes nicks in the circular covalently closed DNA, which is otherwise poorly transferred onto the nitrocellulose sheet. After blotting, the nitrocellulose sheet was washed in 0.3 M NaCl–0.03 M sodium citrate (pH 7.0), air dried, immersed for a few seconds in a 10% (wt/vol) solution of 2,5-diphenyloxazole (3) in toluene, and then air dried. The nitrocellulose sheet was placed at −70°C on Kodak RP Royal X-Omat film which had been preexposed to a background fog increment of 0.15 optical density unit at 540 nm (20).

Isolation of cellular DNA. Cells grown in glass bottles were washed with PBS and then lysed with a buffer containing a 0.1 M EDTA, 0.01 M Tris (pH 8.5), 1% (wt/vol) sodium dodecyl sulfate, and pronase (2 mg/ml) (1 ml of lysis buffer was used for approximately 2×10^6 cells). The lysate was incubated overnight at 37°C with slow shaking. The lysate was extracted twice with phenol and once with 4% (vol/vol) isoamyl alcohol in chloroform. Cold (−20°C) ethanol

(2.5 times the volume of the lysate) was slowly poured on the surface of the lysate. The DNA precipitating at the interphase was collected by winding it on a glass rod (22). The DNA was dissolved in 1 × SSC (150 mM NaCl, 15 mM sodium citrate) and then digested with pancreatic RNase (100 μg/ml) at 37°C for 1.5 h and extracted with 4% (vol/vol) isoamyl alcohol in chloroform. The DNA was then exhaustively dialyzed against 10 mM Tris–1 mM EDTA (pH 7.6).

Restriction endonucleases. The restriction enzymes *Hin*dII+III, *Eco*RI, *Pst*I, *Bam*HI, *Bgl*I, and *Tag*I were purified by the procedure described by Crawford and Robbins (6). *Hpa*I endonuclease was prepared by the method of DeFilippes (9). *Hin*dIII endonuclease was a gift from J. Maat (Laboratory of Biochemistry, University of Leiden, Leiden, The Netherlands).

Heteroduplex analysis. Open circular mutant DNA with a single nick in one of the two strands was prepared from the closed circular form as described by Greenfield et al. (14). Linear molecules were prepared by restriction endonuclease cleavage. Heteroduplexes were spread for electron microscopy essentially by the RNA spreading procedure described by Robberson et al. (30). Open circular PM2 DNA was used as a length marker (33). In the experiments, the length of the spread single-stranded DNA was found to be longer (up to 10%) than the corresponding double-stranded DNA. The results of the length measurements were corrected for this difference.

Preparation of ^{32}P-labeled probes and filter hybridization. Restriction enzyme-generated fragments of SV40 DNA were used for the labeling. SV40 DNA was simultaneously digested with *Eco*RI and *Kpn*I, and the two fragments were separated on a horizontal agarose gel containing ethidium bromide (1). The DNA bands were cut out and placed in dialysis bags, and the DNA was eluted by electrophoresis (15). Purified *Eco*RI-*Kpn*I fragments A and B (1 μg each) were labeled with ^{32}P by the nick translation procedure (21, 29); assuming a 100% recovery of the DNA, its specific activity ranged from 7×10^7 to 11×10^7 cpm/μg in the different preparations. Nitrocellulose filters were loaded with restriction enzyme digests of cellular DNA by the Southern blot technique (34). The prehybridization treatment of the filters, the hybridization, and the posthybridization were done as described by Jeffreys and Flavell (17).

RESULTS

In this study we describe the physical and biological characterization of two evolutionary variants of SV40; ev2200 and ev2201 were derived from size classes of defective SV40 DNA which, respectively, measure 91 and 78% of unit length of the WT genome (33). For the purification and cloning of the variant DNAs, see above.

Physical characterization of ev2200 and ev2201 DNAs. In Fig. 1, lanes a and b show the *Hin*dIII and *Hin*dII+III digests of the SV40, ev2200, and ev2201 DNAs. The estimated sizes of the fragments are presented in Table 1. Fragments of the mutant DNA with the same size as the SV40 fragments were given the same notation; further analysis proved this to have been correct. The *Hin*dII+III digests of the mutant DNA contain two ev2200 fragments or one

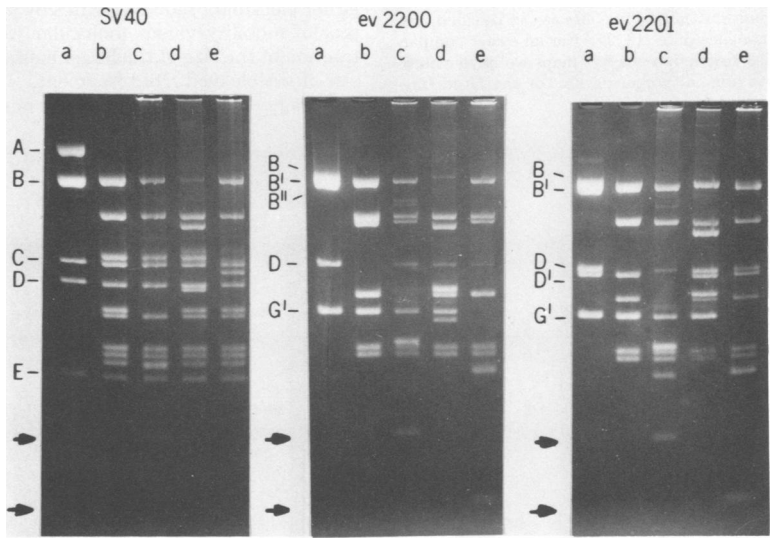


FIG. 1. *Restriction enzyme analysis of SV40 DNA and mutant ev2200 and ev2201 DNAs. Lanes: a, HindIII digests; b, HindII+III digests; c, HindII+III digests of DNA that were first digested with BamHI; d, HindII+III digests of DNA that were first digested with TaqI; and e, HindII+III digests of DNA that were first digested with BglI. The arrows indicate the positions of the 2.5% and 1.3% SV40 genome length fragments that are present in the BamHI-HindII+III and BglI-HindII+III digests, respectively.*

ev2201 fragment in bimolar amounts. This suggested the presence of sequence duplications in the mutant DNA, which was also indicated by the cleavage of the mutant DNA by restriction enzymes that cleave SV40 DNA only once. ev2200 DNA was cleaved twice by both the *Bam*HI and the *Taq*I enzymes by which fragments of 63 and 29% of unit length were formed (Fig. 2). The ev2200 DNA was cleaved four times by *Bgl*I. The smallest fragment (4.4%) was present in a bimolar amount, and the others measured 25 and 58%. ev2201 DNA was also cleaved twice by *Bam*HI (62 and 16%), once by *Taq*I (78%), and four times by *Bgl*I (58, 12, and two 4.4% fragments). The *Bam*HI-, *Taq*I-, and *Bgl*I-restricted DNA was further digested by *Hin*dII+III to determine in which *Hin*dII+III fragments the DNA had been cleaved (Fig. 1; Table 2).

The presence of duplicated sequences was also shown by electron microscopic examination of single-stranded variant DNA. Figure 3 shows the appearance of single-stranded ev2200 DNA. The molecule contains two hairpin structures with a (double-stranded) length of 6% of SV40 DNA. Electron microscopic examination of single-stranded ev2201 DNA also revealed two hairpins separated from each other by a 5% of unit length single-stranded DNA instead of an 18% of unit length single-stranded DNA as in

TABLE 1. *HindIII and HindII+III cleavage products (fragment:size) of SV40, ev2200, and ev2201 DNAs*[a]

*Hin*dIII cleavage product of			*Hin*dII+III cleavage product of:		
SV40	ev2200	ev2201	SV40	ev2200	ev2201
A:33	B′:23	B′:23	A:22.5	A:22.5	A:22.5
B:22.5	B″:22.7	B:22.5	B:15	B:15	B:15
C:21	B:22.5	D:10.5	C:10.5	B′:14.7	D′:9.8
D:10.5	D:10.5	D′:9.8	D:10	E′:8 (2)	E′:8
E:8.5	G′:7 (2)	G′:7 (2)	E:8.5	G′:7 (2)	G′:7 (2)
F:4			F:7(G)[b]	H:5.5	H:5.5
			G:6.5 (F)[b]	I:5	I:5
			H:5.5		
			I:5		
			J:4.5		
			K:4		

[a] The sizes of the fragments of the mutant DNA were calculated from their mobility using a semilogarithmic plot of the mobility of the SV40 fragments, used as standards, versus their molecular weight. Numbers within parentheses indicate the molarity of the fragment.

[b] The *Hin*d F fragment of SV40 DNA migrates in a 4% polyacrylamide gel slower than the *Hin*d G fragment (8). The mobility of the *Hin*d F fragment in 3% agarose, as shown in Fig. 1, is in accordance with its actual size as can be calculated from published sequence data (12, 27). For an easier comparison with published restriction enzyme maps we have continued the use of the original nomenclature for the *Hin*d fragments.

TABLE 2. *HindII+III fragments and fragment lengths of SV40, ev2200, and ev2201 cleaved by BglI, BamHI and TaqI*[a]

DNA	Fragment and length when cleaved by:		
	*Bgl*I	*Bam*HI	*Taq*I
SV40	C: 9 + 1.3	G: 4.5 + 2.5	A: 14 + 8
ev2200	G′: 4.4 + 1.3 + 1.3[b]	E′: 5.5 + 2.5[b]	A: 14 + 8 B′: 7 + 8
ev2201	G′: 4.4 + 1.3 + 1.3[b]	E′: 5.5 + 2.5 D′: 5.5 + 4.3	A: 14 + 8

[a] The sizes of the fragments were calculated from either electrophoretic mobilities by a semilogarithmic plot of mobility versus molecular weight or by subtraction of the size of the larger subfragment from the size of the cleaved *Hin*d fragment.

[b] Fragments present in bimolar amounts.

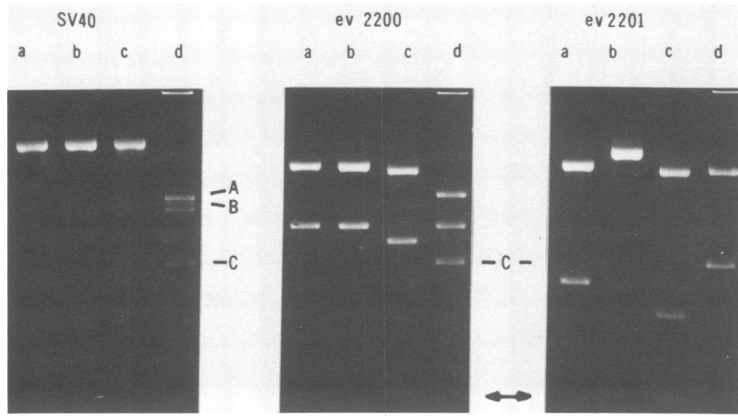


FIG. 2. *Restriction enzyme analysis of the DNAs of the ev2200, ev2201, and WT viruses. Lanes: a, BamHI digests; b, TaqI digests; c, BglI digests; and d, HpaI digests. The arrows indicate the position of a 4.4% SV40 genome length fragment that is present in the BglI digests of ev2200 and ev2201 DNAs. The HpaI fragment C is also present in the digests of the mutant DNA.*

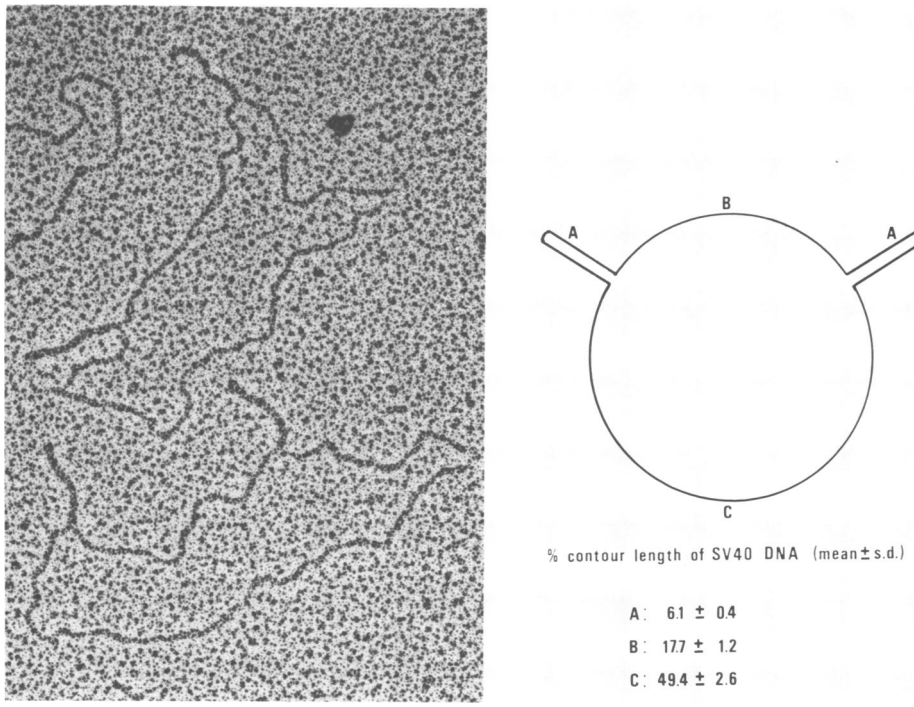
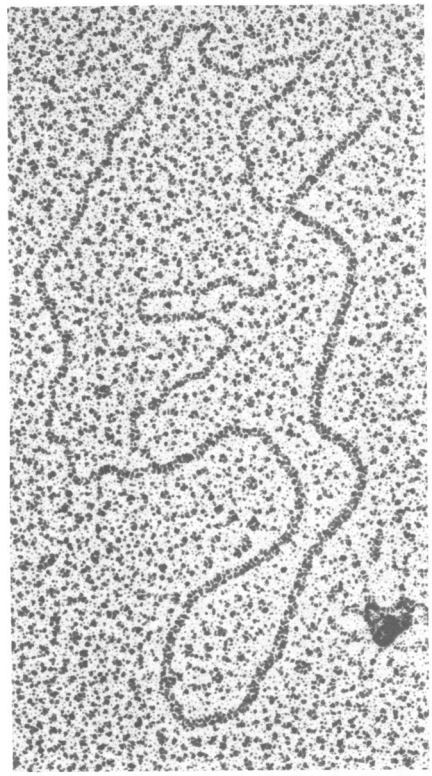
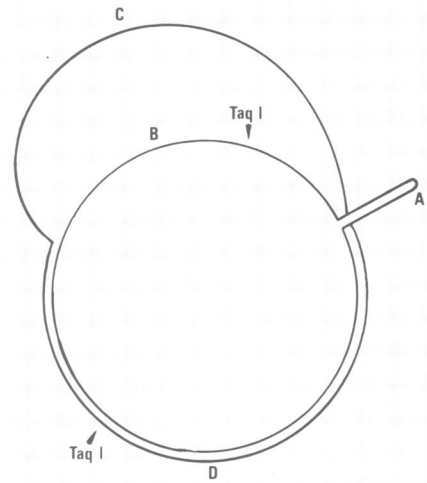


FIG. 3. *Electron micrographs of circular single-stranded molecules of ev2200 DNA. The double-stranded hairpins are the result of pairing between the complementing bases of inverted repeats.*

ev2200 DNA, which is in accordance with the DNA being 13% shorter. To obtain more information about the base sequences of the mutant DNA, heteroduplexes were formed between circular single-stranded ev2200 DNA and linear single-stranded *Taq*I-cleaved SV40 DNA. The two types of heteroduplexes that were found with nearly equal frequency are shown in Fig. 4. Both types show only one hairpin. The other has been lost by zippering out through the heteroduplex formation. From the heteroduplexes, it was concluded that the mutant DNA contains a continuous stretch of sequences identical with SV40 sequences, starting from approximately 0.12 map unit (*Taq*I site, 0.57 minus 0.45 map unit) in the *Hin*dII+III fragment G, through 0.15 map unit (the *Bam*HI site), the *Hin*dII+III fragments B, I, H, A, and 0.66 map unit (the *Bgl*I site) and terminating at approximately 0.69 map unit (*Taq*I site, 0.57 plus 0.12) in the *Hin*dII+III fragment C. Electron microscopic examination of the annealing product of circular single-stranded ev2201 DNA and single-stranded *Taq*I-cleaved SV40 DNA showed essentially one type of heteroduplex like that in Fig. 4A except that the single-strand region of the mutant DNA was 13% shorter, which agrees with the difference in the total length. With the heteroduplex analysis described above, only a part of the sequences present in the short region between the two inverted repeats of ev2200 has been determined.

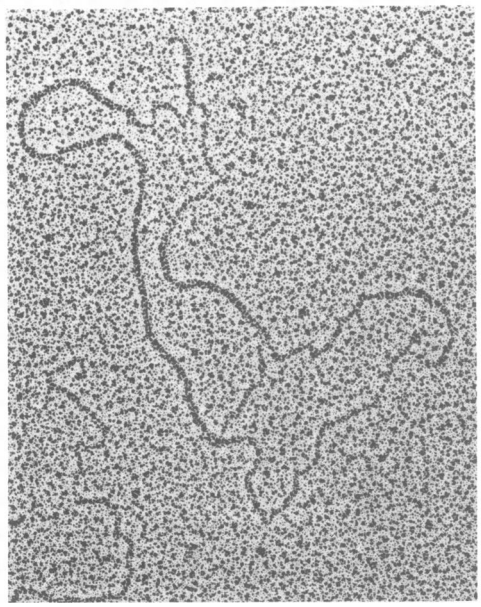
Besides sequences located on the SV40 genome at and around the *Taq*I site, sequences from the region at and around the SV40 *Bam*HI site also were expected to exist in that region. To detect these sequences, heteroduplexes were prepared between circular single-stranded ev2201 DNA and the short (29%) ev2200 *Bam*HI fragment. From the results presented above, it was concluded that this fragment must contain, in addition to one inverted repeat, nearly all of the sequences between the two inverted repeats. An electron micrograph of the single type of heteroduplex found is shown in Fig. 5. From the length measurements it could be concluded that the ev2200 fragment contains sequences from 0.15 to approximately 0.22 map unit, clockwise, which are then linked to sequences located at approximately 0.55 map unit proceeding clockwise through the *Taq*I site (0.57 map unit). The inverted repeat present in the fragment has formed a homoduplex with the inverted repeat of ev2201 DNA. The duplex formation terminates at the second *Bam*HI site. Variant ev2201 DNA is 13% shorter than ev2200 DNA and might be generated from ev2200 by a deletion in the short region between the two inverted re-

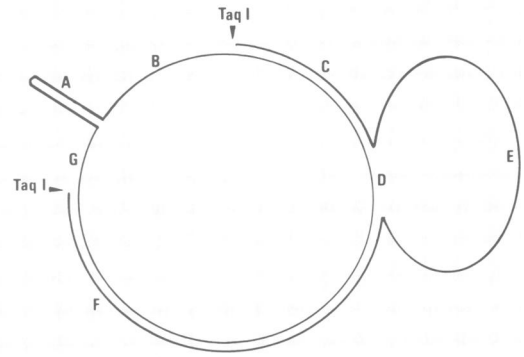




% contour length of SV40 DNA (mean ± s.d.)

A: 6.0 ± 0.3	C: 43.6 ± 1.4
B: 24.2 ± 1.2	D: 55.5 ± 1.6





% contour length of SV40 DNA (mean ± s.d.)

A: 5.9 ± 0.3	E: 44.2 ± 2.5
B: 10.9 ± 1.5	F: 44.6 ± 2.0
C: 11.2 ± 0.8	G: 6.0 ± 0.7
D: 7.1 ± 0.6	

FIG. 4. *Electron micrographs of the main types of heteroduplexes that were formed between circular ev2200 and linear TaqI-cleaved SV40 DNAs.*

peats. To determine the boundaries of the deletion, heteroduplexes were made between single-stranded linear molecules of ev2200 and ev2201. The linear molecules were generated by digestion with the *Pst*I enzyme that cleaves both DNAs once. The cleavage site for *Pst*I that is preserved in the mutant DNA is located on SV40 DNA at 0.28 map unit, in the *Hin*d fragment B.

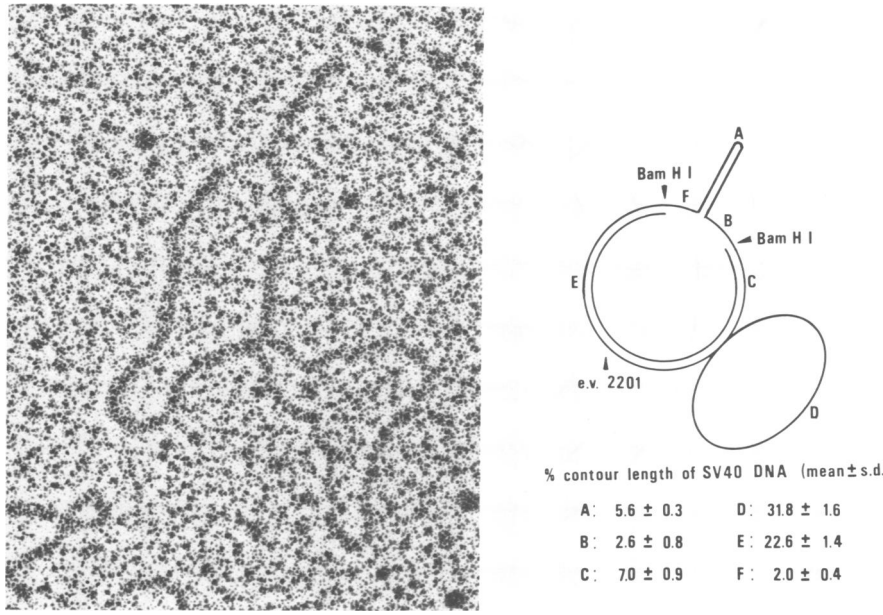


FIG. 5. *Electron micrograph of the heteroduplex formed between the 29% WT genome length BamHI fragment of ev2200 and circular ev2201 DNAs.*

The heteroduplexes found showed that ev2201 lacks sequences that start in ev2200 DNA just next to the inverted repeat which is closest to the *Hin*d fragment B (data not shown).

The combined results of the heteroduplex analysis and the restriction enzyme analysis permitted the construction of the physical maps as they are shown in Fig. 6. As an example of how the construction was done, one way to map the four *Bgl*I sites in ev2200 is given. The heteroduplexes indicated that the SV40 sequences at and around the *Bgl*I site were retained in the mutant DNA. Furthermore the *Hin*dII+III fragment G′ of ev2200 DNA is cleaved twice by *Bgl*I, producing a 4.4% and two 1.3% of unit length fragments. *Bgl*I cleaves the SV40 *Hin*dII+III fragment C in a 9% and a 1.3% fragment, so it was concluded that the *Hin*d fragment G′ of ev2200 is connected to the *Hin*d fragment A with one *Bgl*I site, like the single one in SV40, 1.3% away from the junction. Linkage of the *Hin*d fragment A and one of the *Hin*d fragments G′ was also shown by *Hin*dII+III digestion of the largest *Hpa*I fragment of ev2200. The digest yielded the *Hin*dII+III fragments A, E′, G′, and H (data not shown). Since the fragments G′ and A are produced by the action of *Hin*dIII only (Fig. 1) and the *Hpa*I recognition sequence is also recognized by *Hin*dII, these fragments are situated internally in the *Hpa*I fragment. The second *Bgl*I site is then situated 4.4% clockwise from the first one. From this, the boundary of the inverted repeat could be calculated to be at 0.685 map unit (half of the 4.4% *Bgl*I fragment, clockwise from the *Bgl*I site at 0.663 map unit). The third and fourth *Bgl*I sites in ev2200 DNA are then respectively situated 25% clockwise from the second and 58% counterclockwise from the first, both located in the second *Hin*d fragment G′. Although it is not proven by the above-described analysis, the results strongly suggest the presence of an intact early coding region plus some adjacent sequences in the mutant DNA.

T-antigen induction in BSC-1 cells. We tested whether the mutant DNA was able to induce T antigen in permissive cells. Table 3 shows that the mutant DNA has a strongly reduced T-antigen induction capacity when compared with WT SV40 DNA. The possibility that the observed T antigen was induced by WT DNA that contaminated the mutant DNA was considered unlikely because the contamination is less than 1% as determined by agarose gel electrophoresis. Also, the expression of WT DNA will probably be repressed due to interference by the mutant DNA (40; see below). To obtain more information about the T-antigen induction of the mutant DNA, a dose-response relation was examined. Figure 7 shows that with SV40 DNA, a plateau is reached with low doses of DNA, whereas with mutant DNA, the number of T-antigen-positive nuclei increases in a roughly linear fashion with increasing quantities of mutant DNA. The appearance of the fluorescence staining in the nuclei of the mutant DNA-infected cells was indistinguishable from that of

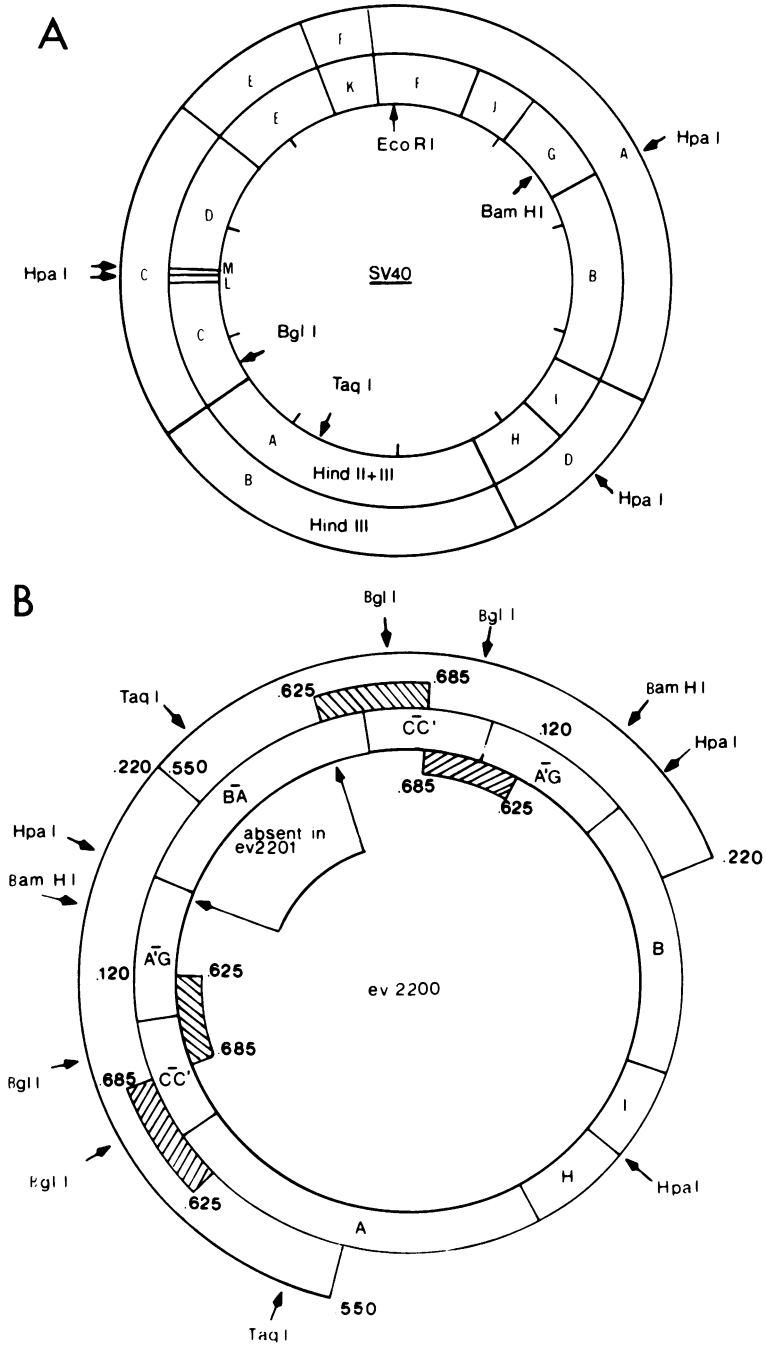


FIG. 6. *A. Physical map of SV40. The positions where the restriction enzymes that were used for the analysis of the evolutionary variants cleaved the DNA are indicated. B. Map of ev2200 and ev2201 DNAs. The HindII+III cleavage sites are indicated by the intersections in the complete double-lined ring. The three HindII sites are also HpaI cleavage sites. Some HindII+III fragments originate from a fusion (indicated by an overbar) of parts of WT SV40 HindII+III fragments. The fragments CC', A'G, and BA are, respectively, the fragments G', E', and B' of Tables 1 and 2. The part of the genome that is duplicated in ev2200 DNA is indicated by the outer arcs. The inverted repeats are indicated by the dashed arcs.*

the WT DNA-infected cells. Also the number of T-antigen-positive nuclei is not changed significantly at 72 h p.i. (data not shown). Substantial differences in the number of T-antigen-positive nuclei per cover slip were found in the different experiments (Table 3; Fig. 7). This may at least partially be caused by the necessary use of a suboptimal DEAE-dextran concentration for the infection (see above).

Complementation analysis. We examined whether the T antigen produced in BSC-1 cells after infection with the mutant DNA was able to complement the *A* gene product of the *tsA*28 mutant at the nonpermissive temperature. To obtain an indication of the quantity of DNA that had to be used in the complementation assay, a dose-response relationship for the infection with WT DNA was determined. The result is shown in Fig. 8, and it is almost identical with that published by Wilson (40). Approximately 3% of the cells could be productively infected. For the complementation assay, semiconfluent BSC-1 cells in 5-cm plastic petri dishes were infected with 100 ng of each DNA preparation. With reference to Fig. 8, it was assumed that, with 100 ng of DNA, the maximum number of cells would be infected. Table 4 shows that infection with ev2200 DNA alone resulted in only a few plaques, possibly caused by contamination with WT SV40 DNA. This low number of plaques is not indicative for the real contamination because

TABLE 3. *T-antigen induction in BSC-1 cells*[a]

DNA	Amt of DNA on cover slip (ng)	No. of T-positive nuclei on cover slip
SV40	10	133
SV40	50	188
ev2200	10	2
ev2200	50	14
ev2201	10	1
ev2201	50	7

[a] BSC-1 cells growing on cover slips were infected with the indicated quantities of SV40 or mutant DNAs. Screening for T antigen was done 48 h p.i.

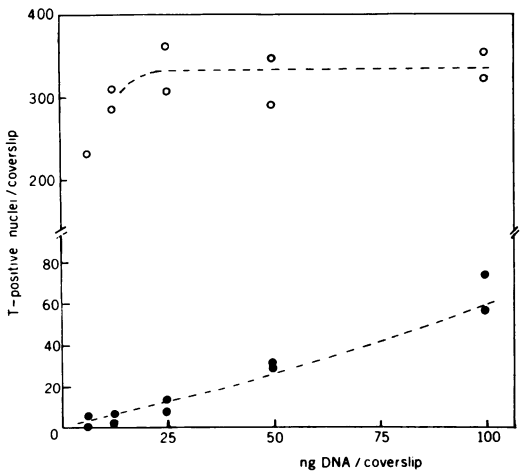


FIG. 7. *Number of T-antigen-positive nuclei per cover slip as a function of increasing amounts of WT (○) or ev2200 DNA (●). Infection with DNA and the detection of T antigen with the immunofluorescence assay are outlined in the text.*

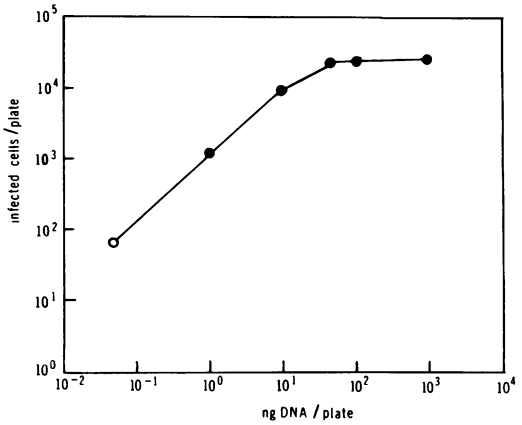


FIG. 8. *Number of productively infected cells as a function of increasing amounts of SV40 DNA. Semiconfluent BSC-1 cells (1.4 × 10^6 cells per plate) were pretreated with a DEAE-dextran solution in PBS (500 μg/ml) before the addition of the SV40 DNA diluted in PBS. With the lowest quantity of DNA, the infected cells were scored in duplicate by a direct plaque assay (○). For the other quantities of DNA, the numbers of infected cells (●) were scored by seeding the infected cells 1 day p.i. in duplicate serial 10-fold dilutions onto a lawn of 4 × 10^5 cells. An agar overlay was applied the next day. Plaques were counted 12 days p.i.*

TABLE 4. *Complementation analysis*

DNA (100 ng each per dish)[a]	No. of plaques[b] at:	
	40.5°C	33°C
*tsA*28	0.5 (0)	7 × 10^3
*tsB*4	7.5	6 × 10^3
SV40	2 × 10^4	7 × 10^3
ev2200	4.5 (2.5)	1
*tsA*28 plus *tsB*4	1 × 10^4	9.5 × 10^3
SV40 plus ev2200	4 × 10^3	10^3
*tsB*4 plus ev2200	3.5	0.8 × 10^3
*tsA*28 plus ev2200	8.5 (2.5)	10^3

[a] Plastic petri dishes (5 cm) were used.

[b] Average of the number of plaques on two duplicate dishes multiplied by the dilution factor (see text). Numbers within parentheses are number of plaques obtained in a second experiment.

of an interference by the mutant DNA with plaque formation by WT DNA, as can be seen in the experiment where coinfection with ev2200 and SV40 DNA was done. An 80% reduction in the number of plaques occurred. Infection with SV40 DNA alone resulted in 2×10^4 plaques at 40.5°C. The DNA had a specific activity of 1.3×10^6 PFU/μg, and, therefore, approximately six times the amount of DNA needed to obtain the maximal number of infected cells was used. Under these experimental conditions it can be expected that in the cells infected with the mutant DNA a few percent of the productively infected cells would show the nuclear T antigen with the immunofluorescence assay (Fig. 7).

If complementation between ev2200 and *tsA*28 is proportional to the number of T-antigen-positive nuclei in the mutant-infected cells, then with a value as low as 1% T-antigen-positive nuclei, as compared with WT infection, approximately 100 to 200 plaques can be expected in the absence of any interference by ev2200 DNA in the coinfection with *tsA*28 DNA. When the interference is as strong as with WT DNA, then 20 to 40 plaques can be expected. However, the actual number of plaques obtained by coinfection with *tsA*28 and ev2200 DNA is not significantly higher than the number of plaques obtained with ev2200 DNA alone.

Replication of the mutant DNA in BSC-1 cells. The low T-antigen induction capacity and the lack of complementation made it unlikely that the mutant DNA replicates as autonomously as WT SV40 DNA, and this was confirmed by the results of the experiment described below. For a confirmation of the results of the complementation analysis, the replication of the ev2200 DNA after coinfection with *tsA*28 DNA at both permissive and nonpermissive temperatures was included in the investigation.

Figure 9 shows the result of the experiment, and it is evident that the replication of ev2200 DNA is very inefficient as compared with SV40 DNA replication. Figure 9 furthermore confirms that the complementation of *tsA*28 by ev2200 is very weak or absent, altogether, and it also shows the interference of ev2200 with the replication of WT and temperature-sensitive DNAs. The relative amount of replicated ev2200 DNA after coinfection with an equal quantity of WT or temperature-sensitive DNA is greater than the amount of replicated WT or temperature-sensitive DNA, and the total quantity of the replicated viral DNA is decreased relative to the infections without ev2200 DNA.

In the cells infected with ev2200 DNA or

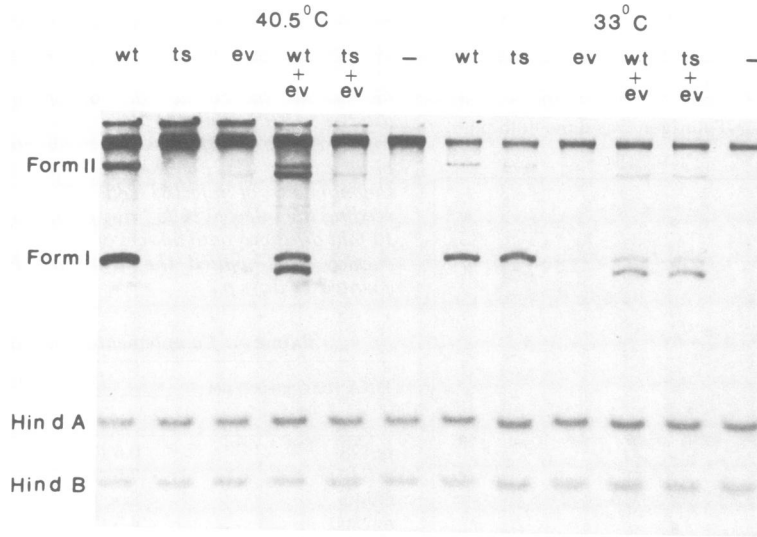


FIG. 9. *Assay of ev2200 for replication and complementation of A gene defect. BSC-1 cells were infected with SV40 DNA (wt), tsA28 DNA (ts), ev2200 DNA (ev), or combinations of the DNAs, or were mock infected (–). The cells were incubated at either the nonpermissive temperature (40.5°C) or the permissive temperature (33°C). The isolated [^{3}H]thymidine-labeled viral DNA was subjected to agarose gel electrophoresis and, after introducing nicks in form I DNA (see text), was blotted on a nitrocellulose filter (34). The phenyloxazole-impregnated filter was exposed to a prefogged X-ray film (see text). Exposure was for 1 week at –70°C. The Hind fragments A and B are a result of the addition of HindII+III-digested ^{14}C-labeled SV40 DNA to the lysis buffer to allow a comparison for the recovery of low-molecular-weight DNA. The faint bands that are not properly in the lanes are artefacts caused by the blotting procedure.*

ev2200 DNA plus *tsA*28 DNA at the nonpermissive temperature, a small quantity of replicated ev2200 DNA is detected. This replication can, at least partially, be a result of a small contamination of WT DNA. Because of the interference by the mutant DNA, the replication of the WT DNA might be not sufficient to be detected.

Transformation of, and T-antigen induction in, BRK cells. We investigated the ability of the mutant DNA to transform BRK cells with the calcium phosphate technique (2). Table 5 shows that the ev2200 and ev2201 DNAs are able to transform BRK cells with approximately the same efficiency as WT DNA. A number of foci, each from a different dish, were established as cell lines. After about 10 passages, the cells were tested for the presence of T antigen with the indirect immunofluorescence assay. In essentially all of the cells of each cell line a bright fluorescence was seen in the nuclei. In an initial experiment we noted that, after infection of BRK cells with either mutant or SV40 DNA, despite the equal transformation efficiency, the number of T-antigen-positive nuclei at 48 h p.i. was considerably lower in the mutant-infected cells than in the WT-infected cells. We investigated this further by infecting BRK cells over a small range of different doses of DNA for both WT SV40 and ev2200 DNAs, followed by screening for both T antigen and transformation.

TABLE 5. *Transformation of BRK cells by SV40 and mutant DNAs*[a]

DNA	Viral DNA (μg/dish)	No. of foci per dish of each of 4 dishes
SV40	1	3, 3, 3, 2
SV40	0.75	9, 5, 5, 4
SV40	0.5	4, 3, 2, 1
ev2200	1	8, 6, 5, 4
ev2200	0.75	9, 4, 2, 2
ev2200	0.5	4, 3, 2, 1
ev2201	1	4, 3, 2, 2

[a] The experiment included dishes with BRK cells that were infected with calf thymus DNA-calcium phosphate precipitate. Foci of densely staining cells did not appear.

Table 6 shows that the capacity of the DNA to induce T antigen and to transform cells follows the same nonlinear dose response. This nonlinearity has been shown earlier by Abrahams and van der Eb (2) for the transformation by SV40 DNA. For the quantities of DNA that we have tested, the number of transformed foci obtained with the mutant DNA is of the same magnitude as that obtained with WT DNA. However, for the four different quantities of DNA used, the number of cells showing immunoreactive T antigen is significantly lower after ev2200 DNA infection (10% of the number obtained after WT DNA infection in this experiment).

Characterization of the viral DNA in the transformed cells. When at least one integrated mutant DNA molecule has retained the early coding region plus some adjacent sequences uninterrupted by the integration event, then it is possible to indicate the presence of the mutant DNA in the transformed cell DNA because of the repeated sequences in the mutant DNA (Fig. 6). For instance, if the incision or integration site on ev2200 DNA is situated in the DNA sequence between (proceeding clockwise) the extra *Bam*HI site and the extra *Taq*I site, then digestion of the transformed cell DNA

TABLE 6. *DNA T-antigen induction in, and transformation of, BRK cells*

DNA	Induction		Transformation	
	Viral DNA (ng/ Leighton tube)[a]	No. of T-positive nuclei in cover slip[b]	Viral DNA (ng/ dish)	No. of foci in each of 2 to 4 dishes[c]
SV40	240	73, 100	1,000	4, 4, 3, 0
SV40	180	88, 91	750	5, 5, 3, 2
SV40	120	69, 79	500	5, 4, 4, 3
SV40	60	50, 66	250	2, 2, 2, 1
ev2200	240	8, 9	1,000	6, 5, 4,
ev2200	180	7, 13	750	5, 4, 4
ev2200	120	4, 5	500	2, 1, 0
ev2200	60	4, 6	250	3, 1, 1, 1
Calf thymus DNA-calcium phosphate precipitate	0	0, 0	0	0, 0

[a] The Leighton tubes have a surface area for cell growth that is about one fourth that of the dishes, and to obtain an equivalent ratio of DNA to cell, a corresponding smaller volume of the DNA calcium solution was used.

[b] T-antigen-positive nuclei were scored at 48 h p.i.

[c] Foci were counted 3 weeks p.i.

with *Bam*HI, *Taq*I, or *Bgl*I must give rise to mutant-specific fragments of, 62, 62, and 58%, respectively, of the WT genome length. These mutant-specific fragments can then be detected by the Southern technique (34). By similar analysis of the DNA of ev2201-transformed cells digested with *Bam*HI or *Bgl*I, fragments of 62 and 58% of the WT genome length, respectively, can be expected. Although it seemed unlikely in view of the efficiency of transformation by the mutant DNA, it remained possible that the transformation might have been caused by contaminating WT DNA in the mutant DNA preparation. The presence of WT DNA in the transformed cells became unlikely, since no infectious virus could be rescued from the transformed cell by employing the method of Sendai virus-mediated cell fusion. The absence of WT DNA and the presence of mutant DNA in the mutant-transformed cells was further studied by the following experiment. Samples of cellular DNA of transformed cells were separately digested with various restriction enzymes, and the digests were split into two equal portions which were simultaneously electrophoresed. After denaturation of the DNA, the gel was cut into two halves, each half containing the same digests. The DNA was then blotted onto nitrocellulose sheets. The DNA on one of the nitrocellulose sheets was subjected to hybridization with the ^{32}P-labeled *Eco*RI-*Kpn*I fragment A of SV40 DNA that contains the sequences, clockwise, from 0.00 to 0.716 map unit on the physical map of SV40. The DNA on the second nitrocellulose sheet was subjected to hybridization with the *Eco*RI-*Kpn*I fragment B of SV40 DNA that contains sequences, clockwise, from 0.716 to 1.00 map unit on the physical map of SV40. If the transformed cell contained only mutant DNA as viral DNA, then hybridization with the *Eco*RI-*Kpn*I fragment B was not expected. When WT DNA was present in the transformed cells, then hybridization with the *Eco*RI-*Kpn*I fragment B was likely to occur, since analysis of a number of SV40-transformed cells have revealed that, besides the early region, the late region is also retained in the transformed cells (4).

Figure 10 shows that, in the digest of cellular DNA of a BRK cell transformed by SV40 DNA, fragments that contain viral DNA hybridized with both *Eco*RI-*Kpn*I fragments or exclusively with one of the two. The viral DNA containing fragments in the digests of the cellular DNA of mutant DNA-transformed cells hybridized predominantly with the *Eco*RI-*Kpn* fragment A (Fig. 11). The fragments characteristic for the

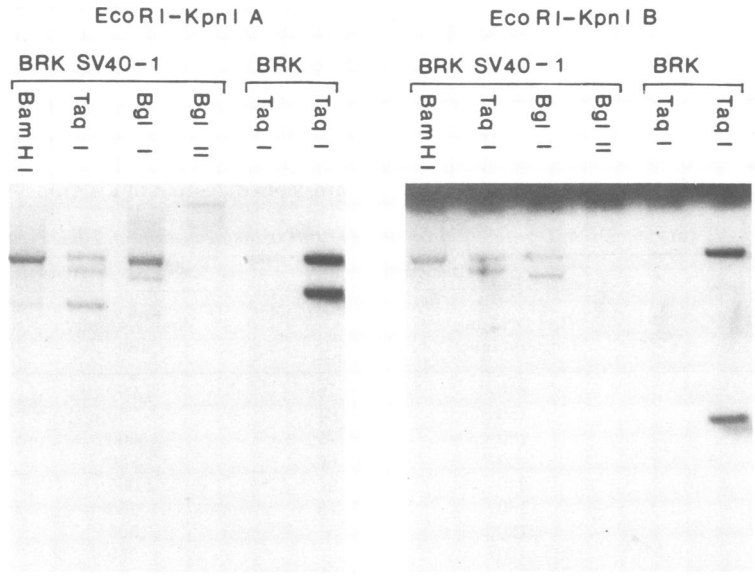


FIG. 10. *Detection of viral DNA-containing fragments in restriction enzyme digests of cellular DNA (7.5 μg per slot) isolated from SV40 DNA-transformed BRK cells. The control BRK lanes contain TaqI digests of control rat DNA. To one digest, 50 pg of EcoRI-cleaved SV40 DNA and 50 pg of EcoRI- and KpnI-cleaved SV40 DNA were added before electrophoresis. The viral DNA-containing fragments were detected by electrophoresis of the digests through 1.5% agarose, transfer of the DNA onto nitrocellulose, and hybridization with the ^{32}P-labeled EcoRI-KpnI fragment A or B of SV 40 DNA (see text). The exposure time was 14 days.*

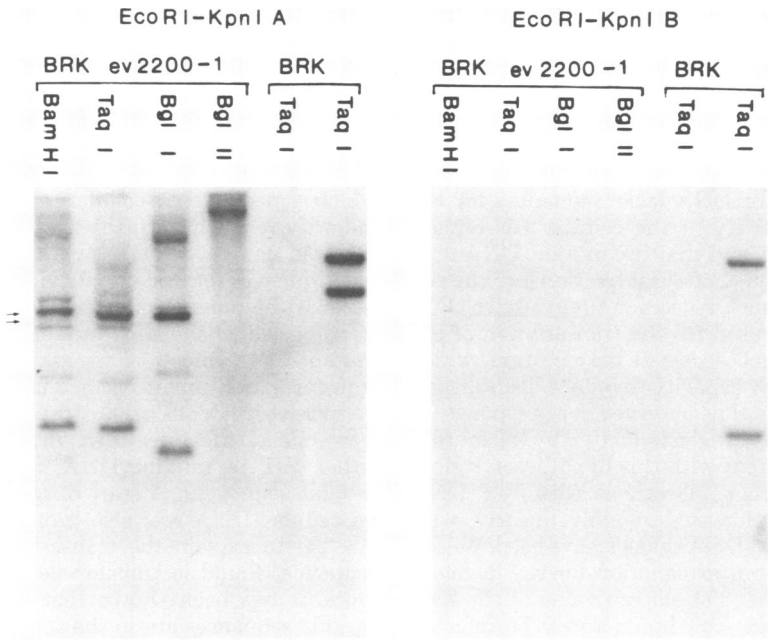


FIG. 11. *Analysis of the cellular DNA of the BRK ev2200-1 cell line. Experimental conditions are as described in the legend of Fig. 10. The arrows indicate fragments that are specific for the mutant DNA.*

mutant DNA are present in the *Bam*HI, *Taq*I, and *Bgl*I digests. The *Taq*I 62% fragment migrates slightly faster than the *Bam*HI 62% fragment because the gel area around the *Taq*I fragment contains more restricted cellular DNA than the corresponding *Bam*HI fragment area, and this slightly influences the mobility. (C. Sol, unpublished data). Analysis of the DNAs of three other mutant DNA-transformed BRK cell lines that were examined yielded similar results. A very weak hybridization with the *Eco*RI-*Kpn*I fragment B probe became evident upon longer exposure of the X-ray films. This was probably caused by a contamination of the *Eco*RI-*Kpn*I fragment A in the *Eco*RI-*Kpn*I fragment B preparation, which was demonstrated by the control experiments. In most experiments a weak hybridization was detected with a fragment of approximately the size of linear SV40 DNA in the *Taq*I digest of cellular rat liver DNA. This was not due to contamination with SV40 DNA because we could not detect hybridization with *Bam*HI-digested DNA. We have no explanation for this finding.

DISCUSSION

In this paper we have described the origin and physical and biological characterization of two evolutionary variants of SV40. The construction of the physical maps (Fig. 6) of the mutants by restriction enzyme and heteroduplex analysis revealed the presence in the mutant genomes of the region from 0.120 to 0.685 map unit, clockwise, on the physical map of SV40. This stretch of DNA contains the entire region that codes for the early proteins, as shown by direct nucleotide sequencing of the SV40 genome (12, 27). Furthermore, ev2200 DNA contains a direct repeat with a length of approximately 29% of the length of SV40 DNA. Within each direct repeat there is a short inverted repeat of sequences which are situated between 0.625 and 0.685 map unit on the physical map of SV40 DNA.

Also, ev2201 DNA contains four repetitions of the sequences from 0.625 to 0.685 map unit. Since the origin of SV40 DNA replication has been determined to be situated close to 0.67 map unit (7, 11), the mutant DNA has four initiation sites for DNA replication; this might be the basis for the observed interference with the replication of SV40 DNA (Fig. 9).

The results of the experiments concerning the biological properties of the mutant DNA are as follows. (i) The capacity of the mutant DNA to induce T antigen in permissive cells is reduced relative to the capacity of WT DNA. The percent reduction is dependent on the quantity of DNA administered to the cells which is caused by a difference in the dose-response relationship for mutant and WT DNA infection. (ii) Complementation between ev2200 DNA and *tsA*28 was not detected. (iii) Replication of the mutant

DNA is helper dependent. (iv) The T-antigen induction by the mutant DNA in nonpermissive BRK cells is significantly lower than the induction by WT DNA. (v) The capacity of the mutant DNA to transform BRK cells is indistinguishable from that of WT DNA. All of these results can be explained with the hypothesis that the mutant DNA lacks sequences for the binding or activity of the cellular RNA polymerase or both and that the mutant DNA only becomes expressed after integration into the cellular DNA at such a location that cellular DNA sequences are used for the transcription of the viral DNA. Such an event may be rare, which explains the low capacity to induce T antigen in permissive cells. The observed dose-response relation for the T-antigen induction by the mutant DNA is consistent with this hypothesis. It does not level off to a plateau as with WT DNA infection but increases, roughly linearly, with the quantities of DNA that we have used. Wilson (40) has given an explanation for the development of the low (3%) plateau value of the fraction of cells that can productively be infected with DNA with DEAE-dextran as facilitator. His viewpoint is that an equilibrium is established between intact DNA molecules reaching the nucleus (potentially PFU) and noninfectious molecules damaged enroute to the nucleus. Both types of molecules compete for a limited amount of cellular component(s) required for productive infection. The expression of the mutant DNA by the mechanism that we propose may be, at least with the quantities of DNA that we used, only dependent on the concentration of viral DNA reaching the nucleus. If this hypothesis for the mechanism of expression of the mutant DNA is true, then the absolute quantity of viral RNA and T antigen in the cells that are positive for T antigen with the indirect immunofluorescence assay probably would be lower in the mutant DNA-infected than in the WT DNA-infected cells. The number of T-antigen molecules in the mutant DNA-infected cells might be similar to that in cells transformed by SV40 and might be too low for an efficient replication of nonintegrated molecules of mutant DNA or *tsA*28 DNA. Alternatively, the T antigen might not be able to reach the nonintegrated viral DNA due, for instance, to a kind of compartementalization.

The number of T-antigen(s)-positive nuclei in the nonpermissive BRK cells is much lower after infection with mutant DNA than with WT DNA, whereas the number of cells that become stably transformed is in the same order of magnitude (Tables 5 and 6). To explain this, we hypothesize that the T antigen(s) in the mutant DNA-infected BRK cells is a result of a transcription of the viral DNA that has become integrated into transcribed regions of the cellular DNA and that the transcription of the viral DNA is mediated via a start on sequences located on the cellular DNA. Part of the T antigen(s) detected by immunofluorescence in the WT DNA-infected BRK cells is also the result of transcription of the integrated viral DNA, which is mediated via cellular sequences, but the majority result from transcription of either integrated or free viral DNA, which is mediated via sequences located on the viral DNA. Then, because the number of cells that became stably transformed is indistinguishable after WT or mutant DNA infection, we assume that stable transformation might occur only in those nonpermissive rat cells where transcription of at least one of the integrated viral DNA copies, either WT or mutant DNA, is mediated via cellular sequences. A start of the transcription on cellular DNA was also proposed by others (18, 24) to explain the transcripts of anti-late sequences found in transformed cells. Furthermore, it has been shown that cell and virus-specific sequences are in the same molecules of nuclear RNA from virus-transformed cells (39). That no stable transformation occurs when the transcription of the viral DNA is mediated via a promoter on the viral DNA may be a result of one or more of the following reasons. (i) The cellular RNA polymerase has, compared with its affinity for the cellular promoter, a low affinity for the viral promoter. Then the number of transcripts might eventually be high enough to give rise to a quantity of T antigen(s) that can be detected by immunofluorescence but that is too low for a stable transformation. (ii) The *A* gene product is an autoregulator (19, 28, 36). It may bind (37) or promote the binding of other proteins at or close to the viral promoter region, thereby preventing the attachment of the RNA polymerase. Again, the quantity of T antigen(s) could be high enough to be detected by immunofluorescence but too low for a stable transformation. (iii) Part or all of the surplus of the T-antigen-positive nuclei in the BRK cells infected with WT DNA, compared with mutant DNA infection, might result from transcription of free viral DNA. It might be argued that the observed biological properties of the mutant DNA are caused not by the absence of sequences necessary for transcription but rather by the structure of the DNA. When the inverted repeat sequences (0.625 to 0.685 map unit on the WT genome map) contain the necessary information to start transcription, then transcription can start at four positions on the mutant genome. Two transcriptions proceed clockwise and two counterclockwise, and then steric hindrance between RNA polymerase could prevent an effi-

cient transcription. We think that this explanation is very unlikely, because unless a very specific incision site on the viral genome is used upon integration (which is not observed with the blotting experiments), such steric hindrance would also occur in the transcription of the integrated mutant DNA and probably would be reflected in the transformation efficiency; this was not observed. Also the immunoprecipitable large and small T antigen in the mutant DNA-transformed cell is of the same quantity and size as in the cells transformed by WT SV40 DNA (P. Schrier and C. Sol, unpublished data). If the mutant DNA indeed lacks sequences necessary for the early transcription, where then are they located on the WT genome?

The in vitro-constructed deletion mutant dl BCDE 1210, which complements *tsA* mutants well, lacks the DNA sequences located between 0.72 and 0.80 map unit (5). The viable deletion mutant dl 1635 has a deletion from 0.712 to 0.727 map unit. The mutant not only lacks the 5′ end of the late 16S mRNA but also lacks a stretch of 52 nucleotides preceeding it (35). The deletion mutant dl 1408, with a deletion spanning the region from 0.675 to 0.69 map unit, grows as well as the WT virus (31). Then, together with what is known about the physical map of the mutant DNA described in this paper, a location for the indispensable sequences is narrowed down to approximately the area of 0.69 to 0.712 map unit on the SV40 map. A perfect tandem repeat of 72 base pairs is located from 0.68 to 0.71 map unit (38). One of the two copies is certainly absent in the mutant DNA described in this paper, and the other is probably truncated. A group of viable mutants with deletions in the region from 0.675 to 0.745 map unit has been described previously (32). By nucleotide sequence analysis, it has been shown (38) that some of these mutants have lost one copy, or part of one copy, of the tandem repeat, but to our knowledge no viable mutant has been described that lacks more than one copy. Although the evidence is only circumstantial, the perfectly repeated 72-base-pair-long DNA segments might contain sequences that are necessary for the RNA polymerase to initiate the early transcription in permissive cells. We have not excluded the possibility that the mutant DNA contains a small deletion or point mutation(s) in the DNA region extending clockwise from the early coding region towards 0.685 map unit. Such a change in the DNA might destroy a sequence necessary for the transcription. Direct DNA sequencing is needed to provide the answer.

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