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Construction and Analysis of Viable Deletion Mutants of Simian Virus 40

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Viable mutants of simian virus 40 (SV40), with deletions ranging in size from 15 to 200 base pairs, have been obtained by infecting CV-1P cells with circularly permuted linear SV40 DNA. The linear DNA was produced by cleavage of closed circular DNA with DNase I in the presence of Mn^{2+}, followed, in some cases, by mild digestion with λ 5'-exonuclease. The SV40 map location and the size of each deletion were determined by using the S1 nuclease mapping procedure (Shenk et al., 1975) and the change in the size of fragments produced by *Hin*d II+III endonuclease cleavage. Deletions in at least three regions of the SV40 chromosome have slight or no effect on the rate or yield of viral multiplication and on viral-induced cellular transformation. These regions are located at the following coordinates on the SV40 physical map: 0.17 to 0.18; 0.54 to 0.59; and 0.68 to 0.74.

Two biochemical techniques have been developed recently that are useful in the genetic analysis of simian virus 40 (SV40): one permits the introduction of small deletions into the viral DNA (2), and the second makes it possible to accurately map such small deletions on the SV40 DNA molecule (18).

This paper describes a class of mutants in which relatively small deletions (15 to 200 base pairs) have been introduced in regions not essential for vegetative growth of the virus. Such mutants were obtained by infecting permissive monkey cells with SV40 DNA that had been cleaved to linear molecules with a relatively nonspecific endonuclease, DNase I in the presence of Mn^{2+}. The map position and size of the deletions in cloned mutants of this type were determined by using the S1 nuclease mapping procedure (18) and the altered fragment pattern produced by *Hin*d II+III endonuclease digestion of the mutant DNA (4). A subsequent paper will describe the isolation of defective, helper virus-dependent mutants of SV40 produced by essentially the same procedure.

MATERIALS AND METHODS

Cells and viruses. The origin and the procedures for growth of the CV-1P and MA-134 monkey kidney cell lines were described previously (12). All virus stocks and DNA were prepared in MA-134 cells. Plaque assays with either SV40 virus or DNA were performed on monolayers of CV-1P cells as described previously (12). The wild-type parent of the SV40 deletion mutants described in this report was a plaque-purified derivative of the SVS strain (20) designated *wt*830 (12).

DNA and enzymes. SV40 DNA was extracted by the method of Hirt (7) from MA-134 cells, which had been infected at a multiplicity of <0.05 PFU per cell, when >90% of the cells showed cytopathic effect. Covalently closed viral DNA [SV40(I) DNA] was purified directly from the Hirt supernatant by centrifugation to equilibrium in a solution containing CsCl (1.56 g/cm^3) and ethidium bromide (200 μg/ml). After the band of SV40(I) DNA was collected, the ethidium bromide was removed with Dowex-50 (16).

*Eco*RI endonuclease, *Hpa*II endonuclease, *Hin*d II+III endonucleases, bacteriophage λ 5'-exonuclease, and S1 nuclease were prepared and used according to published protocols (*Eco*RI [6, 15]; *Hpa*II [17]; *Hin*d II+III [3, 19]; λ exonuclease [8-10]; S1 nuclease [22]). The S1 nuclease mapping procedure (18) and the procedure for the digestion of SV40 linear DNA with λ 5'-exonuclease (2) were as described previously. Deoxyribonuclease I was purchased from Worthington Biochemical Corp.

Double-stranded cleavage of SV40(I) DNA using DNase I. DNase I makes double-stranded cleavages in DNA in the presence of Mn^{2+} (11). SV40(I) DNA (100 μg/ml in a solution of 10 mM Tris [pH 7.5], 1 mM $MnCl_2$, and 100 μg of bovine serum albumin per ml) was digested with DNase I (0.5 ng/ml) for 20 min at room temperature. The reaction was terminated by adding EDTA (to 12 mM), and the solution was extracted once with phenol followed by ethanol precipitation. After digestion with the enzyme, electron microscopy revealed that 31% of the molecules were linear [SV40(L_{DNase}) DNA], 19% were relaxed circles [SV40(II) DNA], and 50% remained as supercoiled circles [SV40(I) DNA]. In addition to full-length

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linear molecules, there was a substantial number of short linear molecules, which probably arose from multiple cleavages of an SV40(I) DNA by DNase I. Incubation of the DNA with DNase I for longer than 20 min reduced the yield of full-length SV40(L_{DNase}) DNA and increased the number of short linear molecules. The majority of the short linear molecules and SV40(I) DNA were separated from the SV40(L_{DNase}) molecules by velocity sedimentation in a 5 to 20% sucrose gradient. The SV40(L_{DNase}) DNA was then subjected to electrophoresis twice through 4% agarose plugs (1 cm long, 6 mm in diameter, 4 mA per plug, 16 h). Since linear DNA, but not SV40(I) and SV40(II) DNA, migrates through 4% agarose (1), most of the remaining SV40(I) and SV40(II) DNA was removed. Electron microscope examination of the final product indicated there was less than 1 circular molecule per 500 SV40(L_{DNase}) DNAs.

Gel electrophoresis. Agarose gels (1.2%, 6 mm in diameter, 200 mm long) were prepared in Tris-borate buffer (89 mM Tris-OH, 89 mM boric acid, 2.5 mM EDTA, pH 8.2) (6). Samples were applied in 50 μl of Tris-borate buffer containing sucrose (20% wt/vol), and electrophoresis was at 40 V for 17 h. DNA bands were stained with ethidium bromide and visualized by using short-wavelength UV light. The fluorescent bands were photographed using a Vivitar orange (02) filter and Polaroid type 105 film. Polyacrylamide gels (6 by 200 mm) contained 5% acrylamide and 0.25% *N,N'*-methylene bis-acrylamide in Tris-borate buffer. Electrophoresis was at 100 V for 16 h. The DNA bands were stained and photographed as described above.

RESULTS

Isolation of viable deletion mutants. When monkey kidney cells are infected with linear SV40 DNA, which has been digested with λ 5′-exonuclease to expose a short single-stranded segment at each 3′ end of the molecule, mutants containing deletions at the site of the double-stranded cleavage are obtained (2). When the SV40(I) DNA has been cleaved with an enzyme that produces short, single-stranded, complementary sequences at the termini, the exonuclease digestion removes the cohesive ends and prevents circularization and regeneration of wild-type DNA molecules (2, 14). However, it is not clear whether exonuclease treatment is required to rejoin the ends of linear DNA molecules that do not contain cohesive termini (as, for example, those produced by DNase I). Therefore, two preparations of linear SV40 DNA were used for the construction of deletion mutants. After cleavage of the SV40(I) DNA to linear molecules with DNase I in the presence of Mn^{2+} [SV40(L_{DNase}) DNA], one aliquot was treated with λ 5′-exonuclease to remove 25 to 30 nucleotides from the 5′ termini [SV40(L_{DNase}exo) DNA]. Both the untreated and the exonuclease-digested DNA preparations produced plaques after infection of CV-1P cell monolayers; the efficiency of plaque formation was only about 1% compared with that found with SV40(I) DNA [SV40(I), 9.5×10^6 PFU/μg; SV40(L_{DNase}), 1.4×10^5 PFU/μg; SV40(L_{DNase}exo), 7.2×10^4 PFU/μg]. Virus obtained from plaques produced by SV40(L_{DNase}) or SV40(L_{DNase}exo) DNAs was purified by two successive plaque isolations, and DNA was prepared from each. Among the 67 DNA preparations, each obtained from an independently arising plaque, 18 contained alterations and 49 were not distinguishable from wild-type DNA. The latter probably resulted from a small amount of SV40(I) DNA that still contaminated the SV40(L) DNAs used to infect the CV-1P cell monolayers.

Since the frequency of deletion mutants was about the same in the SV40(L_{DNase}) and SV40(L_{DNase}exo) DNAs, the exonuclease digestion appears not to be required when DNase I is used to generate the SV40(L) DNA. Possibly, single-stranded termini are not essential for the cell-mediated rejoining process. However, we cannot rule out the presence of contaminating exonuclease activity in our preparation of DNase I, or, more likely, the action of cellular exonucleases to modify the ends of the DNA prior to the cyclization step.

The SV40 map location and size of the deletions were determined by the S1 nuclease mapping procedure (18) and from the change in mobility of fragments produced by *Hin*d II+III endonuclease digestion of the mutant DNA. The putative deletion DNA was cleaved to unit-length linear molecules with *Eco*RI endonuclease (this cleaves SV40 DNA once at 0/1.0 map unit) and mixed with linear wild-type DNA produced by the same enzyme, and the mixture was denatured and reannealed. DNAs that contain a deletion form heteroduplex structures having a single-strand loop at the site of the deletion. These heteroduplex molecules, when digested with the single-strand-specific S1 nuclease, are cleaved at the site of the deletion loop to produce two fragments. DNAs that lack a deletion generate duplex molecules that are homologous over their entire length, and these are not appreciably cleaved by S1 nuclease digestion. For example, neither of the homoduplexes of wild-type and a mutant (*dl*892) DNA (each of which had been denatured and reannealed separately) is cleaved by S1 nuclease (Fig. 1a, gel 2), but the heteroduplex molecules formed from *dl*892 and wild-type DNA are cleaved by S1 nuclease to produce fragments 0.32 and 0.68 of SV40 fractional length (Fig. 1a, gel 3). This locates the mismatch between *dl*892 and its wild-type parent at either 0.32 or 0.68 on the SV40 map. These two alternatives could

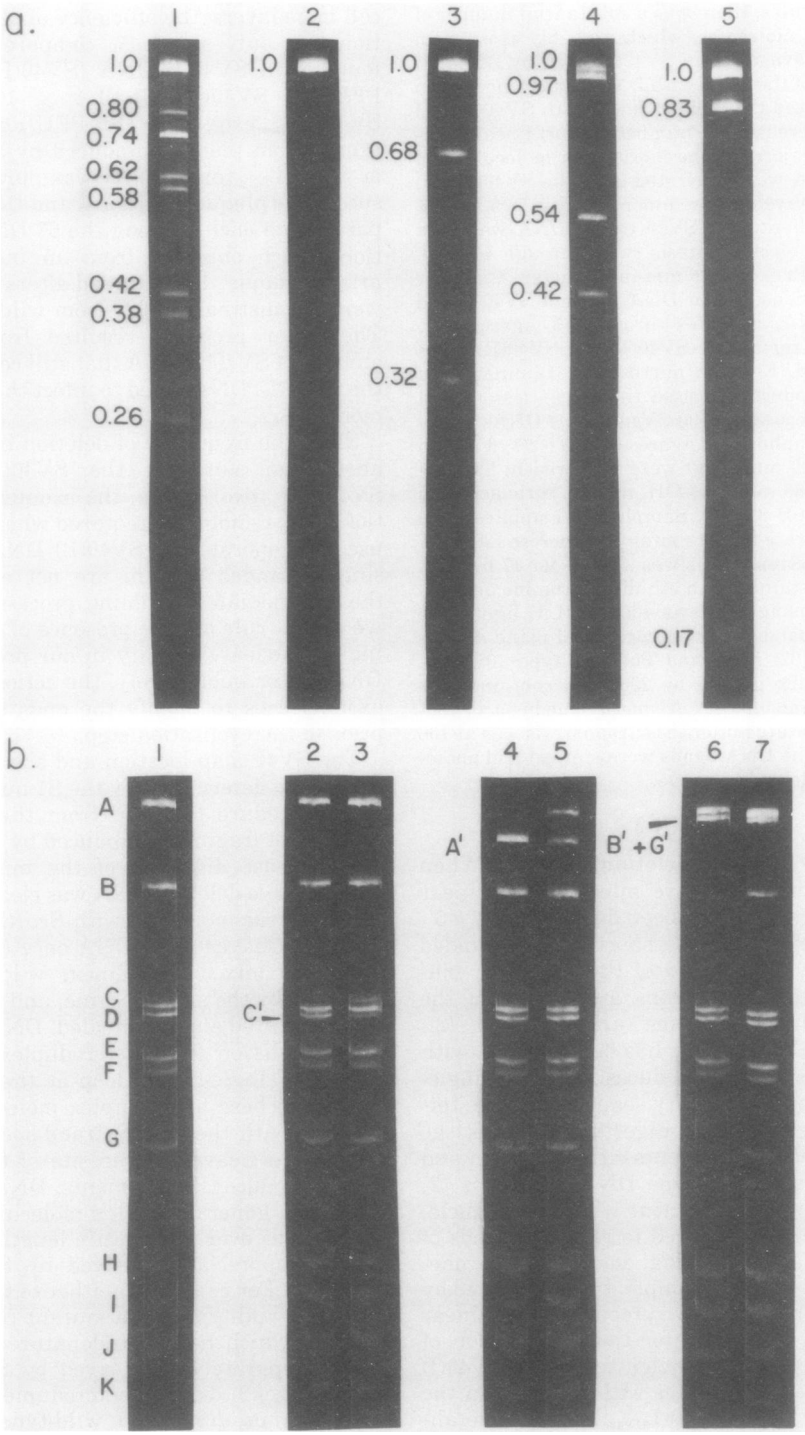

FIG. 1. *Physical characterization of viable deletion mutations. (a) Cleavage by S1 nuclease of heteroduplexes prepared from EcoRI endonuclease-generated linear DNAs of deletion mutants and their wild-type parent. The S1 nuclease reactions contained 5 μg of DNA per ml. Samples of 0.2 μg of DNA were applied to each agarose gel. Gel 1, Marker fragments. These include EcoRI endonuclease-generated SV40 linear DNA,*

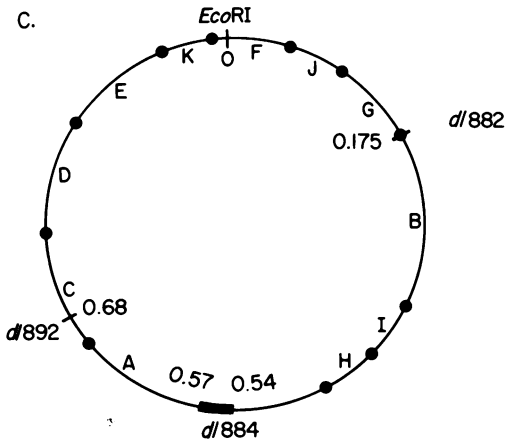

FIG. 1C

be distinguished by comparing the size of the fragments produced by cleavage of the mutant and wild-type DNAs with the *Hin*d II+III endonucleases (these endonucleases together cleave SV40 DNA to 11 major fragments [4]). If the deletion is at map position 0.32, *Hin*d II+III fragment B would be smaller than the wild-type B fragment; alternatively, if the deletion is at 0.68 the C fragment would be smaller. We find that *Hin*d II+III fragment C from *dl*892 migrates faster (Fig. 1b, gel 2) than the corresponding wild-type fragment (Fig. 1b, gel 1) and, therefore, conclude that the deletion in *dl*892 is at 0.68 on the SV40 map (Fig. 1c). Furthermore, using the difference in the relative mobilities of the mutant and wild-type *Hin*d II+III fragment C, we estimate the size of the deletion to be about 48 base pairs (13). The analyses of two additional deletion mutants are also summarized in Fig. 1. Mutant *dl*884 lacks about 184 base pairs between 0.54 and 0.57 map units (S1 digest—Fig. 1a, gel 4; *Hin*d II+III digest—Fig. 1b, gels 4 and 5). Mutant *dl*882 lacks about 16 base pairs at 0.175 map units (Fig. 1a, gel 5; Fig. 1b, gels 6 and 7).

The deletion mutants fall into three groups (Table 1). In the first (three mutants), the deletions are located between 0.17 and 0.18 on the SV40 DNA map; the second group (nine mutants) contains deletions between 0.54 and 0.59; and the third group's deletions (four mutants) occur at 0.68 to 0.73. The deletions range in size from <15 to 200 base pairs. Two of the mutant

TABLE 1. *Physical characteristics of deletion mutants*[a]

Mutant	S1 nuclease-generated fragments (SV40 fractional length)	Altered *Hind* II+III fragment	Net change[b] (base pairs)
*dl*880	0.17, 0.83	G	−21
*dl*881	0.17, 0.83	G	−12
*dl*882[c]	0.17, 0.82	B + G	−16
*dl*883[d]	0.46, 0.54	A	−23
*dl*884	0.42, 0.54	A	−184
*dl*885	0.45, 0.55	A	−38
*dl*886	0.45, 0.56	A	<−15
*dl*887	0.41, 0.56	A	−197
*dl*888	0.45, 0.56	A	−34
*dl*889	0.42, 0.57	A	−46
*dl*890	0.42, 0.58	A	−53
*dl*891	0.41, 0.59	A	−41
dl892	0.32, 0.68	C	−48
*dl*893	0.29, 0.69	C	−136
*dl*894	0.28, 0.70	C	−170
*dl*895	0.28, 0.70	C	−120
Mutant 896	0.17, 0.82	G	+160
Mutant 897	0.32, 0.69	C	+48

[a] Procedures used in the analysis are described in the legend of Fig. 1.

[b] These estimates are accurate to within about ±10 base pairs.

[c] The size of the deletion in *dl*882 was determined from the altered migration of *Hae*III endonuclease fragment C (C. Cole and T. Landers, personal communication).

[d] The symmetry problem discussed in the text (i.e., that the deletion could be at 0.46 or 0.54 SV40 map units) was not solved in all cases by determining which *Hind* II+III fragment became smaller (both 0.46 and 0.54 map in the *Hind* II+III fragment A). In these cases, an unambiguous assignment of location was made by repeating the S1 nuclease analysis using *Hpa*II endonuclease-generated linear molecules rather than *Eco*RI endonuclease-generated linear molecules (18).

fragments obtained by sequential cleavage of SV40 DNA with HpaII and EcoRI endonucleases, and fragments obtained by partial cleavage of SV40 DNA with HpaI endonuclease. Gel 2, S1 nuclease-treated homoduplexes. Gel 3, S1 nuclease-treated heteroduplexes formed from dl892 and wild-type DNAs. Gel 4, S1 nuclease-treated heteroduplexes formed from dl884 and wild-type DNAs. Gel 5, S1 nuclease-treated heteroduplexes formed from dl882 and wild-type DNAs. (b) Hind II+III fragments produced by cleavage of mutant and wild-type DNAs. DNAs (0.2 μg/reaction mixture) were digested with the Hind II+III endonucleases and then subjected to electrophoresis on polyacrylamide gels. Gel 1, Wild-type DNA; gel 2, dl892 DNA; gel 3, dl892 plus wild-type DNAs; gel 4, dl884 DNA; gel 5, dl884 plus wild-type DNAs; gel 6, dl882 DNA; gel 7, dl882 plus wild-type DNAs. (c) Locations of three viable deletion mutations on the SV40 chromosome. The solid circles represent points at which the Hind II+III endonucleases cut, and the letters designate the fragments produced by this cleavage (4).

DNAs, mutants 896 and 897, have larger *Hin*d II+III fragments than the wild type. A similar finding was made previously using this method to construct mutants (2). Neither the origin nor the nature of the modification in these mutants is presently understood.

Deletion mutants are viable. Although each of the deletion mutant isolates was carried through two successive plaque purifications in the absence of added helper virus, it is conceivable that the apparent viability of the mutants results from contamination of the isolated virus and its DNA with small amounts of a complementing wild-type or defective virus, or their DNA, respectively.

Several lines of evidence indicate that this is not so. First, appreciable levels of contamination (>5%) can be eliminated because in the digest produced by *Hin*d II+III endonuclease digestion of the mutant DNAs only one fragment is modified (an exception, *dl*882, is the case in which the deletion fused two fragments together); moreover, a mutant DNA produces either an altered or wild-type fragment but never both. That leaves the possibility that the helper is present in trace quantities. However, if the infectivity of the mutant DNA is due to a small amount of a second, complementing mutant DNA or wild-type DNA, the plaque-forming titer of the mutant DNA would be much lower (<5%) than wild-type DNA. However, the infectivity of three representatives of these mutant DNAs was identical to that of wild-type DNA (Table 2). Moreover, the plaque-forming activity of the mutant DNA showed single-hit kinetics, whereas reconstructed mixtures of two complementing defective mutants yield plaques with two-hit kinetics.

These experiments lead us to conclude that the isolated deletion mutants are indeed viable and that the isolated DNAs are homogeneous preparations of a single genome.

Growth characteristics of viable deletion mutants. It was of interest to compare the kinetics and yield of mutant and wild-type virus multiplication. Single-step growth curves using CV-1P cells were obtained with three representative mutants whose deletions are located between 0.68 and 0.74 on the SV40 map (Fig. 2a). Mutant *dl*861, whose deletion maps at 0.74 (and lacks the *Hpa*II endonuclease cleavage site [2]), has a latent period longer than that of the wild type and produces less than 5% of the wild-type yield at late times after infection. Mutant *dl*895, with a deletion at 0.70 on the SV40 map, multiplies faster than *dl*861 but somewhat slower than the wild-type virus; mutant *dl*892, whose deletion maps at 0.68, multiplies almost as well as its wild-type parent. Although studies with additional mutants having deletions in these regions are needed, there does appear to be some correlation in the position of the deletion and the ability to multiply. It appears that the growth rate is better correlated with the map location than with the size of the deletion: *dl*861 (−32 base pairs) at 0.74 multiplies at the poorest rate, *dl*895 (−120 base pairs) at 0.70 multiplies at an intermediate rate, and *dl*892 (−48 base pairs) at 0.68 on the map multiplies as well as the wild type.

Members of the mutant group with deletions located in the region between 0.54 and 0.59 on the SV40 map all multiplied somewhat more slowly and produced slightly lower yields than their wild-type parent (Fig. 2b), but there was no correlation between the growth rate and the location or size of the deletion mutations. The two mutants mapping between 0.17 and 0.18 on the SV40 chromosome were indistinguishable from the wild type in their ability to multiply.

Viable deletion mutants can transform mouse cells. The viable deletion mutants appear to transform BALB/c 3T3 cells as well as wild-type SV40 virus. Transformants were scored by their ability to form colonies in growth medium containing 2% calf serum (5). There was no significant difference in transforming activity relative to plaque-forming ability between any of the mutants and wild-type SV40 virus (Table 3). Further studies are needed to determine whether other parameters of transformation, such as the ability to grow to high-saturation densities or to grow suspended in agar, are affected differentially.

TABLE 2. *Infectivity of DNA from viable deletion mutants*[a]

DNA	Location of deletion	PFU/μg × 10^{-6}
*wt*830		1.2
*dl*892	0.68	1.4
*dl*884	0.54 → 0.57	1.4
*dl*882	0.175	1.4

[a] Plaque assays were performed on monolayers of CV-1P cells with DNA in the presence of DEAE-dextran (12).

DISCUSSION

Viable deletion mutants have been isolated by infecting monkey kidney cells with a preparation of circularly permuted, linear SV40 DNA. The deletions cluster in three regions of the SV40 chromosome (Fig. 3). The first is located at 0.68 to 0.74; these mutants extend the region discovered earlier with mutants selected for the loss of the *Hpa*II endonuclease cleavage site (0.735 on the SV40 map) (2, 13). Mutants whose deletions extend furthest toward 0.74

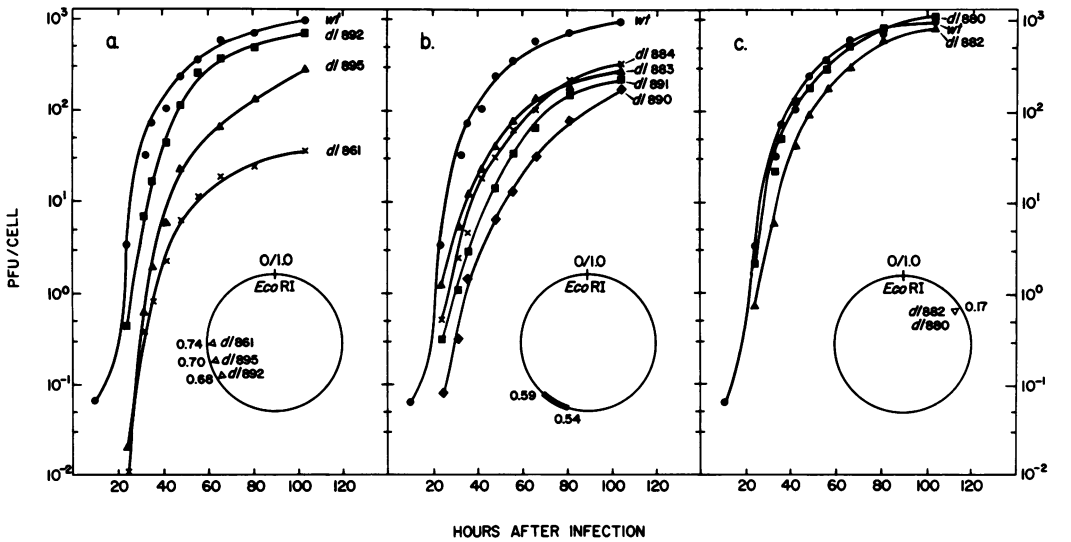

FIG. 2. *Growth curves of SV40 viable deletion mutants in CV-1P cells. Cells were infected at a multiplicity of 10 PFU/cell. After adsorption for 90 min at 37 C, the monolayers were washed three times with Tris-buffered saline, and medium containing 5% fetal calf serum was added. Cultures were harvested at the indicated times, and the virus titer was measured by plaque assay on CV-1P cells. (a) Growth curves of mutants located in the region from 0.68 to 0.74. Symbols: ●, wild type; ■, dl892; ▲, dl895; ×, dl861. (b) Growth curves of mutants located in the region from 0.54 to 0.59. Symbols: ●, wild type; ×, dl884; ▲, dl883; ■, dl891; ◆, dl890. (c) Growth curves of mutants located in the region from 0.17 to 0.18. Symbols: ●, wild type; ■, dl880; ▲, dl882.*

TABLE 3. *Transformation by viable deletion mutants*[a]

Virus	Location of deletion	TU/PFU × 10^5
*wt*830		5.0
*dl*895	0.70 → 0.72	13.0
*dl*892	0.68	6.7
*dl*891	0.59	1.9
*dl*884	0.54 → 0.57	3.3
*dl*883	0.54	2.5
*dl*882	0.175	7.7

[a] Separate plates containing 10^6 BALB/c 3T3/A31 cells were inoculated with 0.2 ml of each virus stock (multiplicity of infection ≥ 10 PFU/cell). Three days after infection, the cells were removed from the plate with trypsin, serially diluted, replated, and allowed to grow into colonies in a medium containing 2% calf serum. Four weeks after infection, the cells were fixed and stained with Giemsa stain, and transformed colonies were counted. The number of colonies that arose on uninfected plates (approximately 1 colony per 10^5 cells plated) was subtracted from the data obtained for infected cells.

multiply poorest, while deletions nearest 0.68 are very nearly normal. The reason for this gradient is not clear, although it would not appear to result from interference of initiation of replication at 0.67. Perhaps comparison of the structure and amount of SV40 mRNA produced by these mutants and wild-type virus should help determine whether these deletions affect the transcription of the late mRNA.

Another region that can be deleted without affecting the viability of the virus occurs between 0.54 and 0.59 on the SV40 map. These mutants grow somewhat more slowly and produce slightly lower yields than their wild-type parent, but we could detect no difference in the amount or distribution of T antigen immunofluorescence in CV-1P cells (*dl*884 and *dl*890 were tested). When T antigen ([^{35}S]methionine labeled) was immunoprecipitated and analyzed by polyacrylamide gel electrophoresis (21), no difference was found in the electrophoretic mobility of T antigens produced after infection by *dl*884, *dl*890, or wild-type virus. We are unable to estimate the effect of such small deletions on the electrophoretic migration of the T antigen, particularly in the absence of detailed information about its chemical and physical nature. Although the extent of this dispensible region is not known, mutants with small deletions at 0.48 and 0.50 on the SV40 map are defective and cannot grow without a helper virus to provide the needed early function (S. Manteuil and P. Berg, unpublished data).

Deletions in the region 0.17 to 0.18 on the SV40 map are also viable. These mutations remove sequences at the 3′ end of the transcript of both the early and late region (Fig. 3). Quite

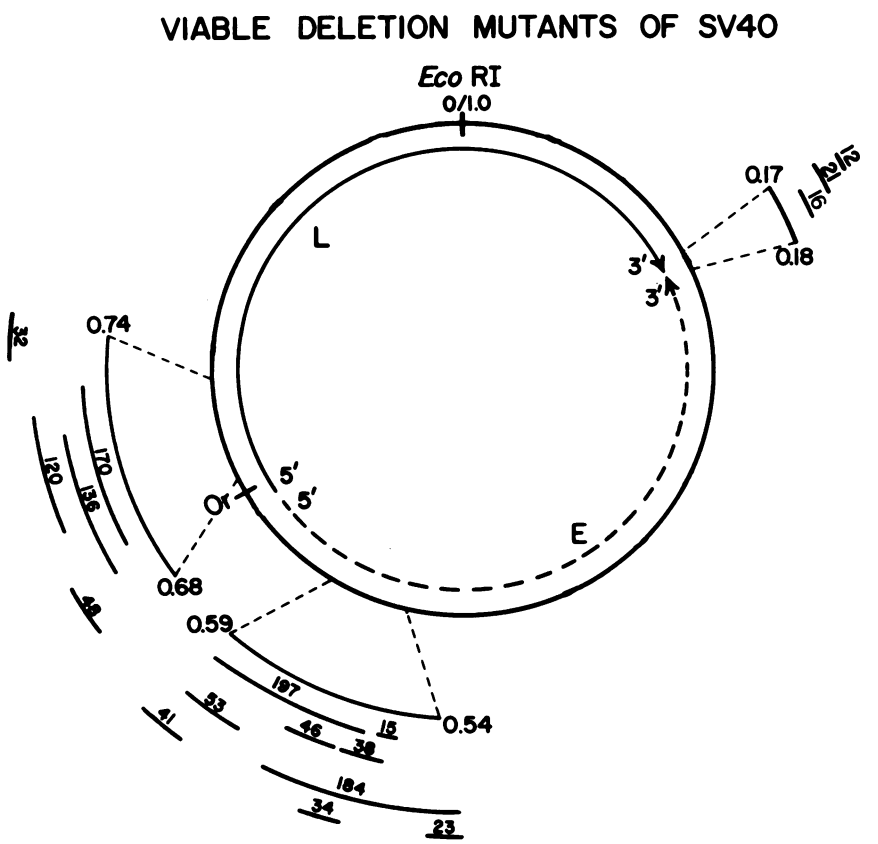

FIG. 3. *Locations of the viable deletion mutations on the SV40 chromosome. The locations and size of the viable deletion mutations, and their positions relative to the EcoRI endonuclease cleavage site, the early (E) and late (L) regions of transcription, and the origin of replication (Or) are indicated.*

possibly these small deletions alter both mRNA's near their 3′ ends. Direct sequence analyses of the mRNA's produced in infections with these mutants are needed to determine whether nucleotides at their 3′ ends are removed, and thereby determine the dispensibility of that portion of the mRNA's.

To define the extent of each of these three dispensible regions studies with additional mutants will be needed. A further question is whether all of the dispensible regions on the SV40 chromosome have been located. Of the 67 plaque isolates obtained by infection with the linear DNA, only 18 contained alterations in their DNA as judged by the S1 nuclease sensitivity of the heteroduplexes formed between *Eco*RI endonuclease-cut wild-type and putative mutant DNAs. This is an extremely sensitive test for deletions (18), but it would probably fail to detect a deletion within about 0.03 map units of the *Eco*RI endonuclease cleavage site. Consequently, each of the DNAs that had been diagnosed as the wild type was digested with the *Hind* II+III endonucleases, and the size of *Hind* II+III fragments K, F, and J (those located about and near the *Eco*RI endonuclease cleavage site) was examined by polyacrylamide gel electrophoresis. Because these fragments are small, deletions of as few as 10 bases would have been detected readily by the alteration in their electrophoretic mobility. However, no changes were found in these fragments from any of the DNAs classed as wild type. Assuming that the DNase I-Mn^{2+} digestion cleaved SV40 DNA randomly, we believe it is unlikely that any other substantial segments (50 base pairs or >1% of the genome) of the DNA can be deleted without rendering the genome defective.

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