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Mutant Carrying Deletions in the Two Simian Virus 40 Early Genes

J. FEUNTEUN,[1]* G. CARMICHAEL,[3] J. C. NICOLAS,[2] AND M. KRESS[1]

Institut de Recherches Scientifiques sur le Cancer, 94802 Villejuif Cédex, France[1]; Institut Pasteur, Virologie Moléculaire, 75724 Paris Cedex 15, France[2]; and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115[3]

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We isolated a simian virus 40 mutant, *dl*2194, which carried deletions in both early genes. One deletion removed 234 base pairs in the 54/59 region within the small-t-antigen coding sequence and the large-T-antigen gene intron. The second deletion removed 57 base pairs at the C terminus of the large-T-antigen coding sequence (0.20 map unit). *dl*2194 was a viable mutant, it carried a normal helper function for adenovirus growth on monkey cells, and it displayed the transformation properties of a small-t-antigen-negative single mutant. Therefore, none of the known large-T-antigen functions seemed to be altered by the C-terminal deletion.

Originally, the early region of the simian virus 40 (SV40) genome was defined as the part of the genome that was expressed in the lytic cycle before the onset of DNA synthesis. Later, it was shown that this region contains all of the information required to establish and maintain malignant transformation (31). The first gene assigned to this region is defined by complementation group A and is involved in the initiation of viral DNA synthesis, as well as in the initiation and maintenance of transformation (31). Missense *tsA* mutations are clustered around nucleotide positions 3,600 through 4,000 on the physical map (14, 25) and render these functions thermosensitive. Several lines of evidence, including sequence data (9, 22) and the existence of a frameshift deletion mutant (23), have established that the product of gene *A* is large T antigen, a 90,000-dalton polypeptide originally shown to be precipitated specifically by sera from SV40-induced tumor-bearing animals. Recently, a second gene in the early region was defined by deletions in the 54/59 region (4, 8, 26, 29); these deletions affect the transforming capability of the virus at least partially. This second gene codes for small t antigen, a 19,000-dalton polypeptide which is also precipitated by sera from SV40-induced tumor-bearing animals. Together with the existence of these two classes of mutants, sequence data and mRNA mapping have contributed to our understanding of the genetic expression of the early region (Fig. 1). Large-T-antigen and small-t-antigen mRNA's originate from a single pre-mRNA by two different splicing events and code for two proteins that share an N-terminal sequence (3, 7, 19).

Polyoma virus is closely related to SV40 in many respects. However, there is a major difference between these two viruses in the expression of their respective early regions. Like SV40, the polyoma virus early region codes for a large T antigen and a small t antigen, but it also codes for an additional, predominantly membrane-associated polypeptide; this is the middle T antigen (molecular weight, 58,000) (11, 12, 23), whose mRNA is also derived from the full transcript of the early region through a third splicing event. Polyoma virus *tsa* mutations are homologous to SV40 *tsA* mutations and also affect large T antigen. The Hr-t class of mutations is unique to polyoma virus and affects both small t antigen and middle T antigen (24). Hr-t mutants have an altered host range for lytic infection and are absolutely defective in the ability to transform cells in culture or to cause tumors in newborn hamsters (28). No middle-T-antigen counterpart has been found in SV40-infected or -transformed cells; in fact, the SV40 sequence shows a single open reading frame in the region which is topologically homologous to the polyoma virus sequence that codes for both middle T antigen and large T antigen. However, there is a second open reading frame, starting at nucleotide 2,920, within the 3′ end of the early region. If this reading frame were used, a new protein would be made, which would be indistinguishable from large T antigen in size. This protein would share most of its amino acid sequence with large T antigen, except for the last 70 residues at the C terminus. Therefore, mutants with mutations that affect the distal part of the early region are of interest. Some of these mutants have been

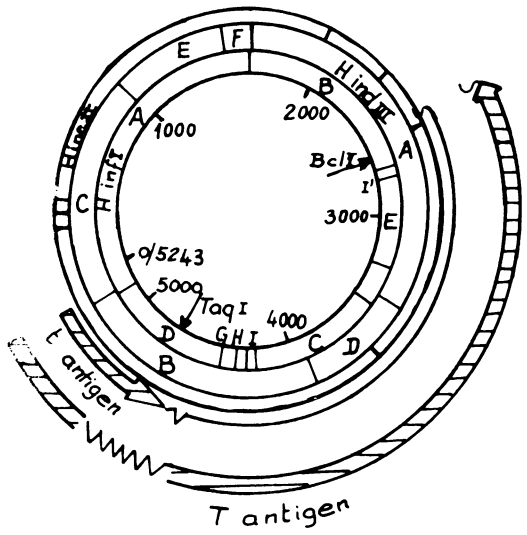

FIG. 1. *SV40 genome. The numbering system is the simian virus system corrected to a total length of 5,243 nucleotides (31). The cross-hatched areas indicate translated sequences, and the wavy line indicates the spliced-out sequence.*

described previously (6, 20, 32). Here we describe a new member of this group.

MATERIALS AND METHODS

Virus and cells. SV40 *dl*2122, a *dl*54/59 derivative of wild-type strain LP, has been described previously (34); *tsA*58 was derived from wild-type strain VA 45-54 (30). SV40 virus stocks were grown on CV1 Cl1 cells and plaqued on CV1P cells. Adenovirus 5 (Ad5) stocks were grown on HeLa cells. Transformation was assayed on NIH 3T3 mouse cells or BALB/c mouse embryo secondary fibroblasts. Cell line 293 is an adenovirus-transformed human cell line (10). All cells were grown on Dulbecco modified Eagle medium (type H21; GIBCO Laboratories) supplemented with 10% calf serum.

Preparation of viral DNAs and heteroduplexes. SV40 viral DNAs were purified from CV1 Cl1 cells infected at a multiplicity of 0.001 PFU/cell. Hirt extraction was followed by two rounds of isopycnic centrifugation in cesium chloride-ethidium bromide gradients.

Heteroduplexes between *tsA*58 and *dl*2122 were made as follows. A 1-μg amount of *tsA*58 *Taq*I linear molecules and 1 μg of *dl*2122 *Eco*RI linear molecules were mixed together in 85 μl of 10 mM Tris (pH 7.4)–1 mM EDTA and denatured by adding 10 μl of 1 N NaOH. After 10 min at room temperature, the solution was neutralized by adding 20 μl of 0.5 N HCl–0.25 M Tris (pH 7.4). Reanneling was performed at 68°C for 3 min, and the sample was loaded onto a 1.4% agarose gel. Two classes of double-stranded molecules were formed; these were linear molecules which were renatured *dl*2122 and *tsA*58 homoduplexes, and circular molecules, which were heteroduplexes made from one *dl*2122 strand and one *tsA*58 strand. Circular molecules were eluted from the gel by electrophoresis.

Enzyme digestion and gel electrophoresis. Restriction enzymes were purchased from New England Biolabs and were used according to the instructions of the supplier.

*Hin*dIII and *Hin*fI digests were electrophoresed on 4% polyacrylamide gels, as described previously (8).

DNA sequencing. DNA sequence analyses were performed by the method of Maxam and Gilbert (18).

Immunoprecipitation and gel electrophoresis. In vivo labeling with [^{35}S]methionine, preparation of extracts, and immunoprecipitation have been described previously (8).

In vitro translation of SV40 mRNA's followed by immunoprecipitation has also been described previously (19).

Adenovirus helper effect. A total of 4×10^5 secondary African green monkey kidney cells per 35-mm^2 plate were coinfected with Ad5 and SV40, each at a multiplicity of infection of 2 PFU/cell. After 2 h of infection, the inocula were removed, and the plates were washed three times with 2 ml of phosphate-buffered saline and fed with fresh medium; the cultures were then incubated at 33°C for 90 h. Crude lysates were prepared from the infected cells, and the adenovirus and SV40 yields were measured by plaquing the lysates at 33°C onto 293 and CV1P cells, respectively.

Transformation assays. The immortalization assay was performed as follows. BALB/c mouse embryo secondary cultures were infected at high multiplicity (100 PFU/cell); these cultures were incubated for 24 h at 37°C, trypsinized, and plated at a high dilution (350 cells/cm^2). Clones were stained and counted after 3 weeks at 33°C in the virus-infected plates, whereas no cell growth was detected in the mock-infected plates.

Transformation of NIH 3T3 cells was assayed in semisolid medium, as described previously (8).

RESULTS

Isolation and characterization of *dl*2194. Mutant *dl*2194 was isolated by chance during an experiment designed for a completely different purpose. The aim of this selection experiment (Fig. 2) was the isolation of a double mutant carrying a gene *A* mutation in *cis* with a deletion in the 54/59 region. To do this, we constructed a circular heteroduplex composed of one strand from a *dl*2122 *Eco*RI linear molecule and one strand from a *tsA*58 *Taq*I linear molecule. We thought that the *tsA*58 54/59 single-stranded sequence tailing out the double-stranded circle would be preferentially accessible to cell nucleases during DNA infections and that the elimination of such a sequence would give rise to progeny which would be 50% *dl*2122 and 50% *dl*2122-*tsA*58 double mutants.

CV1 C11 cells were infected with the purified heteroduplex for 72 h at 33°C, and the virus progeny was cloned on CV1P cells at 33°C. Viruses were grown from five plaques and tested

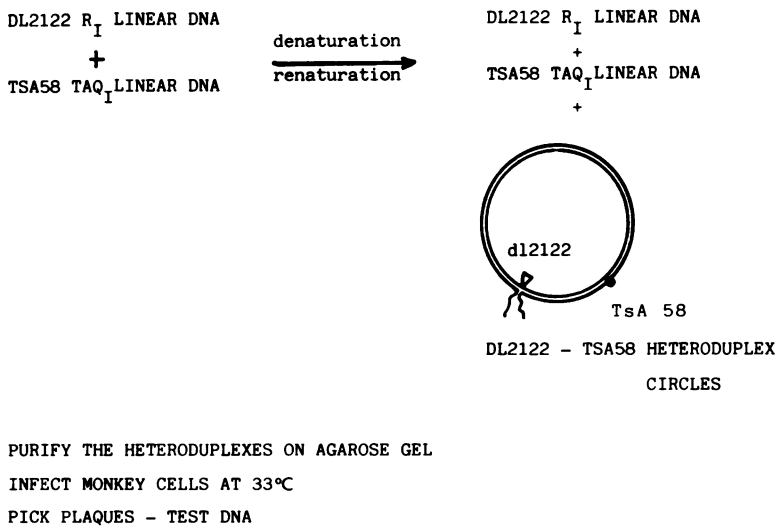

FIG. 2. *Protocol for the isolation of dl2194. The open triangle represents the dl2122 54/59 deletion, and the solid dot represents the tsA58 point mutation.*

for the thermosensitive phenotype; the DNAs of these viruses were prepared and subjected to restriction enzyme analysis.

In a crude assay of plaque suspension titers at 33 versus 41°C, we found that none of the five isolates was clearly thermosensitive; one isolate, *dl*2194, was chosen for a detailed analysis of the virus yields at 33 and 41°C.

Single-cycle infections were performed on CV1 cells at 33 and 41°C, and the titers of virus progeny were determined at 33°C. As Table 1 shows, the virus yields at the two temperatures were not significantly different. The yield of *dl*2194 was similar to the yields of its parent (*dl*2122) and its grandparent (wild-type strain LP), regardless of the temperature. In contrast, *tsA*58 was strongly thermosensitive in the same assay.

The five DNAs described above were compared with the DNAs of both parents (*dl*2122 and *tsA*58) by digestion with *Hin*dIII and *Hin*fI. Surprisingly, the five isolates were identical at this level of analysis; therefore, only *dl*2194 was subjected to further restriction analysis. The *Hin*dIII patterns of *dl*2122 and *dl*2194 were indistinguishable (Fig. 3A). These patterns were characterized by two differences with the *tsA*58 pattern. First, *Hin*dIII fragment B, which carries the 54/59 region, was deleted; and second, *Hin*dIII fragments A and D were fused into a large A-D fragment. The mutation at this *Hin*dIII site occurred spontaneously and was not selected. The *Hin*fI patterns confirmed that the 54/59 deletion of *dl*2122 carried by fragment D′ was conserved in *dl*2194; on the other hand,

TABLE 1. *Lytic growth properties of SV40 mutants*

Infecting virus	SV40 yield (PFU/cell) at:[a]	
	33°C	41°C
SV40 wild type	280	214
SV40 *dl*2122	174	222
SV40 *dl*2194	160	280
SV40 *tsA*58	200	3×10^{-2}

[a] Determined by plaque assay on monkey CV1 cells at 33°C (two plates for each dilution).

these patterns also revealed differences between the two DNAs which were not observed in the *Hin*dIII patterns. In *dl*2194 *Hin*fI fragments B and E were fused into fragment B-E, which was the same size as *Hin*fI-A. This fusion resulted from the deletion of about 60 base pairs, including the two *Hin*fI sites at positions 2,824 and 2,848.

Together, these data showed that *dl*2194 displayed the restriction characteristics of *dl*2122 and did not carry the *tsA*58 mutation. Therefore, *dl*2194 was most likely derived from the *dl*2122 strand of the heteroduplex. It should be emphasized that the C-terminal deletion did not seem to cause a conditional lethal defect.

DNA sequence. The material for sequencing was prepared by digesting strain LP or *dl*2194 DNA with a mixture of *Bcl*I and *Hin*cII. *Hin*cII cleavage left flush ends whereas *Bcl*I created cohesive ends which could be filled with the four ^{32}P-labeled deoxynucleotide triphosphates by using the Klenow fragment of DNA polymerase I. When this procedure was used with the double

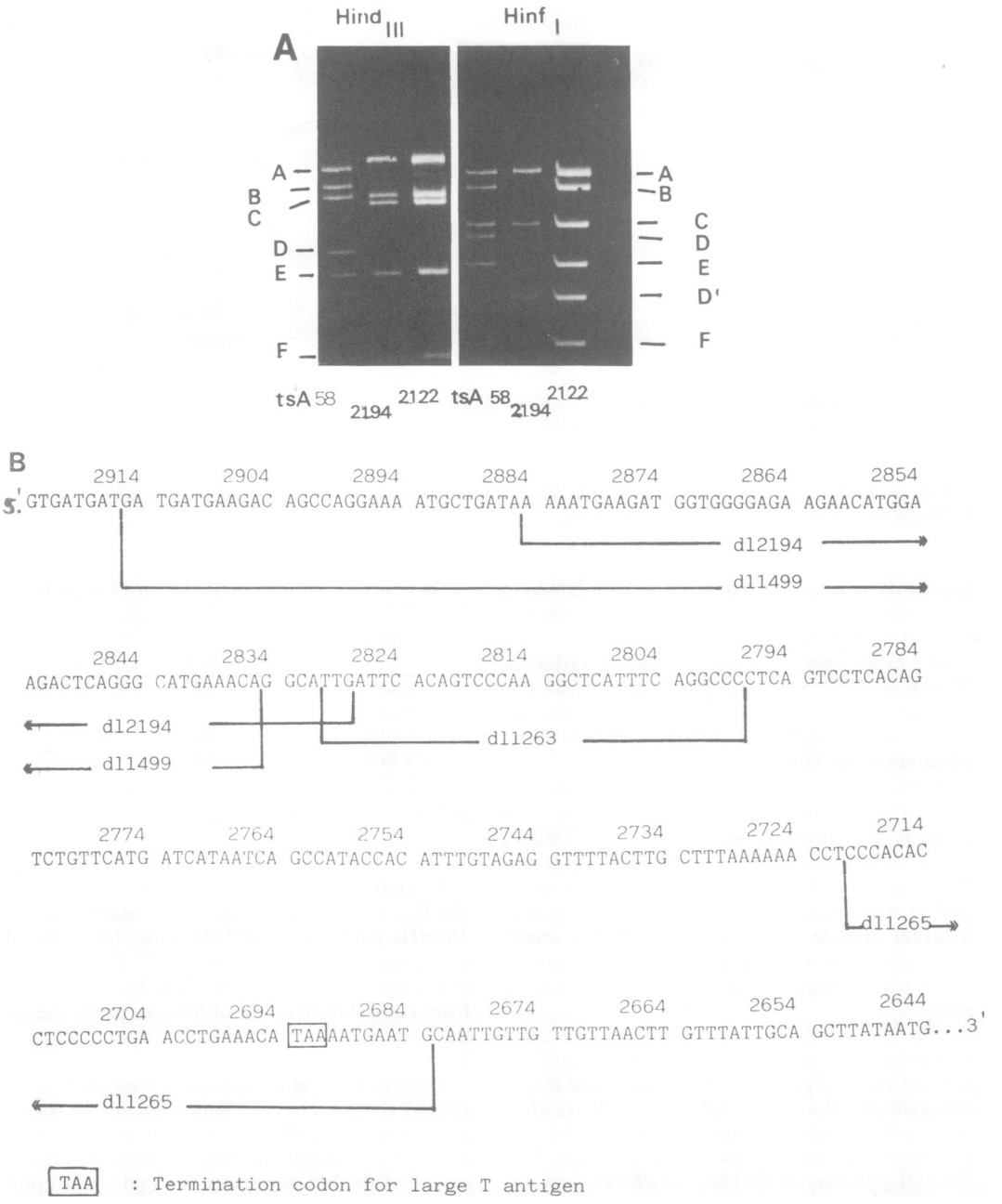

FIG. 3. *(A) HindIII and HinfI restriction patterns of tsA58, dl2194, and dl2122 DNAs on 4% polyacrylamide gels. (B) Sequence of the C-terminal part of large-T-antigen gene, showing the regions deleted in the various mutants.*

digest, it produced only two labeled fragments, which were 963 (positions 2,770 to 3,733) and 106 (positions 2,666 to 2,770) nucleotides long for strain LP (Fig. 1). The large fragments from strain LP or *dl*2194 digests were purified by electrophoresis on a 1.4% agarose gel and sequenced by the method of Maxam and Gilbert by a single loading on a 20% acrylamide gel. Several conclusions could be drawn from the sequence data. First, *dl*2194 DNA contained a 57-base pair deletion between nucleotides 2,828 and 2,884 (Fig. 3B). This deletion removed 19

amino acids from large T antigen that were not essential for lytic growth; it did not change the reading frame and, therefore, did not affect the C-terminal 43 residues. The second observation concerned the sequence difference between the wild-type strains. *dl*2194, as well as wild-type strain LP and *dl*2122 (from which *dl*2194 was derived), showed an almost perfect direct repeat of a 9-base pair sequence at position 2,794; this repeat was present in wild-type strain 776 only as a single copy (Fig. 4). As a consequence of this duplication, the strain LP large T antigen possessed three extra amino acid residues (glutamine, proline, and serine). However, no strong selective pressure seemed to have been exerted in order to keep a perfect repeat, since a thymine-to-cytosine transition in the fourth base of the repeat resulted in a serine-to-proline change without any apparent functional consequence. This observation is similar to the data published recently by Alwine and Khoury (1), who showed that there are sequence differences in the same region between strain 776 and another wild-type strain (VA 45-54). Strain VA 45-54 contains two insertions, one 2 base pairs long at position 2,794 and one 7 base pairs long at position 2,801 (Fig. 4). The 7-base pair insertion is a perfect repeat of a sequence located two nucleotides downstream. In terms of protein sequence, the result of these two insertions is the addition of three amino acid residues and two changes due to the frameshift effect of the 7-base pair addition.

The result of this variability is that *dl*2122 and *tsA*58, which were derived from strains LP and VA 45-54, respectively, differ by 10 base pairs in the region around nucleotide 2,800 (Fig. 4). Therefore, due to the mismatches caused by sequence differences, the heteroduplexes between *dl*2122 and *tsA*58 DNAs carry two contiguous deletion loops. It is difficult to imagine how *dl*2194 was derived from such a heteroduplex. The deletion was generated within a region of perfect match, whereas a mismatch 30 base pairs away was not corrected.

Expression of *dl*2194 early region. We studied the expression of the early region in primary baby mouse kidney cells abortively infected by the wild type (strain LP) and mutants *dl*2194 and *dl*1263. *dl*1263 is another mutant with a deletion in the C-terminal region of the large T antigen gene (6, 32). At 36 h after infection at 37°C, the cells were labeled for 1 h with [^{35}S]methionine. Extracts were prepared and immunoprecipitated as described previously (8). Immune precipitates were electrophoresed on 7.5 or 12.5% sodium dodecyl sulfate-polyacrylamide gels. The 7.5% gels (Fig. 5A) showed the striking effects of the C-terminal deletions on the electrophoretic mobility of large T antigen. The apparent molecular weight of *dl*2194 large T antigen was 82,000, compared with 86,000 for *dl*1263 large T antigen and 90,000 for the wild-type large T antigen. Such shifts in the *dl*2194 and *dl*1263 large-T-antigen molecular weight were completely unexpected since the actual loss of genetic information (57 and 33 base pairs, respectively) suggested molecular weights of 88,000 for *dl*2194 large T antigen and 89,000 for *dl*1263 large T antigen. The abnormal molecular weight of *dl*1263 large T antigen was observed previously (6). In the case of *dl*2194, the same abnormal apparent molecular weight was also demonstrated with large T antigen translated in vitro from polyadenylated mRNA isolated from *dl*2194-infected CV1 cells 48 h after infection (19) (Fig. 5C), suggesting that the mobility shift did not result from unspecific proteolytic degradation. In addition to the large-T-antigen

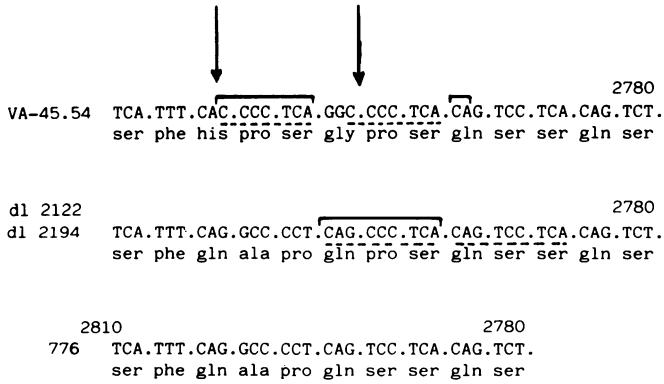

FIG. 4. *Strain comparison of the C-terminal portions of the large-T-antigen gene, showing differences. Wild-type strain 776 is used as a reference. The additions in dl2122 and VA 45-54 are overlined, and the repeats are underlined. The two arrows indicate the limits of the mismatch between these two DNAs.*

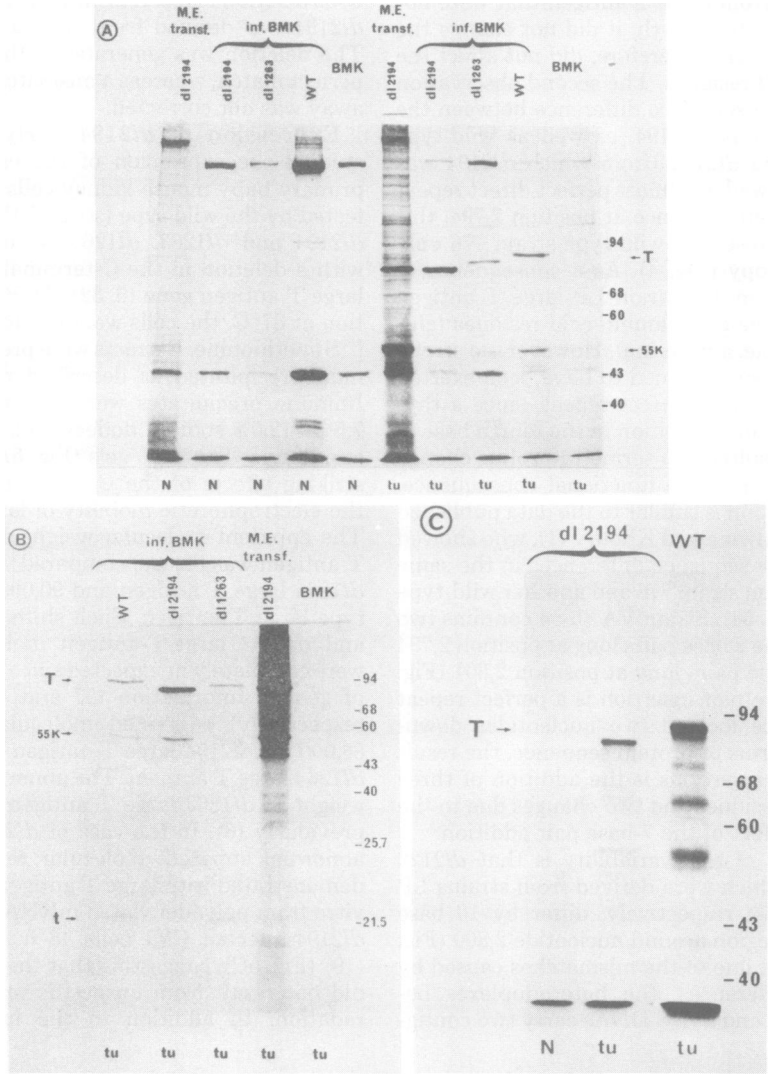

FIG. 5. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immune precipitates from cellular extracts labeled with [^{35}S]methionine. (A) 7.5% gel. Lanes N, Cell extracts precipitated with normal hamster serum; lanes tu, same extracts precipitated with SV40-induced tumor-bearing hamster serum. M.E. transf., Cell extracts from mouse embryo fibroblasts transformed by dl2194; inf. BMK, cell extracts from baby mouse kidney cells infected by various mutants. BMK, uninfected baby mouse kidney cells. An arrow indicates the position of the nonviral 55,000-dalton protein (55K). (B) Same extracts as in (A) analyzed on a 12.5% polyacrylamide gel. (C) 7.5% polyacrylamide gel of in vitro translation products from reticulocyte lysates of polyadenylated mRNA's from CV1 cells infected by wild-type strain LP (WT) and by dl2194.*

bands, Fig. 5C shows multiple bands in the 50,000- to 70,000-dalton range, with comparable relative intensities in both the *dl*2194 and the wild-type extracts. The fact that none of these polypeptides showed the mobility shift characteristic of *dl*2194 large T antigen supported the hypothesis that they were not large T antigen related. The molecular weights of *dl*2194 and *dl*1263 large T antigens were close to the theoretical molecular weight (82,000) expected from the wild-type nucleotide sequence. We postulated that the C-terminal deletions simply removed the source of the discrepancy between the apparent and the theoretical values of large-T-antigen molecular weight. On the 12.5% gels (Fig. 5B) *dl*2194 showed the expected altered form of small t antigen described previously for its parent (*dl*2122) carrying a 54/59 deletion (8).

Biological properties of *dl*2194. (i) Adenovirus helper function. SV40 expresses a function which helps human adenovirus grow in monkey cells (21). This helper function has been mapped at the C terminus of large T antigen (15). Cole et al. (6) have reported that this function is altered severely in mutant *dl*1265, which carries a deletion removing the last 10 amino acids from the C terminus of large T antigen, whereas it is much less affected by the *dl*1263 deletion, which keeps intact the last 35 C-terminal residues (6). We tested the ability of *dl*2194 to help human Ad5 grow on monkey cells. CV1 cells were coinfected with SV40 and Ad5 at a multiplicity of 2 PFU/cell for each of the viruses. Ad5 yields were measured by titration on human 293 cells. The choice of these cells was based on the quality of the plaques which they produced. Because the helper function has been shown to depend on the multiplicity of SV40 infection (6), the titers of the infecting viruses were adjusted carefully. Furthermore, the yields of SV40 from the mixed infections were measured under the assumption that they reflected the level SV40 gene expression accurately. Table 2 shows the results of three experiments. This table shows the SV40 and Ad5 yields, as well as the calculated enhancement factors (expressed as ratios of Ad5 yields in mixed infections to Ad5 yields in single infections). These enhancement factors measured the ability of the various SV40 strains to provide Ad5 with helper functions. It should be pointed out that the yields of the three SV40 strains were reasonably similar, which supported the hypothesis that their genes were expressed at comparable levels. Also, except for the high value of 958-fold enhancement obtained with *dl*2194 in the first experiment, which was not observed in the other experiments, the enhancement factors were similar for the three viruses tested and demonstrated that *dl*2194 was fully competent in helping Ad5 grow on monkey cells. Therefore, in agreement with Cole et al. (6), this function was probably carried out by a portion of large T antigen contained within the C-terminal 43 amino acid residues. The data in Table 1 and in Table 2, experiment 1, were obtained in the same assay, thus allowing quantitative comparisons of the SV40 yields in single infections and mixed infections. This comparison revealed that mixed infections with Ad5 reduced the yield of SV40 four- to fivefold compared with single infections. A similar interference was observed with Ad2 (6, 33) and was shown to be due to an adenovirus early gene (33).

(ii) Transformation. The competence of *dl*2194 for transformation of mouse cells was tested in two different assays. First, we tested the ability of *dl*2194 to immortalize primary whole mouse embryo cells. After SV40 infection, primary rodent cells can acquire an unlimited capacity to divide in vitro and the ability to clone on plastic at a high cell dilution. These properties define an established or immortalized cell line. Sleigh et al. (29) have shown that small-t-antigen-negative mutants are fully competent to immortalize rat embryo primary cells. We reached the same conclusion with mouse embryo primary cells and, in addition, found that *tsA* mutants were defective in this assay at the nonpermissive temperature (C. A. Petit et al., manuscript in preparation). These observations strongly suggested that SV40 gene *A* plays the major role in this immortalization process. Therefore, it was relevant to examine the competence of the altered *dl*2194 large T antigen in this assay. We detected no difference in the frequency of immortalization among wild-type strain LP, *dl*2122 (the small-t-antigen-negative single mutant), and the double mutant *dl*2194 (Table 3).

Induction of the ability to grow in soft agar has been considered the most stringent transformation character (27). The absence of small t

TABLE 2. *SV40 helper effect on Ad5 growth on monkey cells*

Expt	Infecting virus(es)	SV40 yield (PFU/cell)[a]	Ad5 yield (PFU/cell)[b]	Enhancement (-fold)[c]
1	Ad5		0.6	1
	Ad5 + SV40 wild type	65	75	125
	Ad5 + SV40 *dl*2122	45	150	250
	Ad5 + SV40 *dl*2194	34	575	958
2	Ad5	ND[d]	2	1
	Ad5 + SV40 wild type	ND	600	300
	Ad5 + SV40 *dl*2194	ND	300	150
3	Ad5	ND	1	1
	Ad5 + SV40 wild type	ND	72	72
	Ad5 + SV40 *dl*2194	ND	80	80

[a] Determined by plaque assay on monkey CV1 cells at 33°C (two plates for each dilution).

[b] Determined on human 293 cells at 33°C (two plates for each dilution). These yields are expressed as the number of plaque-forming units obtained for the entire harvested culture divided by the number of infected cells.

[c] Enhancement is expressed as the ratio of Ad5 yield in the presence of the SV40 helper to Ad5 yield in the absence of the helper.

[d] ND, Not determined.

antigen or a defect in small t antigen clearly impairs this property (4, 8, 29). In this assay, we expected that *dl*2194 would behave like its parent (*dl*2122); however, the ability to complement the thermosensitive large T antigen from *tsA*58 was a functional test for altered *dl*2194 large T antigen. Table 4 shows the results of such an experiment. The transformation frequency of *dl*2194 was less than 6% of the wild-type frequency regardless of the temperature; at 39°C in single infections *tsA*58 did not transform, but it could be complemented by *dl*2194.

Mouse embryo fibroblasts transformed by *dl*2194 contained both the 82,000-dalton large-T-antigen species and the 55,000-dalton nonviral T antigen which has been shown to accumulate in SV40-transformed or abortively infected rodent cells (13) (Fig. 5A and B).

Therefore, we concluded that the alteration of large T antigen did not cause a defect which was detectable by any of these classical transformation assays.

This conclusion is being tested by a study of the transforming potential of a *dl*2194 derivative which carries only the C-terminal deletion and, therefore, is able to make competent small t antigen.

TABLE 3. *Transformation properties of SV40 mutants: transformation assayed by immortalization of mouse embryo cells*

Infecting virus	Transformation frequency[a]
Wild-type strain LP	2.5×10^{-3}
*dl*2122	0.7×10^{-3}
*dl*2194	1.2×10^{-3}
Mock-infected cells	$<3 \times 10^{-4}$

[a] Each transformation frequency is expressed as the number of colonies growing from 10^4 infected cells per 60-mm petri dish in liquid medium (average of three petri dishes). The value for the mock-infected cells means that no clone was counted in any of the dishes.

TABLE 4. *Transformation properties of SV40 mutants: transformation assayed by anchorage dependence on mouse NIH 3T3 cells*

Infecting virus(es)	Transformation frequency at:[a]	
	33°C	39°C
Wild-type strain LP	5×10^{-3}	5×10^{-3}
*dl*2194	$<3 \times 10^{-4}$	$<3 \times 10^{-4}$
*tsA*58	1×10^{-3}	$<3 \times 10^{-4}$
*dl*2194 + *tsA*58	0.8×10^{-3}	0.5×10^{-3}
Mock-infected cells	$<3 \times 10^{-4}$	$<3 \times 10^{-4}$

[a] Each transformation frequency is expressed as the number of colonies growing from 10^4 infected cells per 60-mm petri dish in soft agar (average of three petri dishes). The values for the mock-infected cells mean that no clone was counted in any of the dishes.

DISCUSSION

*dl*2194 is a double deletion mutant of SV40 in which the two early genes are affected. This mutant belongs to the family of *dl*54/59 deletion mutants and also to a family of C-terminal deletion mutants which were isolated in several different ways. *dl*1263 and *dl*1265 were isolated after treatment of nicked circular molecules by S1 nuclease (5). *dl*1499 was isolated from the progeny of randomly linearized DNA (20); *dl*2194 was the result of an infection with a heteroduplex DNA. The *dl*2194 deletion removes 57 base pairs (positions 2,828 to 2,884); thus, it overlaps three base pairs of the *dl*1263 deletion (32), which removes 33 base pairs (positions 2,798 to 2,830). These deletions remove two regions that are not essential for large-T-antigen function in the lytic cycle. In *dl*1499 (20) 81 base pairs are deleted (positions 2,835 to 2,915). The *dl*2194 deletion is almost totally included in *dl*1499, which extends further toward the 5′ side of the early region. This 5′ difference results in severe phenotypic changes. *dl*1294 is a temperature-independent viable virus. On the basis of its capacity to immortalize primary rodent cells and to complement *tsA*58 in the anchorage dependence assay, *dl*2194 is a fully competent virus. In contrast, *dl*1499 is thermosensitive for lytic growth and cold sensitive for transformation (20). This unique phenotype must result from the deletion of about 30 base pairs from positions 2,885 to 2,915, which are absent only in *dl*1499; 12 nucleotides from this sequence belong to the unique 18-nucleotide sequence coding for six consecutive dicarboxylic residues in large T antigen that might be the SV40 counterpart to the glutamic acid cluster present at the C terminus of polyoma virus middle T antigen.

It is not clear how a single deletion can affect the lytic cycle and transformation in such a reciprocal fashion, unless it also affects a third SV40 early polypeptide which has not been discovered yet. Mark and Berg (17) have suggested that such a polypeptide (large T* antigen) could be coded for by a third early mRNA species, which carries the large-T-antigen splice plus an additional splice about 50 nucleotides long located between positions 2,898 and 2,845. This new splice would be a frameshift splice, allowing the translation to proceed into the only other open frame and giving rise to a large T* antigen differing from large T antigen by the last 70 amino acid residues. Mutants *dl*2194 and *dl*1499

have lost the proposed splicing sites and, therefore, should not produce the putative large T* antigen. Alwine and Khoury (1) have described a small RNA (SAS RNA) which is coded for by the sequence from nucleotide 2,842 to nucleotide 2,908 in the late strand and, therefore, capable of hybridizing to the early mRNA. These authors have proposed that this SAS RNA could be involved in a shift of the reading into the second open frame. This SAS RNA is totally absent in *dl*1499- and *dl*2194-infected monkey cells (1) and thus does not seem to play an essential role in the expression of active early gene products, unless it can be complemented by some host function.

Cells infected or transformed by SV40 express the SV40 tumor-specific transplantation antigen, which has a close molecular relationship to SV40 T antigen (2). The nondefective Ad2 ND hybrids have helped to localize the tumor-specific transplantation antigen at the C terminus of the early region (16). The C-terminal deletion may affect the function of large T antigen involved in tumor rejection. Experiments to test this hypothesis are now in progress in our laboratory.

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