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Simian Virus 40 Deletion Mutants That Transform with Reduced Efficiency

LAUREN SOMPAYRAC* AND KATHLEEN J. DANNA

Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309

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We have constructed two simian virus 40 (SV40) early-region deletion mutants that lack a significant portion of the sequences normally used to encode the SV40 large tumor antigen. Despite these deletions, the mutants were able to transform mouse cells in a focus assay, although with a frequency that was drastically reduced relative to wild-type SV40. Cell lines expanded from the mutant-transformed foci contained integrated mutant DNA, expressed an SV40 tumor antigen (small-t), and exhibited a range of transformed phenotypes, which included the ability to grow while suspended in soft agar. We also present evidence that these mutants are defective for abortive transformation in an assay that tested the transient loss of anchorage dependence. Their ability to stably transform, contrasted with their inability to abortively transform at detectable levels, raises the possibility that the mechanism by which these mutants transform may be different from that of wild-type SV40.

To study the simian virus 40 (SV40) functions required for cellular transformation, we have constructed a series of SV40 deletion mutants. We report here our experiments with the mutants F20*dl* and 536*dl* that lack over half of the early-region sequences normally used to encode the SV40 large-T antigen. We have described the construction of two deletion mutants, F8*dl* and F20*dl*, elsewhere (12). These mutants have deletions between 0.168 and 0.424 and about 0.41 map units, respectively (Fig. 1). In place of the deleted SV40 sequences, both mutant genomes contain a small amount of monkey DNA which was fortuitously acquired in place of the deleted SV40 sequences during the initial growth of these mutants in monkey cells. Although roughly similar in size, the monkey DNA inserts in F8*dl* and F20*dl* are different in sequence. We constructed the mutant 536*dl* by digesting the DNA of F8*dl* with the restriction enzyme *Hin*fI to remove the sequences between the single *Hin*fI site in the inserted monkey DNA and the *Hin*fI site in the SV40 DNA at 0.536 map units (Fig. 1). Thus 536*dl* retains only 83 base pairs of monkey DNA and has a deletion of the SV40 sequences between 0.168 and 0.536 map units (Fig. 2).

We cloned the DNA of F20*dl* and 536*dl* into pBR322 at the *Bam*HI site and used the cloned DNA (pF20*dl* and p536*dl*) to infect mouse C3H10T1/2 cells for a focus assay (8). Both mutants induced the formation of transformed foci in assays in which no foci arose on mock-infected monolayers (Table 1). However, the efficiency of transformation with both mutants was only about 0.2% of that of cloned wild-type SV40 DNA. Foci arising on monolayers infected with pF20*dl* and p536*dl* were picked and expanded into cell lines. High-molecular-weight DNA was extracted from these cells (4) and analyzed for the presence of integrated SV40 DNA by the method of Southern (14). Figure 3 shows the results of an experiment in which cellular DNA was digested with the enzyme, *Eco*RI, subjected to electrophoresis on a 0.7% agarose gel, and blotted onto a nitrocellulose filter. When this *Eco*RI-digested DNA was probed with nick-translated wild-type SV40 DNA (9), bands of SV40-specific DNA could be detected in all four mutant-transformed cell lines. In contrast, when the cellular DNA was subjected to the same kind of analysis without *Eco*RI digestion, no SV40-specific bands were detected. Thus all four mutant-transformed cell lines contain integrated SV40 DNA sequences.

Next we labeled mutant transformants with [^{35}S]methionine and immunoprecipitated labeled proteins with anti-SV40 tumor serum. Figure 4 shows fluorograms of these precipitates after electrophoresis on a sodium dodecyl sulfate-polyacrylamide gel. As expected, we could detect no large-T protein in cells transformed by pF20*dl* or p536*dl*. However, we did observe a protein that comigrated with small-t in immunoprecipitates from all mutant-transformed lines. We have not detected truncated forms of large-T

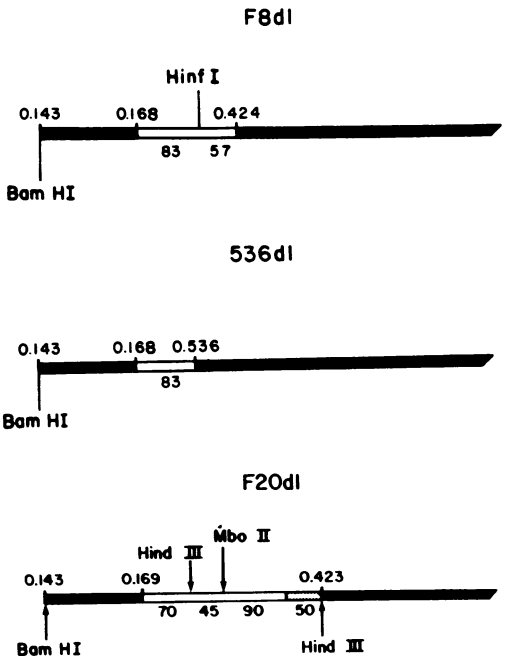

FIG. 1. Restriction enzyme map of the mutated regions of F8*dl*, 536*dl*, and F20*dl*. SV40 DNA is indicated by the black line, monkey DNA by the white line, and sequences that might be either monkey or SV40 by the stippled line. SV40 map units are shown above the line, and numbers of base pairs are given below the line. For F8*dl* and 536*dl*, the endpoints of the SV40 DNA and the sizes of the monkey DNA inserts were determined by sequence analysis. For F20*dl*, these parameters are only approximate since this mutant was mapped by restriction enzyme digestion followed by gel electrophoresis.

in any of the transformants, even with pulses as short as 1 h.

We then tested the mutant-transformed cell lines to determine whether or not they exhibited the transformed phenotypes commonly associated with wild-type SV40 transformants. All four mutant-transformed lines can grow in low serum and to high saturation densities (Table 2). In addition, three of the lines are able to grow while

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                0.536
5'  act  gAT  TCC  TTT  CAA  TCA  TCT  GAG  TGA  GCC  CAG

    TGC  GAT  CTG  AAG  GGT  CCC  TAC  AGG  TGG  AAG  AGG

                                        0.169
    CAG  TGG  CCA  GGA  TCG  CGG  Ttt  aac   3'
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FIG. 2. Sequence of monkey DNA insert in 536*dl*. SV40 map units shown above the line indicate the endpoints of the deleted SV40 sequences. Monkey DNA sequences are shown in large type. SV40 sequences are in small type. Translation termination codons are underlined (7).

TABLE 1. Focus assay with pF20*dl* and p536*dl* DNA

Type of DNA[a]	Foci per μg of DNA per 10^6 cells[b]	Efficiency of transformation relative to wild-type DNA[c]
Salmon sperm	0	0
pF20*dl*	0.13	0.26
p536*dl*	0.08	0.16
pWT	50	100

[a] pWT indicates cloned wild-type DNA.

[b] We seeded 35-mm plates with 2×10^5 10T1/2 mouse cells and infected them the same day with cloned DNA using a modification (18) of the calcium phosphate technique (3). About 16 h after the infection, which was carried out at 37°C, we changed the medium (Dulbecco modified Eagle medium–10% fetal calf serum) and transferred the cultures to 39.5°C. About 48 h after infection we trypsinized the cultures and replated the cells onto 60-mm dishes. Thereafter, we changed the medium every fourth day. Foci were counted without staining at 3 weeks.

[c] (Foci per microgram/foci per microgram of wild-type DNA) × 100.

suspended in soft agar and thus can be considered to be fully transformed (10).

In our hands, C3H10T1/2 cells are very well behaved in focus assays, and spontaneous foci are rarely observed on mock-infected monolayers. In addition, all four cell lines derived from foci on F20*dl*- and 536*dl*-infected cultures ex-

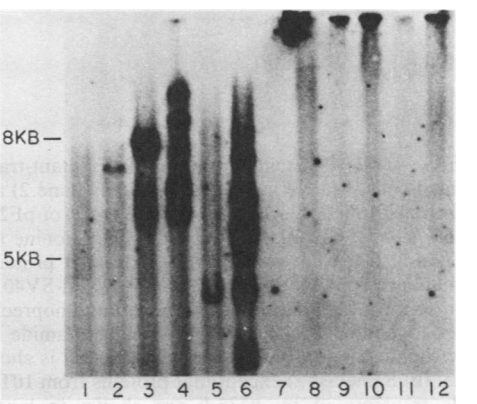

FIG. 3. SV40-specific DNA in mutant-transformed cells. We extracted high-molecular-weight DNA from untransformed 10T1/2 cells (lanes 1 and 7), wild-type-transformed 10T1/2 cells (lanes 2 and 8), 10T1/2 cells transformed by pF20*dl* (Cl55, lanes 3 and 9; Cl56, lanes 4 and 10), or cells transformed by p536*dl* (Cl110, lanes 5 and 11; Cl134, lanes 6 and 12). Cellular DNA digested with *Eco*RI (lanes 1 to 6) or undigested cellular DNA (lanes 7 to 12) was subjected to electrophoresis on a 0.7% agarose gel, blotted onto a nitrocellulose filter, and probed with nick-translated wild-type SV40 DNA according to Southern (14).

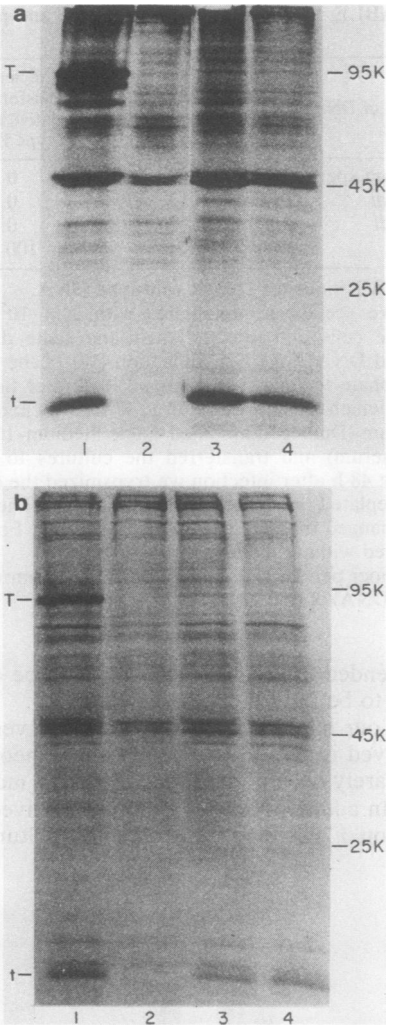

FIG. 4. SV40-specific proteins in mutant-transformed cells. (a) We labeled 10T1/2 cells (lane 2) and cells transformed by wild-type SV40 (lane 1) or pF20*dl* (Cl55, lane 3; Cl56, lane 4) with [^{35}S]methionine (0.4 mCi/ml, 1,000 Ci/mmol) for 2 h, extracted proteins, immunoprecipitated these proteins with anti-SV40 tumor serum (2), and subjected these immunoprecipitates to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6). A fluorogram of this gel is shown here. (b) Same as (a) except that proteins from 10T1/2 cells transformed by p536*dl* are shown in lane 3 (Cl110) and lane 4 (Cl134).

press an SV40 tumor antigen. Thus, if the foci we picked from mutant-infected monolayers were rare spontaneous transformants, these spontaneously transformed cells would also have to express an SV40 tumor antigen to explain our results. Since spontaneous transformants rarely arise on monolayers of 10T1/2 cells, we can assume that each spontaneous focus results from a single cell that has lost growth control. Therefore, we could evaluate the frequency with which a spontaneous transformant might mimic our results if we knew the probability that a cell on an infected monolayer would express a tumor antigen. To estimate this frequency, we infected monolayers of 10T1/2 cells exactly as we would for a focus assay, using an amount of cloned wild-type SV40 DNA equal to that used in assays of mutant DNA. We substituted wild-type DNA for mutant DNA because we wished to use immunofluorescent staining with anti-SV40 tumor serum to evaluate the fraction of the cells expressing a tumor antigen. Cells infected with mutant DNA could not be used for this experiment because they do not exhibit nuclear fluorescence. Table 3 shows that fewer than 1% of the wild-type-infected cells express large-T after a few generations. Thus, if spontaneous foci did arise on monolayers infected according to our standard protocol, we would expect that fewer than 1% of these foci would express an SV40 tumor antigen.

TABLE 2. Phenotypes of mutant-transformed cell lines

Clone	Transformed by	Growth in low serum[a]	Saturation density (cells/cm^2)[b]	Growth in agar[c]
10T1/2	Untransformed	−	5×10^4	−
4	Wild type	+	1.9×10^5	±
98	Wild type	+	3.6×10^5	+
55	F20*dl*	+	2.0×10^5	+
56	F20*dl*	+	2.8×10^5	+
110	536*dl*	+	2.0×10^5	±
134	536*dl*	+	1.6×10^5	−

[a] We plated cells from each line to be tested at 10^4 cells per 35-mm dish in Dulbecco modified Eagle medium (DME)–10% fetal calf serum. The next day, we washed the cells and fed them with DME–1% fetal calf serum. At 4 and 7 days after the seeding, we trypsinized and counted cells in each culture. Untransformed 10T1/2 cells stopped growing by day 4 after seeding. Transformed cells were defined as those that continued to grow after day 4.

[b] We plated cell lines to be tested in 35-mm dishes at a density of 10^5 cells per dish in DME–10% fetal calf serum. Every 4 days we replaced the medium with fresh DME–10% fetal calf serum. We counted cells on replicate plates every few days until saturation was reached or until the transformed cells began to detach from the monolayer. The detachment of transformed cells made an exact quantitation of the saturation density difficult.

[c] We trypsinized cells to be tested, suspended them in 0.32% agar–DME–13% fetal calf serum, and plated the suspension in 60-mm dishes. After incubating the plates at 39.5°C for 14 days, we examined the cultures microscopically for the presence of colonies: −, no colonies; ±, a few small colonies; +, many colonies, some large.

TABLE 3. Tumor antigen expression

Generations after infection	% T-antigen-positive cells[a]
2	3.7
4	0.7
6	0.8
8	0.9

[a] We infected 10T1/2 cells with 6 μg of cloned wild-type SV40 DNA per plate according to the protocol described in Table 1 except that cover slips were included in some of the dishes. Two days after we infected the cells, we fixed the cover slips and replated the cells onto three new dishes, two of which contained cover slips. Every 2 days, cells were replated and cover slips were fixed. We used anti-SV40 tumor serum to detect T-antigen-positive nuclei by indirect immunofluorescence as described elsewhere (11).

Since the probability is less than 10^{-8} that all four mutant-transformed foci could arise spontaneously and express small-t, we feel confident that we are not studying spontaneous transformants.

Taken together, these experiments indicate that both F20*dl* and 536*dl* can stably transform mouse cells in a focus assay. Moreover, cell lines expanded from these foci have integrated SV40 DNA, express an SV40 tumor antigen (small-t), and can exhibit a fully transformed phenotype. However, the efficiency with which these two mutants transform is drastically reduced relative to wild-type SV40.

We believe that the low level of stable transformation we observe with p536*dl* and pF20*dl* is not due to wild-type SV40 contamination for several reasons. All of our experiments were carried out with stocks of cloned mutant DNA that were prepared from well-isolated bacterial colonies. Moreover, we have repeated these focus assays with twice-cloned mutant DNA made by transfecting a different strain of *Escherichia coli* K-12 with our original plasmid DNAs and preparing a new stock of plasmid DNA from well-isolated bacterial colonies. Both preparations of DNA give low, but finite, levels of focus formation. In addition, since wild-type SV40 transformants can be expected to express large-T antigen, the fact that we detect only mutant-encoded small-t antigen in all four mutant-transformed lines argues against transformation by a wild-type contaminant.

It is also at least a formal possibility that the monkey sequences in F20*dl* and 536*dl* are responsible for their ability to transform. We consider this possibility to be remote for several reasons. These mutants are SV40-monkey hybrids and in this sense are analogous to the adenovirus-SV40 hybrids which have been widely used for mapping various SV40 functions (17). In most adenovirus-SV40 hybrids, the SV40 proteins are translated from hybrid SV40-adenovirus mRNAs and likely represent fusions between adenovirus- and SV40-encoded peptides. The presence of these adenovirus sequences does not detract from the usefulness of the hybrids since they were selected only because they had SV40 inserts. Therefore, it would be very unlikely that the flanking adenovirus sequences would encode the unselected SV40 functions that these hybrids have been used to map. By analogy, our SV40-monkey hybrid viruses were selected for study only because they had large early-region deletions. Thus, it would be extremely unlikely that the monkey sequences acquired by two mutants in completely unrelated events would confer on both the unselected property of being able to transform. Further, the monkey insert in 536*dl* is only 83 base pairs in length, and a sequence analysis (Fig. 2) of this DNA reveals that it contains no initiator codons and that it has translational termination codons in all three reading frames. Thus on the basis of size and sequence, it is extremely unlikely that the monkey DNA insert in 536*dl* is responsible for its ability to transform.

As was first suggested by Stoker (15), the functions required for transformation by SV40 can be divided, at least formally, into two classes. The maintenance functions are required to maintain cells in a transformed state and are responsible for the expression of the transformed phenotype. The other class of functions, the initiation functions, are needed for the efficient integration of SV40 DNA into the cellular genome. This integration event, facilitated by the initiation functions, is required if the transformed phenotype is to be stably expressed, generation after generation. Accordingly, our mutants might stably transform poorly either because of a defective initiation function or a defective maintenance function or both. To examine these possibilities, we assayed the ability of F20*dl* and 536*dl* to induce the transient loss of anchorage independence. When SV40 infects nonpermissive cells (e.g., rat fibroblasts), a relatively large proportion of these cells (typically a few percent) initially manifest a transformed phenotype such as growth in soft agar. This abortive transformation continues for several generations (as a result of the expression of the SV40 maintenance functions), but ultimately, only a small fraction of the abortively transformed cells (less than 1%) becomes stably transformed. These stable transformants derive from those few abortively transformed cells in which viral DNA has been successfully integrated into the cellular genome (due to the action of the SV40 initiation functions). Thus, while both the maintenance and initiation functions are

required for stable transformation, only the expression of the SV40 maintenance functions is necessary for abortive transformation (16). Therefore, if our deletion mutants were defective in the initiation but not in the maintenance of transformation, we would expect them to abortively transform as efficiently as wild-type SV40.

To test this, we infected rat F111 monolayers with cloned F20*dl* and 536*dl* DNA using a modification (18) of the calcium phosphate precipitation technique (3). The next day, we trypsinized these infected cells and suspended them in soft agar. Eight days later, we counted the number of small colonies of abortively transformed cells. Table 4 shows that no abortive transformation could be detected with either mutant. We have obtained the same negative result in a similar assay using 10T1/2 mouse cells (data not shown). Thus neither mutant is able to transiently induce anchorage-independent growth with wild-type efficiency. Since this abortive transformation assay tests for the expression of SV40 functions required for the maintenance of transformation, we conclude that both mutants are defective in the ability to maintain transformation. These mutants may also be defective in functions required for the initiation of transformation, but these functions are not tested in an abortive transformation assay.

Thus we are faced with the apparent paradox that these two mutants are able to stably transform mouse cells and yet are deficient in at least one of the functions required for the maintenance of transformation. We have reported elsewhere on transformation experiments carried out with another early-region deletion mutant, F8*dl* (Fig. 1). This mutant transforms mouse cells in a focus assay with a frequency that is only 1 to 2% of that of wild-type SV40 (13) but abortively transforms rat cells with wild-type efficiency (L. Sompayrac and K. J. Danna, submitted for publication). Our interpretation of these results is that F8*dl* has intact maintenance functions but is defective in one or more of the initiation functions. F20*dl* and 536*dl* are clearly different from F8*dl* with respect to transformation, and the interpretation of our experiments with these two mutants is much more difficult.

TABLE 4. Abortive transformation assay

Type of DNA	Amt of viral DNA per dish (μg)[a]	% Abortive transformation[b]
Salmon sperm	0	0.02
pF20*dl*	6	0.02
p536*dl*	6	0.02
pWT	6	0.2

[a] We seeded 35-mm plates with 3×10^5 rat F111 cells (a kind gift from Tom Benjamin), and about 2 h later we infected them with DNA using the modified calcium phosphate technique. We used salmon sperm carrier DNA when necessary to make the total amount of DNA (viral plus carrier) about 6 μg per dish. The infections were carried out at 37°C. At 16 h after infection, we changed the medium on the cultures. The cells were trypsinized 1 day after infection, and one-third of each culture was plated into 0.34% agar–Dulbecco modified Eagle medium–17% calf serum on 100-mm dishes. Eight days later we counted colonies of abortively transformed cells.

[b] Percentage of cells that grew in agar to give colonies with more than three cells. Each entry is the average of the results of duplicate infections. Approximately 5,000 cells were scored for each entry.

It is possible that the maintenance functions encoded by F20*dl* and 536*dl* are so weak that they induce abortive transformation with less than 10% the efficiency of wild type (our limit of detection in the abortive transformation assay) but are strong enough to induce stable transformation on rare occasions. If so, we might expect these rare transformants to overproduce the early mutant proteins. This does not appear to be the case, however, since mutant-transformed cells do not grossly overproduce early proteins as judged by the amount of small-t protein detected (Fig. 4). Another possibility is that a very small fraction of the 10T1/2 cells might be able to supply cellular functions that could complement the weak maintenance functions of the mutants to elicit the transformed phenotype. Again, however, we might expect this type of complementation to be dose dependent, favoring the overproduction of early mutant proteins. Still, our experiments do not rule out either of these explanations.

An alternative explanation for these results is that F20*dl* and 536*dl* transform by a mechanism different from that of wild-type SV40, a novel mechanism that may not be protein mediated. For example, stable transformation with F20*dl* and 536*dl* might be caused by fortuitous integration near a cellular oncogene, thereby influencing its expression. Such a mode of transformation has been observed with avian lymphoid leukosis virus (5). An integration event of this type can be expected to be rare and might be consistent with the low level of stable transformation observed with F20*dl* and 536*dl*. Although this model agrees with our data, we have no direct evidence to support it.

It is also intriguing that a protein that comigrates with authentic small-t is observed in each of the four transformed cell lines. Indeed, we have independent evidence that small-t can perform at least one, but not all, of the functions necessary for the maintenance of transformation (Sompayrac and Danna, submitted for publication). However, since we have examined only a small number of mutant-transformed lines, the

presence of small-t in these lines could certainly be fortuitous.

Clayton et al. have recently reported similar results with a fragment of SV40 DNA rescued from an SV40-transformed mouse cell line (1). This fragment, which encodes only the N-terminal half of large-T antigen, is able to transform rat cells in a focus assay with a frequency that is greatly reduced relative to that of wild-type SV40. Since they do not report whether this DNA fragment is able to induce abortive transformation, we cannot infer that their fragment and our mutants are completely analogous in their transforming properties.

In conclusion, we do not understand why the deletion mutants F20*dl* and 536*dl* are able to stably transform mouse cells even though they lack over half of the sequences normally used to encode large-T. The low transformation efficiency that we observe cannot simply be due to a defective initiation function, since both mutants are defective for abortive transformation. Because these mutants do not resemble wild-type SV40 with respect to their transformation properties, it is possible that they use a mode of transformation different from that of wild-type SV40.

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