Simian Virus 40 Sequences Between 0.168 and 0.424 Map Units Are Not Required for Abortive Transformation

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We have isolated a simian virus 40 deletion mutant, *F8dl*, that lacks the sequences from 0.168 to 0.424 map units. The deleted sequences represent over 60% of the coding region for large T antigen. Despite this deletion, *F8dl* abortively transformed rat cells as efficiently as wild-type simian virus 40. From this result, we conclude that the region of the simian virus 40 genome between 0.168 and 0.424 map units is not essential for abortive transformation. Since abortive transformation requires the expression of the simian virus 40 maintenance functions, we also infer that the sequences deleted from *F8dl* are not required to maintain transformation.

Strong evidence now exists that the action of the large tumor antigen (large T antigen; molecular weight, 85,000 [85K]) of simian virus 40 (SV40) is sufficient to transform cells in culture (1, 4, 5, 14, 21, 22). However, because large T antigen is multifunctional, it has been impossible so far to identify the activity responsible for its ability to transform (2, 9, 10, 12, 13, 18, 20, 23, 27-29). In an attempt to overcome this obstacle, we have constructed several SV40 mutants, each of which has an extensive deletion in the early region sequences that encode the carboxy-terminal half of large T antigen (25). It has been shown that all the SV40 *tsA* mutations map in this region and that these mutations affect many of the SV40 lytic functions (30). Our idea, then, was that mutants lacking the sequences necessary to encode the C-terminal part of large T antigen should have lost many lytic activities not directly required for transformation. We hoped to isolate mutants that still could transform despite this large deletion. Such mutants would be useful tools for simplifying the search for the SV40 maintenance functions.

In 1968, Michael Stoker reported experiments in which he infected baby hamster kidney cells with polyoma virus and suspended the infected cells in methylcellulose (26). He observed that a large proportion of the infected cells continued to grow and divide, even though they were suspended without anchorage. However, for most of these cells, this anchorage-independent growth was ephemeral, lasting just a few generations, and only a small percentage of the abortively transformed cells went on to produce large colonies. Stoker suggested that the stable

transformants originated from those abortively transformed cells in which viral DNA had been successfully integrated into the cellular genome and concluded that the expression of the transformed phenotype does not require a stable association between viral and cellular DNA. More recent experiments have demonstrated that, in all ways tested (e.g., growth in low serum, altered morphology, anchorage independence, susceptibility to agglutination by lectins, increase in mitotic rate), abortively transformed cells exhibit the same transformed phenotypes as their stably transformed counterparts (30). Based on the results of Stoker, the functions required for stable transformation can be divided into two groups. The functions required to facilitate the stable association of viral and cellular genomes are called the initiation functions. The remaining transformation functions are defined as maintenance functions, and they are responsible for the expression of the transformed phenotype. By this analysis, stable transformation requires the expression of both initiation and maintenance functions, whereas abortive transformation requires only the maintenance functions. Therefore, an abortive transformation assay can be used to test for the expression of the SV40 maintenance functions independent of the expression of the initiation functions.

We have reported elsewhere that one of our early region deletion mutants, *F8dl*, is able to transform mouse cells stably in a focus assay, even though this mutant lacks the SV40 sequences between 0.168 and 0.424 map units (25a). Cell lines expanded from *F8dl*-trans-

formed foci contain integrated mutant DNA, express mutant tumor antigens, and exhibit the phenotypes commonly associated with SV40 transformants, including the ability to grow while suspended in soft agar. However, the frequency with which F8*dl* induces focus formation is only about 2% that of wild-type SV40. One possible explanation for this low efficiency of stable transformation (focus formation) is that this mutant might be defective in one of the functions required to initiate transformation. To test this idea, we performed abortive transformation assays with cloned F8*dl* DNA, and we report here that this mutant abortively transforms rat cells to anchorage independence with wild-type efficiency. This result implies that in the mutant F8*dl*, the SV40 maintenance functions are still intact and the SV40 sequences between 0.168 and 0.424 map units are not required for the maintenance of transformation.

MATERIALS AND METHODS

Virus and cells. For our experiments, we used SV40 wild-type strain 776. The construction of the double mutant *dl884/tsB4* and of the early region deletion mutant F8*dl* is described in detail elsewhere (25). We grew BSC-1 monkey cells in Dulbecco modified Eagle medium supplemented with 7% calf serum and grew F111 rat cells (a kind gift from Tom Benjamin) and COSI monkey cells (a kind gift from Y. Gluzman) in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The titer of mutant virus stocks grown on COSI cells was estimated by comparing the cytopathic effects observed when COSI cells were infected with dilutions of either mutant virus or our standard wild-type virus of known titer.

DNA infections. For DNA infections of F111 cells, we used a modification (31) of the calcium phosphate precipitation method of Graham and van der Eb (7). For DNA infections of BSC-1 and COSI cells, we used a modification (24) of the DEAE-dextran method of McCutchan and Pagano (16).

Immunoprecipitations. We used the procedure described by Collett and Erikson (3) with two small modifications. We preincubated our protein extracts with the protein A-containing bacterium *Staphylococcus aureus* for 10 min, spun out the precipitates, and used the supernatant fluid for subsequent precipitations with anti-SV40 tumor serum. In addition, we sonicated our precipitates for 5 min in a jeweler's sonicator during each of the four final washes (suggested by S. Tevethia, personal communication). These two modifications greatly reduced the background that results from nonspecific binding of proteins to staphylococci.

Abortive transformation assays. For virus-induced abortive transformation, we infected 10^5 F111 cells in suspension (1) for 1 h at 37°C and plated these cells into 0.34% agar-Dulbecco modified Eagle medium-17% calf serum on 100-mm dishes at 37°C. Eight days later, we counted the number of small colonies of abortively transformed cells.

For abortive transformation assays that began with

a DNA infection, we seeded 35-mm plates with 3×10^5 F111 cells apiece and infected the cells with DNA about 2 h later by the modified calcium phosphate method. We used salmon sperm DNA as carrier when necessary to make the total amount of DNA (viral plus carrier) about 6 µg per dish. About 16 h after infection, we trypsinized these cells and suspended one-third of them in the same type of agar used for virus-infected cells. Eight days later, we counted colonies of abortively transformed cells.

RESULTS

We constructed the mutant F8*dl* by partially digesting wild-type SV40 DNA with the restriction enzymes *Hpa*I and *Hind*III to yield linear DNA molecules from which the sequences between 0.168 and 0.424 map units had been deleted (25). These linear molecules, which have intact late regions, together with supercoiled DNA from a *dl884/tsB4* double-mutant helper, were used to infect BSC-1 monkey cells in a complementation plaque assay at 40°C (17). A virus stock from one plaque that contained both mutant and helper DNA was grown, and the DNA of the deletion mutant was gel purified and cloned into plasmid pBR322. A sequence analysis (15) revealed that when the deleted linear molecules were circularized during the initial infection of monkey cells, 140 base pairs of monkey DNA were fortuitously inserted at the site of the SV40 deletion (Fig. 1). To examine the early proteins encoded by F8*dl*, we infected BSC-1 cells with a mixed lysate of F8*dl* and the *dl884/tsB4* helper. [³⁵S]methionine-labeled proteins were extracted 48 h after infection, immunoprecipitated with anti-SV40 tumor serum, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Figure 2 shows that F8*dl* makes a protein that comigrates with authentic small t antigen (the helper does not make small t) as well as several new proteins that presumably are truncated forms of large T antigen.

Because abortive transformation experiments have always been done with virus infections (5, 26), we began by making a virus stock of the mutant F8*dl*. To do this, we excised cloned F8*dl* DNA from its plasmid and used this DNA to infect COSI cells, a monkey cell line that can supply the functional large T antigen necessary for a lytic infection with F8*dl* (6). In parallel, we prepared a lysate from mock-infected COSI cells to use as a control. We infected Fischer rat F111 cells in suspension with either the F8*dl* viral lysate or control lysates, suspended the infected cells in soft agar, and incubated the cultures at 37°C. Eight days later, we counted the small colonies of abortively transformed cells that appeared (Table 1) and found that F8*dl* virus grown in COSI cells abortively transformed rat

FIG. 1. (A) Restriction enzyme map of the mutated region of *F8dl*, showing SV40 DNA in black and monkey DNA in white. SV40 map units are shown above the line; numbers of base pairs are given below the line. (B) Sequence of monkey DNA insert in *F8dl*. SV40 map units 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.

cells with an efficiency comparable to that of wild-type SV40 virus.

There are several disadvantages in using viral lysates for abortive transformation experiments. First, it is time consuming to prepare high-titer virus stocks beginning from a DNA infection. Second, because lysates of mock-infected cells can sometimes stimulate cells to grow in agar (Table 1), it is difficult to be sure exactly what kind of mock viral infection to use. Finally, because COSI cells have an integrated SV40 genome, it is possible that in the multiple rounds of infection necessary to obtain a high-titer viral lysate, recombination might occur between mutant DNA and the integrated SV40 genome, producing a novel viral recombinant.

To avoid the difficulties of using virus infections, we decided to try to assay for abortive transformation by infecting rat cells directly with cloned DNA. We used the calcium phosphate technique to infect F111 monolayers with either cloned wild-type DNA (pWT) or cloned mutant DNA (pF8dl). Table 2 shows that infection with cloned wild-type DNA (about $6 \mu\text{g}$ per 3×10^5 rat cells) induced about the same level of abortive transformation as a virus infection at a multiplicity of infection of 200 PFU per cell. In addition, the number of abortively transformed colonies decreased with decreasing amounts of viral DNA, indicating that saturating levels of DNA were not used. This experiment also shows that infection with pF8dl DNA resulted in at least as many abortively transformed colonies as infection with an equal amount of pWT DNA, thus confirming our previous result obtained

with a virion infection. The size of the abortively transformed colonies in these assays was small, usually 3 to 16 cells. Nevertheless, the results we obtained are quite reproducible, and the

FIG. 2. Tumor antigens in mutant-infected cells. Subconfluent monolayers of BSC-1 cells were infected with *d/884/tsB4* helper virus (lane 1), wild-type SV40 virus (lane 2), or a mixed lysate of *F8dl* virus plus *d/884/tsB4* helper (lane 3). About 48 h after infection, cells were washed and labeled with [^{35}S]methionine (1,000 Ci/mmol, 400 $\mu\text{Ci}/\text{ml}$) for 2 h in methionine-free medium. Labeled proteins were extracted, immunoprecipitated with anti-SV40 tumor serum, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6 to 15% gradient), and autoradiographed (11).

TABLE 1. Abortive transformation of virus-infected cells

Expt	Virus infection ^a	Multiplicity of infection	Abortive transformation	
			% ^b	Relative efficiency ^c
1	Mock lysate	0	0.5	
	F8dl	140	1.9	1.9
	Wild-type	150	1.2	1.0
2	Mock lysate	0	.04	
	F8dl	170	0.4	0.8
	Wild-type	200	0.6	1.0

^a For experiment 1, we used a crude lysate of either mock-infected or virus-infected COSI cells. For experiment 2, we pelleted both the viral and mock lysates by ultracentrifugation and suspended these pellets in Dulbecco modified Eagle medium plus 1% calf serum. This partial purification procedure greatly decreased the level of abortive transformation induced by mock lysates.

^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 observed for each entry.

^c Percentage of abortive transformation relative to wild-type SV40, normalized to equal input multiplicities with mock-lysate background subtracted.

number of abortively transformed colonies arising from pWT or pF8dl infections was routinely 10- to 50-fold higher than with mock DNA infections. In addition, infections with pF8dl yielded abortively transformed colonies with the same morphology and size distribution that we observed with pWT infections.

TABLE 2. Abortive transformation of cells infected with cloned DNA

Expt	Type of DNA	Viral DNA (μ g) per dish	Abortive transformation	
			% ^a	Relative efficiency ^b
1	Salmon sperm	0	0.02	
	pF8dl	6	1.8	1.5
	pWT	2	0.4	1.0
2	Salmon sperm	0	0.06	
	pF8dl	6	0.9	1.3
	pWT	6	0.7	1.0
	pWT	1.5	0.3	1.5

^a Percentage of cells that grew in agar to give colonies with more than three cells (experiment 1) or more than two cells (experiment 2). Each entry is the average of the results of duplicate infections. Approximately 5,000 cells were observed for each entry.

^b Percentage of abortive transformation relative to that obtained with pWT DNA, normalized to equal amounts of viral DNA.

FIG. 3. The genomes of mutants of C81 and C98. Stippled bars represent the coding regions for large and small tumor antigens. Solid lines represent early region DNA present in mutants. Wavy lines represent the 140-base-pair monkey DNA insert.

Since we expected the maintenance of transformation to be mediated by an SV40-encoded protein, we wanted to be sure that deleting most of the remaining sequences for large T and small t antigens from F8dl would abolish its ability to transform abortively. To this end, we constructed two new mutants, C81 and C98. We lightly digested pF8dl DNA with the restriction endonuclease *TaqI* to obtain linear molecules cut once at the single SV40 site at 0.564 map units, digested these molecules lightly with the exonuclease *Bal31* to remove about 300 base pairs from each end, and religated the products to obtain circular molecules. This DNA was used to transfect *Escherichia coli*, and bacterial colonies were selected for study. We used restriction enzymes to analyze plasmid DNA from these colonies and determined that two plasmids, pC81 and pC98, contained F8dl DNA with the desired deletions (Fig. 3). Both C81 and C98 retained (i) intact late regions (including the 72-base-pair repeats); (ii) all early region control sequences, including the initiator codons for large T and small t antigens; (iii) all the monkey DNA sequences present in F8dl; and (iv) the early region polyadenylation signals. However, both mutants lacked donor and receptor splice junctions for both large T and small t antigens as well as most of the coding sequences for these two proteins. In experiments with these mutants (Table 3), we found that neither C81 nor C98 was able to abortively transform F111 cells under conditions in which pF8dl transformed with high efficiency. Therefore, at least some of the small t or large T antigen sequences present in F8dl are required for abortive transformation.

DISCUSSION

Taken together, these experiments lead us to the following conclusions. (i) Since F8dl abortively transforms rat cells as efficiently as wild-

TABLE 3. Abortive transformation induced by mutant virus DNA

Expt	Type of DNA	Viral DNA (μ g) per dish	% Abortive transformation ^a
1	Salmon sperm	0	<0.02
	pC98	6	<0.03
	pF8dl	6	1.2
2	Salmon sperm	0	0.02
	pC81a ^b	6	<0.03
	pC81b ^b	6	<0.03
	pC98	6	<0.03
	pF8dl	6	1.0

^a 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 observed for each entry.

^b pC81a and pC81b are two different preparations of pC81 from independently isolated bacterial colonies.

type SV40, the SV40 sequences between 0.168 and about 0.424 map units are not required for abortive transformation. (ii) Since abortive transformation requires the expression of the SV40 maintenance functions, the sequences deleted from F8dl are not required to maintain transformation.

The negative results obtained for the mutants C81 and C98 suggest that one or more of the early proteins encoded by F8dl is required for the maintenance of transformation. In addition, since both C81 and C98 contain all the promoter and enhancer sequences contained in F8dl, we can conclude that F8dl does not transform by a promoter insertion mechanism (8).

F8dl is an SV40-monkey hybrid virus, having acquired 140 base pairs of monkey DNA during the early stages of mutant isolation. We believe that the monkey sequences are not directly responsible for the ability of F8dl to transform abortively for several reasons. First, the mutant F8dl was selected for study only because it had a large deletion in its early region. It would therefore be quite unlikely that the monkey sequences, fortuitously acquired, would be able to confer the unselected property of being able to transform abortively. Second, the mutants C81 and C98 contain the entire monkey insert present in F8dl, and yet neither of these mutants is able to transform abortively. Finally, we have sequenced the monkey insert in F8dl and have determined that it is only 140 base pairs long (Fig. 1). Because this short segment of monkey DNA contains six translational termination codons, having at least one in each reading frame, it is very improbable that the monkey DNA insert could encode a functional peptide.

To date, most attempts to understand the

mechanism by which SV40 transforms cells have focused on the activities associated with large T antigen. However, since large T antigen is a multifunctional protein, most of its functions probably have nothing to do with its ability to transform. Because the mutant F8dl lacks a large part of the early region, it can be expected to have lost many activities of large T antigen. We have shown that F8dl is not able to replicate its own DNA (25) and that the early proteins encoded by F8dl do not stabilize the 53K nonviral tumor antigen (25a). Therefore, since F8dl is able to maintain transformation, we can infer that neither the large T viral DNA replication function nor the stabilization of the 53K protein by large T antigen is required for the maintenance of transformation. Experiments of this type, in which we identify large T antigen functions that F8dl cannot perform, should be very useful in discovering which activities of large T antigen are dispensable for the maintenance of transformation.

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