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Isolation and Characterization of Simian Virus 40 Early Region Deletion Mutants

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We constructed a *ts*B4/*dl*884 double-mutant helper virus and used it to isolate two simian virus 40 early region deletion mutants that lack about half of the DNA sequences normally used to encode the large tumor antigen (T). Both mutants make a normal-sized small t antigen, but neither mutant can replicate its DNA in the absence of a T^+ helper.

Two simian virus 40 (SV40) encoded proteins, large T and small t, can be detected early in the lytic infection of monkey cells and in cells transformed by this virus (7, 8, 14). Because these tumor antigens have 82 amino acids in common at their amino termini (9), it seems likely that some large T functions might also be performed by small t. Mutants with a temperature-sensitive large T antigen (*ts*A mutants) might be used to study such overlapping functions. However, some experiments with *ts*A mutants have produced ambiguous results, perhaps because these mutants make a large T protein that is somewhat active even at the restrictive temperature. To avoid the problem of leakiness of *ts*A mutants, we constructed SV40 deletion mutants that lack the sequences normally used to encode the carboxy-terminal half of large T but that retain all of the coding sequences for small t.

To isolate and propagate t^+T^- SV40 mutants, we constructed a *ts*B4/*dl*884 double mutant to use as helper. The *dl*884 parent does not make a detectable small t protein, although it makes a normal large T antigen (10). The other parent, *ts*B4, has a temperature-sensitive lesion in a structural gene (VP1) (15). A double mutant constructed from these two parental viruses would complement our t^+T^- deletion mutants by providing missing large T functions but would not make a detectable small t protein. Thus, in a mixed infection (mutant plus helper), we would be able to determine whether our deletion mutants were t^+. In addition, because of the *ts*B4 mutation, the double mutant could be used in complementation plaque assays to select for t^+T^- mutants that have intact late genes. We constructed the double mutant by ligating the early region of *dl*884 to the late region of *ts*B4 (Fig. 1). After plaque-purifying the mutant three times at 33°C, we examined its DNA to be sure that restriction enzyme markers characteristic of both parents were present (Fig. 2). We measured the ability of the mutant to form plaques at the restrictive and permissive temperatures and confirmed that it had the temperature-sensitive phenotype of the *ts*B4 parent. Finally, we used anti-SV40 tumor serum to immunoprecipitate [^{35}S]methionine-labeled proteins from extracts of cells infected with the *ts*B4/*dl*884 double mutant and detected no small t protein (Fig. 3).

To construct early region deletion mutants, we lightly digested wild-type SV40 DNA with the restriction endonuclease *Hin*dIII and gel-purified molecules cut at only one site. We then digested these unit-length linear molecules lightly with a second endonuclease, *Hpa*I, and gel-purified molecules of 70 to 80% genome length. We used these subgenomic linear fragments, together with DNA of the *ts*B4/*dl*884 double-mutant helper, to infect BSC-1 cells for a complementation plaque assay at 40°C (6, 11) and used gel electrophoresis to identify two plaques containing both helper DNA and mutant DNA of subgenomic length.

To test whether these two mutants could make small t, we used anti-SV40 tumor serum to immunoprecipitate [^{35}S]methionine-labeled proteins from BSC-1 cells infected with viral stocks grown up from these plaques. In these experiments, we were able to detect both large T (from the helper) and small t (from the mutants), thus confirming that both deletion mutants were t^+ (Fig. 3). In cells infected with one mutant, F8*dl*, we also detected two new proteins that were specifically immunoprecipitated with anti-SV40 tumor serum. These two proteins, which migrated with apparent molecular weights of about 37,000 and 18,000, were presumably truncated forms of large T encoded by the mutant. In contrast, we detected no truncated large T proteins in cells infected with the second mutant, F20*dl* (Fig. 3).

To separate mutant DNA from helper-virus

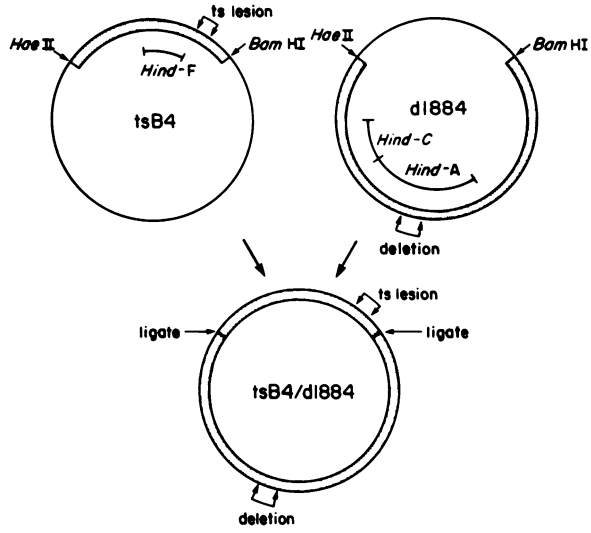

FIG. 1. Construction of the *ts*B4/*dl*884 double mutant. DNA from each of the two mutants, *ts*B4 and *dl*884, was digested with the enzymes *Bam*HI and *Hae*II. The large fragment from *dl*884 (which contains the *dl*884 deletion) and the small fragment from *ts*B4 (which contains the *ts*B4 temperature-sensitive mutation) were gel purified and ligated together to form a *ts*B4/*dl*884 double mutant. To infect cells with DNA, we used a modification (11) of the method of McCutchan and Pagano (5). Briefly, we exposed cells to DNA in Dulbecco modified Eagle medium–50 mM Tris (pH 7.2)–DEAE-dextran (200 μg/ml) at 37°C for 8 h. For complementation plaquing, we infected BSC-1 cells with helper DNA and linear DNA fragments in a molar ratio of 1:10 in a 40°C assay as described by Mertz and Berg (6).

DNA, we cloned gel-purified F8*dl* and F20*dl* DNA into pBR322 at the *Bam*HI site (1). The plasmids that contained these deletion mutants were designated pF8*dl* and pF20*dl*.

We sequenced the DNA of F8*dl* and F20*dl* beginning at the *Bam*HI site (0.143 map units) and found that the DNA sequence of each was colinear with wild-type SV40 DNA up to the *Hpa*I site (0.169 map units [4]). Unexpectedly, the sequences encountered on the far side of this site did not correspond to sequences found anywhere in the SV40 genome. To determine the size of this nonviral DNA, we made a restriction map of the early regions of the two mutant genomes. About 290 base pairs of nonviral DNA were inserted into the genome of F8*dl* between SV40 map units 0.169 and about 0.44 (Fig. 4A). F20*dl* had an insertion of about 230 base pairs of foreign DNA between 0.169 map units and about 0.41 map units (Fig. 4B). Although the segments of foreign DNA in these two mutants were similar in size, their restriction maps were quite different, indicating that different sequences were inserted.

To determine the origin of these DNA inserts, we digested F20*dl* DNA with the restriction endonuclease *Hin*dIII. This enzyme cuts once within the inserted DNA and also at map unit 0.423 in the SV40 genome to yield a fragment

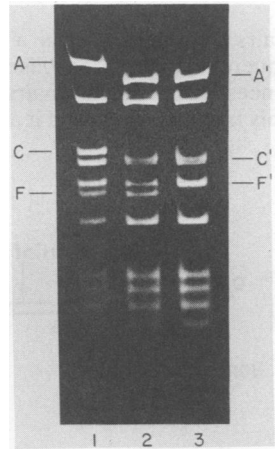

FIG. 2. Comparison of *Hin*dII plus *Hin*dIII digests of *ts*B4, *ts*B4/*dl*884, and *dl*884. Samples of DNA from the *ts*B4/*dl*884 double mutant and from each of its two parents were digested with *Hin*dII plus *Hin*dIII enzymes and subjected to electrophoresis on a 4% polyacrylamide gel. The gel was stained with ethidium bromide and photographed with UV illumination. Lane 1, *ts*B4 DNA; lane 2, *ts*B4/*dl*884 double-mutant DNA; lane 3, *dl*884 DNA. The double mutant had the *Hin*d-A and *Hin*d-C fragments characteristic of its *dl*884 parent and the *Hin*d-F fragment characteristic of its *ts*B4 parent.

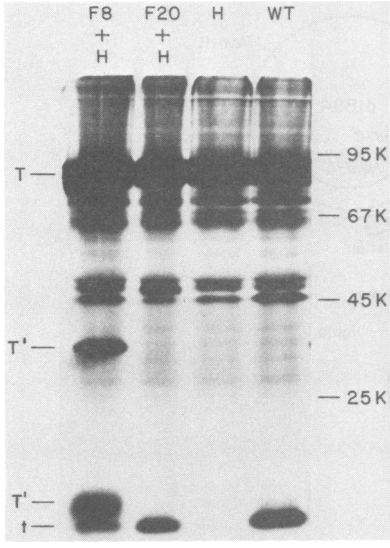

FIG. 3. Tumor antigens in mutant-infected BSC-1 cells. BSC-1 cells were infected with mixed lysates of either F8*dl* plus helper or F20*dl* plus helper, helper alone, or wild-type SV40. At about 48 h after infection, cells were labeled with [^{35}S]methionine (1,000 Ci/mmol, 400 μCi/ml) for 2 h. Labeled proteins were immunoprecipitated with anti-SV40 tumor serum, subjected to electrophoresis on a sodium dodecyl sulfate gradient polyacrylamide gel (6 to 15%), and autoradiographed. We did our immunoprecipitations as described by Collett and Erikson (2).

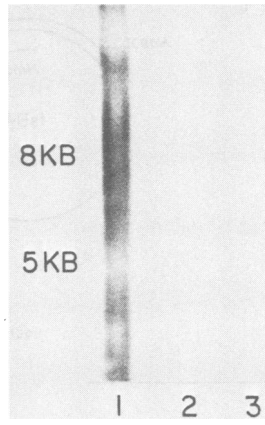

FIG. 5. Hybridization of inserted sequences in F20*dl*. The 185-base-pair *Hin*dIII fragment of F20*dl* that includes at least 135 base pairs of inserted DNA was gel purified, nick-translated, and hybridized to about 5 μg each of *Eco*RI-digested monkey DNA (lane 1), mouse DNA (lane 2), or rat DNA (lane 3) that had been subjected to electrophoresis on a 0.7% agarose gel and blotted onto a nitrocellulose filter (12). In a control experiment, we hybridized a wild-type SV40 DNA probe to an identical filter. This probe showed no detectable hybridization to monkey, mouse, or rat DNA in similar exposures (data not shown).

185 base pairs in length that is a mixture of inserted sequences (about 135 base pairs) and SV40 sequences (about 50 base pairs). We nick-translated this fragment and used it as a probe in a Southern blot analysis of *Eco*RI-digested DNA from monkey, mouse, and rat cells (12). The 175-base-pair fragment from F20*dl* hybridized strongly to monkey DNA but not to mouse or rat DNA (Fig. 5). In a similar experiment, an 80-base-pair fragment from the inserted DNA in F8*dl* also hybridized strongly to monkey DNA

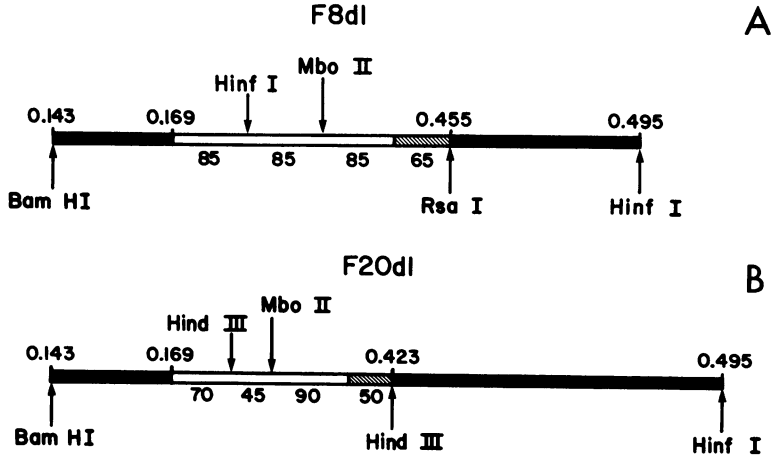

FIG. 4. Restriction enzyme maps of monkey DNA inserts in F8*dl* (A) and F20*dl* (B). Black areas indicate SV40 DNA; white areas indicate monkey DNA; stippled areas indicate sequences that might be either monkey or SV40 DNA. SV40 map units are shown above the line, and approximate numbers of base pairs are given below the line. Enzymes that did not cleave the inserted DNA in F8*dl*: *Hin*dIII, *Eco*RII, *Mbo*I, *Fnu*4HI, *Bgl*I, *Hin*cII, *Bam*HI, *Sal*I, *Rsa*I. Enzymes that did not cleave the F20*dl* insert: *Hin*fI, *Fnu*4HI, *Bgl*I, *Hin*cII, *Bam*HI, *Sal*I.

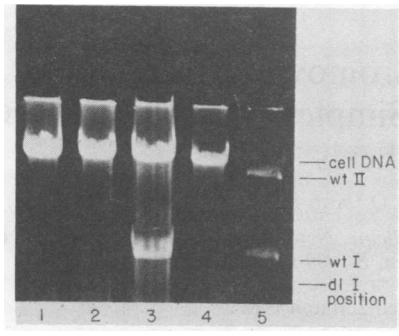

FIG. 6. Analysis of viral DNA replication in cells infected with F8*dl* or F20*dl*. We infected plates of BSC-1 monkey cells with equal amounts of F8*dl*, F20*dl*, or wild-type SV40 DNA that had been excised from their respective plasmids (0.07 $\mu g/10^5$ cells). After 24 h, we harvested one plate of wild-type infected cells by the Hirt procedure (3) as a control. After 72 h, we harvested the remaining plates. The Hirt supernatants were phenol extracted, ethanol precipitated, and subjected to electrophoresis on a 1.4% agarose gel. Lane 1, F8*dl*; lane 2, F20*dl*; lane 3, wild type at 72 h; lane 4, wild type at 24 h; lane 5, wild type form I and form II markers. A band of cellular DNA is present in each lane, as indicated.

but not to mouse DNA, rat DNA, or F20*dl* DNA (data not shown).

From these studies, we concluded that F8*dl* and F20*dl* have deletions that extend from 0.169 to about 0.44 and 0.41, respectively, and have insertions of about 260 base pairs of monkey DNA, probably acquired at the stage when linear SV40 DNA used to make these mutants was circularized during infection of BSC-1 monkey cells.

To test the ability of F8*dl* and F20*dl* to replicate their own DNAs, we excised the DNA of each mutant from its respective plasmid, religated it, and infected BSC-1 cells with these circular DNA molecules. Although large amounts of DNA replication could be detected in control cells infected with religated wild-type DNA, both mutants failed to synthesize DNA (Fig. 6).

In summary, we have constructed a *ts*B4/*dl*884 double mutant and have used it to isolate two early region deletion mutants of SV40. One mutant, F8*dl*, lacks the sequences between 0.17 and 0.44 map units, and the other, F20*dl*, lacks sequences between 0.17 and about 0.41 map units. Both mutants make a normal-sized small t and can replicate only when grown with a helper that supplies a functional large T. Thus, at least one activity commonly associated with large T antigen, the viral DNA replication function (13), is missing in these early region deletion mutants. Because they lack about half of the coding sequences for large T, our t^+T^- deletion mutants are likely to be less leaky than *ts*A mutants and therefore should be useful tools for studying functions that small t and large T may have in common.

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