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Flat Revertants of Temperature-Insensitive Transformants Induced by Simian Virus 40 *tsA* Mutants Lose Their Ability to Express T-Antigen

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Temperature-insensitive transformants that contained simian virus 40 sequences at only one or a few sites in the rat chromosome and that were induced by a temperature-sensitive *A* gene mutant of simian virus 40 were used to select flat revertants (revertants that had lost the transformed phenotype). The isolation was performed at the nonpermissive temperature so as not to select against temperature-sensitive transformants. Nonetheless, all of the revertants examined had lost their ability to express the T-antigen at both temperatures, and all contained rearrangements of the integrated simian virus 40 sequences. These results are most compatible with the hypothesis that the T-antigen of simian virus 40 is required for the maintenance of the transformed state even in temperature-insensitive cell lines.

Some cells transformed by simian virus 40 (SV40) *tsA* mutants are temperature sensitive (6), and some are not (for a recent review see reference 5). The basis for this difference is unclear. However, whether the T-antigen of SV40 (encoded by the *A* gene) has only one function or is multifunctional is immaterial. If only the replicative function of the T-antigen were temperature sensitive in *tsA* mutants, and the transformation function were temperature insensitive, then no cell line transformed by a *tsA* mutant should ever be temperature sensitive.

There are, therefore, only two likely explanations for the existence of temperature-sensitive and temperature-insensitive lines. The first is that temperature-insensitive lines arise by leakiness—possibly due to overexpression of the T-antigen (1–4, 8, 12). The second is that temperature-insensitive lines arise as a result of cellular mutation. In this paper we attempt to distinguish between these two possibilities.

Flat revertants of transformed cell lines can be isolated by selectively killing with fluorodeoxyuridine those cells that continue replication at high cell densities (7). When this selection was performed at the nonpermissive temperature with temperature-insensitive transformants induced by a *tsA* mutant, all of the revertants characterized had lost their ability to express the SV40 T-antigen.

MATERIALS AND METHODS

The cell lines and blotting techniques have been described previously (3, 9), except that the partial acid hydrolysis procedure of Wahl et al. (13) was added. The selection for flat revertants was carried out at 40°C precisely as described by Steinberg et al. (11).

RESULTS

Fisher rat 3T3 cell lines that were transformed by a temperature-sensitive mutant of SV40, but that were temperature insensitive and that contained SV40 sequences at one (FR3T3 A12) or a few (FR3T3 A24 and FR3T3 A84) sites (3, 9), were grown at the nonpermissive temperature, and flat revertants were selected. (No attempt was made to determine precisely the frequency of reversion, but calculating from the number of cells used and the number of flat revertants obtained, the frequency was roughly 1 in 10^7.) The flat revertants were cloned and then tested for their ability to express T-antigen at the permissive (34°C) or nonpermissive (40°C) temperatures (Table 1). All of the flat revertants examined had lost their ability to express T-antigen at both temperatures, and all had the nontransformed phenotype at both temperatures.

Southern blots (10) and hybridizations were performed with DNA isolated from several of the revertants and digested with a series of restriction enzymes. Rearrangements of the in-

TABLE 1. T-antigen expression in temperature-insensitive transformants and their revertants

Cell line	T-Antigen[a]	
	33°C	40°C
FR3T3 A12	+	+
FR3T3 A12 R2	−	−
FR3T3 A12 R3	−	−
FR3T3 A12 R4	−	−
FR3T3 A12 R5	−	−
FR3T3 A24	+	+
FR3T3 A24 R2	−	−
FR3T3 A84	+	+
FR3T3 A84 R1	−	−

[a] T-antigen was assessed by immunofluorescence (7, 12).

tegrated SV40 sequences were apparent in all of the cell lines even when a restriction enzyme that does not cut SV40 (*Xba*I) was used (Fig. 1). Similar results were obtained with four revertants of FR3T3 A24 (data not shown).

When one of these revertant cell lines, A12R3, was analyzed with the *iso*-schizomers *Mbo*I (recognition site GATC) and *Sau*IIIA (recognition site GATC and G^{Me}ATC), it was observed that a slowly migrating band was found with *Mbo*I, but not with *Sau*IIIA, whereas no difference was obtained between *Mbo*I and *Sau*IIIA in the patterns for cell line A12R2, A12R4, or A12R5 (Fig. 2). This result suggests that a G^{Me}A site had been generated at or near the SV40 site of integration during the reversion event. We cannot, however, be certain that the methylation event in A12R3 is causally related to the reversion since rearrangements of the SV40 sequences can also be detected in this line (see the *Pst*I digestion pattern in Fig. 2).

DISCUSSION

If T-antigen were not required for the maintenance of the transformed state in temperature-insensitive cell lines induced by *tsA* mutants, then rarely, if ever, should revertant cell lines be T-antigen negative. This would be the case because the selection is for correction of the transformed phenotype, not for the loss of T-antigen. The selection was performed at the nonpermissive temperature in order not to prejudice the results since correction of the hypothetical cellular lesion might then have left the cell temperature sensitive for the transformed phenotype by virtue of the expression of the temperature-sensitive T-antigen (2, 6). Nonetheless, no temperature-sensitive revertants were obtained.

Since all (six of six) of the revertant cell lines examined had lost their ability to express T-antigen even at the permissive temperature, the most reasonable explanation is that the expression of T-antigen is required for the maintenance of the transformed state even at the nonpermissive temperature in temperature-insensitive, *tsA*-induced transformants.

The results obtained are those that would be expected if the failure of these lines to be temperature-sensitive were a manifestation of leakiness (due to overproduction of T-antigen as previously proposed [2, 12]). These results do

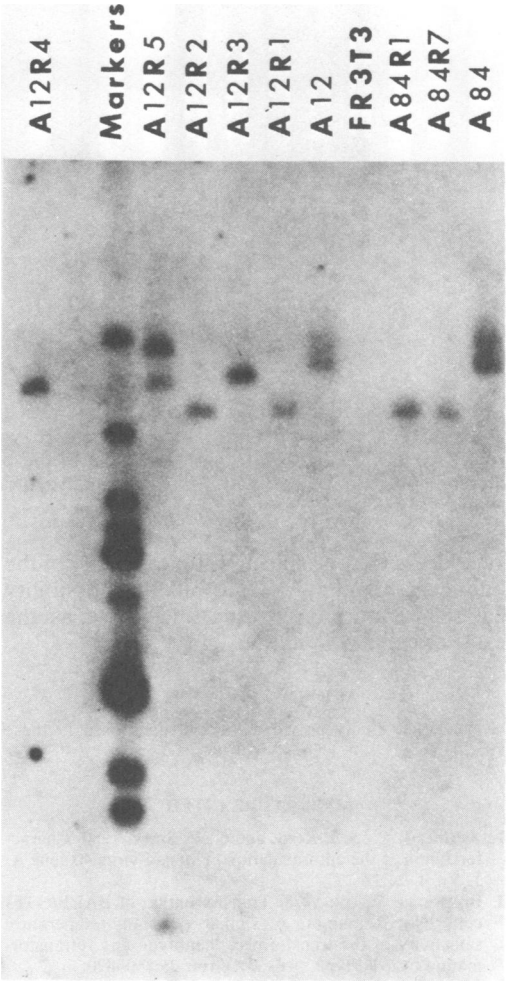

FIG. 1. Southern blot (10) of *Xba*I-digested DNA from transformed and revertant cells. High-molecular-weight DNA was isolated from a number of cell lines and digested with *Xba*I, and electrophoresis was performed in an agarose gel. The DNA was blotted onto nitrocellulose and hybridized with a probe of nick-translated SV40. Although we had previously reported that the SV40 sequences in A12 migrated at 7.9 kilobases (kb), we now find it migrating as a single band closer to 12 kb at the present passage state of the cells. The markers (from top to bottom) are 23.7 kb, 9.5 kb, 6.6 kb, SV40 form II, SV40 form III, 4.3 kb, SV40 form I, 2.3 kb, and 1.9 kb.

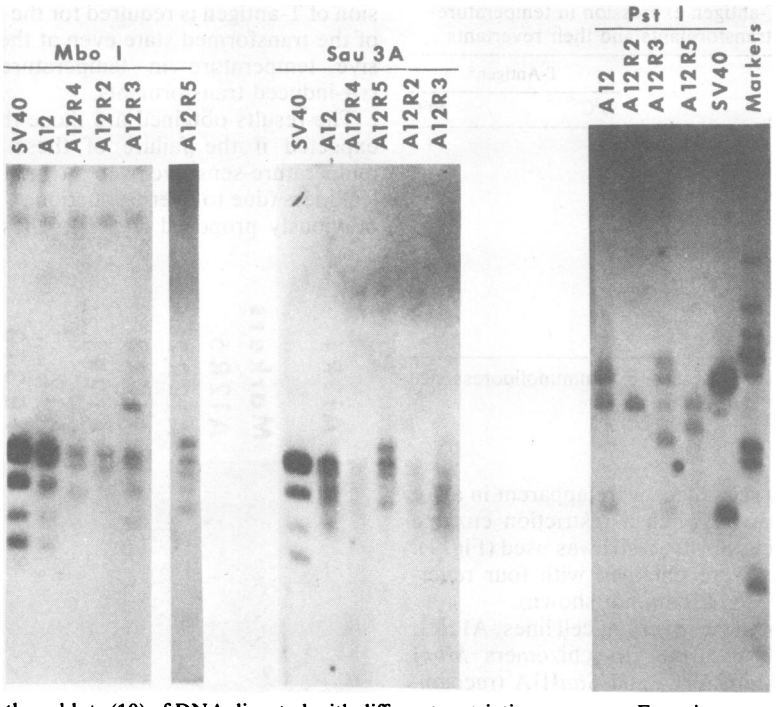

FIG. 2. Southern blots (10) of DNA digested with different restriction enzymes. Experiments were performed as in Fig. 1, except that the indicated restriction enzymes were used. Note that a slow-migrating band in sample A12R3 (row 5) of the *Mbo*I digest is absent in the corresponding sample (row 6) of the *Sau*IIIA digest. The sample of A12R5 digested with *Mbo*I was analyzed on a different gel than the others of this group.

not address the possible multifunctionality of the T-antigen, but they do rule out the possibility that temperature-insensitive lines arise as the result of cellular mutation.

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