Separation of Lytic and Transforming Functions of the Simian Virus 40 A Region: Two Mutants Which Are Temperature Sensitive for Lytic Functions Have Opposite Effects on Transformation

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Received 23 October 1980/Accepted 30 January 1981

Thirty-six of 40 rat cell clones transformed to anchorage independence at low multiplicity of infection by simian virus 40 *tsA58* were heat sensitive for continued expression of the transformed phenotype. *tsA1499* is an 81-base-pair deletion at 21 map units which is like *tsA58* in that it is also heat sensitive for lytic growth, belongs to the A complementation group, and produces rat cell transformants which contain a thermolabile T antigen. Unlike *tsA58*, however, *tsA1499* generated rat cell transformants efficiently at the temperature at which it was lytically defective, and 10 of 17 clones transformed by *tsA1499* were cold rather than heat sensitive for the continued maintenance of the transformed phenotype. The lytic and transforming activities of the A region thus appeared to function independently in mutant *tsA1499*.

The ability of simian virus 40 (SV40) to malignantly transform cells depends upon the early half of the genome (1, 17). Via partially overlapping spliced mRNA's (4), this early region codes for two known proteins, a 94,000-dalton (94K) large T antigen (35), which is required for lytic growth, and a 17K small t antigen (9; Fig. 1).

Both proteins influence viral transformation. Analysis of deletion mutants suggests that the small t antigen is required for the efficient initiation of transformation when growth is restricted soon after infection (5, 11, 28, 42) but plays a minimal role in the subsequent maintenance of the transformed phenotype either in vitro or in vivo (22, 38). Large T antigen, on the other hand, may play a continuingly active role in transformation. It is required for the induction of stable transformants by SV40 virions (18, 44) and has often appeared to be continuously required for the maintenance of transformation, since clones of rat, hamster, rabbit, or mouse cells transformed by mutants with temperature-sensitive lesions in large T may lose various transformed characteristics when grown at the nonpermissive temperature (6, 7, 19, 27, 29, 30, 32, 33, 38, 45, 47).

In this paper we report the effect of two dif-

ferent temperature-sensitive T-antigen mutants on transformation as measured by anchorage independence. Mutant *tsA58*, first isolated by P. Tegtmeier, maps by marker rescue with the bulk of the *tsA* mutants studied to date between 32.4 and 37.3 map units (19; Fig. 1) in the middle of the coding region for large T antigen. Mutant *tsA1499* is a temperature-sensitive deletion mutant isolated by T. Shenk which lacks 81 base pairs at 21 map units lying within the carboxy-terminal portion of the T-antigen-coding region (31). Both mutants fall into complementation group A for lytic growth (31, 46); both can be marker rescued by fragments falling within the known coding region of the large T antigen (20, 31); both direct the synthesis of a thermolabile large T antigen in permissive and nonpermissive cells; and both are severely heat sensitive for lytic growth. Despite these similarities, when these two mutants are assayed for transformation under identical conditions on the same cell line, opposite results are obtained. *tsA58* conforms to its lytic phenotype, being defective in generating and maintaining transformants at the high temperature where lytic growth is inhibited. Conversely, *tsA1499* generates and maintains transformants efficiently at the temperature where lytic growth is inhibited and is severely defective for transformation at the lytically permissive low temperature. This dissociation of the lytic and transforming phenotypes as

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FIG. 1. Map of the SV40 genome showing the location of the *tsA58* and *tsA1499* lesions.

expressed by mutant *tsA1499* reveals that the transforming and the lytic activities of the A region of SV40 virus can function independently.

MATERIALS AND METHODS

Cells and virus. Cells were routinely grown in Dulbecco modified Eagle medium (DME) supplemented with 10% fetal calf serum. The F111 rat cell line (12) was used for transformation experiments. Viral stocks were grown and assayed on CVC, CV-1P, or BSC-1 monkey kidney lines. SV40 mutant *tsA58* and its parent, wild-type 45-54, were a gift from P. Tegtmeyer. *tsA1499* (31) was obtained from T. Shenk, and its wild-type parent, *wt830*, a plaque-purified derivative of the SVS strain, was from P. Berg. *dl1410* (40) contains a large deletion in small t (54 to 58.5 map units) and a smaller deletion at 70 to 72 map units.

Direct transformation. For direct transformations, cells suspended at 10^6 /ml in Tris-buffered saline without Ca^{2+} or Mg^{2+} containing 1% filtered calf serum were infected with various multiplicities of infection (MOIs) of either appropriately diluted virus lysates or CsCl-banded virions while being stirred in suspension at 37°C for 1 h. After disruption of any clumps, infected cells were diluted and plated in soft agar at 2×10^4 to 4×10^4 cells per 60-mm² dish (25), using DME containing 5% dog serum and 5% fetal calf serum solidified with 0.30% agar. Colonies arising in agar (diameter, >0.2 mm) were counted after 4 to 5 weeks of incubation at 38.5 or 39.3°C and after 8 to 9 weeks of incubation at 32°C. At these cell densities, no spontaneously arising colonies have been observed in over 2×10^7 normal F111 cells plated. Percentage of transformation was calculated as the number of colonies arising in soft agar divided by the number of cells plated times 100. All transformed colonies were isolated by picking colonies from the soft agar, growing them in 35-mm² dishes containing DME with 20% fetal calf serum, and recloning them in 0.30% soft agar.

Agar assay for the maintenance of transformation. Cells growing at selected temperatures were assayed in soft agar as in direct transformation but at 10^3 cells/dish and simultaneously assayed for their cloning ability on plastic by plating at 200 cells per 60-

mm² dish in DME plus 20% fetal calf serum. Colonies arising in liquid medium were stained with 1% aqueous methylene blue-methanol (1:1). Data are presented as the relative agar plating efficiency, which is the number of cells able to clone in soft agar divided by the number of cells able to clone on plastic in liquid media times 100. Temperatures chosen represent those which are effectively permissive and nonpermissive for the viral lytic cycle.

Growth curves. A total of 5×10^4 cells were seeded into 100-mm² dishes containing DME plus 10% fetal calf serum and incubated at selected temperatures. At various times, duplicate plates were harvested and counted. This procedure was repeated until plateau values were reached.

Labeling, extraction, and immunoprecipitation. Cells to be labeled were washed twice with Hanks salt solution or with DME without methionine and labeled for the indicated time with [³⁵S]methionine in methionine-free DME at the indicated temperature. Labeled cells were cooled to 0°C, washed twice in iced Tris-buffered saline, pH 8, containing 1 mM CaCl_2 and 1 mM MgCl_2 , and extracted in this buffer with 0.5% Nonidet P-40 for 5 min at 0°C. After clearing at $45,000 \times g$ for 20 min. The extracts were immunoprecipitated as described by Yang et al. (48), using hamster antitumor serum, a generous gift from M. K. Rundell or John Cole, National Cancer Institute. The immunoprecipitates were analyzed on Ortec 20% acrylamide gels (35) by fluorography (21).

RESULTS

Direct transformation by *tsA58* and *tsA1499* virions. Both *tsA58* and *tsA1499* are unusually tight heat-sensitive mutants for lytic growth (31, 44). Isolates used in these experiments varied 10^5 -fold in plaque-forming ability between the permissive and nonpermissive temperatures. When rat cells were infected by the mutants and then assayed at high and low temperatures for cloning ability in a soft-agar transformation assay, *tsA58*, like other *tsA* mutants studied to date, was heat sensitive for viral transformation (Fig. 2A), producing many more transformants at 32 than at 39.3°C.

When mutant *tsA1499* was tested in the same assay with the same rat cells it behaved quite differently, generating many more transformants at 38.5 than at 31.5°C (Fig. 2B). This phenotype is the reverse of that observed in the lytic cycle and in transformation experiments with *tsA58*. At low multiplicity at the nonpermissive temperature for lytic growth, transformation by *tsA1499* was depressed only 7-fold compared with the wild type, whereas it was reduced 90-fold at the temperature permissive for lytic growth. The mutant appeared to be slightly heat sensitive and severely cold sensitive for viral transformation.

Maintenance of transformation in cloned lines initiated by *tsA58*. The direct transformation experiments with newly infected cells

FIG. 2. Direct transformation of F111 cells. F111 rat cells were infected with increasing multiplicities of (A) *tsA58* or wild-type virions or (B) *tsA1499* or wild-type virions and assayed at the permissive and nonpermissive temperatures.

reflect the combined effects of the virus on the initiation and on the maintenance of the transformed phenotype. To assess the role of the mutants in maintenance alone, clones which arose in soft agar in the course of direct transformation experiments at the permissive temperature for transformation were picked and recloned. Each clone was grown on plastic in parallel at the permissive (32°C) and nonpermissive (39.3°C) temperatures for a week and then tested for its ability to maintain the transformed phenotype by measuring at each temperature the relative plating efficiency in agar, i.e., the number of cells able to clone in soft agar divided by the number of cells able to clone on plastic times 100. Only in this way was it possible to distinguish the trait of anchorage independence from the ability to clone with anchorage which, for the cell lines and high serum levels used here, is not related to neoplastic transformation.

When analyzed in this way, the majority of rat cell clones transformed by *tsA58* were clearly heat sensitive for the maintenance of transformation. Table 1 shows data for 42 cloned lines transformed by *tsA58*; Table 2 shows data for 20 wild type-transformed control clones. Figure 3

plots the average ratios of the relative agar plating efficiency at the permissive temperature divided by that at the nonpermissive temperature for each clone. These ratios varied tremendously among the 42 independent *tsA58*-transformed clones, whereas 19 of the 20 wild type-transformed clones varied less than 10-fold. We have therefore considered those clones with a more than 10-fold differential in relative agar plating efficiency at the two temperatures to be temperature dependent (heat sensitive in the case of *tsA58*) for the transformed phenotype. Of the 42 clones transformed by *tsA58*, 36 (86%) were temperature dependent and 6 (14%) were unrestricted by temperature. Thirty-four of 45 (81%) of the *tsA58*-transformed clones had agar plating differentials falling outside the third standard deviation of the mean of the wild-type population (Fig. 3).

Although many of the mutant-transformed lines showed a wide variability from experiment to experiment in the ratios of their relative agar plating efficiencies at the two temperatures (Table 1), each clone behaved consistently in either a temperature-dependent or temperature-independent manner. The temperature inhibition for growth in agar of clones transformed by *tsA58* was fully reversible. Cells that lost their ability to plate in agar at 39.3°C regained that (quantitatively similar) ability when transferred to 32.5°C and lost it again when shifted to 39.3°C (data not shown).

The *tsA58*-transformed clones were indeed shifting their phenotype for transformation in response to temperature shift and not simply changing in growth rate or cloning ability. Both the *tsA58*- and the wild type-transformed lines were able to clone on plastic as efficiently at high as at low temperatures, achieving a colony size of greater than 500 cells at both temperatures. This number of cells was sufficient to form a visible colony in soft agar. In addition, more extensive growth curves on representative temperature-dependent and temperature-independent clones (Fig. 4) demonstrated that these lines could divide rapidly at permissive and nonpermissive temperatures. There seemed to be little significant difference between temperature-dependent and temperature-independent *tsA58*-transformed lines in their growth properties on plastic plates in liquid media.

Virus rescued from seven representative temperature-dependent and three temperature-independent *tsA58*-transformed clones was in all cases temperature sensitive for lytic growth to the same degree as the original *tsA58* stock. In addition, the plating efficiency in agar at 39.3°C of representative *tsA58*-transformed temperature-dependent lines could be increased signifi-

TABLE 1. Temperature dependence of relative agar plating efficiencies for *tsA58*-transformed F111 cell lines^a

Clone	MOI	Avg absolute agar plating efficiency (%)		Ratio of relative plating efficiencies in agar (32.5/39.3°C)								Transformation	
		32°C	39.3°C	1 ^b	2	3	4	5	6	7	8		Avg
A	1	17	<0.1	>325	>332	57	38	131	35			>153	Heat sensitive
B	1	4	<0.1	56	200	127	375	125				>177	Heat sensitive
D	1	11	<0.5	17	123	49	88					69	Heat sensitive
E	1	11	<0.1	80	200	136	113	223	243			166	Heat sensitive
G	1	21	0.2	29	49	32						37	Heat sensitive
H	0.1	15	<0.7	47	10	73						>20	Heat sensitive
I	10	11	0.2	39								39	Heat sensitive
1a	0.5	22	0.03	83	33							58	Heat sensitive
2	10	2	0.4	3								3	Unrestricted
5	5	6	<0.1	32	39							36	Heat sensitive
6	5	8	<0.9	235	33	2	32	8				62	Heat sensitive
8	5	8	<0.2	245	>9	2	>211	>92	75			>106	Heat sensitive
9	5	10	<0.1	214	100	29	>571	324	825			>343	Heat sensitive
10	10	14	<0.3	40	104	>258	24	10	227	21		>98	Heat sensitive
12	10	3	<0.4	7	3	38	0.4					12	Heat sensitive
13	10	15	0.05	319								319	Heat sensitive
15	10	13	<0.1	>372	24							198	Heat sensitive
16	10	36	0.2	17	103							60	Heat sensitive
17	10	27	0.2	136	120							128	Heat sensitive
18	10	21	2	11	11	6	10					10	Heat sensitive
14	10	5	0.5	12								12	Heat sensitive
29	25	7	<0.1	>13	88	34						>45	Heat sensitive
30	25	6	0.6	40	3	10	5	3	4			12	Heat sensitive
35	5	13	3	6	6	4	9	5	4			6	Unrestricted
36	5	4	<0.4	267	>190	>66	36	62				>124	Heat sensitive
37	5	15	1	49	36	410	67	7	43	5	32	>78	Heat sensitive
38	5	20	<0.04	>74	512	59	18					>166	Heat sensitive
43	10	16	<0.4	686	>13	>46	31	192	11			>163	Heat sensitive
44	10	17	<1	7	136	172	66	8	10			67	Heat sensitive
57	5	16	18	1	1	4	1	2	2			2	Unrestricted
59	5	14	2	15	5	2	6	8	25			10	Heat sensitive
60	5	15	8	1	13	1	2	1				4	Unrestricted
90	10	18	<0.3	>638								>638	Heat sensitive
115	3	13	<0.1	109	>133							>121	Heat sensitive
120	6	5	<0.1	>16								>16	Heat sensitive
121	6	13	0.2	46	80							63	Heat sensitive
122	6	18	2	2	37							20	Heat sensitive
130	12	12	11	2								2	Unrestricted
131	12	5	<0.03	103	>124							>114	Heat sensitive
133	12	2	5	1								1	Unrestricted
134	12	29	0.5	43	106							75	Heat sensitive
137	12	10	1.5	7	4	39						17	Heat sensitive

^a Individual clones of transformed cells were grown in parallel at the indicated temperatures and assayed at the temperature grown for absolute agar plating efficiency (percentage of cells plated which clone in agar) and relative agar efficiency (percentage of cells able to clone on plastic which can clone in agar). The ratio of the relative agar plating efficiencies at the two temperatures indicates the degree of heat or cold sensitivity, or, when within 10 of unity, indicates a transformed phenotype which is unrestricted by temperature.

^b Experiment number.

cantly by superinfection with wild-type virus, suggesting that these clones can be retransformed by wild-type virus at the temperature at which they express the normal phenotype.

Maintenance of transformation in cloned lines initiated by *tsA1499*. To determine whether the unusual cold-sensitive phenotype for transformation displayed in direct transformation experiments by *tsA1499* reflected a viral maintenance function, transformed clones arising at 38.5°C were picked, recombined, and tested for their ability to maintain transformation at

high and low temperatures as described above for the *tsA58* transformants. Ten of the 17 clones analyzed (59%) exhibited a cold-sensitive phenotype for growth in soft agar, producing colonies much more efficiently at 38.5°C than at 32.5°C (Table 3 and Fig. 3), although all cloned efficiently at both temperatures on plastic. Seven of the clones tested (41%) were not temperature restricted for transformation, cloning equally well in soft agar at both temperatures.

Virus was rescued by fusion with permissive BSC-1 cells from two cold-sensitive and two

TABLE 2. Temperature dependence of relative agar plating efficiencies for wild type-transformed F111 cell lines^a

Clone	MOI	Avg absolute agar plating efficiency (%)		Ratio of relative plating efficiencies in agar (32.5/39.3°C)									Transformation
		32°C	39.3°C	1 ^b	2	3	4	5	6	7	8	Avg	
A	13	12	13	1	1	1	1	1	1	2	3	1	Unrestricted
D	13	24	8	2								2	Unrestricted
E	13	33	19	2	5	1						3	Unrestricted
1	25	19	7	5	1	2	8	5	4			4	Unrestricted
1a	14	12	21	1								1	Unrestricted
2	25	4	4	10	6	0.1	3					2	Unrestricted
2a	14	22	14	2								2	Unrestricted
3	25	23	6	6								6	Unrestricted
32	5	21	6	14	8	5	5	1				7	Unrestricted
33	5	13	5	13	1							7	Unrestricted
40	25	27	12	1	2							2	Unrestricted
41	25	21	4	4								4	Unrestricted
42	25	35	13	0.2								0.2	Unrestricted
51	10	4	2	1								1	Unrestricted
52	10	16	9	7								7	Unrestricted
53	10	18	11	7								7	Unrestricted
54	10	28	12	0.07								0.07	Cold sensitive
103	18	14	2	2	2							2	Unrestricted
104	18	7	6	0.5	3	2	9					3	Unrestricted
105	18	15	7	2	3							3	Unrestricted

^{a,b} See Table 1. FIG. 3. Histogram of the ratio of relative agar plating efficiencies at high and low temperatures for individual clones. Each box represents a single clone transformed by either *tsA58* (top), wild type (middle), or *tsA1499* (bottom) with data taken from Tables 1, 2, and 3. *m*, Mean of wild type population; *s*, standard deviation of the wild-type means.

unrestricted clones transformed by *tsA1499* and in all cases was heat sensitive for plaque formation, as was the original infecting virus. Cold-sensitive transformants induced by *tsA1499* could be retransformed at the temperature at which they expressed the normal phenotype either by wild-type virus or by a small t antigen

deletion mutant *dl1410* (Table 4). It thus appeared that this cold-sensitive transformant did not shift its phenotype for transformation because the cell was inherently incapable of expressing SV40 transformation at the low temperature.

Heat lability of T antigen in cloned lines

FIG. 4. Growth curves for representative *tsA58*-transformed temperature-dependent [FR(*tsA58*)36, FR(*tsA58*)38] and temperature-independent [FR(*tsA58*)57] clones as well as wild type-transformed [FR(*wt648*)1] and parent F111 at 39.3°C (A) and 32.5°C (B) as described in Materials and Methods.

derived from either mutant. In lytic infections, *tsA58* produces a T antigen of wild-type size at both the permissive and nonpermissive temperatures which is more heat labile in vivo (46) and in vitro (3) than is wild-type SV40 T antigen. In the F111 cell clones transformed by *tsA58*, T antigen is also produced over a wide

range of temperatures both in clones which are heat sensitive for expression of transformation and in clones which are unrestricted by temperature. That this T antigen is heat labile in both types of clones is demonstrated in Fig. 5. When cells were pulsed at low temperature and chased at low and high temperatures, T antigen in all *tsA58* transformants appeared to be heat labile compared with T antigen in parallel wild type-transformed clones. No visible difference in T antigen levels or heat lability was evident when clones heat sensitive for the expression of transformation (clones A, 10, and E) were compared with those which were not restricted by temperature (clones 57 and 35). Similar findings have been reported by Fluck and Benjamin (11).

As expected for a deletion mutant, cells lytically infected with *tsA1499* produced a shortened T antigen, which migrated on the gel system used here at about 76,000 molecular weight (Fig. 6). In clones of F111 cells transformed by *tsA1499*, this deleted T antigen (*dT*) was more heat labile than wild-type T antigen. Whether cells were pulsed at 32 or 38°C, label disappeared from *dT* more rapidly at 38 than at 32°C (Fig. 7). In parallel cultures of wild type-transformed cells, T antigen was quite stable; in parallel cultures of *tsA58*-transformed cells, it was labile as in the *tsA1499*-transformed clones. As with the *tsA58*-transformed clones, no obvious difference in *dT* labeling was seen between clones which were temperature limited in the expression of transformation (e.g., clone 31; Fig. 7) and those which were not (e.g., clone 46; Fig. 7).

DISCUSSION

Results with the mutant *tsA58* reaffirm that in an SV40-transformed cell line the maintenance of the anchorage-independent transformed state can be controlled by the A gene. Even when unusually stringent conditions are used to produce and analyze transformed clones, including infecting at low MOI, testing a large number of clones, controlling for temperature effects on anchorage-dependent cloning frequency, and using the tumor-correlated (41) soft-agar transformation assay throughout, the *tsA58* transformants are still clearly a statistically different population from those transformants generated under identical conditions by wild-type virus. The *tsA58* transformants are primarily heat sensitive for the maintenance of transformation, reflecting the phenotype of this mutant in the lytic cycle, whereas none of the wild type-transformed clones are heat sensitive. Concordant results have been reported for other *tsA* mutants and with other cell types (6, 11, 16, 27, 32, 38).

TABLE 3. Temperature dependence of relative agar plating efficiencies for *tsA1499*-transformed F111 cell lines^a

Clone	MOI	Avg absolute agar plating efficiency (%)		Ratio of relative plating efficiencies in agar (38.5/32.5°C)						Transformation
		32.5°C	38.5°C	1 ^b	2	3	4	5	Avg	
FR(<i>tsA1499</i>)										
1	1	0.7	37	40	16	44	238	1,460	>360	Cold sensitive
2	2-8	<0.025	3	257	>56				>157	Cold sensitive
5	2-8	36	21	0.9	0.4	1.1			0.8	Unrestricted
7	2-8	7	35	8	2				5	Unrestricted
15	2-8	<0.05	5	125	6	>32			>54	Cold sensitive
23	2-8	11	30	3	4				4	Unrestricted
30	2-8	0.2	28	34	13				24	Cold sensitive
31	2-8	<0.02	6	>169	>35				>102	Cold sensitive
32	2-8	0.03	7	154					154	Cold sensitive
33	2-8	5	5	30	0.2				0.6	Unrestricted
34	2-8	5	4	2	0.06				0.1	Unrestricted
36	2-8	0.1	6	333	25				179	Cold sensitive
40	2-8	<0.025	2	>53	>180	>6	>2		>60	Cold sensitive
43	2-8	3	12	4	4				4	Unrestricted
46	2-8	33	43	2	2				2	Unrestricted
48	2-8	0.03	15	446	>38	>175			>220	Cold sensitive
51	2-8	0.1	10	427	36				232	Cold sensitive
FR(<i>wt830</i>)										
1	10	43	26	0.6	0.8				0.7	Unrestricted

^{a,b} See Table 1.TABLE 4. Retransformation of a *tsA1499*-transformed temperature-dependent line at its normal temperature^a

Virus	MOI	Transformation frequency	
		F111	FR(<i>tsA1499</i>)48
<i>wt830</i>	0	<0.0025	0.02
	30	0.0065	0.027
	60	0.014	0.03
	100	0.034	0.05
<i>dl1410</i>	0	<0.0008	0.02
	30	<0.0008	0.027
	60	<0.0008	0.033
	100	<0.0008	0.044

^a F111 cells or a cold-sensitive clone of F111 cells transformed by *tsA1499* were infected with various multiplicities of either wild-type virus or small t deletion mutant *dl1410* and assayed for transformation in soft agar at 32.5°C.

The straightforward interpretation of these data suggests that the 94K large T antigen is the A gene product which is responsible for the maintenance of transformation. However, there are two problems with this hypothesis. First, almost all surveys of *tsA*-transformed clones have found, as we have, that although the majority of clones tested are heat sensitive for the maintenance of transformation, a significant minority are not restricted by temperature although the T antigen in these unrestricted clones is heat labile and the rescuable virus they carry is invariably temperature sensitive and not

wild type. A possible explanation of these clones is that they contain higher levels of the heat-labile T antigen than their heat-sensitive counterparts, as has been demonstrated for one clone of CHL cells (10, 47) and one clone of 3T3 mouse cells (6). This explanation correlates with the observations that temperature-independent clones occur more frequently when rat cells are infected at a high MOI (33) or when rat cells (although not mouse) (16, 29) or semipermissive CHL cells (27) are unable to grow freely immediately after infection (33, 38). Our failure and that of others (11) to detect in rat cells an increased quantity of T antigen in unrestricted clones compared with restricted clones might be due to the inadequate sensitivity of quantitation by immunoprecipitation. A second possibility is that there is only a slight difference between the amount of T antigen that is adequate and that which is inadequate to maintain transformation in F111 cells. Indeed, some *ts src* mutants may show little change in amount or synthesis of pp60^{src} protein (7, 36) and very little difference in functional activity (37) when *ts* mutant-infected cells are shifted between temperatures where they display their normal and transformed phenotypes. A third possible explanation could be that transformation is dependent on a second minor 94K large T antigen (see below) present in low concentration whose changes are masked by the major T antigen.

A second problem with the hypothesis that large T antigen controls the maintenance of

FIG. 5. Pulse-chase immunoprecipitations of representative *tsA58*-transformed lines. Temperature-dependent *tsA58*-transformed lines *FR(tsA58)A*, *FR(tsA58)10*, and *FR(tsA58)E*, temperature-independent *tsA58*-transformed lines *FR(tsA58)57* and *FR(tsA58)35*, and wild type-transformed *FR(wt648)40* were grown at 32°C for longer than 1 week and pulsed for 3 h at 32°C with 40 μ Ci of [35 S]methionine per ml. One plate was harvested at once (P-32), and the other two were refed with unlabeled media, chased for 21 h at either 32°C (Ch-32) or 39°C (Ch-39), extracted, and immunoprecipitated.

SV40 transformation is more difficult to dismiss. This is the finding that a second *tsA* mutant, *tsA1499*, which, like *tsA58*, is *ts* in the lytic cycle, belongs to the A complementation group, produces a heat-labile T antigen, and maps in the coding region of large T, uniquely produces transformed clones which are cold sensitive rather than heat sensitive for the maintenance of transformation. These cold-sensitive lines clone well in soft agar at high temperature when T antigen is labile and the lytic cycle is inhibited and clone poorly or not at all in soft agar at low temperature when T antigen appears to be stable and the lytic cycle proceeds.

The cold-sensitive transformants resulting from *tsA1499* infection probably do not arise from transformation by some variant cold-sensitive virus in our cloned mutant stock, for (i) the mutant *tsA1499* is a deletion, and revertants and variants should be rare; (ii) in all clones

FIG. 6. Immunoprecipitation of *tsA1499*-infected permissive cells. Just confluent CV-C cells were infected at an MOI of 10 to 30 PFU/cell, grown for 3 days at the temperature indicated, labeled with 50 μ Ci of [35 S]methionine per ml for 1.5 h at 38.5°C or for 3 h at 31°C, extracted, and immunoprecipitated.

FIG. 7. Pulse-chase immunoprecipitation of *tsA1499*-transformed clones. Temperature-dependent [*FR(tsA1499)31*], temperature-independent [*FR(tsA1499)46*], as well as *tsA58*-transformed temperature-dependent [*FR(tsA58)10*] and wild type-transformed [*FR(wt)40*] lines were grown for 6 days at the temperature indicated for a pulse, labeled for 1 h at 38.5°C or for 2 h at 32°C with 100 μ Ci of [35 S]methionine, extracted at once in the case of the pulse (P) or rinsed and refed with medium containing a 10-fold excess of cold methionine and incubated at the indicated temperature for a chase period of 23 h (Ch), extracted, and immunoprecipitated.

examined the majority of the large T antigen is heat labile and is not cold labile; and (iii) only temperature-sensitive virus can be rescued from these clones. Nor can all of these cold-sensitive clones be explained as the result of transformation of rare cellular mutants unable to express the SV40 transformation at low temperature because almost no wild-type clones (1 of 20) and no *tsA58* transformants (0 of 42) show a cold-sensitive phenotype and, more important, the *tsA1499* clones can be retransformed at their normal temperature both by wild-type virus and by a small t-antigen deletion mutant. The retransformation of a cold-sensitive clone by the small t deletion mutant probably results from complementation with the integrated genome, which makes it difficult to invoke a cold-sensitive regulatory defect which alters the level of small t antigen (11) as the explanation of the cold-sensitive phenotype of these cells.

tsA1499 appears to be a single lesion because both the heat-sensitive lytic growth and cold-sensitive transformation phenotypes can be rescued with a wild-type *Pst*I-B fragment which extends from 3.9 to 27.2 map units, and nucleotide sequencing of *tsA1499* from 17.5 to 22 map units has revealed no additional alteration except the 81-base-pair deletion (31). *tsA1499* dif-

fers from *tsA58* and most other A mutants in that it is a deletion and lies in the carboxyl end rather than toward the middle of the T-antigen coding region. Its unique opposite phenotypes in the lytic cycle and for transformation could arise from the multifunctional nature of T antigen. SV40-transformed lines have been previously described that contain a large T antigen which is not competent for lytic functions (14). Although it is difficult to be certain that the large T in these lines is indeed maintaining transformation, these results are consistent with the suggestion that the lytic function of T antigen may be completely independent from the transforming function, with copious quantities of nuclear T being required for lytic growth, whereas minor amounts of T interacting with cell DNA (R. G. Martin, Adv. Cancer Res., in press), cell membranes, or a host protein (21, 24) are crucial to the maintenance of transformation. A single lesion could result in a T antigen physically and functionally heat sensitive in the lytic cycle but cold sensitive for stabilizing interactions with a cellular protein, membrane, or other constituent in the transformed cell.

Alternatively, the unusual phenotype of *tsA1499* could be explained by invoking a hypothetical third early SV40 protein. Mark and

Berg (26) have suggested that SV40 may code for a polypeptide identical to large T antigen except for the final 70 amino acids, which would be coded for by the alternate reading frame available beyond 22 map units (34) and result in a second large T antigen with a new hydrophobic tail. Antigenically distinct classes of 94K large T antigen have been identified recently (15, 43) which could be due to two 94K proteins which differ in primary structure. Discontinuities in SV40 early mRNA near the start of the alternate reading frame have also been reported and attributed to the binding of a small 65-nucleotide RNA in this region (2) and possibly to a splice junction here (26). Mutant *tsA1499* removes the entire binding site for the small RNA and lacks the proximal end of the proposed splice junction. If the *tsA1499* deletion could render either the splice process itself or the undiscovered third early polypeptide cold sensitive, then the unique *tsA1499* cold-sensitive transformation phenotype could be explained. However, the significant proportion of *tsA1499* transformants which are not temperature sensitive remains difficult to explain satisfactorily.

Whatever the final explanation for the phenotype differences between *tsA58* and *tsA1499*, it is clear from the data presented here that the function of the A gene in SV40 lytic infection may in some instances be clearly dissociated from its function in the maintenance of transformation. What is not yet clear is whether this dissociation reflects unusually independent multiple functions of a single gene A product, the well-known large T antigen, or indicates the existence of an as yet unidentified early gene product or process crucial to the maintenance of transformation.

ACKNOWLEDGMENTS

We express our appreciation to M. K. Rundell, in whose laboratory some of the immunoprecipitations were performed, for her generous hospitality and useful advice, to T. Shenk for the gift of our original stock of *tsA1499* and helpful discussion, and to A. Glenn, L. Zeigwied, and R. Huang for amiable technical help.

This work was supported by Public Health Service grants to N. Bouck and G. di Mayorca and in part by the Charles Edison Fund and the Metropolitan Life Foundation.

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