Temperature Dependency for Maintenance of Transformation in Mouse Cells Transformed by Simian Virus 40 *tsA* mutants

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Mouse embryo fibroblasts and 3T3 cells were transformed by wild-type, *tsB4*, *tsA7*, *tsA58*, and *tsA209* simian virus 40. Clones of transformants were generated both in soft agar and in liquid medium by focus formation and at both high and relatively low multiplicities of infection. All transformants were assayed for three phenotypes of transformation: (i) the ability to form highly multinucleated cells in cytochalasin B-supplemented medium, i.e., uncontrolled nuclear division; (ii) the capacity to continue DNA synthesis at increasing cell density; and (iii) the ability to form colonies in soft agar. The great majority of mouse embryo fibroblast transformants generated with *tsA* mutant virus were temperature sensitive for transformation in all three assays, regardless of the input multiplicity or whether they were generated in liquid medium or soft agar. These transformants exhibited a normal or near-normal phenotype at the nonpermissive temperature of 40°C. All but one of the transformants which appeared transformed at both temperatures were in the A209 group. In contrast to mouse embryo fibroblasts, transformants generated with 3T3 cells and *tsA* virus were often not temperature sensitive, exhibiting the transformation phenotypes at both temperatures. This phenomenon was more often observed when 3T3 transformants were generated in soft agar. These results, along with other published data, suggest that uncontrolled nuclear division and uncontrolled DNA synthesis are a function of the simian virus 40 A gene. Finally, with the 3T3 transformants, there was often discordance in the expression of transformation among the three phenotypes. Some *tsA* transformants were temperature sensitive in one or two assays but were transformed at both 33 and 40°C in the remaining assay(s). Other transformants exhibited a normal cytochalasin B response at either temperature but were temperature sensitive in the other assays.

Temperature-sensitive simian virus 40 (SV40) mutants in complementation group A (*tsA*) are defective in initiating either viral or cellular DNA synthesis at the nonpermissive temperature (7, 8, 42). Much of the early work (3, 17, 18, 21, 31, 43) and some of the more recent work (2, 4, 44) with rodent cells transformed by SV40 *tsA* mutants suggested that many transformants were temperature sensitive for the transformed phenotypes. This suggested a direct relationship between the SV40 gene A function or T-antigen and the maintenance of the transformed phenotype (3, 4, 21, 31). However, one of the earlier reports and some of the recent work have suggested that some cells transformed by SV40 *tsA* mutants are transformed at both temperatures (33, 34, 37-39, 43). Work done with rat 3T3 cells has shown that temperature sensitivity for the maintenance of transformation is expressed (type N) or not expressed (type A) depending on how transformants are generated (33, 34, 38, 39). The generation of these type N transformants

may require that cells be permitted to pass through the cell cycle for a few days after infection (34, 39). However, recent studies in mouse 3T3 cells did not reveal type A transformants (15).

The mold metabolite cytochalasin B (CB) can prevent cytoplasmic cleavage without directly affecting nuclear division (5, 10). Normal and a variety of transformed cells (including cultured tumors and DNA virus-transformed cells) show a differential response to CB. CB-treated normal cells become arrested for growth (probably in G1) after a maximum of only one nuclear division, resulting in the formation of binucleated cells (14, 16, 23, 26, 30). Nuclear division in CB-treated normal cells is controlled (26). In DNA virus-transformed and many cultured tumor cells, both DNA synthesis and nuclear division are uncontrolled, and highly multinucleated cells are formed in CB medium (14, 16, 30, 47). Similarly, DNA virus-transformed cells showed an uncontrolled growth phenotype with respect

to other parameters of transformation, namely, the ability to grow to high saturation densities with continued DNA synthesis (26, 27) and continued DNA synthesis in caffeine-supplemented medium (26). Many RNA tumor virus-transformed cells show a CB phenotype intermediate between normal and DNA virus transformants (30). Whereas mostly binucleated cells are formed, there is a relatively higher rate (as compared with untransformed cells) of cellular DNA synthesis in the presence of CB. When examined by other methods, the degree of transformation exhibited by RNA virus-transformed cells is, again, intermediate between that of normal and DNA virus-transformed cells (26-28). Because of this, it has been suggested that this form of uncontrolled growth, exclusive to DNA virus-transformed cells, represents a persistence of the property of DNA tumor viruses to directly induce cellular DNA synthesis (25, 27, 28).

If uncontrolled nuclear division (UND) and continued cellular DNA synthesis at high densities are reliable parameters of transformation by DNA tumor viruses, then we would expect temperature sensitivity for these phenotypes in cells transformed by SV40 *tsA* mutants. In this report, we have analyzed this and the appearance of type N and A SV40 transformants in both primary and established lines of mouse cells.

MATERIALS AND METHODS

Cells and viruses. Mouse embryo fibroblasts (MEF) were generated by mincing and trypsinizing decapitated embryos to produce cell suspensions. Medium-washed cells were plated in basal Eagle medium with 10% fetal bovine serum in petri dishes or culture flasks and infected with wild-type (WT) or *tsA* SV40. TC-7 is a line of African green monkey cells used for the propagation of SV40. SV40 *tsA7*, *tsA58*, *tsA209*, and *tsB4* were secured from Fred Rapp, Peter Tegtmeier, Janice Chou, and Claudio Basilico, respectively. The large-plaque strain of WT SV40 has been described in earlier publications (11, 29). Stocks of all viruses were prepared from TC-7 cells which were infected with a 1:1,000 dilution of SV40 (29).

Infection and generation of transformants. MEF and 3T3 cells were infected with both WT and *tsA* mutants at approximately 25 (unconcentrated virus) and 500 (concentrated virus) PFU per cell. SV40 stocks were concentrated by pelleting viruses in an SW27 rotor of a Beckman ultracentrifuge at 25,000 rpm for 3 h. The viral pellets were suspended in small volumes of culture media and sterilized by a 15-min treatment of a 20% ether suspension, which also served to remove clumps of pellet material. The ether was removed by bubbling nitrogen into virus suspensions. It was necessary to concentrate the virus since, with some, *tsA* mutants were unable to obtain MEF transformants in agar consistently, and the number of transformants was often low.

Virus was adsorbed to infected cells for 90 to 120

min at 37°C, and the cultures were incubated in culture media. After 14 to 16 h, the cells were detached with trypsin-EDTA (24, 28, 30), divided, and plated in agar or liquid medium. Approximately 5×10^4 cells were seeded into 6-mm petri dishes or 25-cm² flasks and cultured at 37°C (WT or *B* mutant virus) or at 33°C (*tsA* virus). With WT and *tsB4* mutants, transformed foci usually appeared within 3 to 4 weeks. *tsA* transformants usually appeared within 4 to 5 weeks. The foci were isolated by covering them with stainless-steel cloning wells, trypsinizing the cells into the cylinders, and growing the cells up in culture flasks, as described previously (24). To accommodate the cloning cylinders, the tops of culture flasks were cut off with sterile scissors.

The remaining infected cells were suspended in 0.33% agar (containing culture medium) and seeded into 75-cm² Lux culture flasks containing a pre-hardened 15-ml layer of 0.7% agar (also containing culture medium). Each flask was seeded with approximately 5×10^5 cells. The flasks, with loose lids, were incubated overnight in a CO₂ incubator and then with the lids tight in a dry incubator at 33°C for *tsA* transformants and at 37°C for WT and *tsB4* virus transformants. Once weekly, the flasks were fed with 10 ml of 0.33% agar-culture medium. Colonies of WT and *tsB4* transformants usually appeared within 5 to 7 weeks, whereas those of *tsA* transformants appeared within 6 to 8 weeks. These colonies were picked by drawing each colony into a Pasteur pipette, passing the colony plus agar through a 26-gauge needle, and plating the cells into 35-mm petri dishes where the cells were grown up. All transformants were negative for mycoplasma contamination by Hoechst 33258 fluorescence staining (6).

T-antigen analysis. All colonies and foci were analyzed for intranuclear T-antigen by immunofluorescence with serum from tumor-bearing hamsters induced by SV40-transformed hamster cells (29, 32). Monolayers of putative transformants were air dried, fixed in acetone, air dried again, and incubated with a 1:20 dilution of tumor-bearing hamster serum at 37°C for 30 min. They were washed twice with phosphate-buffered saline and incubated in a 1:10 dilution of fluorescein-conjugated goat anti-hamster serum for 30 min at 37°C. Controls utilized normal hamster serum or untransformed cells or both. The cells were then examined for intranuclear T-antigen with a Zeiss epifluorescence photomicroscope. All of the transformants were T-antigen positive in at least 85 to 90% of the cells. Some 3T3 transformants generated in liquid medium contained 10 to 15% negative cells, but all other transformants were 95% T-antigen positive.

Transformation maintenance assays. Maintenance of transformation was assayed by the following methods: (i) the capacity to form highly multinucleated cells in CB medium, (ii) the capacity to continue DNA synthesis as a function of high cell density, and (iii) the ability to grow in soft agar. For method i, approximately 2×10^5 cells were seeded into 35-mm petri dishes containing cover glasses (22 by 22 mm) and incubated in CB (1.5 µg/ml) medium for 7 days. The cultures were then processed as described previously (27). For method ii, replicate 35-mm dishes, half of which contained cover glasses, were seeded with 1×10^5 to 2×10^5 cells and incubated. Periodically

(usually days 1, 2, 3, 5, 6, 7, and 8 after seeding), two dishes with and two dishes without cover glasses were harvested and assayed for cell growth or DNA synthesis (autoradiography) as described previously (27). For method iii, growth in agar was assayed similarly to that described above for the generation of agar transformants. Replicate 60-mm petri dishes with a bottom layer of 0.7% agar-medium mixture were inoculated with 10^3 , 10^4 , 10^5 , or 10^6 cells suspended in a 0.33% agar-culture medium mixture and incubated for 3 to 4 weeks, and the number of colonies was counted. Dishes were fed once weekly with a 0.33% agar-culture medium mixture. All three assays were performed at both 33 and 40°C with SV40 *tsA*, *tsB4*, and WT transformants and untransformed 3T3 cells and MEF.

RESULTS

A large number of transformants have been generated in these studies. Tables 1 and 2 list all of the transformants for both MEF and 3T3 cells with WT and *tsB4* mutant viruses and with each of *tsA7*, *tsA58*, and *tsA209* viruses. The nomenclature describing the generation of transformants is as follows. Transformants generated in agar are denoted a, whereas those generated in liquid medium by focus assay are denoted l. Transformants generated with concentrated virus are named c, and those generated with unconcentrated virus are named u. For instance, clone 1 of *tsA58*-transformed MEF generated in liquid medium with unconcentrated virus is described as A58 lu clone 1.

The transformants analyzed in the following three assays were assayed also for the ability to form colonies in liquid medium after seeding of 100 cells per 60-mm petri dish. WT transformants showed a plating efficiency of 48 to 77% at 33°C and 5 to 10% at 40°C. Similarly, the *tsA* transformants exhibited a plating efficiency of 40 to 75% at 33°C. However, all but a very few of the *tsA* transformants showed a plating efficiency of less than 1% at 40°C.

MEF transformants. (i) CB assay. MEF transformants were assayed for UND (multinucleation) at both 33 and 40°C. Figures 1 and 2 show the results for a representative group of MEF transformants. Untransformed MEF were predominantly binucleated and mononucleated at either temperature, although at 40°C the frequency of binucleation was significantly increased. With the *tsA* transformants, there was a large number of cells with four or more nuclei and a significant number with seven or more nuclei when the cultures were CB treated at 33°C. However, at 40°C there were very few cells with four or more nuclei, and the CB response was similar to that seen with untransformed cells at either 40 or 33°C. This temperature sensitivity for UND was seen with *tsA* transformants generated in agar or liquid medium and after both high and low multiplicities

of infection. WT transformants showed UND at both temperatures. The data for all of the MEF transformants are summarized in Table 1. The great majority of *tsA* SV40 MEF transformants were temperature sensitive in the CB assay, whereas WT transformants were uncontrolled (multinucleated) at both temperatures. The only clones not exhibiting temperature sensitivity were A7 ac clone 3 and four clones in the two groups of *tsA209* transformants.

(ii) DNA synthesis as a function of cell density. All WT transformants at 33 and 40°C and *tsA* transformants at 33°C grew to high saturation densities, although there was some variability among different clones (Fig. 3). These transformants, unlike normal cells, also showed a substantial proportion of cells continuing DNA synthesis at saturation density. Untransformed MEF performed somewhat better at 33°C than at 40°C. At 40°C, the *tsA*-transformed MEF clones uniformly showed a lower saturation density, and the frequency of DNA synthetic cells at saturation density was significantly reduced (Fig. 3). Also, the slope of the curve was significantly greater at 40 than at 33°C. This was in contrast to WT transformants which often performed at 40°C as well as or better than they did 33°C. Therefore, with these MEF transformants, the ability to synthesize DNA at increasing densities was again temperature sensitive.

(iii) Growth in soft agar. A selected group of MEF transformants was assayed for growth in soft agar. Untransformed MEF did not form colonies. WT transformants formed colonies at both temperatures, although in most of the clones growth was 10-fold more efficient at 33°C (Table 3). Therefore, a 10-fold-lower number of colonies appearing at 40°C was not considered indicative of virus-controlled temperature sensitivity. Most of the *tsA*-transformed MEF grew well in soft agar at 33°C, but not at 40°C, regardless of how they were generated (agar versus liquid medium [Table 3]). With a few exceptions, there was virtually no growth at 40°C, even in dishes seeded with 10^6 cells.

3T3 transformants. (i) CB assay. The results with 3T3 transformants were different. A representative array of these transformants is shown in Fig. 4. Many of the *tsA* transformants generated in agar or liquid medium exhibited the so-called type A phenotype, showing high degrees of multinucleation at both 40 and 33°C, whereas others were predominantly binucleated at both temperatures. Of all the liquid medium clones, only A58 lu clone 1 and A58 lc clone 2 appeared temperature sensitive in the CB assay. A58 lu clone 3 and A58 lc clone 5 were not significantly more multinucleated than were untransformed 3T3 cells at either 33 or 40°C. A7 lu clone 2 and A58 lc clone 7 were only slightly

TABLE 1. *MEF SV40 tsA transformants: temperature dependency for maintenance of three transformation phenotypes*

Cell line	Assay		
	CB	DNA synthesis vs density	Colonies in agar
WT lu clone 1	UND at 33 and 40°C	Transformed at 33 and 40°C	Colonies at 33 and 40°C, but more at 33°C
WT lu clone 2	UND at 33 and 40°C	ND ^b	ND
WT lu clone 3	Binucleated at 33 and 40°C	ND	ND
WT au clone 1	UND at 33 and 40°C	Transformed at 33 and 40°C	ND
A7 lu clone 1	ts ^a	ts	ts
A7 lu clone 2	ts	ND	ND
A7 lu clone 3	ts	ND	ND
A7 lu clone 4	ts	ND	ND
A7 lu clone 5	ts	ND	ND
A58 lu clone 1	ts	ts	ND
A58 lu clone 2	ts	ND	ND
A58 lu clone 3	ts	ND	ts
A7 lc clone 1	ts	ts	ts
A7 lc clone 2	ts	ts	ts
A7 lc clone 3	ts	ND	ND
A58 lc clone 1	ts	ts	ts
A58 lc clone 2	ts	ND	ND
A58 lc clone 3	ts	ND	ts
A58 lc clone 4	ts	ND	ND
A58 lc clone 5	ts	ND	ND
A58 lc clone 6	ts	ts	ts
A58 lc clone 7	ts	ND	ND
A209 lc clone 1	ts	ND	ND
A209 lc clone 2	ts	ND	ts
A209 lc clone 3	UND at 33 and 40°C	Transformed at 33 and 40°C	ts
A209 lc clone 4	ts	ts	ts
A209 lc clone 5	ts	ND	ND
A209 lc clone 6	ts	ND	ND
A209 lc clone 8	ts	ND	ND
A209 lc clone 9	UND at 33 and 40°C	ND	ND
WT ac clone 1	UND at 33 and 40°C	Transformed at 33 and 40°C	Colonies at 33 and 40°C
WT ac clone 2	UND at 33 and 40°C	Transformed at 33 and 40°C	Colonies at 33 and 40°C
WT ac clone 3	UND at 33 and 40°C	ND	Colonies at 33 and 40°C
WT ac clone 4	UND at 33 and 40°C	ND	Colonies at 33 and 40°C
WT ac clone 5	Marginally ts	Transformed at 33 and 40°C	Colonies at 33 and 40°C
A7 ac clone 1	ts	ts	ts
A7 ac clone 2	ts	ts	ND
A7 ac clone 3	UND at 33 and 40°C	Transformed at 33 and 40°C	Colonies at 33 and 40°C
A7 ac clone 4	ts	ts	ND
A58 ac clone 1	ts	ts	ND
A58 ac clone 2	ts	ts	ND
A58 ac clone 3	ts	ND	ND
A58 ac clone 4	ts	ND	ts
A58 ac clone 6	ts	ts	ts
A58 ac clone 7	ts	ND	ND
A209 ac clone 2	ts	ND	ND
A209 ac clone 3	UND at 33 and 40°C	ND	ts
A209 ac clone 4	ts	ND	ND
A209 ac clone 5	UND at 33 and 40°C	ND	Colonies at 33 and 40°C
A209 ac clone 6	Binucleated at 33 and 40°C	ts	ts
A209 ac clone 8	ts	ND	ND

^a ts, Temperature sensitive.

^b ND, Not done.

TABLE 2. 3T3 SV40 *tsA* transformants: temperature dependency for maintenance of three transformation phenotypes

Cell line	Transformation assay at 33 and 40°C		
	CB	DNA synthesis vs density	Colonies in agar
WT lu clone 1	UND at 33 and 40°C	ND ^a	Colonies at 33 and 40°C
WT lu clone 3	UND at 33 and 40°C	Transformed at 33 and 40°C	Colonies at 33 and 40°C
WT lu clone 4	UND at 33 and 40°C	Transformed at 33 and 40°C	Colonies at 33 and 40°C
WT ac clone 1	UND at 33 and 40°C	Transformed at 33 and 40°C	ND
WT ac clone 2	UND at 33 and 40°C	Transformed at 33 and 40°C	Colonies at 33 and 40°C
WT ac clone 3	UND at 33 and 40°C	ND	ND
WT ac clone 4	UND at 33 and 40°C	ND	ND
WT ac clone 5	UND at 33 and 40°C	ND	ND
WT ac clone 6	UND at 33 and 40°C but more at 40°C	ND	ND
WT ac clone 7	Binucleated at 33 and 40°C	ND	Colonies at 33 and 40°C
WT ac clone 8	Binucleated at 33 and 40°C	ND	ND
WT ac clone 9	UND at 33 and 40°C	ND	ND
B4 lu clone 1	UND at 33 and 40°C	Transformed at 33 and 40°C	Colonies at 33 and 40°C
B4 lu clone 2	UND at 33 and 40°C	Transformed at 33 and 40°C	Colonies at 33 and 40°C
A7 lu clone 1	UND at 33 and 40°C	ts ^b	Colonies at 33 and 40°C
A7 lu clone 2	Binucleated at 33 and 40°C	ts	ts
A58 lu clone 1	ts	ND	ND
A58 lu clone 3	Binucleated at 33 and 40°C	ND	ND
A58 lc clone 2	Moderately ts	ts	ts
A58 lc clone 5	Binucleated at 33 and 40°C	ts	ts
A58 lc clone 7		ND	ND
A209 lc clone 1	UND at 33 and 40°C (more at 40°C)	ND	ts
A7 ac clone 1	Binucleated at 33 and 40°C	ND	ND
A7 ac clone 2	ts	Moderately ts	ts
A7 ac clone 3	ts	ND	ND
A7 ac clone 4	UND at 33 and 40°C	ND	ND
A7 ac clone 5	UND at 33 and 40°C	ts	ts
A58 ac clone 1	Marginally ts	Marginally ts	ts
A58 ac clone 2	Moderate UND at 33 and 40°C	Transformed at 33 and 40°C	Colonies at 33 and 40°C
A209 ac clone 1	ts	ts	Colonies at 33 and 40°C
A209 ac clone 2	Binucleated at 33 and 40°C	ts	Colonies at 33 and 40°C
A209 ac clone 3	Binucleated at 33 and 40°C	ND	ND

^a ND, Not done.^b ts, Temperature sensitive.

more multinucleated than were untransformed 3T3 cells. Some of the agar clones were multinucleated at both 33 and 40°C, and some were binucleated at both temperatures. Only A7 ac clones 2 and 3 and A209 ac clone 1 showed significant temperature sensitivity. WT transformants were usually multinucleated at 33 and 40°C; few clones were binucleated at either temperature. Table 2 summarizes data from all of the 3T3 transformants; again, unlike the MEF transformants, only some of the clones were temperature sensitive for the CB phenotype.

(ii) **DNA synthesis versus density.** Untransformed 3T3 cells performed equally at both 33 and 40°C, but untransformed MEF were somewhat temperature sensitive. WT and *tsB4* transformants usually performed at 40°C as well as or better than they did at 33°C. Many of the *tsA* 3T3 transformants were partly temperature sensitive in this assay (Fig. 5). These transformants often propagated to equally high densities at both temperatures, but at 40°C, the DNA synthetic activity fell to lower levels, as was the case with untransformed 3T3 cells. Some of the

FIG. 1. Photomicrographs of SV40 WT- and *tsA*-transformed MEF after CB treatment at 33 and 40°C. (a) WT lu clone 1 at 33°C; (b) WT lu clone 1 at 40°C; (c) A58 ac clone 7 at 33°C; (d) A58 ac clone 7 at 40°C.

clones which exhibited this partial temperature sensitivity (A7 lu clones 1 and 2, A58 lc clone 5, A7 ac clone 5, and A209 ac clone 2) were not temperature sensitive in the CB assay (Table 2). However, other *tsA* 3T3 transformants, unlike transformed MEF, were not temperature sensitive and continued DNA synthesis at saturation

density at 33 or 40°C (Fig. 5 and Table 2).

(iii) **Growth in soft agar.** Untransformed 3T3 cells did not form colonies in agar under our conditions; WT and *tsB4* did, but at 33°C the efficiency was often 10-fold greater. The *tsA*-transformed lines generated in agar or liquid medium often formed colonies in soft agar at

FIG. 2. Bar graphs of CB-treated WT- and *tsA*-transformed MEF showing temperature sensitivity for UND in *tsA* transformants.

both 33 and 40°C if enough cells were seeded, but with most of the transformants the efficiency was significantly reduced at 40°C, even more so than WT and *tsB4* transformants (Tables 2 and 4). More of the liquid transformants were temperature sensitive for growth in agar, and the differences at 33 and 40°C were greater. Again, some of the transformants showed discordance with the other phenotypes (Table 2).

DISCUSSION

MEF transformed by SV40 *tsA* mutants were temperature sensitive for the maintenance of transformation in all three assays utilized. At least two of the three assays (CB and DNA synthesis as a function of density) measured the capacity to continue DNA synthesis under conditions in which normal cell growth is arrested. This is consistent with the observation that at the permissive temperature *tsA* transformants show a greater number of DNA replicating forks (22). These observations of the temperature-sensitive nature of both the CB and density phenotypes support earlier observations in cells abortively transformed by SV40 that UND represents an SV40 A gene function (25). They would also lend further support to the hypoth-

esis (27) that the ability of SV40 to induce cellular DNA synthesis early after infection persists in transformed cells and is responsible for UND.

With a few exceptions, the MEF transformants showed concordance in expression of the three transformation phenotypes (Table 1). If a particular transformant was temperature sensitive in one assay, it was often temperature sensitive in all three assays.

All but one of the transformants not exhibiting temperature sensitivity were in the two groups (both agar and liquid medium generated) of *tsA209* transformants. Perhaps there is a significant amount of leakiness at 40°C which permits some cells to pass through the cell division cycle. Normally, the nonpermissive temperature for SV40 *tsA* mutants and transformants is 40.5 to 41.5°C but varies according to the cell type (2-4, 21, 31, 43). However, mouse cells are more sensitive to the toxic effects at elevated temperatures (43); therefore, we employed a relatively low nonpermissive temperature of 40°C.

In contrast to the results with MEF, 3T3 transformants generated in agar often exhibited the transformed phenotypes at both temperatures or appeared untransformed in at least one

FIG. 3. MEF transformants: graphs of increasing cell density versus thymidine incorporation in untransformed (a) and SV40 WT (b and c) and *tsA* (d to f) transformants at 33 and 40°C.

of the assays at both temperatures. Some clones, however, were indeed temperature sensitive. In the CB and DNA synthesis-versus-density assays, the failure to show temperature sensitivity was somewhat more common in the agar-generated transformants. This is partly consistent with the development of type A transformants in rat 3T3 cells as described by Seif and Cuzin (38), Rassoulzadegan et al. (33, 34), Gaudray et al. (12), and Seif and Martin (39). However, in the assay for growth in soft agar, many agar-generated transformants were partly temperature sensitive.

When generated in liquid medium by focus formation, *tsA*-transformed 3T3 cells showed a similar display of transformation expression. Most clones were not temperature sensitive for transformation by at least one criterion, some clones were transformed at both temperatures, and some did not show the transformed phenotypes at either temperature. The failure to express UND in SV40-transformed 3T3 cells has

been observed previously (24). Since a somewhat greater number of cells (5×10^4 cells per 25-cm² flask) were plated after infection for liquid medium transformants than that found optimal for the generation of type N transformants by Rassoulzadegan et al. (34), our conditions may have allowed for the generation of some type A transformants in liquid medium. This, however, would not explain the transformants which were only weakly transformed or the results with MEF in which the great majority of both agar and liquid medium transformants were temperature sensitive.

Another unusual feature of the 3T3 transformants was the apparent discordance in the expression of temperature sensitivity for the three transformation phenotypes. Some clones expressed one or two transformation phenotypes, and others expressed temperature sensitivity for one or two different transformation phenotypes (Table 2, A7 lu clone 2, A58 lc clone 5, A209 lc clone 1, A7 ac clone 5, and A209 ac clone 2).

TABLE 3. Colony-forming ability of untransformed and SV40-transformed MEF cells in soft agar

Cell line	Temp (°C)	No. of colonies per dish in dishes plated with the following no. of cells:			
		10 ³	10 ⁴	10 ⁵	10 ⁶
MEF	33	0	0	0	0
MEF	40	0	0	0	0
WT lu clone 1	33	10	79	1,020	TNTC ^a
WT lu clone 1	40	0	5	120	1,116
WT lc clone 2	33	30	250	2,000	TNTC
WT lc clone 2	40	0	90	1,500	TNTC
WT ac clone 5	33	24	320	3,580	ND ^b
WT ac clone 5	40	0	35	277	ND
WT ac clone 7	33	6	340	3,050	ND
WT ac clone 7	40	0	21	396	ND
A7 lc clone 2	33	2	31	180	ND
A7 lc clone 2	40	0	0	0	0
A58 lu clone 3	33	4	63	TNTC	TNTC
A58 lu clone 3	40	0	0	0	0
A58 lc clone 1	33	12	66	240	4,240
A58 lc clone 1	40	0	0	0	0
A58 lc clone 6	33	0	0	0	0
A58 lc clone 6	40	0	0	0	0
A209 lc clone 2	33	2	32	1,060	TNTC
A209 lc clone 2	40	0	0	0	68
A209 lc clone 4	33	60	930	2,720	TNTC
A209 lc clone 4	40	0	0	17	140
A7 ac clone 1	33	64	269	1,580	TNTC
A7 ac clone 1	40	0	0	9	270
A7 ac clone 3	33	21	640	2,370	TNTC
A7 ac clone 3	40	8	320	1,130	TNTC
A58 ac clone 4	33	2	28	260	TNTC
A58 ac clone 4	40	0	0	0	0
A58 ac clone 6	33	11	512	7,100	TNTC
A58 ac clone 6	40	0	0	0	0
A209 ac clone 3	33	24	530	7,000	TNTC
A209 ac clone 3	40	0	0	0	0
A209 ac clone 5	33	7	413	2,950	TNTC
A209 ac clone 5	40	8	130	2,420	TNTC
A209 ac clone 6	33	3	50	734	TNTC
A209 ac clone 6	40	0	0	0	0

^a TNTC, Colonies were too numerous to count.^b ND, Not done.

FIG. 4. Bar graphs of CB-treated WT-, *tsB4*-, and *tsA*-transformed 3T3 cells showing temperature sensitivity for UND in only some *tsA* transformants.

Some clones failed to express at least one of the transformation phenotypes at either 33 or 40°C. This phenomenon was seen in only two clones of MEF transformants (Table 1). Discordance of transformation phenotypes has been shown previously in SV40-transformed 3T3 cells (13, 35, 42) and in somatic cell hybrids of normal and neoplastic cells (41; F. J. O'Neill, J. Natl. Cancer Inst., in press).

The lack of temperature sensitivity exhibited by the 3T3 transformants may be a result of the fact that mouse 3T3 cells are not entirely normal (1, 45, 46). Under special conditions, 3T3 cells are tumorigenic (1). Tegtmeyer (43) has suggested that with 3T3 cells spontaneous transformation may be superimposed on some cells after virus-induced changes. This could lead to the expression of transformation at the nonpermissive temperature. In addition, the nonviral middle T-antigen, although absent from primary cell cultures, is present in small amounts in some established or tumor cell lines (9, 19), including 3T3 cells (20) and 3T6 and 3T12 cells (E. G. Gurney, personal communication). Viral infection may induce a further increase in this antigen

in some cells under some conditions, resulting in a constitutive type of transformation. This possibility is being investigated. However, this hypothesis cannot explain those 3T3 transformants not exhibiting one or more of the transformed phenotypes.

The contrasting results obtained with MEF and 3T3 cells suggest that, in addition to the importance of carefully defining the conditions of transformation (33, 34, 38, 39), the type of cells (primary versus established lines) used to generate transformants is also of importance since only *tsA*-transformed MEF consistently showed temperature dependency for the maintenance of the transformed phenotypes.

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TABLE 4. Colony-forming ability of untransformed and SV40-transformed 3T3 cells in soft agar

Cell line	Temp (°C)	No. of colonies per dish in dishes plated with following no. of cells:			
		10 ³	10 ⁴	10 ⁵	10 ⁶
3T3	33	0	0	0	0
3T3	40	0	0	0	0
WT lc clone 1	33	200	2,500	TNTC ^a	TNTC
WT lc clone 1	40	20	200	3,400	TNTC
WT lc clone 3	33	140	2,100	TNTC	TNTC
WT lc clone 3	40	20	230	2,500	TNTC
WT lc clone 4	33	300	3,000	TNTC	TNTC
WT lc clone 4	40	32	280	3,200	TNTC
WT ac clone 2	33	32	387	6,167	TNTC
WT ac clone 2	40	7	24	2,230	TNTC
WT ac clone 7	33	15	58	338	TNTC
WT ac clone 7	40	0	5	180	TNTC
B4 lu clone 1	33	6	53	TNTC	TNTC
B4 lu clone 1	40	7	34	TNTC	TNTC
B4 lu clone 2	33	22	295	2,700	TNTC
B4 lu clone 2	40	3	10	800	6,000
A7 lc clone 1	33	75	1,200	TNTC	TNTC
A7 lc clone 1	40	0	40	1,500	TNTC
A7 lc clone 2	33	0	20	250	7,000
A7 lc clone 2	40	0	0	0	500
A58 lc clone 2	33	500	1,500	TNTC	TNTC
A58 lc clone 2	40	0	0	0	500
A58 lc clone 5	33	120	1,250	TNTC	TNTC
A58 lc clone 5	40	9	9	550	5,000
A7 ac clone 2	33	5	171	3,560	TNTC
A7 ac clone 2	40	0	0	0	0
A7 ac clone 5	33	34	840	TNTC	TNTC
A7 ac clone 5	40	0	0	107	2,400
A58 ac clone 1	33	0	10	960	TNTC
A58 ac clone 1	40	0	0	8	94
A58 ac clone 2	33	0	0	14	1,940
A58 ac clone 2	40	0	0	0	103
A209 ac clone 1	33	0	68	307	4,300
A209 ac clone 1	40	0	30	125	TNTC
A209 ac clone 2	33	0	8	264	3,700
A209 ac clone 2	40	0	14	247	3,500
A209 lc clone 1	33	0	0	660	TNTC
A209 lc clone 1	40	0	0	0	0

^a TNTC, Colonies were too numerous to count.

FIG. 5. 3T3 transformants: graphs of increasing cell density versus thymidine incorporation in untransformed (a) and SV40 WT (b), tsB4 (c), and tsA (d to f) transformants at 33 and 40°C.

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