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Temperature-Sensitive Mutants of Chemically Transformed Epithelial Cells

(carcinogenesis/transformation *in vitro*/reversion/liver cell cultures)

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ABSTRACT **The first temperature-sensitive mutants of epithelial cells transformed with chemical carcinogens have been isolated. Like the wild-type transformed parental cells, the mutants readily grow in agar suspension at 36°, but in contrast to the wild type, they do not do so at 40°. Detailed studies of one of these mutants, TS-223, indicate that at high temperature it also has reduced cloning efficiency in monolayer culture and a lower saturation density. Scanning electron microscopy revealed that at 40° confluent cultures of TS-223 consist of a monolayer of generally flat polygonal cells, whereas 36° cultures contain many patches of piled-up cells that are spherical and have rougher surface membranes. All of these cellular changes are reversible with upward or downward temperature shifts. The temperature-sensitive lesion appears to reside in a host cell gene which modulates expression of the transformed cell phenotype. These mutants may provide a useful system for elucidating the minimal biochemical changes required for expression of the transformed phenotype in epithelial cells.**

Mutants that are temperature sensitive (TS) in the expression of cell transformation afford a powerful tool for analyzing mechanisms of transformation since one can, at will, turn off or on the tumor phenotype. Several TS mutants of RNA and DNA tumor viruses have been isolated (1–10). The cells transformed by these viruses have characteristics of normal cells when grown at high temperature and of tumor cells when grown at lower temperature. Cell mutants of this type have also been isolated from 3T3 mouse fibroblasts transformed by simian virus 40 (11).

It is important to develop similar mutants of cells transformed by chemical carcinogens, since the phenotype of chemically transformed cells may differ from that of virally transformed cells. Aside from the cold-sensitive chemically transformed BHK cells described by DiMayorca *et al.* (12), there are no published reports of TS mutants in cells transformed by chemical carcinogens.

In the present study we report the isolation of five mutants of epithelial cells transformed by the carcinogen, *N*-acetoxyacetylaminofluorene, which are temperature sensitive in the maintenance of several properties associated with transformation. Our laboratory has emphasized the use of epithelial cells because about 80% of human tumors arise from epithelial tissues (13), and the growth properties of tumorigenic epithelial cultures (14–16) differ in several respects from those of transformed fibroblast cultures (17).

MATERIALS AND METHODS

Cell Lines and Media. The source of our TS mutants was a transformed cell line, W-8, which was obtained by treating K-16, a normal epithelial rat liver cell line (kindly supplied by Dr. E. M. Kaighn), with a single exposure to 0.5 μg/ml of *N*-acetoxy-2-acetylaminofluorene. W-8 cells, in contrast to K-16, form colonies in agar and are tumorigenic. When 10^5 or 10^6 W-8 cells were injected subcutaneously into newborn Sprague–Dawley rats, within 3–4 weeks 1- to 2-cm locally invasive tumors composed of epithelioid cells appeared. Because of their epithelial origin, W-8 cultures do not display the criss-cross growth pattern seen with transformed fibroblasts (17) and they show "piling up" only when kept as confluent cultures for several days and only when the growth medium is changed repeatedly.

The growth medium used throughout these experiments, unless stated otherwise, was Dulbecco's modification of Eagle's medium (GIBCO) supplemented with 10% fetal calf serum. This medium was also used when cells were grown in 0.4% agar suspension or 1.2% methyl cellulose.

Mutagenesis and Selection. A cloned strain of W-8 cells was obtained by isolating a colony at 41° in agar and then recloning these cells at 41° in 1.2% methyl cellulose. The clonal isolate, referred to as "wild-type W-8", was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (18) at 1 μg/ml for 16 hr at 36°. With this dose, the survival, as measured by plating efficiency, was 34%. Subsequent selection of clones with decreased growth at high cell density was done by a slight modification of the procedure of Renger and Basilico (11). The cultures treated with *N'*-methyl-*N'*-nitro-*N*-nitrosoguanidine were washed free of the mutagen and incubated at 36° for three generations, to allow full expression of the mutant phenotype (19). Cells were then subcultured at a density of 2.5×10^5/90-mm petri dish and shifted to 41°. After 2 days, when the cultures had almost reached confluence, FdU (25 μg/ml) was added in the presence of an excess of uridine (125 μg/ml). After 4–5 days at 41° the cells were washed free of the drug and incubated at 36° in Ham's F12 medium (GIBCO) with 10% fetal calf serum. Less than $1/10^3$ of cells treated with nitrosoguanidine survived this treatment. The survivors were grown up and passed through the FdU selection two more times. After the last FdU treatment and removal of the drug, the surviving cells were allowed to grow for 1 week at 36°. They were then shifted to 41°. After 4–6 days, colonies that did not show piling-up were considered possible TS mutants, and 150 of these were isolated. Each of these was then tested for growth in agar at 36° and 41°. Five clones exhibited significantly higher cloning efficiency at 36° than 41°, indicating that they were probably TS mutants in maintenance of transformation. The TS mutants were recloned in agar at 36°, propagated, and stored in liquid nitrogen. The following experiments were done within 4 months after the cell stocks were thawed. The phenotypes of these mutants were stable during this period.

Abbreviations: TS, temperature-sensitive; FdU, 5-fluoro-2-deoxyuridine.

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TABLE 1. *Growth of TS mutants in agar suspension*

Cells	Cloning efficiency (%) 36°	41°	Ratio (41°/36°)
Wild-type W-8	46.0	18.0	0.3900
TS-171	3.4	0.007	0.0021
TS-212	14.2	0.01	0.0007
TS-223	8.1	0.007	0.0008
TS-241	2.0	0.003	0.0015
TS-387	1.7	0.0005	0.0003

Cells (10^3–10^5) were suspended in 4 ml of 0.4% agar in growth medium containing 10% fetal calf serum and plated on top of a 4-ml layer of 0.8% agar in 5-cm petri dishes. Plates were incubated at 36° or 41°. An additional 4 ml of 0.4% agar medium was overlayed on day 8. Colonies larger than 40 μm in diameter were counted on day 19. The few colonies seen with TS mutants at 41° were much smaller in size than those seen with W-8 at 41° or the TS mutants at 36°.

RESULTS

The cloning efficiencies in agar of the parental cells and the TS mutants grown at 36° and 41° are compared in Table 1. As expected, the normal epithelial K-16 cells failed to yield a single colony in agar at either temperature, even when 10^6 cells were inoculated per plate. The wild-type transformed cells derived from K-16, after treatment with *N*-acetoxyacetylaminofluorene, readily grew in agar at both temperatures. On the other hand, all the TS mutants of W-8 suffered 100-fold or greater reductions in cloning efficiencies in agar when grown at 41° rather than 36°. The cloning efficiencies in agar at 36° of the TS mutants were also lower by 2- to 10-fold than those obtained under comparable conditions with wild-type W-8 cells (Table 1), indicating that even at 36° the function of the TS gene was not completely restored. Growth studies in monolayer culture (see below) indicated that this is not simply a function of decreased viability of the TS mutants.

When grown in monolayer culture, the saturation density of transformed cells, i.e., the maximum number of cells attached to the dish at the end of a growth curve, is generally higher than that of the related normal cell (17). Indeed, this is, in part, the basis of the FdU procedure for selecting revertants of transformed cells (11). It was of interest, therefore, to determine the saturation densities of our TS mutants at 36° and 41° (Table 2). The saturation density of the wild-type W-8 cells grown at 41° was only slightly less than that obtained at 36°, indicating that the high temperature is not simply toxic to these cells. With the exception of TS-212, the saturation densities of the TS mutants at 41° were much lower than those obtained at 36°. It is of interest that when individual mutants were compared, there was not an exact correlation between the effects of high temperature on reduction of cloning efficiency in agar and decrease in saturation density in liquid medium (compare Tables 1 and 2). For example, TS-212 had a very low cloning efficiency in agar at 41° but a relatively high saturation density at 41°. These results are consistent with other types of evidence indicating that individual cell properties frequently associated with transformation can be dissociated either during forward transformation or reversion (20, 21). Since TS-223 displayed the most extreme response to temperature when scored for growth in both agar suspension and monolayer culture, this mutant was chosen for more detailed studies.

TABLE 2. *Growth of TS mutants in monolayer culture*

Cells	Saturation density (cells/dish × 10^{-6}) 36°	41°	Ratio (41°/36°)
Wild-type W-8	14.0	10.0	0.72
TS-171	7.1	1.1	0.15
TS-212	9.4	7.1	0.75
TS-223	5.7	0.59	0.10
TS-241	6.8	1.8	0.26
TS-387	3.0	1.6	0.53

Cells (2×10^5) were plated in 5-cm dishes. After overnight incubation at 36°, to allow the cells to attach to the dishes, half of the dishes were incubated at 41° and the remainder were left at 36°. At days 4 and 7, the growth medium was changed. The number of cells attached to replicate plates was determined every other day, after removal by trypsinization. Saturation density designates the maximum number of cells attached to the dish at the end of the growth curve.

Although it is not a highly reliable criterion, the cloning efficiency of cells in monolayer culture frequently increases after transformation, particularly if one starts out with normal cells that have a very low cloning efficiency (22, 23). Reversion of the tumor phenotype may, therefore, be accompanied by a decreased cloning efficiency in monolayer culture. Table 3 indicates that growth of the W-8 cells at elevated temperatures (39.5° or 41°) has relatively little effect on their cloning efficiency in monolayer culture. With TS-223 cells, however, increases in temperature progressively inhibit not only the cloning efficiency in agar suspension and the saturation density, but also the cloning efficiency in monolayer culture. In these experiments the cloning efficiency of TS-223 was about the same (30–40%) as that of W-8 when both were compared at 36°. This indicates that TS-223 does not simply have a defect in viability or replicative capacity.

The effects of temperature on growth curves of TS-223 are shown in Fig. 1. The exponential growth rate at 40° was somewhat less than at 36°. The major difference was that the

TABLE 3. *Effects of temperature variation on cloning efficiencies and saturation densities*

	Ratios			
	Wild-type W-8		TS-223	
	39.5°/36°	41°/36°	39.5°/36°	41°/36°
Cloning efficiency in agar suspension	0.57	0.39	0.013	0.009
Cloning efficiency in monolayer culture	1.1	0.96	0.74	0.019
Saturation density in monolayer culture	1.0	0.72	1.0	0.1

These data were pooled from six experiments. To determine cloning efficiency of cells in monolayer culture, 10^2–10^4 cells were plated in 5-cm dishes and incubated at 36°, 39.5°, and 41°. At day 5 the medium was changed, and at day 9 the cells were fixed with 10% formaldehyde solution and the colonies (more than 20 cells) were counted. The assays for cloning efficiency in agar suspension and saturation density in monolayer culture are described in the legends of Tables 1 and 2.

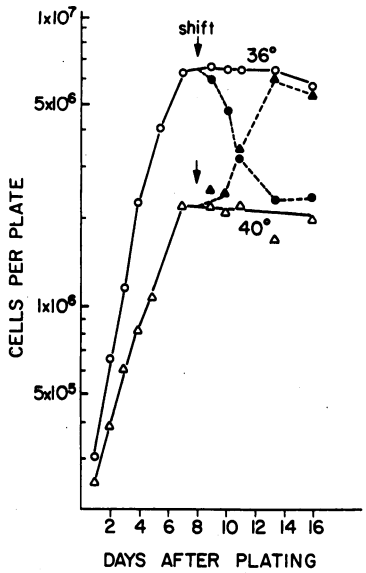

FIG. 1. Effect of temperature shift on the cell density of TS-223. Cells (2×10^5) were plated in 5-cm petri dishes, and incubated at 36° (○) and 40° (△). Medium was changed at day 3, day 6, and then every other day. At day 8 several plates were shifted from 36° to 40° (●), and from 40° to 36° (▲). Cells adherent to the dishes were counted, after trypsinization, on the indicated days.

saturation density at 40° was 2.5×10^6 cells per 5-cm plate, whereas at 36° the corresponding value was 6.5×10^6. A growth curve at 39° gave a saturation density that was closer to that obtained at 36°, whereas growth at 41° gave a much lower value (5.0×10^5). Studies with the wild-type W-8 cells revealed a much less striking effect of temperature on the shape of the growth curve and saturation density than that obtained with the TS mutants (see Table 2). The saturation densities of TS-223 at 36° and 40° remained at their plateau values even though fresh medium was added. If, however, the temperature of the 40° culture was shifted to 36°, after a lag of about 1 day, the saturation density began to increase and within 3 days reached the level obtained by the culture grown at 36°. When the culture originally grown at 36° was allowed to reach saturation and then the temperature was shifted to 40°, the opposite effect was observed. Within 1 day the number of cells attached to the plate began to decrease, and continued to do so until this number approached the cell density of the culture that was grown entirely at 40°. These results demonstrate that the temperature-sensitive lesion in this mutant is readily reversible by upward or downward shifts in temperature, as would be expected for a mutation that affects expression of the transformed state rather than cell viability.

Microscopy indicated that at 40° the plateau in saturation density of TS-223 occurred about two doublings after the culture had reached a confluent monolayer. At saturation the cells were, therefore, a tightly packed monolayer but did not show piling-up. At 36°, however, the cell number continued to increase for about four doublings after the cells were confluent. The latter phase of growth was associated with a decrease in cell size and the appearance of distinct patches of piled-up cells. When the temperature was subsequently increased from 36° to 40°, these piled-up regions gradually disappeared.

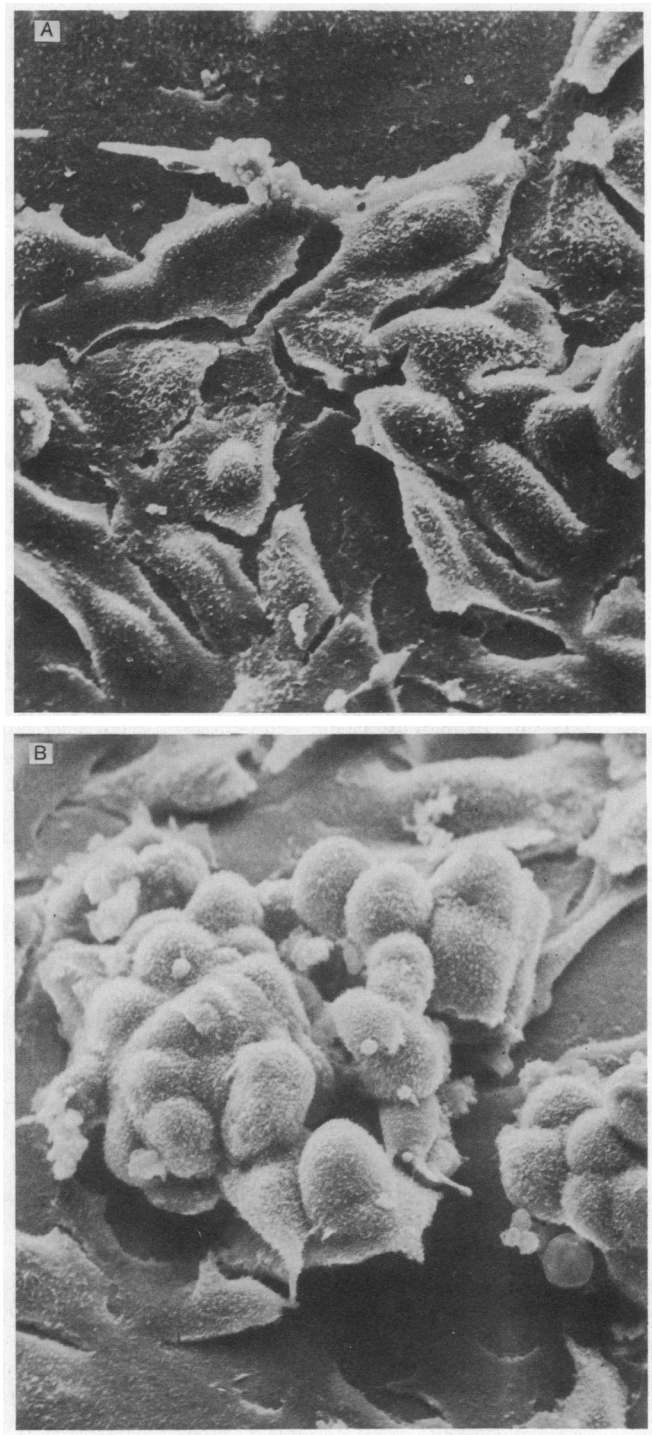

FIG. 2. Scanning electron microscopy of TS-223 grown at 36° and 40°. Cells were grown on coverslips at 36° and 40° and the medium was changed every other day. Four days after they had formed confluent monolayers, the cells were fixed for scanning electron microscopy. (A) A monolayer of TS-223 cells grown at 40°. The cells have separated from each other during fixation. (B) A piled-up region of TS-223 cells grown at 36°.

Scanning electron micrographs of TS-223 cells grown to saturation at 40° and of a patch of piled-up cells seen in the dense cultures grown at 36° are shown in Fig. 2. The cells grown at 40° are generally flat, polygonal, have a fairly smooth surface, and are arranged as a monolayer (Fig. 2A). On the other

hand, the piled-up cells seen in the 36° culture are more spherical and have a rough surface due to a larger number of microvilli (Fig. 2B). Scanning electron micrographs of the normal epithelial cells K-16, grown at either 36° or 40°, resembled TS-223 grown at 40°. The transformed wild-type W-8 cells, grown at either 36° or 40°, revealed piled-up patches of cells which, by scanning electron microscopy, resembled the TS-223 cells grown at 36°.

DNA synthesis usually ceases in normal fibroblast cultures when the cultures become confluent (24, 25), although addition of excess serum may allow them to continue to synthesize DNA and "pile-up" at high cell density (26). On the other hand, transformed fibroblasts frequently continue to synthesize DNA and pile-up when the culture reaches a high cell density, even at low serum concentrations (26). In this respect, our wild-type W-8 cells, despite the fact that they are transformed, resemble normal rather than transformed fibroblast cultures (unpublished studies). Dulbecco has also described differences between epithelial and fibroblast cells in their response to serum concentration and cell density (27). It was of interest, therefore, to study DNA synthesis in dense cultures of TS-223 at 36° and 40°. We found that when the 36° and 40° cultures were at their plateaus in saturation density, there was a very low level of [^{3}H]thymidine incorporation. A subsequent change to fresh medium led to a burst of [^{3}H]-thymidine incorporation at both temperatures (Fig. 3). Nevertheless, the number of cells attached to the dishes did not increase because of a compensatory shedding of cells into the medium. For reasons that are not clear, the cells that are shed into the medium do not replicate when replated.

It is apparent, therefore, that when these cultures are at a plateau in saturation density (in terms of maximum number of cells attached to the plate), cell replication does not necessarily cease. It can be stimulated by fresh medium, and the increase in cell number is balanced by a loss of cells from the growth surface. What is most intriguing is that the cell density at which this change in population dynamics occurs appears to be controlled by a specific gene whose function is temperature-dependent in our mutant.

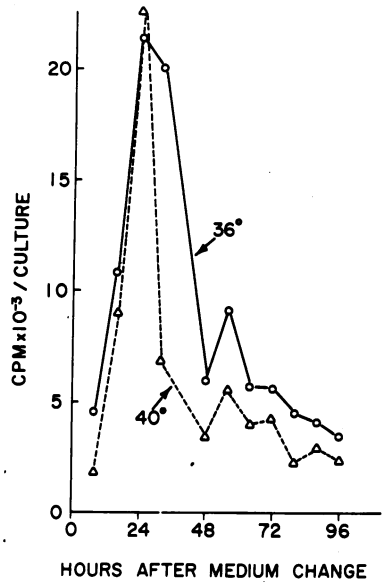

FIG. 3. Rate of DNA synthesis after medium change. TS-223 cells (5×10^4) were plated in 3-cm petri dishes, and incubated at 36° (○) or 40° (△). Medium was changed on days 3 and 6. After the last medium change (time 0) and at the times indicated, DNA synthesis was measured by pulse-labeling the cells for 1 hr with [^{3}H]thymidine (2 μCi/ml, 51.5 Ci/mmol). Immediately after the 1-hr labeling, the cultures were washed twice with phosphate-buffered saline and lysed in 0.5% sodium dodecyl sulfate. Trichloroacetic acid-insoluble material was collected on glass fiber filters (GS/A, Whatman) and counted in a Nuclear-Chicago liquid scintillation spectrometer.

DISCUSSION

The present study describes the first isolation of conditional mutants of chemically transformed epithelial cells. Most of the previously described mutants that are temperature sensitive in the maintenance of transformation were obtained from cells transformed with oncogenic viruses, and the cells were either embryonic cultures of mixed cell morphology or fibroblastic cell lines (1–11).

The transformed W-8 cells from which our TS mutants were derived are negative for evidence of oncornavirus production when examined by [^{3}H]uridine labeling (28) or electron microscopy. Studies in progress indicate, however, that infection of these cells, but not the normal K-16 cell line, with Rauscher leukemia virus results in the rescue of an agent that will transform NRK and 3T3 cells. A similar transforming agent can be rescued from all five of our TS mutants. The cells transformed with this agent are not, however, temperature sensitive with respect to morphology or growth in agar. The TS lesion present in our mutants appears, therefore, to reside in a host cell gene(s) which modulates expression of the transformed cell phenotype, rather than in this latent "viral genome." In this sense our mutants are similar to the TS mutant described by Renger and Basilico (11). At the present time it is not clear whether or not the transforming agent rescuable from our cells bears any mechanistic relationship to the transformation of the normal parental K-16 cell line that occurred after exposure to the carcinogen *N*-acetoxy-acetylaminofluorene.

Our wild type transformed cell line W-8, and TS mutants grown at permissive temperatures, do not reveal the full spectrum of characteristics typically associated with transformed cells in culture (17). W-8 and TS-223 cells are tumorigenic and can grow in agar. On the other hand, in liquid medium they stop growing after reaching a confluent monolayer and resume one cycle of synchronized DNA synthesis if fresh medium is supplied. Therefore, they appear to retain the "restriction point control" more characteristic of normal cells (29). At the permissive temperature (36°), a dense culture of the TS-223 mutant did show piling-up of cells after frequent medium changes, but this can also be seen with normal fibroblast cultures (26). Because of their epithelial origin, our transformed cells retain a round or polygonal shape. Therefore, the criss-cross or jack-straw pattern frequently used to identify transformed fibroblasts is also not applicable in this system (14–16). Studies in progress further indicate that neither the W-8 cell line nor the TS-223 mutant, when grown at either 36° or 40°, synthesize the large amounts of plasminogen activator (30) seen with other transformed cell lines (unpublished studies in collaboration with D. Rifkin and E. Reich). The fact that our TS mutants show minimal features of transformation complicates their further characterization. On the other hand, these mutants may be useful for elucidating the

minimal biochemical changes required for expression of the transformed phenotype in epithelial cells.

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