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Conditional Immortalization of Human Thyroid Epithelial Cells: A Tool for Analysis of Oncogene Action

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To overcome the difficulty of assessing oncogene action in human epithelial cell types, such as thyroid, which have limited proliferative potential in culture, we have explored the use of temperature-sensitive (*ts*) mutants of simian virus 40 (SV40) early region to create conditionally immortalized epithelial cell lines. Normal primary cultures of human thyroid follicular cells were transfected with a plasmid containing the SV40 early region from mutant *tsA*58. Expanding epithelial colonies were observed after 2 to 3 months, all of which grew to >200 population doublings without crisis. All showed tight temperature dependence for growth. After switch-up to the restrictive temperature (40.5°C), no further increase in cell number was seen after 1 to 2 days. However, DNA synthesis declined much more slowly; the dissociation from cell division led to marked polyploidy. Viability was maintained for up to 2 weeks. Introduction of an inducible mutant *ras* gene into *ts* thyroid cells led, as expected, to morphological transformation at the permissive temperature when *ras* was induced. Interestingly, this was associated with a marked reduction in net growth rate. At the restrictive temperature, induction of mutant *ras* caused rapid cell death. These results demonstrate the utility of a *ts* SV40 mutant to permit the study of oncogene action in an otherwise nonproliferative target cell and reveal important differences in the interaction between *ras* and SV40 T in these epithelial cells compared with previously studied cell types.

Activated oncogenes, particularly the *ras* family, have now been identified at high incidences in a number of human epithelial tumor types. In the thyroid, for example, we have shown that more than 50% of both benign adenomas and malignant follicular carcinomas contain a mutant *ras* gene (21, 48), and in another pathological subtype of thyroid cancer, rearrangements of the *ret* and *trk* proto-oncogenes have recently been reported at similar frequencies (5). A major question now concerns the mechanisms by which such genes perturb cell proliferation, particularly in epithelial cells, since ~90% of human cancers are epithelial rather than mesodermal in origin (28). Direct investigation of the biochemical effects of introducing rearranged or mutant oncogenes into epithelial cells is often severely hindered by the limited proliferative capacities of such cells in culture. Thyroid follicular cells, for example, will undergo at most two to three doublings.

Dominantly acting "immortalizing" genes, such as simian virus 40 (SV40) T and adenovirus E1a, can be used to overcome growth limitation, but at the risk of perturbing growth control to the extent that subsequent observations on the effects of introduced oncogenes are invalidated.

The existence of temperature-sensitive (*ts*) mutants of the SV40 early region large T encoding gene (11, 28, 29) offers the possibility of reversibly overcoming the growth limitations of normal epithelial cultures. Such a conditionally immortalized line could in principle be transfected with the activated oncogene under study, expanded to mass culture at the permissive temperature, and then switched to the restrictive temperature for analysis of oncogene actions, hopefully free of any interfering effects of large T.

Since we had recently demonstrated the ability of wild-type SV40 DNA to immortalize normal human thyroid epithelial cells, with retention of several differentiated functions (20), we explored the feasibility of using a *ts* mutant to generate corresponding reversibly immortalized epithelial cell lines.

MATERIALS AND METHODS

Primary human cell cultures. Monolayer cultures of human thyroid follicular cells were prepared from histologically normal surgical samples of fresh thyroid tissue, as described previously (45). Briefly, suspensions of follicles (epithelial cell clusters) were obtained by collagenase-dispase digestion with gentle, mechanical disaggregation and separation from contaminating, single stromal cells by differential sedimentation. On plating, the resulting follicle preparations gave primary monolayers that were >99% epithelial, as judged by cytokeratin and thyroglobulin immunostaining.

Cells were grown routinely in RPMI 1640 medium (GIBCO Laboratories) supplemented with 10% fetal calf serum (FCS; Imperial Laboratories) in a humidified atmosphere containing 5% CO_2.

DNA transfection. Primary monolayers were plated at 5×10^5 cells per 60-mm dish and transfected 4 days later with plasmid pUCSVtsA58 (kindly donated by H. Land, ICRF, London, United Kingdom) containing the early region of the *ts* SV40 mutant *tsA*58 (40). Transfection was performed by the strontium phosphate coprecipitation method (6). Six hours before transfection, RPMI medium was replaced with 5 ml of warm SF-12 medium (Flow Laboratories) containing

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10% FCS. Plasmid pUCSVtsA58 (5 μg) in 220 μl of water was mixed with 30 μl of 2 M strontium chloride and was then added dropwise to 250 μl of 2× HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-balanced salt solution. The resulting coprecipitate was left in contact with the monolayer for 90 min, after which the dishes were rinsed and then incubated with 1.5 ml of 15% glycerol in Hanks buffered salt solution (Flow) for 30 s. After washing with Hanks balanced salt solution, the cultures were fed with RPMI 1640 plus 10% FCS.

Immunocytochemistry. Cells growing on Thermanox cover slips (Flow) were fixed with acetone (10 min, −20°C) and air dried. Expression of SV40 large T protein was assessed by an indirect immunoperoxidase procedure by using monoclonal antibodies PAb419 (16) or PAb205 (13) (kindly provided by D. Lane, ICRF, London, United Kingdom) as a primary antibody, followed by rabbit anti-mouse immunoglobulin-peroxidase conjugate as a secondary antibody. Sites of antibody binding were visualized by the deposition of brown polymer following incubation in diaminobenzidine-hydrogen peroxide solution (15). Expression of thyroglobulin was assessed similarly by using rabbit anti-human thyroglobulin polyclonal antibody (Dako) followed by sheep anti-rabbit immunoglobulin G-peroxidase. Cytokeratin immunostaining was carried out on acetone-methanol (1:1)-fixed cells by using mouse monoclonals LE41 and LE61 (19).

Iodide uptake assay. Iodide uptake assays were based on the methods of Weiss et al. (43) and Reader et al. (32). Cells were seeded at 2×10^5 per well in 24-well plates in RPMI 1640 plus 1 or 10% FCS or Coon modified F-12 plus insulin, transferrin, and 5% calf serum (2). After 1 or 2 days, wells were fed with fresh medium containing 0, 0.1, 1.0, or 10 mU of bovine thyroid stimulating hormone (Calbiochem-Behring) per ml. After a period of 1 to 3 days, ^{131}I (0.2 μCi in 3.5 μM sodium iodide) was added to each well, and the wells were incubated for 4 to 6 h at 37°C. Finally, cells were washed quickly with ice-cold Hanks balanced salt solution and lysed overnight in 0.1 M sodium hydroxide. ^{131}I uptake was assessed by counting the activity of the lysates in a gamma scintillation counter.

[^{3}H]thymidine autoradiography. For assessment of the proportion of nuclei in cell cycle S phase, monolayers growing in 35-mm dishes were labeled with 10 μCi of [^{3}H]thymidine ([^{3}H]TdR) (41 Ci/mmol; Amersham Corp.) per ml for 1 h. After being fixed in methanol-acetic acid (3:1), the monolayers were then coated with Ilford K2 emulsion, exposed in the dark for 4 days, developed, and counterstained with Giemsa. For each datum point, the labeling index (LI) was determined in a random count of >1,000 nuclei from each of three replicate dishes.

Flow cytofluorimetry. Nuclear DNA content was assessed by propidium iodide fluorescence by using standard techniques (27) on a FACS 400 flow cytometer (Becton Dickinson and Co.).

Growth curves. Dishes (35 mm) were seeded with 1×10^4 or 5×10^4 cells each in RPMI 1640 plus 10% FCS. After allowing 1 day for attachment, dishes were washed twice with serum-free medium and fed with RPMI 1640 containing either no or 10% FCS. One pair of dishes (taken on day 0) was trypsinized, and cells were counted immediately; the other pairs of dishes were counted at intervals up to 10 days. Parallel sets of dishes were treated identically, except that they were transferred to 40.5°C and/or to medium containing zinc sulfate (up to 100 μM) on day 0.

Assessment of anchorage dependence. Growth in methocel was assayed by using a published method (37). Briefly, 35-mm dishes coated with 0.9% agar were seeded with 10^2, 10^3, 10^4, or 10^5 cells dispersed in RPMI 1640 plus 10% FCS containing a final concentration of 1.17% methocel (Fluka). Colonies greater than ~0.1 mm in diameter were counted in Giemsa-stained dishes 2 to 4 weeks later. The plating efficiency in methocel was compared with that on parallel cultures set up on plastic. The spontaneously transformed Chinese hamster fibroblast line V79 (49) was used as a positive control.

Tumorigenicity. Athymic (nude) mice were injected subcutaneously with 1×10^6 or 5×10^6 cells suspended in 0.2 ml of growth medium, and mice were monitored for appearance of tumors for 4 months. V79 cells were used as a positive control.

Chromosome analysis. Chromosomes were prepared by standard techniques (42) and Giemsa banded with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C for 15 min and Wright stain (diluted 1:3 in 50% Sorenson buffer). For each cell line, 20 metaphases were counted but only those with clear banding were fully analyzed.

Retroviral infection. Retroviral infection was carried out as described previously (8). Briefly, filtered, virus-containing medium from ~90% confluent producer cells was applied to target cells for 2 h together with 8 μg of polybrene per ml. After the cells were washed, 2 days were allowed for proviral integration and expression, following which cultures were passed at appropriate dilutions into medium containing 400 μg of G418 per ml to select for infectants.

Production of amphotropic retroviral *ras* vector. A high-titer amphotropic retroviral vector encoding mutant human H-*ras* was constructed as follows. Plasmid SVX1 (36), kindly provided by M. W. Lieberman (Fox Chase Cancer Center, Philadelphia, Pa.) and containing the valine 12 mutant H-*ras* gene from the EJ/T24 bladder cancer line (39) under the control of the zinc-inducible metallothionein I gene promoter (38), was cloned into the vector plasmid pZIPneoSVX (9). We first transfected SVX1 into the ecotropic packaging line, psi-CRE (14), and screened resulting G418-resistant clones for retroviral production by determining titers for transduction of G418 resistance on murine NIH 3T3 fibroblasts. Supernatant from one high-titer producer clone (>10^6 CFU/ml) was next used to infect the amphotropic packaging line psi-CRIP (14). Again, G418-resistant colonies were screened to obtain the highest titer producers, but this time both NIH 3T3 and a human epithelial line (A431) were used as the target cells. Of 10 clones tested, titers ranged from ~10^1 to ~10^3 CFU/ml on A431 cells and, in most cases, approximately 100-fold higher on NIH 3T3. The highest titer producer (3×10^3 CFU/ml on A431), designated psiCRIP-MT.T24.1, was used for subsequent experiments.

RESULTS

Isolation of *ts*-SV40 transfectants. Six separate transfections were carried out on a total of 35 dishes. In all experiments, 2 to 3 weeks after transfection, foci of mitotically active cells with an epithelial morphology were observed in most dishes at a frequency of ~10^{-5}. Two dishes immunostained at this stage with PAb419 showed clusters of 20 to 50 cells with strong nuclear large T expression.

Although the majority of these early foci failed to progress beyond around five population doublings (p.d.) and finally disappeared after 2 to 3 months, in one dish from transfection no. 2 (with cells from a 44-year-old subject), a rapidly expanding colony was observed after 2 months. This clone

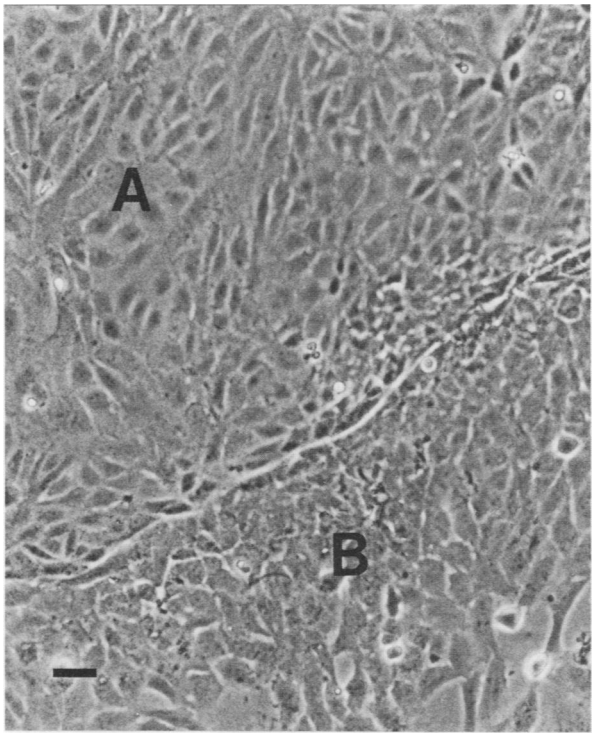


FIG. 1. Early passage *tsA*58-transfected human thyroid epithelial cells. A colony of cells prior to first subculture showing characteristic dedifferentiation event is shown. Compare initial morphology seen at center of colony (A) with morphologically less well-differentiated, more rapidly growing variant arising at periphery (B). Bar, 50 μm.

was successfully isolated and passaged to give rise to a stable line, which was designated N-thy-ts.1 (see below).

Multiple variations in culture conditions were explored in an attempt to increase the yield of progressively growing transfectants. Cultures were transferred to the permissive temperature (33°C) just before or 3 or 7 days after transfection or were kept at 36°C (intermediate between permissive and restrictive temperatures) throughout. Cultures were either split 3 days after transfection (to avoid possible density inhibition) or left in original dishes (in case close contact between early transfectant cells was required). Reduced serum concentrations (5 or 1%) were also tried in an attempt to select for transfectants (since wild-type SV40 had been found to reduce serum requirement [20]). None of these manipulations appeared to influence the outcome.

However, a later transfection (no. 5) employing cells from a younger patient (13 years old) gave strikingly different results. Of seven dishes transfected, more than 30 expanding colonies similar to N-thy-ts.1 were observed at 2 to 3 months. Of these, one was picked from each separate dish (to ensure the uniqueness of clones), and the seven were designated N-thy-ts.2 to N-thy-ts.8.

Behavior of *ts* clones in early passage. When first isolated, all colonies were composed of closely packed polyhedral cells forming the cobblestone appearance typical of epithelial cell monolayers. However, by the first subculture (corresponding to ~15 p.d.), multiple segregant foci of more angular, morphologically less-differentiated cells began to appear, often at the edges of cell islands (Fig. 1). These variants grew more rapidly than the well-differentiated parent cells and arose with such frequency that, despite repeated subcloning and/or mechanical scraping of dishes, they became the predominant cell type in all lines within a few passages.

The final, stable phenotype of all eight lines was closely similar, with respect to the parameters analyzed as described below. For simplicity, N-thy-ts.1 is presented as an example. All lines continued to grow for >200 p.d. with no sign of later crisis.

Temperature-dependent phenotype. N-thy-ts.1 cells grew with a doubling time of ~2 days in 10% FCS. Growth began to slow at temperatures above 36°C and ceased at 40.5°C (Fig. 2a), which was used as the restrictive temperature in subsequent experiments. After switch-up to 40.5°C, a marked change in morphology was evident within 2 days and consisted of a flattening of the cytoplasm of all cells together with enlargement of the nucleus and/or appearance of binucleate cells (Fig. 3); average nuclear diameter continued to increase over the next few days. Provided that cultures were more than 25% confluent at the time of temperature switch, however, there was no sign of toxicity or cell death until at least day 10, and cell numbers remained constant over this time. (In sparse cultures, dead cells became noticeable much sooner and cell numbers began to decline within the first week at 40.5°C [data not shown].) Control HTori-3 (20) cells expressing wild-type T grew even more rapidly at 40.5 than at 33°C, thus making unlikely any nonspecific growth inhibitory effect of the raised temperature (Fig. 2a). As a further check of viability at 40.5°C, cells were replated to 33°C at clonal densities after 5 days of culture at 40.5°C. Plating efficiencies averaged 3.2% (32 colonies per 10^3 cells plated) compared with 9.8% in parallel cultures never exposed to the restrictive temperature.

Expression of SV40 large T. By using antibody PAb419, more than 99% of the nuclei were strongly immunostained in *ts*.1 cells maintained at 33°C, and more than 90% were stained after 5 days at 40.5°C. In contrast, by using PAb205, which discriminates between the conformation of T at permissive and restrictive temperatures, only ~5% of nuclei were positive at 40.5°C compared with more than 99% at 33°C (Fig. 4).

Proliferative behavior of ts.1 cells. As expected from the growth curves (Fig. 2a), DNA synthesis (assessed by [^{3}H]TdR labeling) was clearly temperature dependent. On switching to 40.5°C, the nuclear LI in 10% FCS fell from 44.1% ± 5.2% at day 0 to 16.8% ± 2.4% after day 5 and reached a lower limit of between 4 and 5% by day 7 (Fig. 2b). However, the rate of this reduction was insufficient to account for the completeness and rapidity of growth cessation at 40.5°C (Fig. 2a). To explain the existence of a labeling index of >15% for the first 4 days despite no increase in cell numbers, we analyzed the cell cycle phase distributions of the population by flow cytofluorimetry of P_I-stained cells.

Logarithmically growing cultures of ts.1 cells at 33°C showed the expected DNA histogram for a diploid population (Fig. 5a). After a switch to 40.5°C, by day 2 there was a diminution in the G1 and S phase regions coupled with a marked increase in the G2 (4 *n* DNA) fraction, which by day 3 became the predominant peak (Fig. 5b). By day 4, a third peak began to appear corresponding to an 8 *n* DNA content. The final, stable pattern (reached by day 7) consisted of three roughly equal peaks at the 2, 4, and 8 *n* level (Fig. 5c). We conclude that, during this first week at 40.5°C, much of the [^{3}H]TdR labeling represented ongoing DNA synthesis in the absence of cell (or nuclear) division, leading as a result to polyploidy.

Growth factor dependence. To assess growth factor depen-

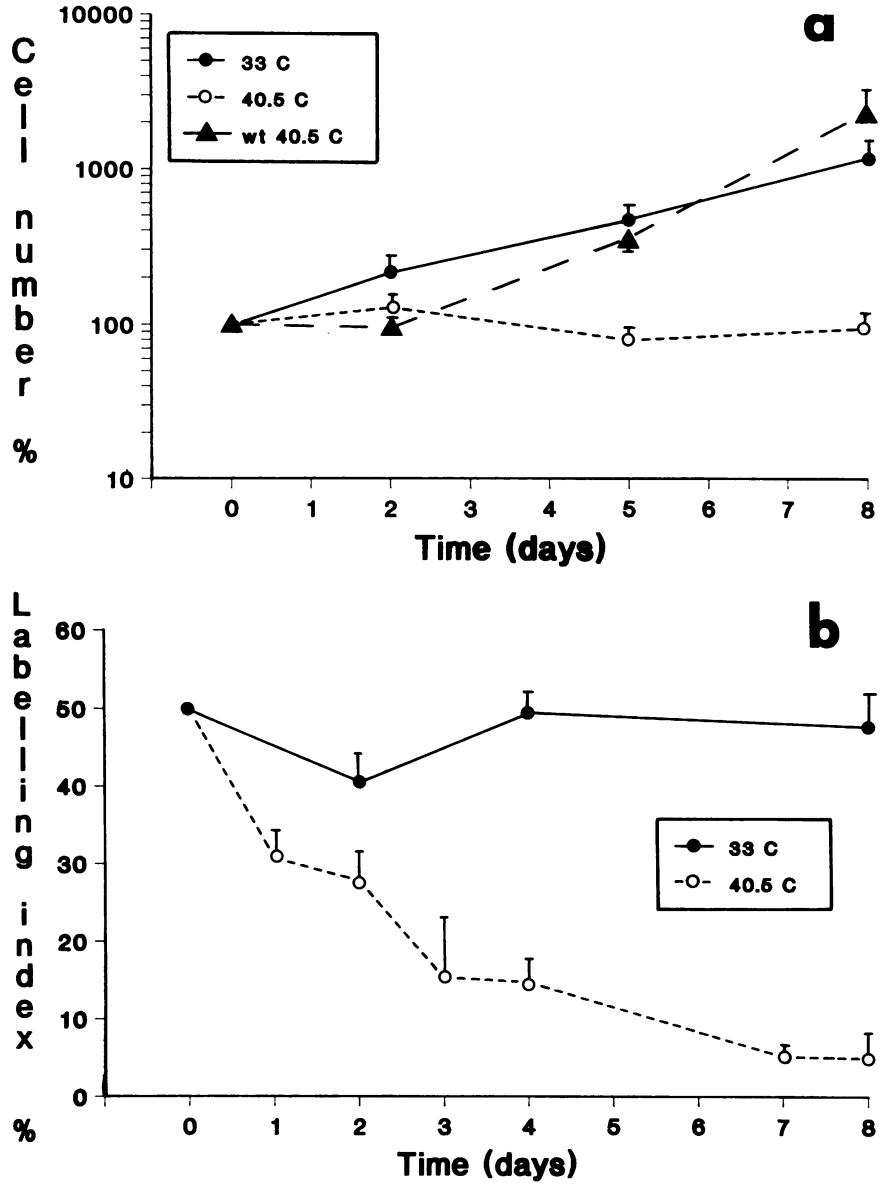


FIG. 2. Temperature dependence of immortalized thyroid epithelial cell line N-thy-ts.1. (a) Growth curves (in 10% FCS) at permissive (33°C) and at restrictive (40.5°C) temperatures demonstrating high degree of temperature dependence with rapid cessation of growth after temperature switch-up. (Growth of a comparable line immortalized by wild-type SV40 [Ht-ori3] at 40.5°C is shown for comparison.) Cell number is expressed in each case as percentage of the seeding value; each datum point is the mean of duplicate samples (the vertical bar indicates the range of values). (b) Corresponding time course of nuclear labeling indices (after pulse-labeling with [^{3}H]TdR). Note much slower decline in DNA synthesis after temperature switch-up. Each datum point is the mean of three samples (vertical bar, standard error of the mean).

dence, cells were replated at 33 or 40.5°C in 10% FCS and, after 1 day was allowed for attachment, washed and fed various concentrations (from 0 to 10%) of FCS. Proliferation was assessed by [^{3}H]TdR labeling 5 days later. Growth curves were also measured in 10 versus 0% FCS. At 33°C, there was only minimal dependence of DNA synthesis on serum; the LI was 44.1% ± 5.2% in 10% FCS, compared with 31.9% ± 3.6% after 5 days in serum-free medium (Fig. 6b). Cell numbers continued to increase in 0% FCS (Fig. 6a), but more slowly than could be accounted for by the modest reduction in the LI, indicating that an increase in cell death rate occurred in serum-free conditions.

At 40.5°C, there was a more marked serum dependence; LI (assessed after 5 days at 40.5°C) fell from 16.8% ± 2.4% in 10% FCS to 4.8% ± 2.2% in 0% FCS (Fig. 6b). Furthermore, marked cell loss occurred after 3 days in 0% FCS (Fig. 6a). This was expected, given the increased cell death rate inferred from the information presented above.

Anchorage dependence and tumorigenicity. No proliferation of ts.1 cells was observed at any cell density in 1.17% methocel at 33 or 40.5°C. Control V79 cultures gave a plating efficiency in methocel of ~40% of that obtained on plastic.

No tumors were seen in six nude mice injected with 1×10^6 and 5×10^6 ts.1 cells (mice received two injections, one of each inoculum size) after 4 months of observation. Control V79 cells gave tumors at each site in <4 weeks.

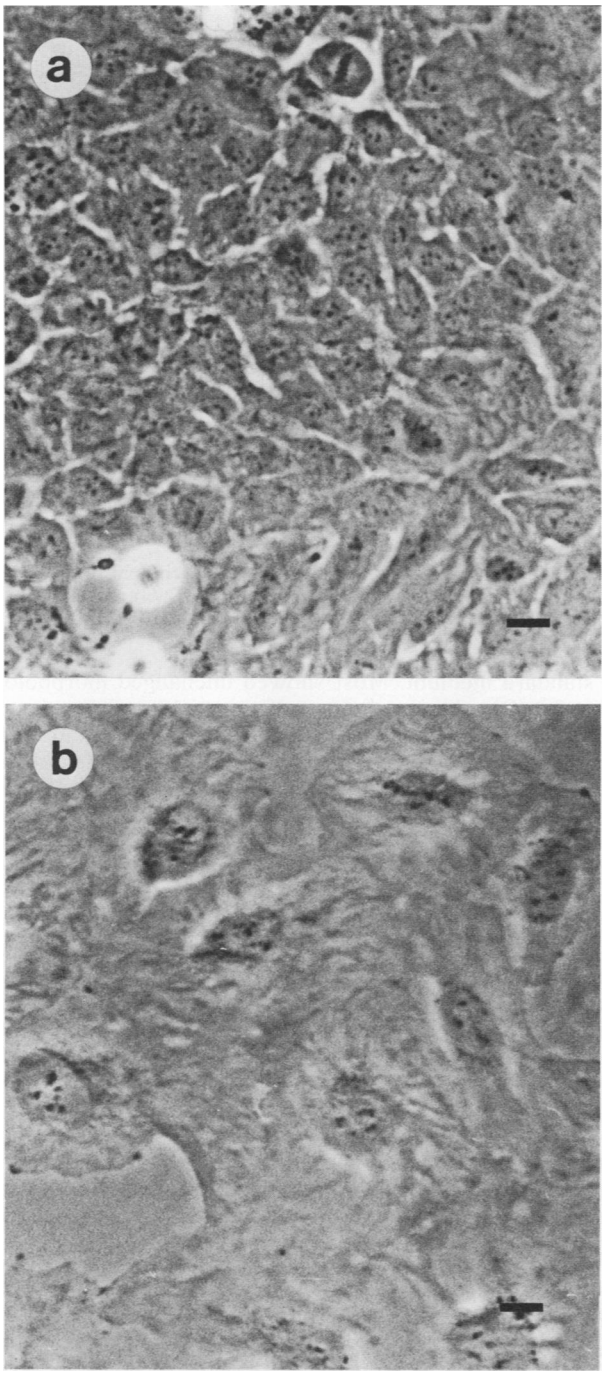


FIG. 3. Morphology of N-thy-ts.1 cells after ~150 p.d. at permissive temperature (33°C) (a) or after 5 days at restrictive temperature (40.5°C) (b). Note flattening of cells and enlargement of nuclei. Bar, 20 μm.

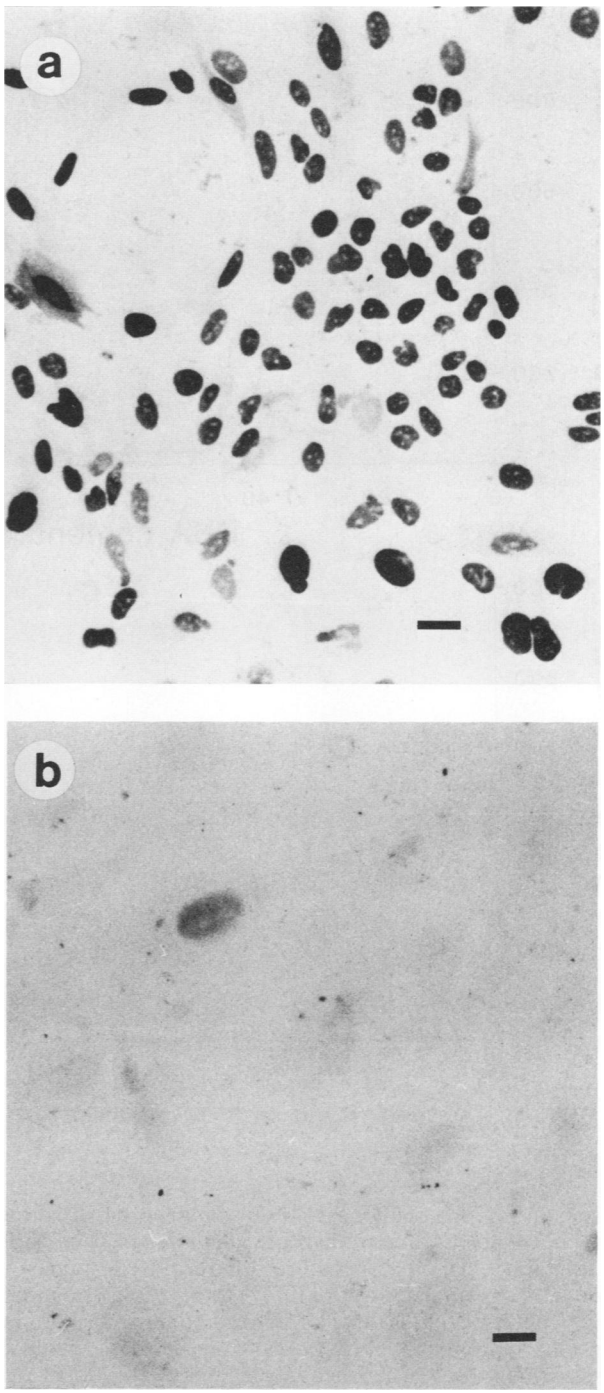
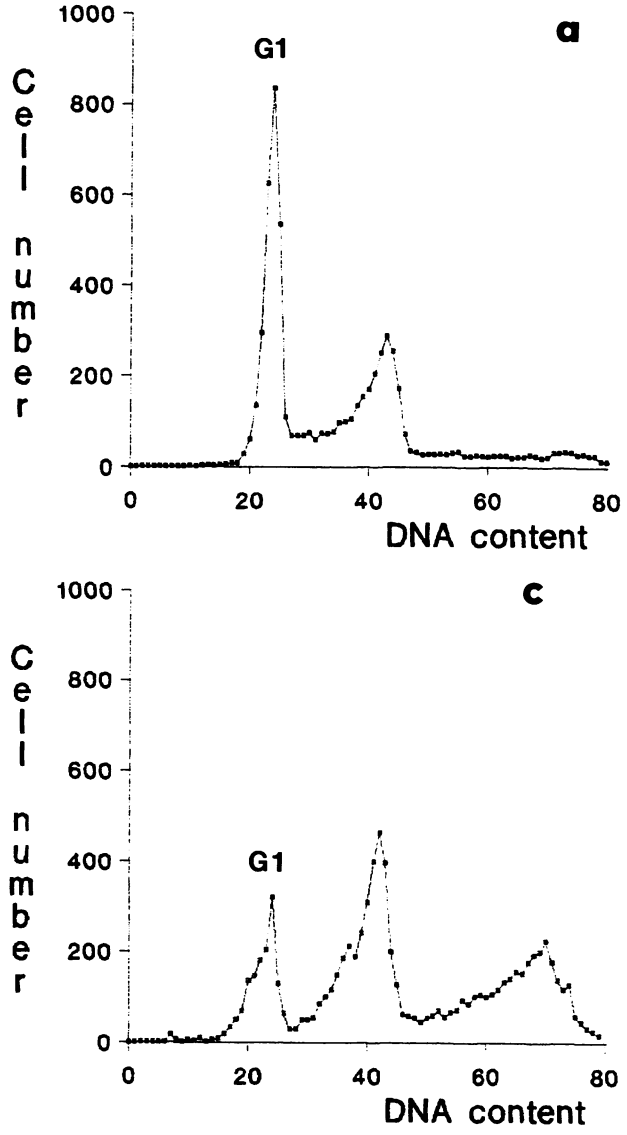


FIG. 4. Temperature-dependent expression of the SV40 T epitope recognized by monoclonal PAb205. N-thy-ts.1 cells were immunostained with PAb205 at 33°C (a) and after 5 days at 40.5°C (b). Bar, 30 μm.

Differentiation markers. (i) Cytokeratin and thyroglobulin expression. When first isolated, ts.1 cells showed strong cytoplasmic immunostaining with monoclonal antibodies specific for both cytokeratin 8 (LE41) and cytokeratin 18 (LE61) and thyroglobulin, comparable in intensity with that seen in normal primary follicular cells. Concurrent with the observed change to a less epithelial morphology after further passage, there was a loss of detectable thyroglobulin and a marked reduction in expression of cytokeratin 8; expression of cytokeratin 18 was, however, undiminished (Fig. 7). These patterns of immunoreactivity were not influenced by temperature; in particular, there was no reexpression of cytokeratin 8 in late-passage cultures on switching to 40.5°C.

(ii) Iodine uptake. Despite varying several parameters, including medium composition (RPMI 1640 or Coon; 1, 5, or 10% serum) and timing of assays, no significant trapping of radioiodide compared with the negative controls (V79 fibro-



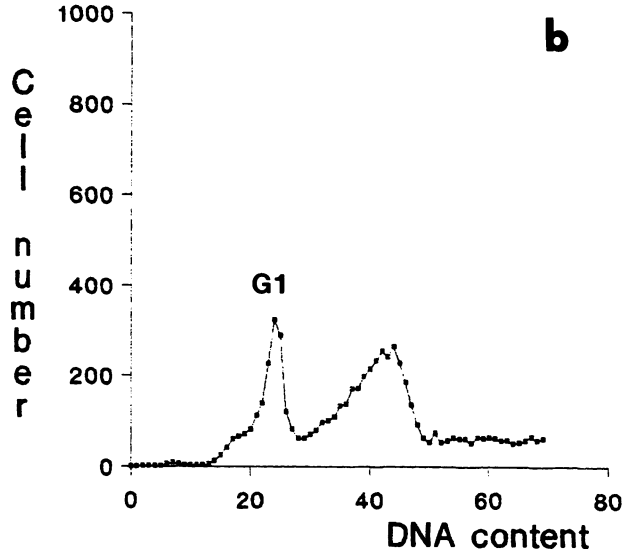


FIG. 5. Flow cytometric analysis of DNA content of N-thy-ts.1 cells in logarithmically growing culture at 33°C (a) and after being switched to 40.5°C for 3 days (b) and 7 days (c).

blasts or thyroid epithelia at 4°C) could be demonstrated with any concentration of thyroid stimulating hormone up to 10 mU/ml. Positive control rat thyroid FRTL cells (8) assayed in parallel showed a three- to eightfold stimulation of ^{131}I uptake with 0.1 mU of thyroid stimulating hormone per ml. (Only established, late-passage *ts* cells could be assessed for I^- uptake, since insufficient cell numbers were available from the early, well-differentiated stage).

Karyotype. N-thy-ts.1 and N-thy-ts.7 lines analyzed after >100 p.d. were predominantly diploid; 85 and 65% of metaphases, respectively, had 46 chromosomes, with no marker chromosomes. N-thy-ts.2 and N-thy-ts.8 showed no clear modal number (N-thy-ts.3, -4, -5, and -6 were not analyzed).

Transformation of ts.1 cells by mutant *ras*. As a preliminary test of the usefulness of *ts* thyroid cells for analyzing oncogene effects, we infected ts.1 cells with an amphotropic retroviral vector (psi-CRIP-MT-T24.1) containing the codon 12 valine mutant human H-*ras* derived from the EJ bladder cancer line, under the control of the zinc-inducible metallothionein promoter. Colonies were selected in G418 at 33°C in standard medium. Most showed unchanged morphology (Fig. 8a). On addition of zinc sulfate to a 100 μM concentration, there was a dramatic morphological response in >90% of colonies. Within 2 to 3 days, many cells rounded up and became more loosely attached, giant multinucleate cells appeared, and widespread cytoplasmic vacuolation was evident. A large number of aberrant mitoses were also observed (Fig. 8c). (These changes were also seen to a less marked extent in a minority [<5%] of colonies maintained in Zn^{2+}-free medium.)

On switching to 40.5°C, colonies in Zn^{2+}-free medium (showing parental ts.1 morphology) simply underwent the expected flattening and growth arrest seen previously in the parent line (Fig. 8b). In contrast, the effect of temperature switch-up on colonies in 100 μM Zn^{2+} showing transformed morphology was to induce rapid cell death; >99% of cells had detached and died within 5 days at 40.5° (Fig. 8d).

Further analysis of mutant *ras* effects. To assess mutant *ras* effects in more detail, we studied a representative line, designated ts.T24-1, which was derived by transfecting ts.1 cells with the SVX1 plasmid (containing metallothionein-driven mutant H-*ras*).

In the absence of zinc, ts.T24-1 showed a stable phenotype closely similar to that of the parent, ts.1. Northern (RNA) blot analysis of total cellular RNA showed, as expected, a 1.4-kilobase (kb) transcript hybridizing to a human H-*ras* probe the intensity of which increased markedly from barely detectable in the absence of Zn^{2+} to a stable maximum reached after 2 days in 100 μM Zn^{2+} (Fig. 9). At 33°C, addition of 100 μM Zn^{2+} induced morphological changes similar to those seen in the early colonies (described above), e.g., cytoplasmic vacuolation and nuclear pleomorphism, within 3 days. [^{3}H]TdR labeling showed a minor but statistically significant decline from 44.9% ± 3.2% in zinc-free medium to 33.0% ± 3.7% ($P < 0.05$) after 2 days in Zn^{2+}, which was sustained thereafter (33.6% ± 4.2% at day 10). There also appeared to be little observable change in mitotic activity in dense (>25% confluent) cultures. Nevertheless, serial cell counts revealed a marked and early reduction in net growth rate compared with Zn^{2+}-free cultures (Fig. 10) to a much greater extent than could be

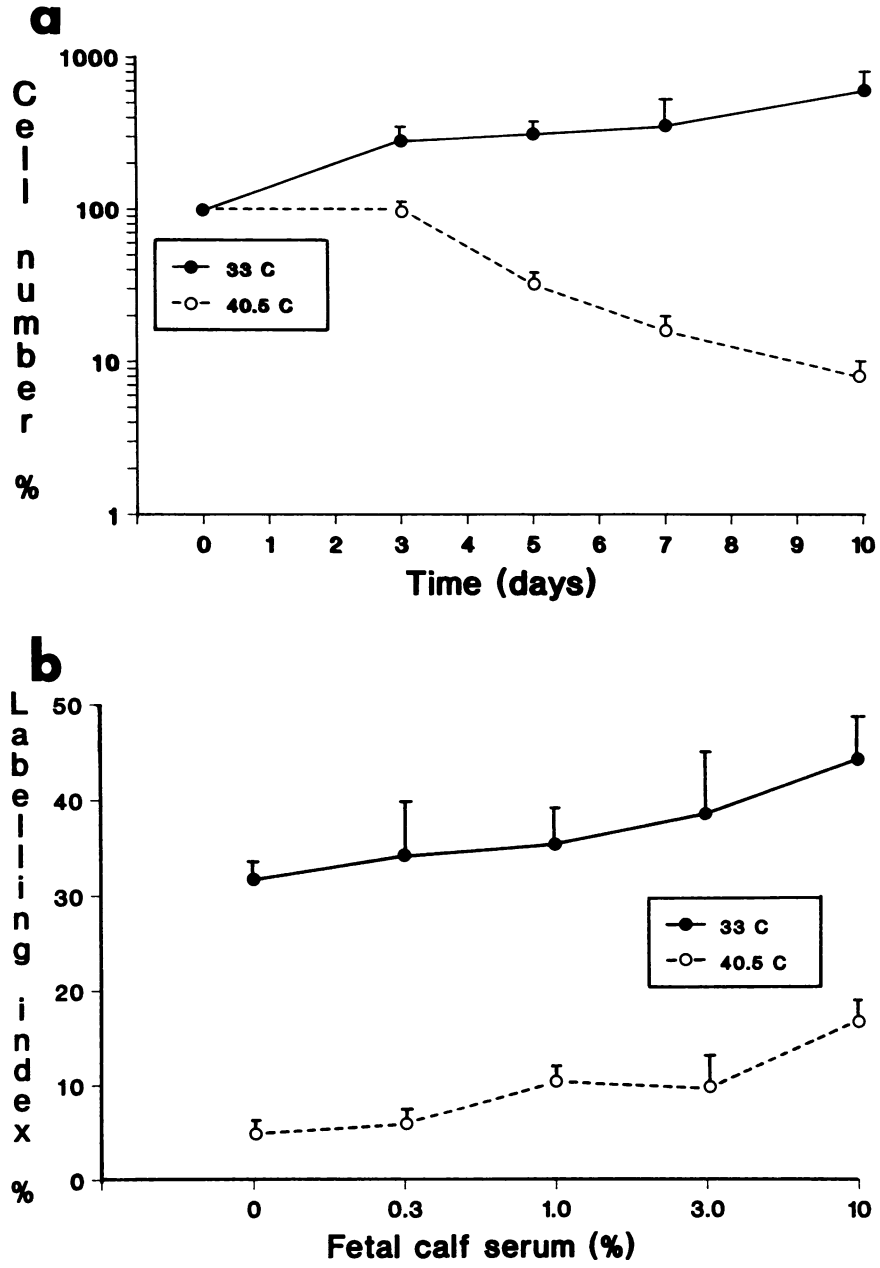


FIG. 6. (a) Temperature-dependent survival of ts.1 cells in 0% FCS. Cell number is expressed as a percentage of seeding value. Each datum point is the mean of duplicate samples (the bar indicates the range of values). (b) Serum dependence of DNA synthesis in N-thy-ts.1 cells. [^{3}H]TdR LI after 5 days in medium containing 0 to 10% FCS at 33 and at 40.5°C. Each datum point is the mean of three samples (vertical bar, standard error of the mean).

explained by the reduction in the LI and therefore indicating an increased death rate. At a low density (<5% confluent), addition of Zn^{2+} led to morphologically obvious cell death and cell numbers fell rapidly to <10% within 7 days (data not shown).

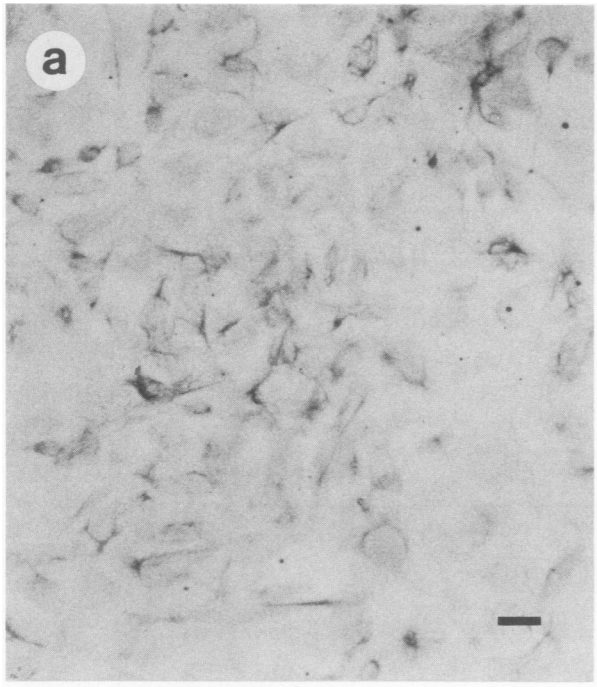
Switch-up to 40.5°C in the absence of zinc resulted in viable growth arrest with the same morphological changes as seen in ts.1 (although survival was more dependent on high cell density and was sustained for a shorter period) (Fig. 10). The [^{3}H]TdR LI fell as in ts.1 (from 42.4% ± 5.3% at 33°C to 11.2% ± 2.1% after 6 days at 40.5°C and <5% by day 8). In the presence of 100 μM Zn^{2+}, however, transfer to 40.5°C resulted in dramatic rounding up and detachment of cells; more than 95% were dead within 7 days of switch-up in high-density cultures (Fig. 10) and in less than 3 days at low-seeding densities (<5% confluent) (data not shown).

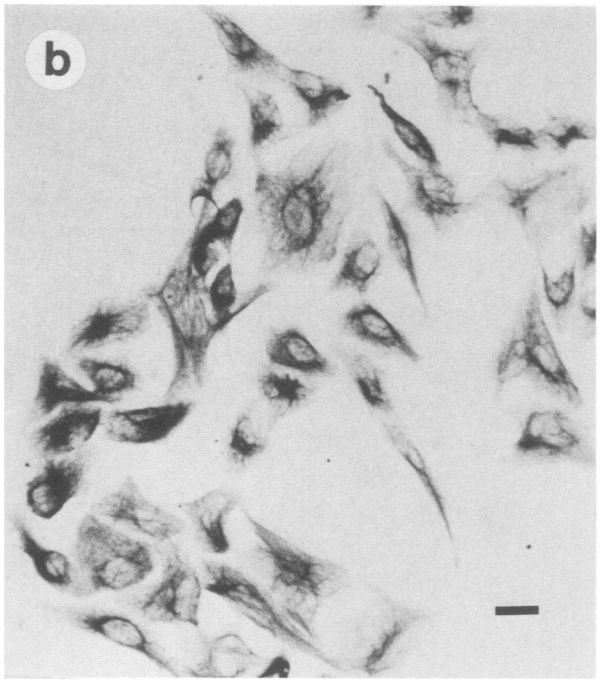
No colony formation occurred in methocel at either temperature in the presence or absence of zinc.

Addition of Zn^{2+} had no detectable effects on morphology or growth of the parent ts.1 line.

DISCUSSION

Our results show that differentiated normal human thyroid epithelial cells can be immortalized by transfection with a plasmid containing the SV40 early region encoding a tem-





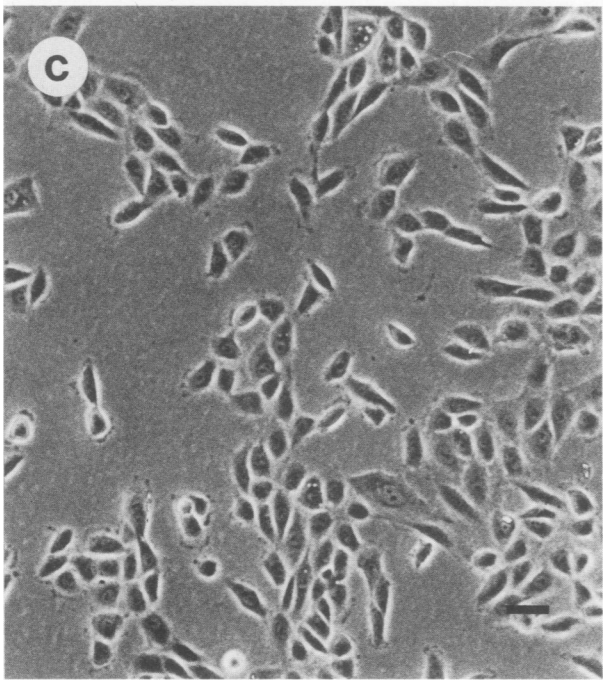


FIG. 7. Expression of epithelial cytokeratins by N-thy-ts.1 cells. (a) Immunostaining with Ab LE41 specific for cytokeratin 8; (b) Ab LE61 specific for cytokeratin 18. Bar, 30 μm. (c) Phase contrast of parallel culture. Bar, 50 μm.

perature-sensitive large T protein. The evolution of these lines showed several interesting features.

Only a small proportion of the initial presumptive transfectant foci developed beyond 4 to 5 p.d. The exact relationship between these foci and the rare, progressively growing colonies cannot be clearly established from these experiments, since it is not known whether the latter grew continuously or whether there was an intermediate period of stasis from which a subclone emerged. The latter possibility is more likely, however, since in all cases large colonies appeared quite sharply after more than 2 months of observation. This, together with the finding that the abortive foci appeared to be undergoing continuous mitotic division without net growth (followed ultimately by net death), suggests that thyroid cells go through a crisis stage, as is often observed in other SV40-transformed human cells (for example, breast [10] and colon [4]). If this is the case, however, it occurred much earlier in thyroid; in breast and colon, it is seen only after at least 30 p.d. Repetition of these experiments with a more painstaking follow-up of all early foci should resolve this point.

What is clear from our observations is that the progressively growing colonies, which at first showed good epithelial differentiation, underwent a frequent dedifferentiation event after 10 to 15 p.d. The frequency with which less-differentiated, independent segregant foci appeared in all first passage lines was much higher than would be expected from mutation or chromosome rearrangement, even given the increase in these events characteristic of SV40-transformed cells (22, 25, 41), and there were no cytogenetic markers, as have often been associated with rarer progression events in SV40 transfectants (7, 24). Although multiple copies of SV40 early region were present (estimated at 7 to 10 per cell in ts.1 from Southern blot analysis [data not shown]), replication of SV40 sequences is also an unlikely cause, since the same phenomenon was clearly seen in previous experiments by using wild-type pSV40ori$^-$, which lacks a viral origin of replication and typically gave lines containing one copy per cell. It has also since been observed by using a U19 (non-origin-binding) variant of *tsA*58, which is also incapable of replication.

In view of the remarkably similar morphology and growth characteristics of all the *ts* lines, we suggest an epigenetic mechanism in which a predetermined switch in differentiation program occurs to a less-differentiated phenotype. This switch is most likely a spontaneous event, to which the cells are predisposed by continuous growth in culture. If, how-

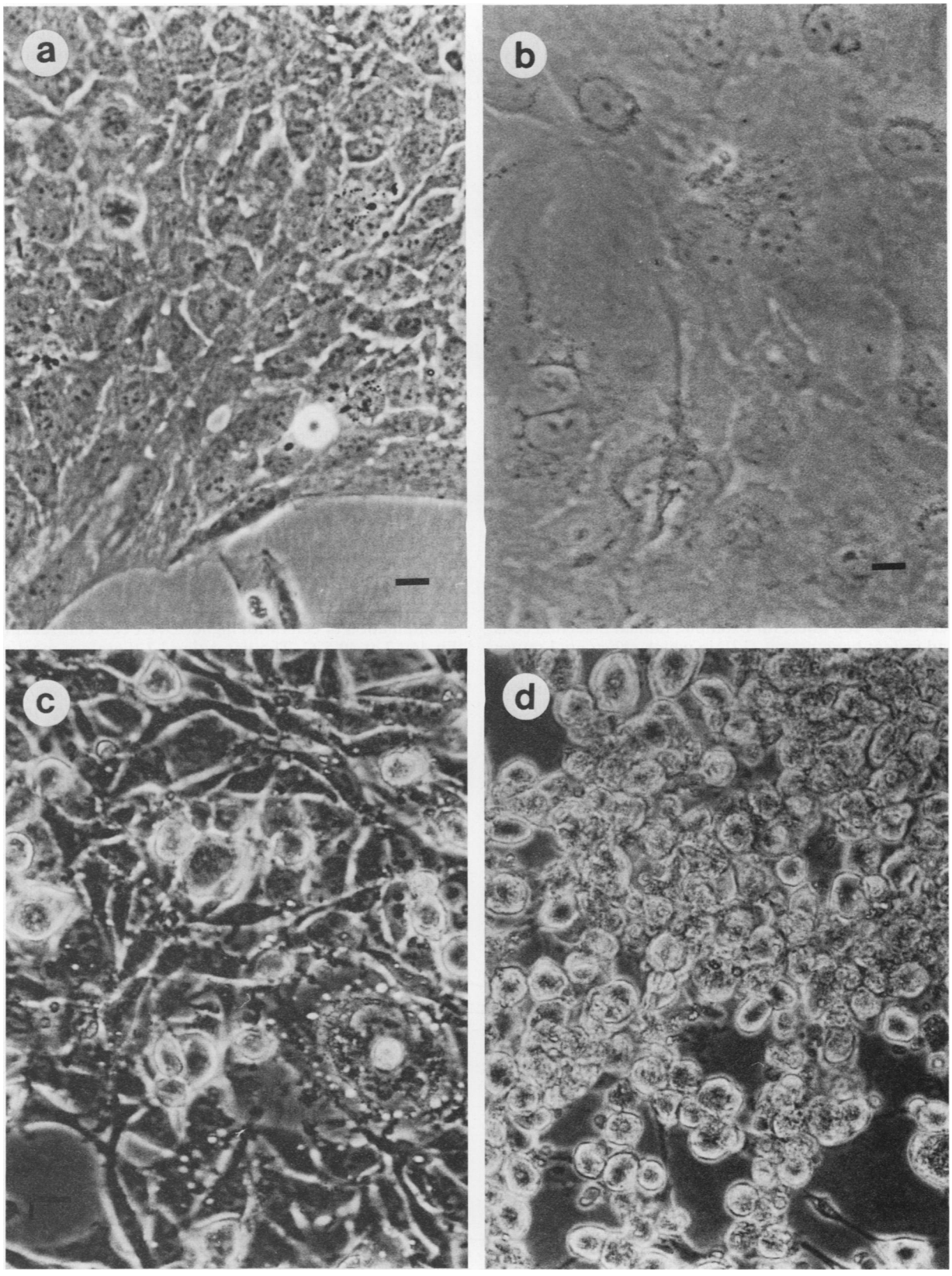


FIG. 8. Morphology of *ts*.1 cells containing an inducible mutant *ras* vector. (a) Uninduced (Zn^{2+}-free medium) at 33°C; (b) uninduced at 40.5°C; (c) mutant *ras* induced (100 μM Zn^{2+}) at 33°C showing large numbers of mitoses (some aberrant), giant cells, and prominent cytoplasmic vacuoles; (d) *ras* induced at 40.5°C showing nearly all cells rounded up in clumps. These subsequently detached and were not viable on return to 33°C either with or without Zn^{2+}. Bar, 20 μm.

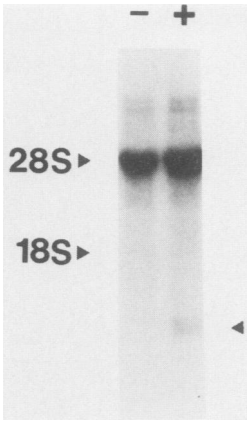


FIG. 9. Northern blot showing Zn^{2+}-inducible expression of mutant H-*ras* from the MT-T24 vector. Total cellular RNA (20 μg per lane) from ts.T24.1 cells growing in the absence (lane −) or presence (lane +) of zinc sulfate (100 μM, 2 days) was separated on a 1% agarose-formaldehyde gel, blotted to Zetaprobe (Bio-Rad Laboratories), and hybridized to ^{32}P-labeled insert of pEJ (6.6-kilobase human genomic H-*ras* fragment). A Zn^{2+}-inducible transcript of ~1.4 kilobases was observed (arrow). (Several much larger, nonspecific bands, including 28 S RNA, cross-hybridized to this probe.)

ever, SV40 T is directly involved, it must be through a hit-and-run mechanism, since the new phenotype is not reversed at the restrictive temperature. This is in contrast to several other *ts* cell lines, for example, human placental cells (11) and rodent hepatocytes (12), in which substantial return to normal phenotypes is seen at the higher temperature. In some cases, choice of culture conditions has been found to preserve differentiation (or its reversibility), e.g., use of serum-free media and specific hormone supplements for wild-type and *ts* SV40-immortalized rodent hepatocytes (12, 46). Such manipulations have not so far met with success in our hands. However, our experience with wild-type SV40 thyroid lines in which a minority (HT ori-3 and ori-5 [20]) were found to be much better differentiated suggests that screening of a larger series of lines should be rewarding.

Although this dedifferentiation event currently restricts the usefulness of our cells for studies of thyroid differentiation, it is of potential interest for studies of thyroid growth and tumorigenesis. Well-differentiated thyroid cancers occasionally undergo progression to a less- or undifferentiated phenotype associated with a much worsened prognosis. The dedifferentiation event observed in this study may be an in vitro analog of this step, a possibility all the more interesting if it does indeed occur by an epigenetic mechanism.

Whereas loss of differentiation was not reversible in our lines, growth, on the contrary, showed excellent temperature dependence. Growth ceased rapidly on switch-up to the restrictive temperature, and a constant population size could be maintained for more than 10 days. No revertant (non-*ts*) segregant clones were observed in more than 50 passages. This completeness and stability of the *ts* phenotype observed in thyroid cells is to our knowledge superior to any previous *ts* SV40 human lines, which commonly either continue to grow slowly (3) at the restrictive temperature or else rapidly die (31).

The kinetics of growth arrest were, however, more complex than anticipated, in that although net population growth ceased within one day, DNA synthesis as measured by [^3H]TdR LI declined much more slowly. Persisting DNA synthesis without increase in cell number could clearly be the result of a counterbalancing death rate, but there was no evidence of cell death on this scale over the first week. The explanation for the discrepancy was provided by flow cytofluorimetric analysis, which demonstrated the progressive accumulation of cells with higher DNA content, reaching 8 *n* by day 7 at 40.5°C. The persisting LI therefore most probably reflects DNA synthesis occurring in the absence of mitosis, resulting in polyploidy. Dissociation of DNA synthesis from cell division also explains two prominent features observed early after temperature switch-up, namely the development of binucleate cells and the increase in average nuclear size. Binucleate cells arise through DNA replication and mitosis occurring without subsequent cytokinesis and are a frequent accompaniment, and probably a precursor, of mononucleate polyploid cells in a variety of cell systems (23). Mononuclear polyploidy is due to either

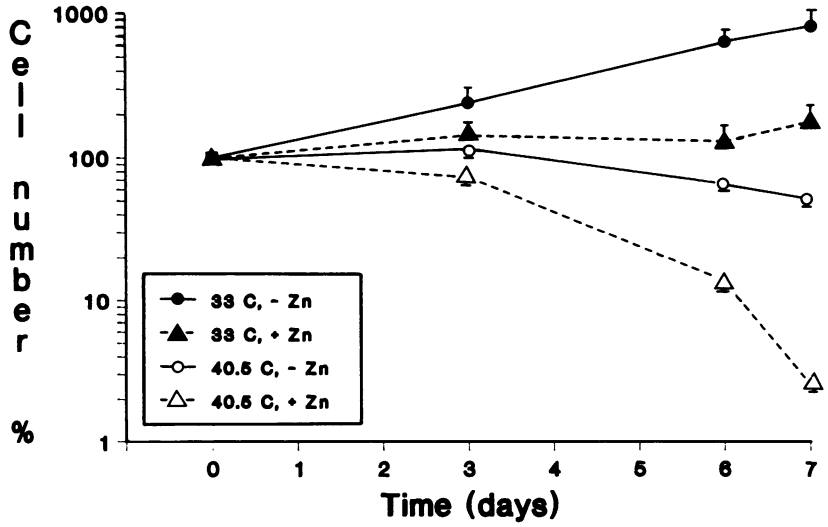


FIG. 10. Growth characteristics of ts.T24.1 cells. Growth curves showing effects of induction of mutant *ras* by Zn^{2+} (100 μM) at 33 and 40.5°C. Note inhibition of growth at 33°C and marked cell loss at 40.5°C in presence of Zn^{2+}. (Growth curves were performed as described in Fig. 2a.)

DNA replication (with or without chromatid separation) occurring without mitosis or nuclear fusion in binucleate cells and is usually associated with a proportional increase in nuclear size (47).

Increased nuclear DNA content to the 4 *n* level in primary fibroblasts was reported in the analogous *ts* experiments of Jat and co-workers (18). Although this was interpreted as G2 arrest, it is not possible in those or in our experiments to distinguish, on the basis of DNA content, a G2 phase of a diploid cycle from a G1 phase of a tetraploid cycle (the essential difference being whether chromatid separation has taken place). The subsequent development of an 8 *n* subpopulation however, confirms that in our system at least, a proportion of cells achieve a true tetraploid state. In fibroblasts (18) one notable difference was that DNA synthesis ceased much more quickly after temperature switch (within ~3 days) than in ts.1, which may have limited the degree of polyploidy attainable.

The existence of a period of dissociation between DNA synthesis and cell division has several interpretations. It may simply reflect a more stringent dependence on SV40 T for cytokinesis than for DNA synthesis, the dissociation occurring while intracellular T activity falls between the levels required for the two processes. Further immunochemical and functional analysis of T after temperature switch-up will be needed to address this possibility.

Alternatively, there may be an inherent potential of thyroid epithelial cells to undergo polyploidization during growth stimulation in the absence of T. It is well established that other conditional renewal tissues, notably liver and salivary gland epithelia, develop 4 and 8 *n* nuclei when subjected to hyperplasia in vivo (23, 30) and, more gradually, as part of the natural aging process (23). It appears that when such cells near the end of their proliferative potential, cell division stops before DNA synthesis stops. It is possible, therefore, that when the stimulus provided by T is removed, ts.1 cells may recapitulate this dissociation. Unfortunately, we do not yet know whether the arrest state of *ts* thyroid lines at 40.5°C is equivalent to the terminal state of senescence in which primary thyroid epithelial cells normally arrest after a few divisions in culture, since the ploidy status of the latter is not known.

The ability to use *ts* thyroid epithelial cells to investigate the effect of activated oncogenes was demonstrated by using a retroviral vector containing a mutant H-*ras* gene driven by an inducible promoter. Similar results were also obtained with the same construct introduced by transfection.

The overall net effect of mutant *ras* expression (at least at the levels achievable by the MT promoter) was in all conditions inhibitory. In the presence of functional T (and at high cell density), mutant *ras* induced morphological signs of transformation but reduced net growth rate. Since little effect on cell proliferation (as judged by LI and mitotic activity) was seen, this implied an increase in death rate. Furthermore, increased cell death was clearly evident by direct observation at low cell density. In the absence of functional T, mutant *ras* induced cell death and net cell loss at all cell densities.

These data reveal important differences from previous reports of the effect of mutant *ras* expression and its interaction with SV40 T.

In the presence of functional T (or an equivalent, cooperating oncogene), introduction of a mutant *ras* gene (whether by transfection or infection) has been shown to produce morphological transformation in a variety of cell types, including epithelial (keratinocytes) (33, 34), fibroblasts (17), and Schwann cells (35), but with no indication of reduced growth. Indeed, in Schwann cells, the *ras* transformants appeared to grow more quickly (35). Our finding of growth inhibition due to increased death rate may reflect a true difference in *ras* action due to differences in cellular context. Alternatively, it may simply be due to the use in our experiments of an inducible *ras* construct, thereby permitting selection of potential transformants under neutral conditions (G418, in zinc-free medium with *ras* expression switched off), which avoids the possibility inherent in earlier studies of bias towards atypical uninhibited variants. What is clear is that, at least in thyroid epithelial cells, SV40 T is not able to fully override the inhibitory, toxic effect of mutant *ras* in the majority of clones.

A further striking contrast with previous reports was the finding of rapid cell loss when mutant *ras* is expressed in the absence of functional T in thyroid cells. In other cell types where an inhibitory effect of *ras* has been observed in the absence of a cooperating oncogene (e.g., primary fibroblasts [17], Schwann cells [35], and the neuroendocrine [PC12] cell line [26]), this has merely involved growth arrest leading to a prolonged state of viable quiesence. We do not know why mutant *ras* has a more dramatic toxic effect on thyroid cells, but it may be related to their different proliferative capacities. Whereas all previously studied cells were proliferatively active in the absence of mutant *ras* (and T), the corresponding state for thyroid epithelial cells is that of quiescence associated with terminal differentiation. In general, then, in culture, mutant *ras* may arrest growing cells but kill terminally differentiated or senescent cells.

Clearly, given the known importance of *ras* as an early event in tumorigenesis in thyroid (21) as well as in several other epithelial tissues with similar cell kinetics (notably pancreas [1]) it will be important to resolve the apparent paradox presented by these in vitro findings. The unexpected direction of *ras* effects on growth and survival should not, however, detract from the potential of ts.1 cells as a tool for investigating the biochemical actions of *ras* (and even less so for other oncogenes). There is no reason to suspect that the signals induced by *ras* in ts.1 at the restrictive temperature are different from those acting in vivo in thyroid tumor epithelium, and cell death is sufficiently delayed at 40.5°C (particularly in dense cultures) to permit meaningful analysis of early biochemical responses to *ras* (at least up to day 4) in the absence of functional T. In particular, it will be of interest to use this model to examine the possible causal relationship between *ras* mutation and autocrine IGF-1 expression suggested by recent studies (44).

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