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Human placental cells transformed by *tsA* mutants of simian virus 40: A model system for the study of placental functions

(human chorionic gonadotropin/placental alkaline phosphatase/thymidine kinase)

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ABSTRACT **Human placental cells were transformed with wild-type simian virus 40 (SV40) and temperature-sensitive SV40 mutants of the *A* and *B* classes. Four criteria for transformation were used: decreased generation time, increased saturation density, increased efficiency of growth on plastic, and ability to overgrow a nontransformed monolayer. Cell lines transformed by *tsA* mutants lost the transformed phenotype at the restrictive temperature (40°); therefore, the *A* function of SV40 is required for the maintenance of the transformed phenotype in human placental cells. A decrease in alkaline phosphatase activity, an inhibition of human chorionic gonadotropin synthesis, and an increase in thymidine kinase activity were seen when human placental cells transformed by wild-type or *tsB* mutants of SV40 were grown at 33° or 40° and when *tsA* transformants were grown at 33°. When *tsA* transformants were grown at 40°, alkaline phosphatase activity and human chorionic gonadotropin synthesis were greatly stimulated and thymidine kinase activity was greatly reduced, approximating their levels in the placenta.**

Placental functions *in vitro* have usually been studied by using explants and primary cultures which are difficult to establish and maintain in tissue culture. Choriocarcinoma cells (malignant cells derived from the placenta) have been used extensively as an *in vitro* system to study placental functions (1–4); these cells do not, however, control the synthesis of certain proteins as does the placenta *in vivo*. Because neither placental cells *in vitro* nor choriocarcinoma cells have proven to be a suitable model for placental cells *in vivo*, I thought that placental cells transformed by temperature-sensitive *A* (*tsA*) mutants of simian virus 40 (SV40) might be a better system. The *A* function of SV40 is required for the initiation of viral DNA replication (5, 6), for the induction of host DNA synthesis (7), for the stimulation of cellular thymidine kinase activity (8), and for the maintenance of the transformed phenotype (9–12). Cell lines induced by *tsA* mutants should behave like transformed cells at the permissive temperature, allowing propagation and cloning. At the nonpermissive temperature, these cells should regain their nontransformed phenotype.

Placental cell lines transformed by wild-type SV40 and its temperature-sensitive mutants have therefore been established. This report demonstrates that the *A* function of SV40 is required for the maintenance of the transformed phenotype in human placental cells and that normal placental functions are expressed when *tsA*-transformed cells are incubated at the restrictive temperature.

MATERIALS AND METHODS

Virus. The *ts* mutants and wild-type SV40 virus have been described (13).

Cells and Culture Conditions. Primary placental cells were obtained by collagenase digestion (0.1%, Worthington Biochemical Co., Freehold, NJ) of human term placenta. The primary cultures were grown at 37° in α modified minimal essential medium supplemented with 10% fetal bovine serum (αMEM-10, Flow Laboratories, Rockville, MD), streptomycin (100 μg/ml), and penicillin (100 units/ml). At confluence, the cells were trypsinized and subcultured. These secondary placental cells in medium supplemented with 8% dimethyl sulfoxide were stored in a liquid nitrogen freezer for subsequent use. These cells, designated "TP" cells (term placental cells), were used for transformation.

Transformation. TP cells (passage 2) were grown to 10–20% of confluence (approximately 2×10^5 cells per 25-cm^2 flask), the medium was removed, and wild-type SV40 or a *ts* mutant of SV40 was allowed to adsorb for 2–3 hr at 33° (multiplicity of infection = 5–10). Medium supplemented with 4% fetal bovine serum was then added to the infected flask, and the cells were incubated at 33°. After 24 hr of incubation, the cells were suspended after incubation with trypsin and serially diluted. Medium (αMEM-4) was replaced twice weekly. Clones were identifiable after 4–5 weeks and were selected and purified by growing in low-serum medium (αMEM-4) at low cell density. The transformation efficiency was 0.5–1%. More than 50 clones were isolated. Recloning of wild-type transformed and mutant-transformed cells was generally successful; however, for unknown reasons, the life-span of the doubly cloned cells was considerably shortened. Therefore, only singly cloned cultures were used in this study. Cultures of TP cells from the same placenta were transformed by viral strains *A30*, *A209*, *B201*, and *WT*. Mutants *tsA30* and *tsA209* are independent mutants of the SV40 *A* gene and mutant *tsB201* is a mutant of the *B* gene. The *A* function of SV40, but not the *B* function, is required for the maintenance of transformed phenotype; thus, cells induced by *tsB* mutants manifest the transformed phenotype at both 33° and 40°. The transformed cell lines are identified by their TP origin, the virus by which they were transformed, and their isolation rumber. The transformed lines were thus designated, *TPA30-1*, *TPA30-6*, *TPA209-9*, *TPWT-2*, and *TPB201-1*.

Efficiency of Growth on Plastic. Cells were serially diluted starting with approximately 10^5 cells per 25-cm^2 flask. Duplicate dilutions were prepared, and the efficiencies of clone formation at 40° and 33° were determined. Medium was replaced twice weekly at 33° (αMEM-4) and every other day at 40° (αMEM-10). After 2 weeks, cells were fixed and stained.

Overgrowth of Nontransformed Placental Cell Layer by Transformed Cells. TP cells were treated with trypsin and

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Abbreviations: *ts*, temperature-sensitive; SV40, simian virus 40; TP cells, term placental cells; αMEM, α minimal essential medium; hCG, human chorionic gonadotropin.

suspended in αMEM-4 at a density of 2×10^5 cells per ml. Each well of the 24-well plates (Cooke Laboratory Products, Dynatech Laboratories, Inc., Alexandria, VA) received enough of the suspension (1.5 ml) to produce a confluent monolayer immediately after attachment. Each well also received one of four serial (1:3) dilutions of a transformed cell culture. The first well of each series received $5–10 \times 10^4$ transformed cells. Medium was replaced twice weekly at 33° (αMEM-4) and every other day at 40° (αMEM-10). After 2 weeks, the cells were fixed and stained.

Fixing and Staining. After removal of the medium, the flasks or plates were washed with phosphate-buffered saline, rinsed with absolute methanol, fixed in absolute methanol for 20 min at 25°, and allowed to dry. The cells were stained with 0.1% Evans blue in phosphate-buffered saline for 20 min at room temperature, rinsed with water, and dried.

Glucose-6-Phosphate Dehydrogenase. The electrophoretic mobility of these enzyme species in SV40-transformed placental cells was determined by using Cellogel electrophoresis as described by Rattazzi *et al.* (14). Normal A and B types, kindly provided by L. Corash, were used as standards.

Preparation of Cell Extracts. Cells were harvested by scraping with a rubber policeman. The pellet was suspended in buffer (0.01 M Tris·HCl, pH 7.4/0.15 M KCl/3mM 2-mercaptoethanol/1 mM EDTA) and the cells were ruptured by sonication (Raytheon Magnetostrictive Oscillator, model DF-101, 250-W, 10 kHz, Raytheon Company, Manchester, NH) for 2 min at maximal power. The sonicates were centrifuged at $10,000 \times g$ for 15 min; the supernatant solutions were used immediately for measurement of alkaline phosphatase and thymidine kinase activities.

For gel electrophoresis, heat denaturation, and immunoprecipitation studies, alkaline phosphatase was partially purified by butanol extraction and acetone precipitation of the *tsA*-transformed cells grown at 40° (15).

Assay of Alkaline Phosphatase and Thymidine Kinase. Alkaline phosphatase activity was measured by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at pH 10.7 and 37° (16).

Thymidine kinase was assayed by the method of Kit and Dubbs (17), which measures the rate of conversion of [2-^{14}C]-thymidine to a form that can be adsorbed by polyethyleneimine-cellulose. Protein was determined by the method of Lowry *et al.* (18).

Radioimmunoassays. A double-antibody radioimmunoassay using human chorionic gonadotropin (hCG) and hCG-α as reference standard was used to measure hCG and hCG-α in unfractionated medium (19). Antisera used were: anti-hCG-β (Sb6) for hCG (20), and anti-hCG-α (CA3) for hCG-α. Purified preparations of hCG (CR119, 11,600 international units/mg, ventral prostate assay, Second International Standard hCG) and of hCG-α (CR117) were radioiodinated and used to determine a standard curve for the assay. Complete hCG (CR119) has slight (1–3%) crossreactivity in the hCG-α assay. hCG-α has no crossreactivity in the hCG assay. Complete medium not exposed to cells had no detectable hCG or hCG-α. The limit of detection for hCG or hCG-α in the radioimmunoassays was 0.1 ng. Antisera, standard hCG, and hCG-α were kindly provided by K. Catt, G. Hodgen, and H. Chen.

RESULTS

Establishment and Transformation of Human Placental Cells. Four to 5 weeks after infection of secondary TP cells with SV40 wild-type or *ts* mutants, clones were identifiable. Virtually no clones were observed in mock-infected cultures. All transformation assays were performed at 33°. Transformed clones were selected and grown in low-serum medium (αMEM-4) at low cell density.

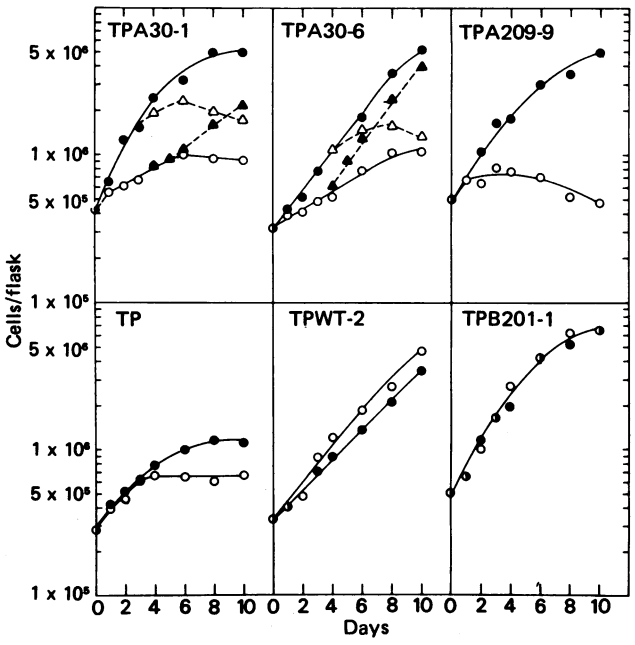

FIG. 1. Growth of transformed cells at permissive (33°) and restrictive (40°) temperature. Cells were grown in αMEM-10 and medium was changed every other day. After 3 days, some of the cultures were shifted from 40° to 33° or from 33° to 40°. Cells were counted with a Celloscope 112TH (Particle Data, Inc., Elmhurst, IL). ●, 33°; ○, 40°; ▲, cultures shifted from 40° to 33°; △, cultures shifted from 33° to 40°.

The glucose-6-phosphate dehydrogenase in these SV40-transformed placental cell lines was of the B type, as demonstrated by Cellogel electrophoresis.

Transformed lines of human placental cells were found to shed virus, as do other human cells transformed by SV40. The *tsA*-transformed lines shed much more virus at 33° than at 40°. These were SV40, because the addition of 1% anti-SV40 serum (Flow Laboratories, Rockville, MD) inhibited plaque formation. The phenotype of the virus released from each transformant corresponded to the phenotype of the virus that induced the transformation. Because significant amounts of infectious particles were produced by these cells, anti-SV40 serum was routinely added to culture media.

Growth Curves. Cells transformed by wild-type or *tsB201* virus grew equally well at 40° and 33° but more rapidly than the nontransformed TP cells (Fig. 1). Although the TP cells grew at the same rate at both temperatures, the saturation density was lower at 40°. At 33° the cells transformed by *tsA* mutants of SV40 behaved like the wild-type transformants; they grew rapidly and achieved high saturation densities. At 40°, however, they behaved more nearly like the nontransformed TP cells; they grew slowly and stopped growing at low saturation densities.

When cultures in the mid-exponential phase were shifted from 33° to 40°, growth of the *tsA*-mutant transformed cells was inhibited within 24 hr (Fig. 1). When cultures grown at 40° for 3 days were shifted to 33°, an increase in the growth rate was seen in the *tsA*-transformed cell lines after a 24 to 48-hr lag.

Cloning Efficiency on Plastic and Overgrowth of Nontransformed TP Monolayers. Cells transformed by wild-type

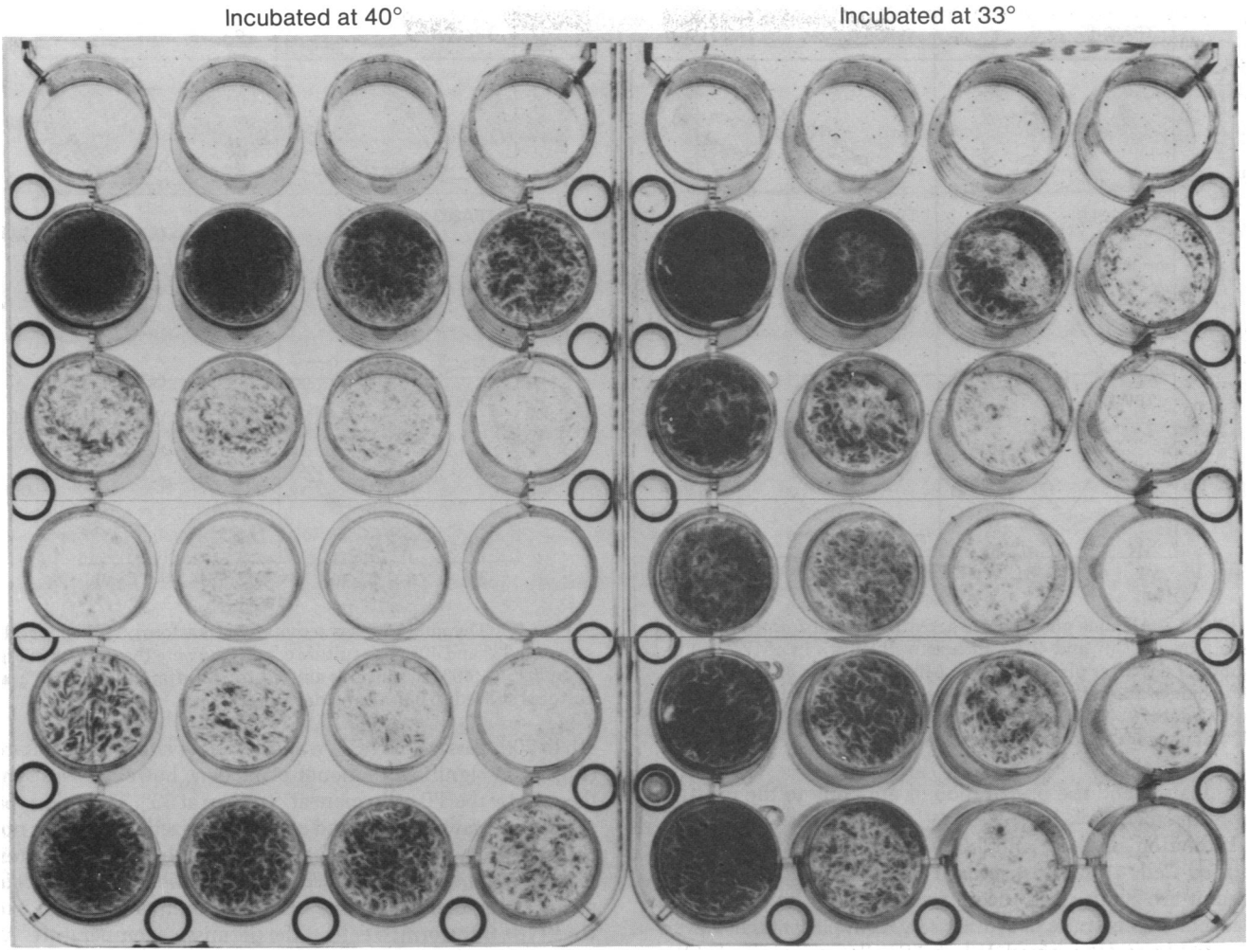

FIG. 2. Ability of transformed cultures to overgrow a monolayer of nontransformed TP cells at 33° and 40°. Each well contained a confluent monolayer of nontransformed TP cells to which serial (1:3) dilutions of the transformants were added. Two identical plates were prepared, using a different transformant for each row of wells. The *Left* plate was incubated at 40° and the *Right* plate was incubated at 33°. Both were incubated for 2 weeks. The transformed lines were, from bottom to top, *TPB201-1, TPA30-6, TPA209-9, TPA30-1, TPWT-2,* and *TP.*

SV40 or the late mutant *tsB201* had similar cloning efficiencies at 33° and 40°. In contrast, cell lines transformed by the *tsA* mutants were temperature-sensitive; at 40°, colony formation was markedly inhibited (data not given). Overgrowth of normal cell layers was found to be temperature-sensitive for the three cell lines transformed by the *tsA* mutants (Fig. 2). Wild-type and *tsB201*-transformed cells overgrew nontransformed TP monolayers at 40° as well as at 33°.

Increase of Alkaline Phosphatase Activity in *tsA*-Transformed Cells at the Restrictive Temperature. Placental alkaline phosphatase is an enzyme synthesized by trophoblastic cells. At least two forms of alkaline phosphatases occur in placenta: one characteristic of term placenta and one characteristic of subterm (first-trimester) placenta. These two phosphatases differ in their physicochemical and immunologic properties (T. Sakiyama and J. Y. Chou, unpublished data). The term placental enzyme is heat-stable whereas the subterm enzyme is heat-labile. The term alkaline phosphatase was clearly separated from the subterm placental enzyme on polyacrylamide electrophoretic gels. The subterm enzyme cannot be inactivated by antiserum to term placental alkaline phosphatase. The specific enzyme activities in term placenta and subterm placenta are approximately 1000 nmol P_i released/min per mg of protein and 1 nmol P_i released/min per mg of protein, respectively.

Alkaline phosphatase activity in wild-type- and *ts*-mutant-transformed cells grown at the permissive temperature was lower than that in term placenta (Fig. 3). Enzyme activity in cells transformed by the wild-type and the late mutant *tsB201* was low and was almost the same at the two temperatures. In contrast, phosphatase activity in cell lines transformed by *tsA* mutants of SV40 was greatly increased at the restrictive temperature. This increase in enzyme activity was reversible: activity in the *tsA*-mutant transformed cells continued to increase for 2–4 days after a shift from 40° to 33° but then decreased as the cells regained their transformed phenotype. Curiously, alkaline phosphatase activity was also low in the nontransformed TP cells by the 6th to 20th passage (approximately 0.1% of the activity in term placenta) and may reflect a dedifferentiation of functional cells *in vitro* (transformation assays were performed at the second passage).

The alkaline phosphatases synthesized by the *tsA*-transformants grown at 40° comigrated with the term placental alkaline phosphatase on polyacrylamide electrophoretic gels. However, these phosphatases from the transformants were as heat-labile as the subterm enzyme and were not inactivated by antiserum to term placental alkaline phosphatase. It is possible that the subterm placental enzyme was induced in term placental transformants. Immunologic identification of these phosphatases must await purification of the subterm placental alkaline

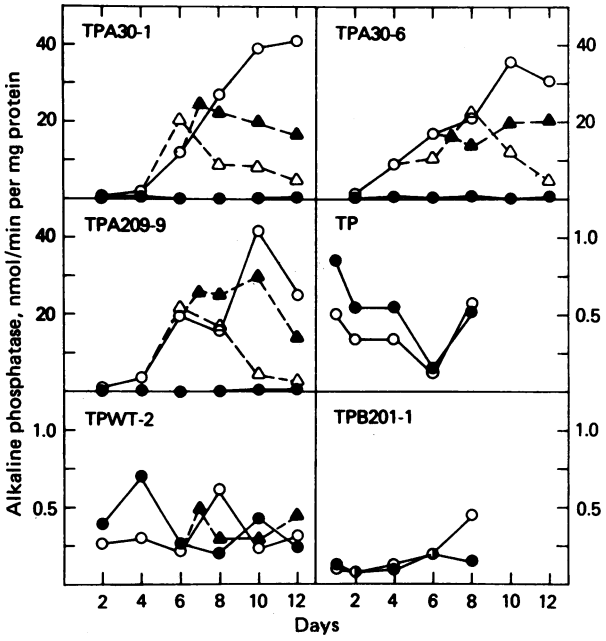

FIG. 3. Alkaline phosphatase activities in transformed placental cells grown at 33° and 40°. Cultures were grown in αMEM-10 beginning 1 day after plating (day 0). After 4 or 6 days of incubation at 40°, some of the cultures were shifted to 33°. Medium was changed every 2 days. ●, 33°; ○, 40°; △, cultures shifted to 33° after 4 days at 40°; ▲, cultures shifted to 33° after 6 days at 40°.

phosphatase and the preparation of antibody to the purified subterm enzyme.

Induction of hCG and hCG-α Synthesis in *tsA*-Transformed Cells at the Restrictive Temperature. hCG and its α subunit are synthesized throughout gestation by the normal placenta. Precise rates of synthesis by term placenta, however, have not been determined. When TP cells were assayed at the

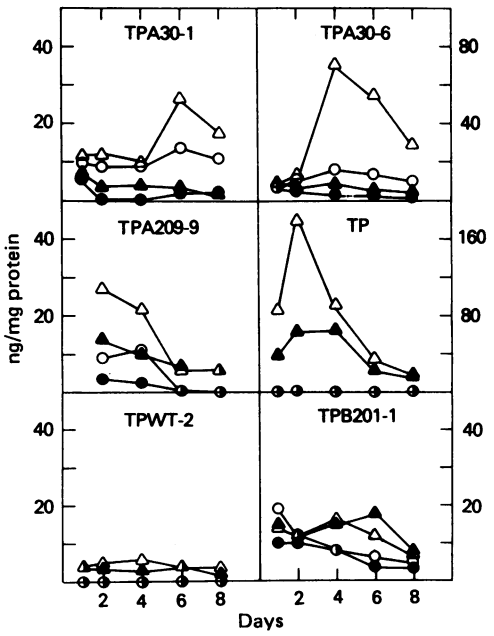

FIG. 4. Synthesis of hCG and hCG-α in transformed placental cells grown at 33° and 40°. Cultures were grown in αMEM-10. Beginning 1 day after plating (day 0), cultures were incubated at 33° or 40°. Medium was changed every 2 days. At the indicated times, medium from each flask was assayed for hCG and for hCG-α separately. ●, hCG, 33°; ○, hCG, 40°; ▲, hCG-α, 33°; △, hCG-α, 40°.

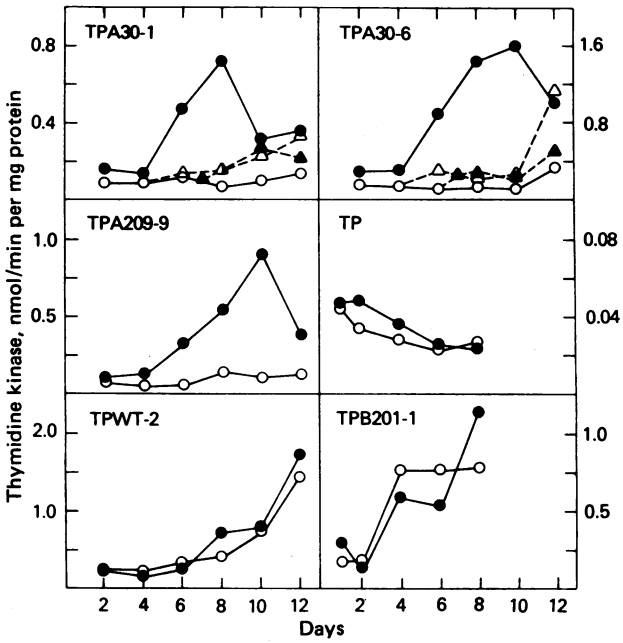

FIG. 5. Thymidine kinase activities in transformed placental cells grown at 33° and 40°. The culture conditions were the same as in Fig. 3. ●, 33°; ○, 40°; △, cultures shifted to 33° after 4 days at 40°; ▲, cultures shifted to 33° after 6 days at 40°.

6th to 20th passage, no hCG was detected at either temperature (Fig. 4). A significant amount of hCG-α, however, was synthesized by the TP cells, more at 40° than at 33°. The wild-type and *tsB*-mutant transformed cells synthesized very low or not detectable levels of both hCG and hCG-α at both temperatures. Again, the *tsA*-mutant transformed cells behaved more like normal placental cells at the restrictive temperature and like wild-type transformed cells at the permissive temperature. At 33°, very low levels of hCG and hCG-α were detected; at 40°, much higher levels were found.

The SV40-transformed TP cells synthesized more hCG-α than hCG; placentas at term also synthesize more hCG-α than hCG. The slope of dose-response curves for the hCG-α of TP cells and the transformed cells were indistinguishable from those for urinary hCG-α. Furthermore, the hCG-α produced by these cells was coeluted with urinary hCG-α on the Sephadex G-100 column.

Decrease in Thymidine Kinase Activity in *tsA*-Transformed Cells at the Restrictive Temperature. It has been demonstrated previously that infection or transformation by SV40 induces thymidine kinase activity (8, 17, 21). Thymidine kinase activities in nontransformed TP cells, term placenta, and subterm placenta were low; they were greatly enhanced by SV40 transformation (Fig. 5; unpublished data). At 33°, enzyme activities in wild-type transformed and mutant-transformed cells were higher than in nontransformed TP cells. At 40°, thymidine kinase activities in nontransformed TP cells were low and similar to the levels found at 33°. Thymidine kinase activities at 40° in cells transformed by wild-type and *tsB* mutant virus were high and similar to the levels found at 33°. In contrast, enzyme activities decreased rapidly after the *tsA*-mutant transformed cells were shifted to the restrictive temperature. This effect was reversible: a shift from 40° to 33° restored thymidine kinase activities after a lag period.

DISCUSSION

The available evidence suggests that the regulation of placental marker proteins (alkaline phosphatase, hCG) in choriocarci-

noma cells differs from their regulation in placental cells *in vivo*. First, alkaline phosphatase activity in choriocarcinoma cells is lower than it is in term placenta (16, 22) and the phosphatases in choriocarcinoma cells differ from the placental enzyme in physicochemical and kinetic properties (T. Sakiyama and J. Y. Chou, unpublished data). Second, the level of hCG is much higher in patients with choriocarcinoma than in women during normal pregnancy (23).

Secondary cultures were established from term placenta; however, these nontransformed TP cultures seemed to be partially dedifferentiated cells that had lost some of the characteristic properties of normal trophoblasts, as indicated by the following observations. Cultured TP cells had extremely low alkaline phosphatase activity, synthesized small amounts of hCG-α, and produced no hCG. By comparison, placenta *in vivo* synthesizes higher quantities of alkaline phosphatase, hCG, and the α subunit of hCG. It is possible that a certain cell population was preferentially selected during establishment and maintenance of secondary TP cultures and that the ability to synthesize hCG, hCG-α, and alkaline phosphatase was impaired in these cells.

Early-passage placental cells were transformed with *tsA* mutants of SV40. At the permissive temperature these cells were indistinguishable from wild-type transformed cells, permitting propagation and cloning. At the restrictive temperature, however, these cells regained their normal trophoblastic behavior: they did not form clones, did not overgrow a secondary TP monolayer, and morphologically appeared nontransformed. Likewise, with respect to hCG, hCG-α, placental alkaline phosphatase, and thymidine kinase, the *tsA*-mutant transformed cell lines behaved like transformed (dedifferentiated) cells at the permissive temperature and like normal TP cells at the restrictive temperature.

The hCG-α made by the transformed cells at the restrictive temperature was indistinguishable from urinary hCG-α in its gel chromatographic and immunologic properties. The alkaline phosphatase produced by these cells appeared to approximate that from subterm rather than TP cells. The proteins made by the transformed cells at 40° therefore appear to be authentic placental proteins, but additional confirmation will be necessary for alkaline phosphatase.

The approach used here should be universal to any cell type that can be transformed by SV40. If clearly differentiated cells can be transformed by SV40 *tsA* mutants, the specialized functions characteristic of each cell type might be studied more easily than was previously possible.

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