Study of Liver Differentiation in Vitro

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ABSTRACT A clonal rat fetal liver cell line that expresses the functions of differentiated liver cells under controllable conditions has been established. Normal fetal liver cells were transformed by a temperature-sensitive A (*tsA*) mutant (*tsA209*) of simian virus 40. At the permissive temperature (33°C), the *tsA209*-transformed liver cell line (*RLA209-15*) can be cultured indefinitely and cloned readily. The *RLA209-15* cells were temperature sensitive for maintenance of the transformed phenotype. These transformed liver cells selectively lost four characteristics of the transformed phenotype at the restrictive temperature (40°C): generation time of the cells increased, the saturation density decreased, the efficiency of growth on nontransformed cell layers decreased, and the ability to clone in soft agar was lost. The transformation can be reversed simply by a shift in temperature. *RLA209-15* fetal liver cells synthesized α-fetoprotein, albumin, and transferrin. At 33°C, the levels of these liver proteins were relatively low. At 40°C, the transformed phenotype was lost and the levels of α-fetoprotein, albumin, and transferrin were greatly increased. At the restrictive temperature, maximal induction of the synthesis of α-fetoprotein, albumin, and transferrin was achieved 3–4 d after the upward shift in temperature.The synthesis of α-fetoprotein then decreased; the synthesis of albumin and transferrin, however, was maintained. A second phase of albumin and transferrin synthesis was observed in all cultures after 6 d or more at 40°C. α-Fetoprotein, albumin, and transferrin secreted by *RLA209-15* cells were immunologically indistinguishable from authentic α-fetoprotein, albumin, and transferrin, respectively. *RLA209-15* cells, like primary cultures of hepatocytes and a simian virus 40 *tsA255*-transformed fetal liver cell line (*RLA255-4*) reported earlier from this laboratory, responded to glucagon with markedly elevated levels of cyclic AMP. Thus, it appears that glucagon receptors characteristic of hepatocytes are retained in the simian virus 40 *tsA*-transformed fetal liver cells.

One of the goals of molecular cell biology is to study normal gene regulation. Studies in vivo are complicated by our inability to control and to manipulate the environment of tissues having specialized functions. Attempts to transfer differentiated tissues to rigorously defined cell culture conditions are often frustrating. In general, the normal differentiated cells fail to proliferate in culture or they cease to express the differentiated function. Chicken embryo retinal melanoblasts (2) and chicken chondroblasts (14) that retain differentiated functions can be established by the transformation of normal melanoblasts and chondroblasts with temperature-sensitive mutants of Rous sarcoma virus. Human placental trophoblasts that retain differentiated placental functions can be established by the transformation of normal placental cells with temperature-sensitive *A* (*tsA*) mutants of simian virus 40 (SV40; 3, 4). The transformed phenotype and the differentiated phenotype of all three types of cells are temperature-dependent. At the permissive temperature, the transformed melanoblasts cease melanin synthesis and degrade their melanosomes, the transformed chondroblasts stop synthesizing sulfated proteoglycan, and the transformed human placental cells synthesize low levels of human chorionic gonadotropin (hCG) and its alpha subunit (hCGα) and have low alkaline phosphatase activities. At the nonpermissive temperature, the transformed melanoblasts actively synthesize melanin, the transformed chondroblasts reinitiate the synthesis of sulfated proteoglycan, and the transformed placental cells synthesize high levels of hCG and hCGα and have high alkaline phosphatase activities. Therefore, the effects of the transforming virus are readily reversible, and the viral activity does not appear to interfere with the genetic controls maintaining the differentiated state of the cells.

Using a similar approach, we have established a rat fetal liver cell line (*RLA255-4*) by the transformation of normal fetal liver cells with SV40 *tsA255* virus (19). *RLA255-4* cells synthe-

size albumin and transferrin and contain functional receptors for glucagon. However, cells of this line do not synthesize measurable amounts of α-fetoprotein in culture. α-Fetoprotein is a major plasma glycoprotein synthesized by mammalian fetal liver (8, 20). Elevated serum concentrations of α-fetoprotein have been associated with developmental, regenerative, and carcinogenic events (1, 21). Although regulation of albumin and transferrin synthesis in *RLA255-4* cells is temperature-dependent, these cells become detached from the plastic surface after growth at 40°C for 5 d or longer. Therefore, it is not possible to carry out extensive studies on the synthesis and regulation of these liver proteins.

In this report, a new rat fetal liver cell line induced by SV40 *tsA209* virus (*RLA209-15*) is described. *RLA209-15* cells, like *RLA255-4* cells, synthesize albumin and transferrin in culture and contain functional receptors for glucagon. In addition, *RLA209-15* cells synthesize α-fetoprotein and can be maintained in culture for 2 wk or longer at both permissive and restrictive temperatures. Synthesis of all three differentiated hepatic proteins in *RLA209-15* cells is temperature regulated. The availability of *RLA209-15* cells enables us to study, in great detail, the kinetics of the synthesis of α-fetoprotein, albumin, and transferrin.

MATERIALS AND METHODS

Transformation and Culture Conditions

Primary fetal rat liver cells (RL cells) were obtained by collagenase (0.2%; Worthington Biochemical Co., Freehold, N. J.) digestion of fetal rat livers from 18-d-old embryos. The dispersed cells were collected by centrifugation, resuspended in α-modified minimal essential medium (αMEM, National Institutes of Health Media Unit, Bethesda, Md.) without arginine, supplemented with 0.4 mM ornithine, 100 μg/ml streptomycin, 100 U/ml penicillin, 10% fetal bovine serum (Flow Laboratories, McLean, Va.), and gassed with 5% CO_2 and 95% air. After 24 h at 37°C, the attached cells (~2 × 10^5 cells/25-cm^2 flask) were washed three times with medium (10 ml/flask) and were infected with 1 ml of SV40 *tsA209* virus (multiplicity of infection = 10–20). After 2–3 h at 33°C with occasional shaking, standard αMEM medium supplemented with 4% fetal bovine serum (αMEM-4; 6 ml/flask) was added to the infected flask, and the cells were incubated at 33°C. After 20 h of incubation, the cells were subcultured and serially diluted (1:5). The first flask received 4 × 10^4 cells. Medium (αMEM-4; 6 ml/flask) was replaced twice weekly. After 2–3 wk at 33°C, transformed clones were isolated and purified by growth in medium with low amounts of serum (αMEM-4) at low cell density (3, 12). *RLA209-15* cell line is identified by its RL origin, the transforming virus, and the isolation number.

Growth Studies

Three parallel sets of *RLA209-15* cultures were grown at 33°C initially. The first set of cultures was maintained at 33°C throughout growth. The second set of cultures was shifted from 33°C to 40°C after various periods of growth at 33°C. The third set of cultures was shifted from 33°C to 40°C as was the second set but was shifted back to 33°C after 4 d at 40°C. Cells were grown in 25-cm^2 flasks in αMEM containing 10% fetal bovine serum (αMEM-10). Culture medium (6 ml/flask) was changed and collected every 24 h. Cells were counted with a Celloscope 112TH (Particle Data, Inc., Elmhurst, Ill.).

Overgrowth of Nontransformed Cell Layer and Cloning in Soft Agar

Overgrowth of nontransformed fibroblasts was tested as described (3). A confluent layer of nontransformed fibroblasts in 24-well plates (Cooke Laboratory Products, Dynatech Laboratories, Inc. Alexandria, Va.) was overlaid with one of six serial dilutions (1:5) of the transformed cells in αMEM-10. The first well received 10^5 transformed cells. Medium was replaced twice weekly at 33°C and 40°C. After 2 wk, the cells were fixed and stained.

Colony formation in soft agar was tested by suspending transformed cells in 1 ml of cloning agar (αMEM supplemented with 27.5% fetal bovine serum and 0.3% Noble agar) at concentrations of 10^6, 10^5, 10^4, and 10^3 cells/ml. The agar-cell suspension was added to each well of the 24-well plates on top of a 0.5-ml layer of solidified 0.7% agar in αMEM supplemented with 17% fetal bovine serum. Cultures were fed with 0.5 ml of cloning agar every 7 d at 33°C and every 5 d at 40°C. Colonies grown in soft agar were stained and counted 2 wk after seeding.

Chromosome Analysis

The analysis was done with conventional Giemsa staining (23) by Biotech Research Laboratories, Inc., Rockville, Md.

Radioimmunoassays

α-Fetoprotein, albumin, and transferrin in the culture media were determined by double-antibody radioimmunoassays. Purified preparations of rat α-fetoprotein, rat albumin, and rat transferrin were used as standards and also radioiodinated for use as tracers in the respective assays. The final volume was 1 ml. All dilutions were made with phosphate-buffered saline containing 1 mg/ml of bovine serum albumin (PBS-BSA). Reaction mixtures contained PBS-BSA, normal rabbit serum, standards or samples, ^{125}I-labeled tracer, and the respective antiserum. The reaction mixtures were incubated for 2 h at 37°C and for 16 h at 4°C. Sheep antiserum against rabbit γ-globulin was used as the precipitating antibody. Rat α-fetoprotein and rabbit antiserum against α-fetoprotein were kindly provided by Dr. J. F. Chiu. Rat albumin, rat transferrin, rabbit antiserum against rat albumin, and rabbit antiserum against rat transferrin were obtained from Cappel Laboratories Inc., Cochranville, Pa. The sensitivities of the assays were 0.2–10 ng for α-fetoprotein, 0.5–25 ng for albumin, and 0.1–5 ng for transferrin. Less than 0.01% cross-reactivity exists between α-fetoprotein, albumin, and transferrin in the respective radioimmunoassays. Complete medium not exposed to cells had no detectable α-fetoprotein, albumin, or transferrin.

Preparation of Cell Extracts

Cultures in 75-cm^2 flasks were grown for 5 d at 33°C initially, and medium was changed every other day. Then half of the cultures were shifted to 40°C and incubation was continued at 33°C and 40°C for an additional 3 d with daily medium change. The cells were harvested and sonicated as previously described (5). The sonicates were centrifuged at 10,000 *g* for 15 min; the supernatant solutions were used for radioimmunoassays of α-fetoprotein, albumin, and transferrin. Protein was determined by the method of Lowry et al. (11).

Intracellular Accumulation of Cyclic AMP

Cells in 24-well plates grown either at 33°C or at 40°C were washed with medium containing no serum (αMEM-0). 1 ml of αMEM-0 was added to each well and the cells were incubated for 20 min at 25°C. Glucagon (Eli Lilly Corp., Indianapolis, Ind.) was added to give a final concentration of 10^{-7} M. Papaverine was included in the cultures to inhibit phosphodiesterase. Incubation was carried out at 25°C, and the reaction was stopped by replacing the medium with 1 ml of boiling water. Finally, cyclic AMP (cAMP) levels in the water extracts were determined by radioimmunoassay with a cAMP radioimmunoassay kit (New England Nuclear Corp., Boston, Mass.).

RESULTS

Establishment of Temperature-sensitive Transformed Fetal Hepatocytes

Normal rat fetal liver cells (1 d after plating) from 18-d-old embryos were infected at 33°C with *tsA* mutants of SV40. Approximately 40 transformants were isolated. Most of the transformed cell lines (80%) synthesized rat transferrin in culture, although only one synthesized measurable amounts of α-fetoprotein. *RLA209-15* cells, which did synthesize significant amounts of α-fetoprotein, albumin, and transferrin, were extensively characterized.

In nearly 100% of the *RLA209-15* cells, T antigen was clearly localized within the nucleus, as revealed by immunofluorescence. These transformed hepatocytes did not shed any detectable SV40 virus as demonstrated by plaque assay (6). *RLA209-15* cells are aneuploid with a modal chromosome number of 56.

Growth Curves

At 33°C, *RLA209-15* cells grew like transformed cells; they grew rapidly to high cell densities (Fig. 1). The doubling time for these cells at 33°C was ~24 h. The high saturation density (~1.4 × 10^6 cells/cm^2) could be maintained for a long time in culture as long as medium was renewed daily (the total growth period in Fig. 1 was 16 d). A temperature-shift experiment was then performed to test *RLA209-15* cells for temperature sensitivity in maintenance of the transformed phenotype at the nonpermissive temperature (40°C).

When cultures of *RLA209-15* cells that had been grown at 33°C for 1, 2, 3, or 4 d (Fig. 1 *A–D*) with cell densities of 0.5, 1.5, 2.1, or 3.6 × 10^5/cm^2, respectively, were shifted from 33°C to 40°C, cell densities continued to increase for another 24–48 h. Cell densities then decreased for the next 24–72 h until constant cell densities were maintained. When cultures that had been grown for 4 d at 40°C were shifted to 33°C, an increase in the growth rate was seen without a lag (Fig. 1 *C* and *D*) or after a 24-h (Fig. 1 *B*) or 72-h (Fig. 1 A) lag. The final cell densities of *RLA209-15* cells at 40°C and the length of the lag observed in these cells after a downward-temperature-shift experiment were a function of the initial cell densities at 33°C before shifting to 40°C.

When cells that had been grown at 33°C for 5 or 6 d and had a cell density >4 × 10^5 cells/cm^2 (cell density was 5.2 in Fig. 1 *E*, 6.8 × 10^5/cm^2 in Fig. 1 *F*) were shifted from 33°C to 40°C, growth was immediately inhibited and a constant cell density was reached within 24 h. When these cultures were shifted back to 33°C after a 4-d incubation at 40°C, growth was resumed immediately without a lag.

Colony Formation in Agar and Overgrowth of Nontransformed Liver Fibroblasts

The temperature sensitivity of the expression of the transformed phenotype of *RLA209-15* cells was examined by comparison of the relative efficiency of colony formation in agar at the permissive and nonpermissive temperatures. *RLA209-15* cells were capable of forming colonies in agar at 33°C (efficiency of cloning was 0.05%). At 40°C, however, no colonies could be detected in agar (efficiency of cloning was <0.0001%).

RLA209-15 cells were temperature sensitive for overgrowth of normal cell layers. At 33°C, 20 clones overgrew the confluent monolayers after inoculation with 160 transformed cells. At 40°C, overgrowth could be detected only after inoculation with 10^5 or more transformed cells.

Increase of α-Fetoprotein Synthesis in RLA209-15 Cells at the Restrictive Temperature

At the permissive temperature (33°C), *RLA209-15* cells synthesized low levels of α-fetoprotein (Fig. 2). At 40°C, however, α-fetoprotein production was greatly increased. The maximal α-fetoprotein level at 40°C was controlled by the initial cell density at 33°C before shifting, if the cell densities at 33°C

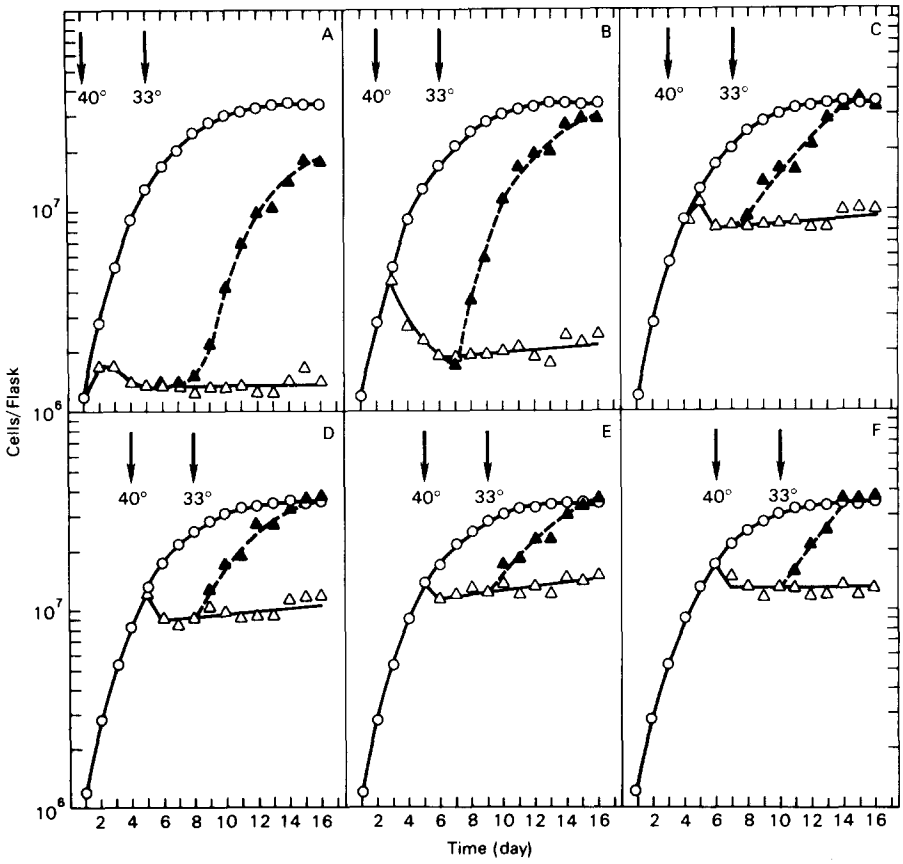

FIGURE 1 Growth of *RLA209-15* cells at the permissive (33°C) and restrictive (40°C) temperatures. The culture conditions were described under Materials and Methods. Cultures were plated at 33°C and were shifted from 33°C to 40°C after 1 (*A*), 2 (*B*), 3 (*C*), 4 (*D*), 5 (*E*), or 6 (*F*) d of growth at 33°C. After incubating at 40°C for 4 d, some of the cultures were shifted back to 33°C. Medium was changed every 24 h. Arrows indicate time of temperature shift. ○, 33°C; △, 40°C; ▲, cultures shifted from 40°C to 33°C after 4 d at 40°C.

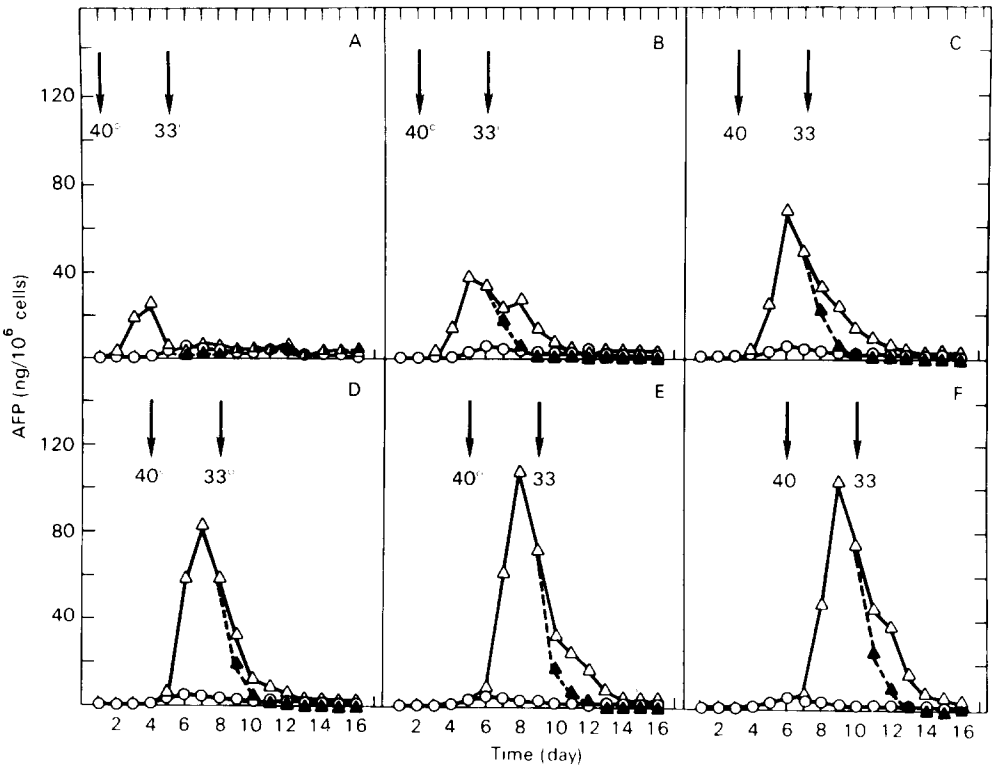

FIGURE 2 Synthesis of α-fetoprotein (*AFP*) in *RLA209-15* cells grown at 33°C and 40°C. The experimental conditions were the same as in Fig. 1. Cells were grown in αMEM-10 and medium was changed every 24 h. Each value (expressed as nanograms per 10^6 cells per 24 h) represents the average of data from two cultures, each analyzed in duplicate. ○, 33°C; △, 40°C; ▲, cultures shifted from 40°C to 33°C after 4 d at 40°C. Arrows indicate time of temperature shift.

were 4×10^5 cells/cm^2 or lower (Fig. 2*A–D*; cell densities at 33°C before shifting were the same as those for Fig. 1). However, if the initial cell densities at 33°C were $>4 \times 10^5$ cells/cm^2, the maximal α-fetoprotein level at 40°C in these cells was independent of the initial cell densities at 33°C (Fig. 2*E* and *F*). This observation agrees with the growth properties of *RLA209-15* cells at 40°C, where similar saturation densities were reached at 40°C when the cell density before shifting was 4×10^5 cells/cm^2 or higher (cf. Figs. 1 and 2).

Regardless of the culture densities at 33°C, maximal increase in α-fetoprotein synthesis at 40°C was achieved 3 d after an upward shift in temperature (Fig. 2). α-Fetoprotein synthesis in these cultures then decreased, even when the cells remained at the restrictive temperature. When cultures that had been incubated at 40°C for 4 d were shifted back to 33°C, a decrease in α-fetoprotein synthesis was seen.

Increase of Albumin Synthesis in RLA209-15 Cells at the Restrictive Temperature

The production of serum albumin is a major specialized function of the liver (7, 15, 18). *RLA209-15* cells grown at 33°C synthesized low levels of albumin (Fig. 3). Like α-fetoprotein synthesis, production of albumin by these cells was greatly increased at 40°C. Increase in albumin synthesis at 40°C was also studied with cultures at various cell densities (Fig. 3*A–F*; cell densities at 33°C before shifting were the same as those for Fig. 1). It appears that the rate of albumin production at 40°C was also dependent upon the initial cell density at 33°C before shifting.

The increase in albumin synthesis after shifting to 40°C was reversible; a decrease in albumin synthesis was seen after a shift from 40°C to 33°C. Although optimal increase in albumin level was reached when cells were grown at 40°C for 3–4 d, the induced levels were maintained for a longer period of time than those of α-fetoprotein. A second phase of albumin synthesis was observed in all cultures after 6 d or more at 40°C. Whereas α-fetoprotein is a fetal protein and synthesized mainly by replicating cells, albumin is synthesized by both fetal and adult nonreplicating liver cells (22). Therefore, the second phase of albumin synthesis may indicate that, at 40°C, *RLA209-15* cells could differentiate further and that an adult liver phenotype was finally reached.

Increase of Transferrin Synthesis in RLA209-15 Cells at the Restrictive Temperature

Transferrin, the major serum iron-binding protein, is a glycoprotein. Liver hepatocytes are the principal site of synthesis of serum transferrin (9, 10, 13). *RLA209-15* cells grown at 33°C synthesized very low levels of transferrin. The maximal level of transferrin synthesized by these cells at 33°C was ~0.01 ng/10^6 cells per d (Fig. 4). At 40°C, however, the levels of transferrin were increased 250- to 700-fold. There is no clear correlation between the maximal rate of transferrin production at 40°C and the initial cell densities at 33°C before shifting, unlike the rates of α-fetoprotein or albumin synthesis. The cell densities at 33°C before shifting were 0.5, 1.1, 2.1, 3.6, 5.2, and 6.8×10^5/cm^2 (Fig. 4*A–F*, respectively). The maximal levels of transferrin production at these cell densities were, however, 6.7, 5.7, 2.4, 3.6, 5.8, and 4.5 ng/10^6 cells per d, respectively. Like albumin synthesis at 40°C, transferrin synthesis by *RLA209-15* cells at the restrictive temperature was biphasic. The increase in transferrin synthesis was reversible; a decrease

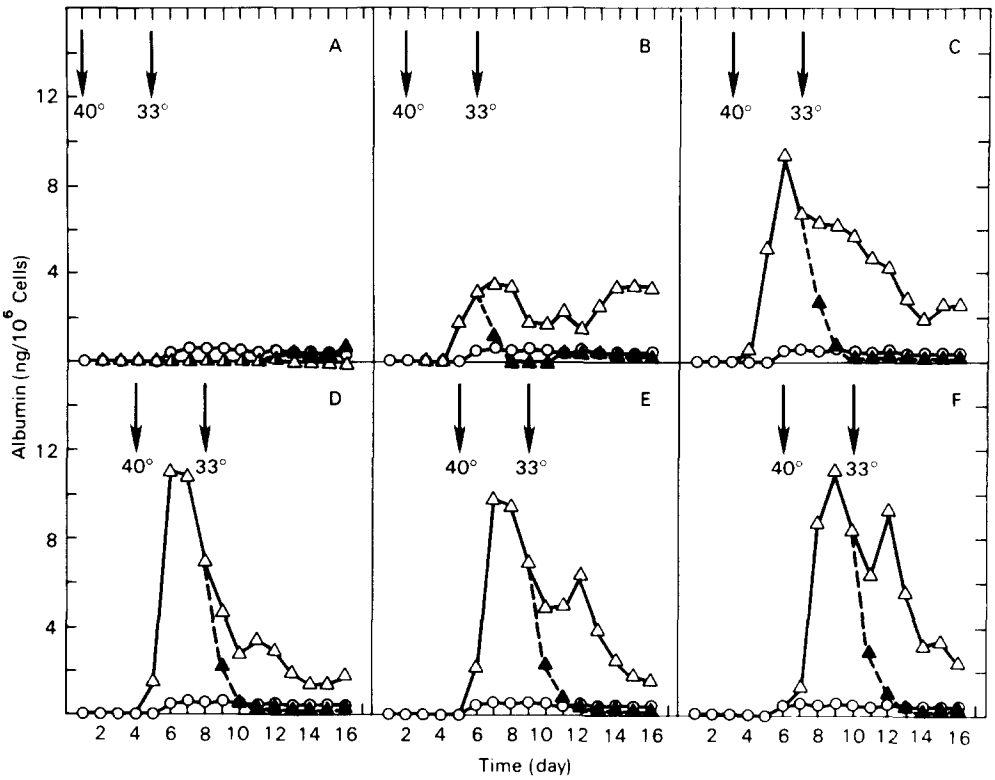

FIGURE 3 Synthesis of albumin in *RLA209-15* cells grown at 33°C and 40°C. The experimental conditions were the same as in Fig. 1. Cells were grown in αMEM-10 and medium was changed every 24 h. Each value (expressed as nanograms per 10^6 cells per 24 h) represents the average of data from two cultures, each analyzed in duplicate. ○, 33°C; △, 40°C; ▲, cultures shifted from 40°C to 33°C after 4 d at 40°C. Arrows indicate time of temperature shift.

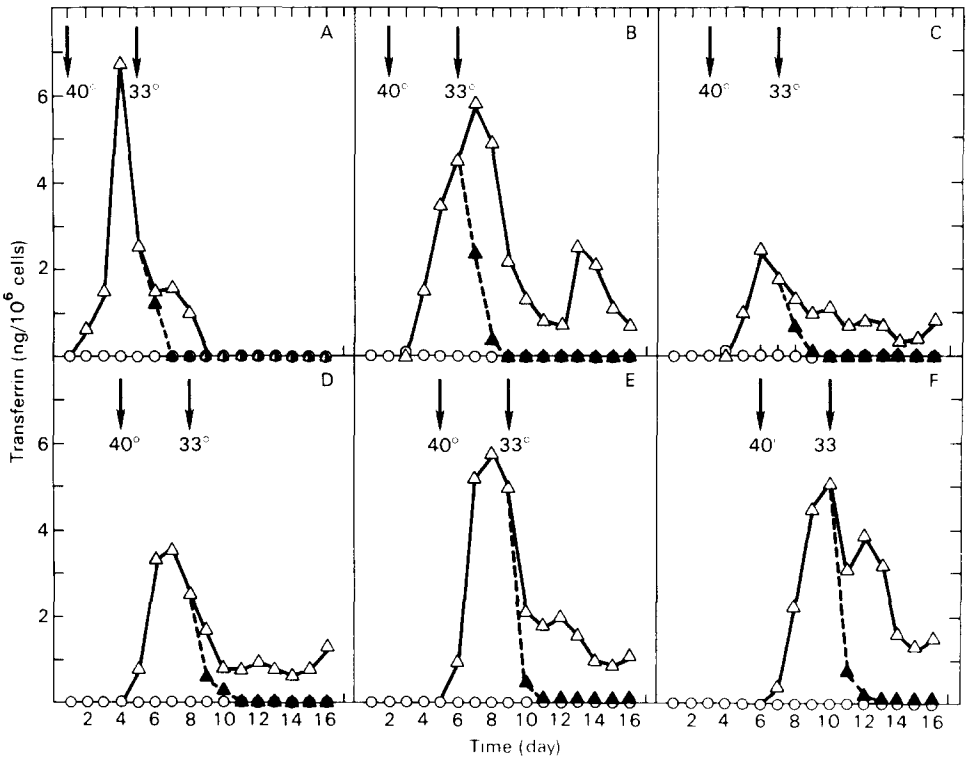

FIGURE 4 Synthesis of transferrin in *RLA209-15* cells grown at 33°C and 40°C. The experimental conditions were the same as in Fig. 1. Cells were grown in αMEM-10 and medium was changed every 24 h. Each value (expressed as nanograms per 10^6 cells per 24 h) represents the average of data from two cultures, each analyzed in duplicate. ○, 33°C; △, 40°C; ▲, cultures shifted from 40°C to 33°C after 4 d at 40°C. Arrows indicate time of temperature shift.

in transferrin synthesis was seen after a shift from 40°C to 33°C (Fig. 4).

Intracellular and Extracellular Accumulation and the Immunological Properties of RLA209-15 α-Fetoprotein, Albumin, and Transferrin

The increase in α-fetoprotein, albumin, and transferrin at 40°C was not attributable to effects on secretion, because intracellular levels of these proteins were also increased (Table I).

The immunological determinants of α-fetoprotein, albumin, and transferrin synthesized by *RLA209-15* cells were compared with the determinants of authentic rat α-fetoprotein, albumin, and transferrin (Fig. 5). The slopes of immunotitration curves for these proteins from *RLA209-15* cells were indistinguishable from those of authentic rat α-fetoprotein, albumin, and transferrin.

Increase of cAMP Levels in Response to Glucagon

RLA209-15 cells, like primary cultures of hepatocytes, responded to glucagon. In the presence of glucagon, the cAMP levels were markedly increased in the transformed cells (Fig. 6). The glucagon response occurred equally well at the permissive and the restrictive temperatures, as was demonstrated previously in *RLA255-4* cells (19). It appears that transformation of the fetal liver cells by SV40 *tsA* mutants has not interfered with the normal control mechanisms for response to this peptide hormone.

TABLE I

Intracellular and Extracellular Accumulation of α-Fetoprotein, Albumin, and Transferrin by RLA209-15 Cells at 33 °C and 40 °C

	Temperature	Cell	Medium
	°C	*ng/mg protein*	
α-Fetoprotein	33	0.8 ± 0.1*	45 ± 2
	40	10.9 ± 0.5	1,278 ± 45
Albumin	33	ND‡	21 ± 1
	40	4.2 ± 0.3	165 ± 10
Transferrin	33	0.3 ± 0.02	15 ± 0.8
	40	3.7 ± 0.2	217 ± 8

Cells in 75-cm² flasks were grown at 33°C or 40°C for 3 d with daily medium change. α-Fetoprotein, albumin, and transferrin were determined in cells and media collected on day 3.

* Mean ± SE.

‡ Not detectable (<1 ng/flask).

DISCUSSION

The SV40 *tsA* mutant-transformed fetal liver cells (*RLA209-15* cells) offer an excellent in-vitro model system for the study of the differentiated functions of liver. *RLA209-15* cells were temperature sensitive for the maintenance of the transformed phenotype. At the permissive temperature (33°C) these cells

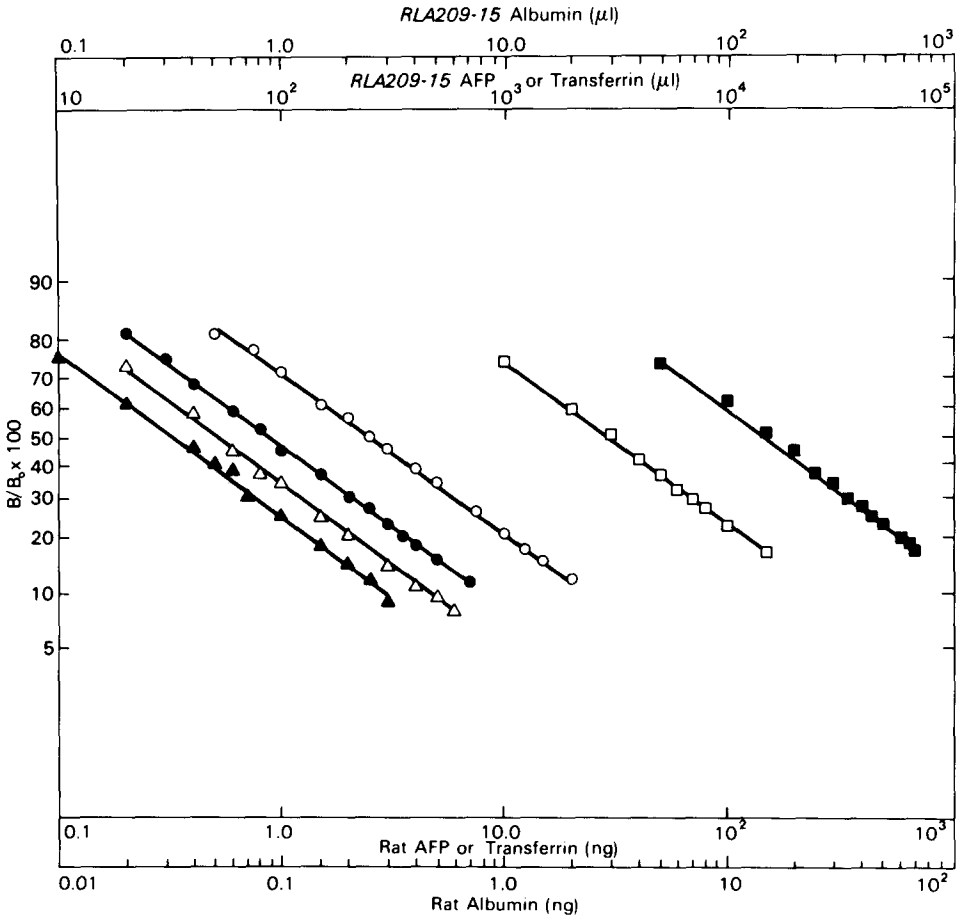

FIGURE 5 Immunotitration curves of α-fetoprotein (AFP), albumin, and transferrin synthesized by *RLA209-15* cells in the respective radioimmunoassay system. ▲, *RLA209-15*-transferrin; △, rat transferrin; ●, *RLA209-15*AFP; ○, rat AFP; □, rat albumin; ■, *RLA209-15*-albumin.

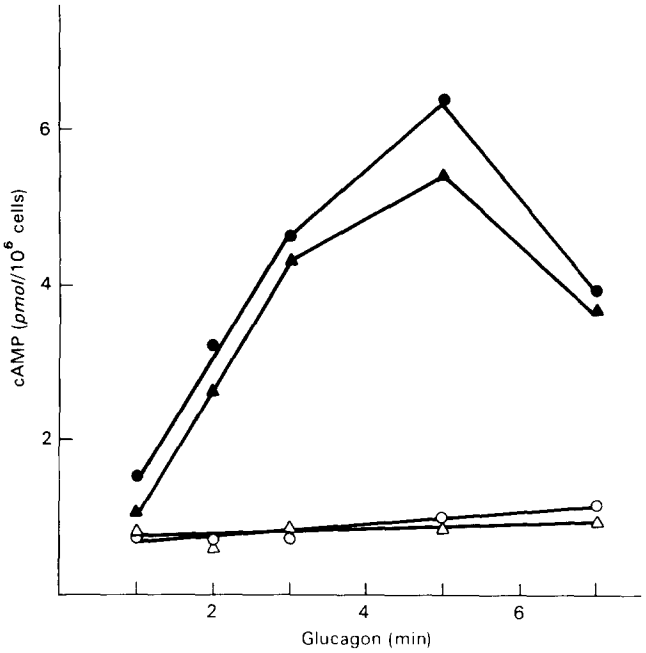

FIGURE 6 cAMP levels in *RLA209-15* cells after exposure to glucagon. Cultures in 24-well plates were grown in αMEM-10. Medium was changed every 2 d. Half of the cultures were shifted to 40°C after growth at 33°C for 5 d. After incubation for an additional 4 d at 33°C or 40°C, cAMP levels in these cultures were measured, in the absence and presence of glucagon. Before glucagon (10^{-7} M) addition, cultures were washed and serum-free medium (1 ml) was added to each well. Glucagon was added to cultures that had been preincubated with papaverine (0.5 mM) for 5 min. Reactions were stopped by the addition of boiling water (1 ml/well). cAMP was estimated by radioimmunoassay. ○, papaverine, 33°C; ●, glucagon plus papaverine, 33°C; △, papaverine, 40°C; ▲, glucagon plus papaverine, 40°C.

behaved like transformed cells, permitting propagation and cloning. At the restrictive temperature (40°C) the transformed phenotype was lost, and *RLA209-15* cells regained their normal differentiated phenotype. These cells synthesized the serum proteins α-fetoprotein, albumin, and transferrin, which are unique to hepatocytes. Regulation of the synthesis of these proteins was also temperature dependent. At 33°C, *RLA209-15* cells synthesized low levels of α-fetoprotein, albumin, and transferrin. At 40°C, the synthesis of all three proteins was greatly increased.

The serum proteins synthesized by *RLA209-15* cells were immunologically indistinguishable from purified α-fetoprotein, albumin, and transferrin from rat. The proteins made by the transformed cells, therefore, appear identical to authentic rat liver proteins.

The monophasic induction of α-fetoprotein synthesis and the biphasic induction of albumin synthesis at 40°C agreed with the patterns observed for the synthesis of α-fetoprotein and albumin in primary rat fetal hepatocytes (22). In the isolated hepatocytes, Sell and co-workers (22) found that the production of α-fetoprotein is proportional to the number of replicating cells and, therefore, is closely coupled to the transitions of cells from quiescent to growing states. On the other hand, albumin production is independent of these transitions. When *RLA209-15* cells grown at 33°C were shifted to 40°C, cells changed gradually from a state of rapid growth to a state of slow growth and, finally, to a quiescent state. Increase in α-fetoprotein synthesis could only be achieved during slow growth, whereas increase in albumin synthesis was achieved during both slow growth and quiescent states. It is possible that the synthesis of albumin during the quiescent state represents the transition of fetal cells to adult liver cells. The biphasic increase in transferrin synthesis at 40°C suggests that the synthesis of transferrin, like that of albumin, was not coupled with rapid growth.

The retention of a glucagon response has been demonstrated in a SV40 *tsA255* transformed liver cell line (19). *RLA209-15* cells also contained functional receptors for glucagon. This indicates that the glucagon receptors characteristic of hepatocytes (16, 17, 24) may be retained in all *tsA*-transformed liver cells. These stable cell lines should prove advantageous in studying the molecular basis of malignant transformation and the process of cellular differentiation.

The establishment of two types of clearly differentiated cells, placental and liver cells, by the transformation of mammalian cells with SV40 *tsA* mutants suggests that the approach used here may be applicable to any cell type that can be transformed by SV40.

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