# TRANSFORMATION OF ISOLATED RAT HEPATOCYTES WITH SIMIAN VIRUS 40

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ABSTRACT

Rat hepatocytes were transformed by simian virus 40 (SV40). Hepatocytes from two different strains of rats and a temperature-sensitive mutant (SV40tsA1609), as well as wild-type virus were used. In all cases, transformed cells arose from ~50% of the cultures containing hepatocytes on collagen gels or a collagen gel–nylon mesh substratum. Cells did not proliferate in mock-infected cultures. SV40-transformed hepatocytes were epithelial in morphology, retained large numbers of mitochondria, acquired an increased nucleus to cytoplasm ratio, and contained cytoplasmic vacuoles. Evidence that these cells were transformed by SV40 came from the findings that transformants were 100% positive for SV40 tumor antigen expression, and that SV40 was rescued when transformed hepatocytes were fused with monkey cells. All SV40-transformed cell lines tested formed clones in soft agarose. Several cell lines transformed by SV40tsA1609 were temperature dependent for colony formation on plastic dishes. Transformants were diverse in the expression of characteristic liver gene functions. Of eight cell lines tested, one secreted 24% of total protein as albumin, which was comparable to albumin production by freshly plated hepatocytes; two other cell lines produced 4.2 and 5.7%, respectively. Tyrosine aminotransferase activity was present in five cell lines tested but was inducible by dexamethasone treatment in only two. We conclude from these studies that adult, nonproliferating rat hepatocytes are competent for virus transformation.

The objectives in transforming an adult hepatocyte are multifold. Firstly, adult, isolated hepatocytes, in contrast to fibroblasts of embryonic origin, are differentiated and express many liver-specific proteins and enzymes that permit analysis of the effects of transformation on gene expression. Secondly, because isolated hepatocytes are epithelial, transformation may produce cells that, when inoculated into syngeneic hosts, will cause carcinomas or hepatomas instead of sarcomas. Thirdly, transformation of isolated hepatocytes should generate a proliferating adult liver cell line. In contrast to transformation of rat cells by chemical carcinogens (13, 28), in virus-induced transformation, the gene information added to the normal cell is known; therefore, by using temperature-sensitive virus mutants, expression of the added information can be controlled by experimental manipulation.

Adult rat liver cell cultures include hepatoma cell lines, cloned rat liver epithelial cells, and nonproliferating hepatocytes isolated by collagenase perfusion. Isolated hepatocytes closely resemble in vivo adult liver cells both in ultrastructure (17) and in biochemical function (22), i.e., they do

not normally divide but, to a limited extent, can be triggered to stimulate DNA synthesis when exposed to appropriate hormones (16) or when infected with virus (H. C. Isom, manuscript submitted). Seeding hepatocytes on floating collagen gels (12) or on collagen gel–nylon mesh substratum (21) increases the longevity of the cells in culture. In monolayer, hepatocytes synthesize ornithine transcarbamylase (H. C. Isom, unpublished results) and albumin (21) for at least 3 d and begin to synthesize α-fetoprotein and γ-glutamyl transpeptidase after 6 d in culture (21). However, monolayer cultures do not proliferate and studies have been limited, therefore, to short time periods.

Our studies show that adult, nonproliferating, differentiated rat hepatocytes are competent for virus transformation. We also describe initial characteristics of the transformants with regard to their properties as transformed cells and their expression of virus and liver gene products.

## MATERIALS AND METHODS

### *Hepatocyte Monolayers*

Male Sprague-Dawley rats, purchased from Charles River Breeding Laboratories (Wilmington, Mass.), were used when they had attained a weight of 165–185 g. Livers were perfused *in situ* at 37°C by the technique described by Berry and Friend (1), as modified by Feldhoff et al. (8). To reduce the effects of diurnal variation, rats were perfused between 1:30 and 3:00 p.m. The perfusion medium was Krebs-Henseleit bicarbonate buffer (pH 7.4) without calcium ions but supplemented with 10 mM glucose, 5 mM monosodium glutamate, 5 mM sodium pyruvate, penicillin (500 U/ml), streptomycin (500 μg/ml), and sufficient bovine erythrocytes to yield a hematocrit of 10%. The components of the buffer were autoclaved and combined under sterile conditions. Before use, the buffer was gassed for 30–45 min with oxygen:carbon dioxide (95:5%) and sterilized with a Nalgene disposable filter (Nalge Sybron Corp., Rochester, N. Y.). Bovine erythrocytes, obtained by sterile bleeding of healthy calves, were washed four times in sterile phosphate-buffered saline (PBS) and filtered through glass wool. Anesthetized rats were shaved, and their skin was painted with antiseptic. All surgical instruments were autoclaved. The lucite drums and tubing from the liver perfusion box (Vanderbilt Apparatus Shop, Nashville, Tenn.) were gas (ethylene oxide) sterilized. Livers were perfused in a nonrecirculating system for 15 min at 14 ml/min. The system was then set to recirculate at the same flow rate with 150 ml of perfusate containing 40 mg of collagenase (Type II, Worthington Biochemical Corp., Freehold, N. J.). The liver was excised after 35–40 min. The excised, digested liver was suspended in 50 ml of gassed Krebs-Henseleit buffer lacking bovine erythrocytes but supplemented with 2.4 mM calcium, penicillin (500 U/ml), streptomycin (500 μg/ml), and 3% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo.), gently forced through a stainless steel screen, and filtered through four layers of cotton gauze. The cell suspension was placed in a 40-ml glass conical centrifuge tube, which was then accelerated rapidly in a nonrefrigerated tabletop International Centrifuge (Damon Corp., I. E. C. Div., Needham Heights, Mass.) to 1,000 rpm and allowed to stop without breaking. The supernatant fluid (containing Kupfer cells and damaged hepatocytes) was removed by aspiration with the top 10% of the pellet. The remainder of the pellet was resuspended in Krebs-Henseleit-BSA, and the washing procedure was repeated four more times. The final two washes were carried out in gassed growth medium instead of Krebs-Henseleit-BSA. Cell count and viability were determined in trypan blue; cell viabilities ranged from 80–95%.

Isolated hepatocytes were plated in plastic culture dishes (60-mm), on a collagen gel (12), or a collagen gel–nylon mesh substratum (21) in L-15 medium supplemented with 18 mM HEPES, 0.2% BSA, 0.15% glucose, penicillin (500 U/ml), streptomycin (500 μg/ml), 5% fetal calf serum, and insulin (0.5 μg/ml). Hepatocyte monolayers were plated at a density of 2–5 × $10^6$ cells per 60-mm dish or mesh.

### *Transformation with Simian Virus 40 (SV40)*

At 6 h after plating, hepatocyte monolayers were infected with wild-type SV40 or the temperature-sensitive SV40tsA1609 mutant at multiplicities of 50–100 plaque-forming units (PFU) per cell; virus was allowed to adsorb for 1–2 h. Wild-type SV40 strain VA45-54 was propagated in African green monkey kidney TC-7 cells (24). SV40tsA1609 was isolated after hydroxylamine mutagenesis and was assigned to the A group (23).

### *Electron Microscopy*

For ultrastructural analysis, 1–2 × $10^7$ SV40-transformed hepatocytes were harvested near confluence, washed, and pelleted. The cell pellet was fixed in Karnovksy's fixative for 12 h at 4°C (10), washed with sodium cacodylate buffer, postfixed in Dalton's chrome-osmium containing 2% osmium (4), washed in buffer, stained *en bloc* with 2% uranyl acetate in 35% ethanol, dehydrated in a graded series of ethanol, and embedded in Epon 812 epoxy resin. Sections were cut on a Sorvall MT-2-B ultratome (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) at 600–800 Å, stained with 2% aqueous uranyl acetate and lead citrate, and examined with a Hitachi HU-12 electron microscope at 75 kV.

### *Detection of SV40 Tumor (T) Antigen*

Isolated hepatocytes were plated on plastic coverslips infected with SV40 6 h after plating and harvested at various times postinfection. Transformed hepatocytes were seeded on glass coverslips and harvested 24–48 h after plating. Cells on both plastic and glass coverslips were harvested by placing them in a fresh 60-mm dish and washing them twice in PBS. To each dish was added 3 ml of absolute ethanol precooled to −20°C. The dish was cooled at −70°C for 30–45 min, the ethanol was removed, and the opened dishes were maintained overnight at 4°C so that the coverslips could dry. The dishes were closed, sealed, and stored at −20°C. This fixation procedure resulted in minimal distortion of hepatocyte structure. Sera containing antibody to SV40 T antigen (kindly provided by Dr. S. S. Tevethia, The Pennsylvania State University College of Medicine, Hershey, Pa.) were obtained from either BALB/c mice or hamsters bearing tumors induced by virus-free SV40 tumor cells and were allowed to react with fixed cells at 37°C for 45 min. The cultures were then washed in PBS and allowed to react for 45 min with fluorescein isothiocyanate-conjugated goat antiglobulin to mouse or hamster γ-globulin. Coverslips were then washed, dried, mounted, and examined under a fluorescence microscope.

### *Albumin Determinations*

Secreted albumin was measured with specific antibodies as a percent of total secreted protein. For freshly plated hepatocytes, isolated hepatocytes were seeded at a density of $1.2 \times 10^5$ cells/$cm^2$ in a 150-$cm^2$ plastic petri dish and fed 10 ml of leucine-free medium. When the cells had attached (1 h after plating), [$^3$H]leucine (50 $\mu$Ci/ml) was added to the medium. Cultures were incubated at 37°C, and the medium was harvested 3 h later. For heptocytes on meshes, hepatocytes were plated on a collagen gel–nylon mesh substratum at the same density but in 21-$cm^2$ petri dishes. At 24, 48, and 72 h after plating, the meshes were washed twice in PBS and placed in 1.5 ml of leucine-free medium for 2 h and labeled for 3 h with [$^3$H]leucine. For albumin secretion studies of SV40hp cell lines, cells were subcultured 1:4 and grown to 80% confluence. Cultures were washed, incubated for 2 h in 1.5 ml of leucine-free medium, labeled for 3 h, and the medium was collected. Medium from all cell types (10-$\mu$l aliquots) was assayed for radioactivity in total protein by trichloroacetic acid precipitation on filter paper disks (8). Incorporation into albumin was determined by immunoprecipitation with specific albumin antibodies as described by Feldhoff et al. (8).

### *Tyrosine Aminotransferase (TAT) Activity*

Cells were grown to confluence in petri dishes (150-$cm^2$) and placed in serum-free "induction" medium (26) with or without $10^{-5}$ M dexamethasone. Cultures were harvested 16 h after treatment, and the cells were washed three times in PBS. Pelleted cells were frozen at −20°C, suspended in 0.5 ml of potassium phosphate buffer supplemented with 2.5 mM tyrosine, and disrupted by sonication. The homogenate was centrifuged at 20,000 *g* for 10 min, and the supernate was assayed for TAT activity according to the method of Diamondstone (7) as modified by Thompson et al. (26). Protein determinations were carried out using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine γ-globulin as a standard.

## RESULTS

### *Transformation of Isolated Hepatocytes with SV40*

Hepatocyte monolayers used for the initial transformation experiment were prepared from outbred (Sprague-Dawley) rats and plated in plastic tissue culture dishes on collagen gels and collagen gel–nylon meshes. The three types of monolayer conditions provided different surfaces for hepatocyte attachment, with the result that, in each condition, the hepatocytes assumed a different morphology. These factors may be critical to the initiation of transformation. Microscopic observation of meshes, gels, and plastic petri dishes containing hepatocytes infected with SV40 (multiplicity of 100 PFU per cell) at 6 h after plating initially showed no cytopathology and no apparent difference when compared with mock-infected control cultures. By 14 d postinfection, both virus-infected and mock-treated cultures had begun to deteriorate, and 40–60% of the cells were left in the medium. At 18 d postinfection, some of the SV40-infected cultures on meshes and gels showed areas of dividing cells, and, in a few cultures, acid formation was detected in the medium. To determine whether these cells would continue to proliferate in the absence of an artificial collagen substratum, cells were recovered from two meshes and two gels by incubation with collagenase (1 mg/ml for 10 min), centrifugation to pellet cells, and placement (after resuspension in fresh medium) into 35-mm plastic dishes. At subsequent 2-d intervals, two to three meshes or gels were treated in a similar fashion. In one dish initially harvested from a mesh, after 6 d the medium became slightly acidic, and a patch of 10–12 epithelial cells growing in a cluster appeared. These cells continued to proliferate and were trypsinized and passaged to a tissue culture flask (25-mm). In the following week, several other plates yielded cultures that contained morphologically similar proliferating epithelial liver cells. In two plates, outgrowth of fibroblasts was observed, but the fibroblasts failed to continue to proliferate when subcultured by trypsinization. None of the cultures plated directly on plastic tissue culture dishes and infected with SV40 yielded proliferating cells.

Of 10 meshes and 10 gels initially infected, nine yielded cell lines (SV40hp1 to SV40hp9). Four of these cell lines arose from hepatocytes initially plated on collagen gels, and five arose from cells plated on meshes. One cell line (SV40hp6) died before passage 3; another (SV40hp9) had the ultrastructure of macrophagelike cells and died by passage 9. Cloning by dilution of a third cell line (SV40hp8) that was initially contaminated with macrophage cells resulted in outgrowth of only epithelial liver cells. The remaining cell lines grew in clonal fashion until passage 4 or 5 and then formed monolayers. These lines (SV40hp1, SV40hp2, SV40hp3, SV40hp4, SV40hp5, SV40hp7, and SV40hp8cl1) appear to be stably transformed inasmuch as they have been subcultured over 40 times.

Fig. 1 shows the morphology of SV40hp5 as observed using phase-contrast light microscopy. SV40hp2, SV40hp7, and SV40hp8cl1 were morphologically indistinguishable from SV40hp5. All of the cell lines were epithelial; however, the growth pattern and cell size varied. SV40hp1 cells grew in tighter "islandlike" formation, and SV40hp3 cells were slightly larger than SV40hp5 cells. Ultrastructural examination indicated that all cell lines retained large numbers of mitochon-

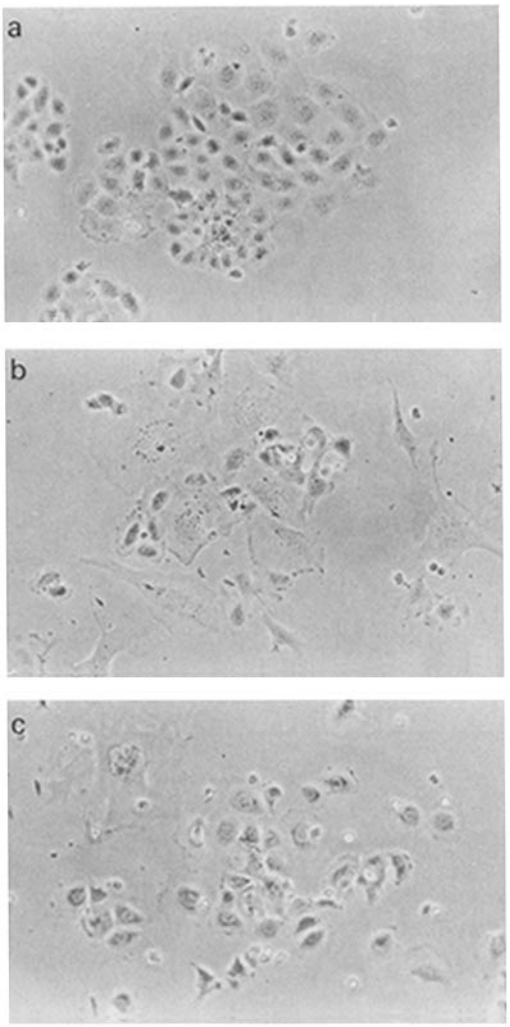


FIGURE 1 Morphology of SV40-transformed rat hepatocytes. Photomicrographs of dividing SV40hp1 (*a*), SV40hp3 (*b*), and SV40hp5 (*c*) cells at passages 5, 5, and 3, respectively. (*a–c*) × 200.

dria, acquired an increased nucleus to cytoplasm ratio and an irregular nuclear profile, and contained vacuoles in the cytoplasm. An electron micrograph of a typical SV40-transformed hepatocyte is seen in Fig. 2.

### *Expression of SV40 T Antigen*

Transformed hepatocytes were characterized for expression of SV40 genetic information. When transformation was initiated, hepatocytes on coverslips were infected with SV40, harvested at various times postinfection, and analyzed for SV40 T antigen by immunofluorescence. At 84 h postinfection, 80% of the infected cells were T antigen-positive (Fig. 3 *a*), indicating that the virus genetic information had not only entered the hepatocyte but also was expressed. All the SV40-transformed hepatocyte cell lines were 100% positive for T antigen (Fig. 3 *b*). Individual cell lines were tested further for expression of virus genes. For example, SV40hp3 was 100% positive for T antigen not only after the 7th subculture but also after the 21st subculture. Expression of T antigen in SV40hp1 and SV40hp3 measured by immunofluorescence was confirmed by immunoprecipitation and SDS polyacrylamide gel electrophoresis. Both cell lines contained large T antigen, which migrated identically with T antigens from well-established SV40-transformed fibroblast cell lines (data not shown). Further studies using SV40hp1 and SV40hp3 detected no infectious SV40 in the medium or in the total cell extract. However, SV40 was rescued from these two cell lines when they were fused using polyethylene glycol (5) with African green monkey (TC-7) cells (data not shown). Virus rescue demonstrated that at least one copy of the entire virus genome was present in SV40hp1 and SV40hp3 cell lines. Both expression of T antigen and virus rescue provided evidence that these cells were indeed transformed by SV40.

### *Properties of SV40-transformed Hepatocytes Isolated from Liver of Inbred Fischer Rats*

Transformation was also performed using hepatocytes isolated by collagenase perfusion of inbred Fischer rats for the purpose of establishing SV40-transformed hepatocyte tumor lines. Of 10 gels and 10 meshes of SV40-infected hepatocytes, subsequent transfer of cells 2–3 wk postinfection resulted in outgrowth of epithelial cells from four gels and six meshes. These cell lines did not differ in gross morphology or ultrastructure from SV40-transformed outbred hepatocytes. All cell lines derived from Fischer rat hepatocytes expressed T antigen in 100% of the cells, as determined by immunofluorescence.

Three of the SV40-transformed inbred Fischer rat hepatocyte cell lines were tested for anchorage independence because it has been previously shown that this property of transformed epithelial cells correlates well with tumorigenicity in syngeneic hosts (13). All three of these cell lines (SV40hpD, SV40hpO, and SV40hpI) formed macroscopically visible clones when grown in soft agarose (Table I). The number of clones formed

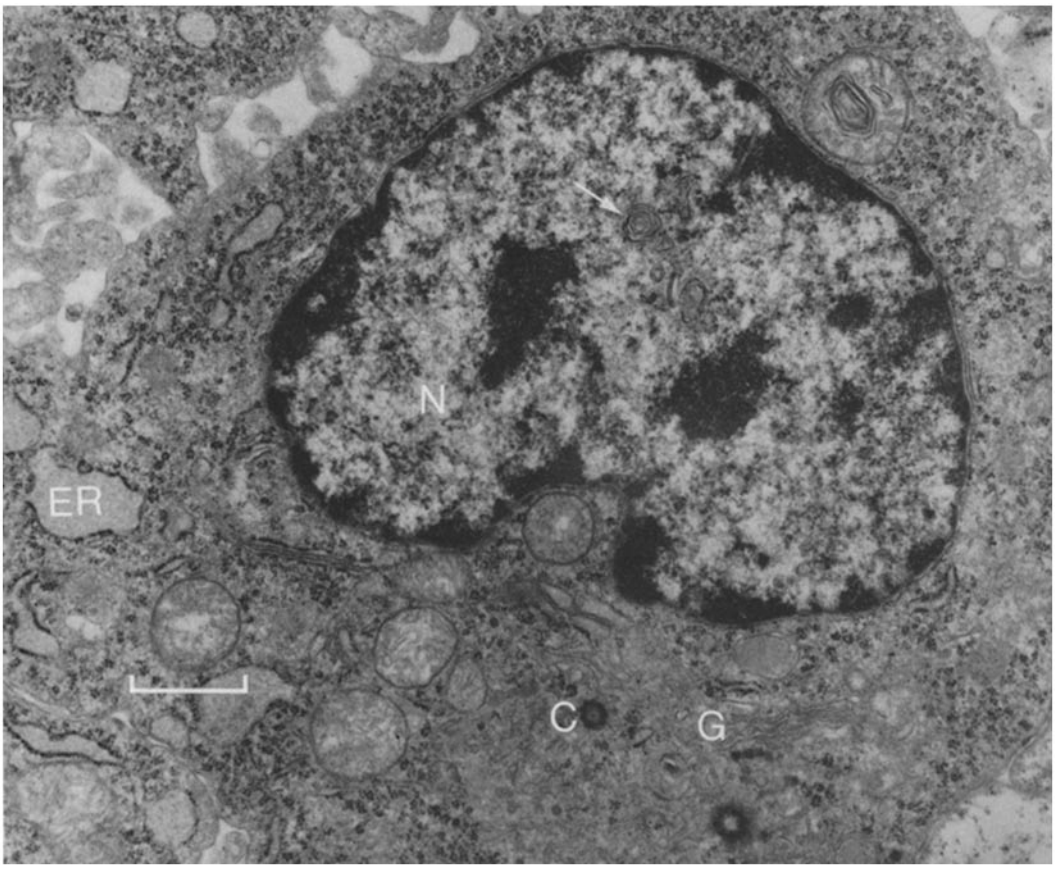


FIGURE 2 Ultrastructure of SV40-transformed rat hepatocytes. The electron micrograph of SV40hpts1 was taken at passage 8. Note that the nucleus is generally large and convoluted and contains membranous inclusion bodies (arrow). Abundant dilated rough endoplasmic reticulum (*ER*) is present, and the golgi (*G*) is relatively prominent. In this particular cell, centrioles (*C*) also can be observed. Bar, 0.8 μm. × 19,750.

per number of cells plated was approximately a magnitude lower with SV40hpI than with SV40hpD or SV40hpO. A normal brown Norwegian rat kidney cell line (kindly provided by Dr. M. Fluck, East Lansing, Mich.) was used as a control, and it formed microscopically visible clones at a frequency of 8 clones per $10^4$ cells plated. Since anchorage independence is only suggestive of tumor induction, animal studies are currently in progress to establish whether SV40-transformed Fischer rat hepatocytes will form tumors when inoculated into the syngeneic host.

### *Transformation of Fischer Rat Hepatocytes with an SV40tsA Mutant*

Temprature-sensitive mutations of the SV40 A gene effect accumulation of T antigen and the ability to induce stable transformation. Cells transformed by SV40tsA mutants in some instances express the transformed phenotype at low (33°C) but not at high (40.5°C) temperatures. SV40tsA1609 transforms as efficiently as wild-type SV40 at 33°C (23; M. J. Tevethia, unpublished data), and it was used in these experiments. When 12 plates of meshes were infected with SV40tsA1609 at a multiplicity of 50 PFU per cell, epithelial cells arose from six of the gels (SV40hpts1 to SV40hpts6). These cell lines were subcultured at both 37 and 33°C and frozen at passages 5, 10, and every 10th passage thereafter. The electron micrograph of SV40hpts1 shown (Fig. 2) is representative of all the SV40-transformed hepatocytes.

Three cell lines transformed with SV40tsA1609

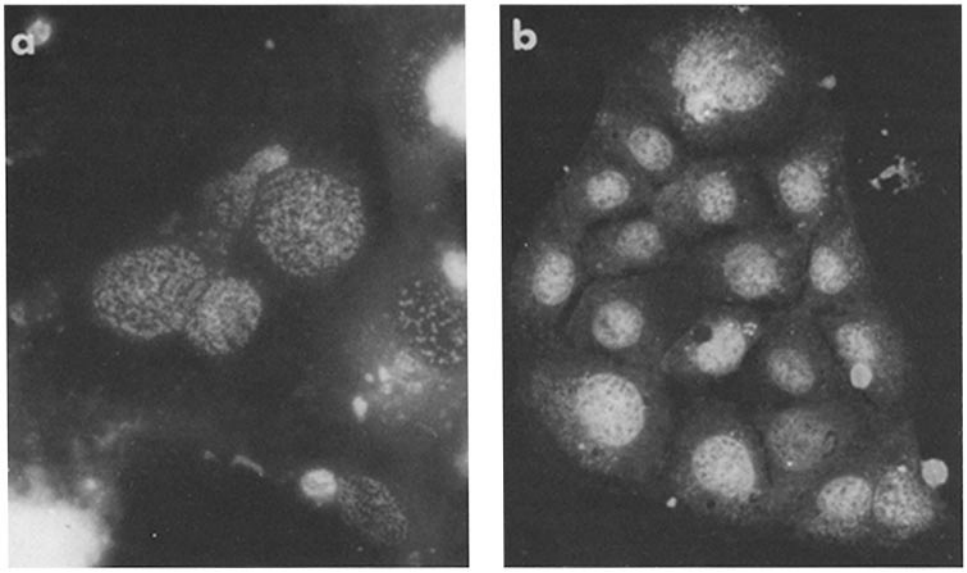


FIGURE 3 Immunofluorescence of SV40 T antigen in SV40-infected (*a*) and transformed (*b*) hepatocytes. SV40 infection was for 84 h. Cells in *b* are SV40hpI at passage 16. Nuclear fluorescence was not observed in uninfected hepatocytes treated with anti-T serum or in hepatocytes and SV40hpI cells treated with normal serum. (*a*) × 500. (*b*) × 320.

(SV40hpts1, SV40hpts4, and SV40hpts5) were tested for efficiency of cloning on plastic at 33, 37, and 40.5°C (Table II). The cloning efficiency of SV40hpts1 was temperature independent, whereas that of SV40hpts4 and SV40hpts5 was temperature dependent and was significantly higher at 33°C (permissive temperature for SV40tsA1609) than at 40.5°C (nonpermissive temperature for SV40tsA1609). Studies on the metabolism of SV40 T antigen in hepatocytes transformed by SV40tsA mutants are currently in progress.

### *Biochemical Properties of SV40-transformed Hepatocytes*

Specific biochemical properties of SV40-transformed hepatocytes were studied to determine what liver-specific functions were retained after virus transformation. The production of albumin is a characteristic liver function that is usually altered in neoplasia (20, 27). In normal rat liver in vivo, albumin accounts for about 11% of total protein synthesis (14). In perfused liver and in freshly isolated hepatocytes in suspension, albumin synthesis accounts for 11–13% of total protein synthesis and remains constant for at least 4 h. Newly synthesized albumin requires at least 12–15 min secretion time (8).

TABLE I

*Growth of SV40-transformed Inbred Fischer Rat Hepatocytes in Soft Agarose*

| Cell line | Number of cells plated* | Average number of clones |
|---|---|---|
| SV40hpD | $10^2$ | 12 ± 3 |
| SV40hpO | $10^3$ | 24 ± 7 |
| SV40hpI | $10^4$ | 17 ± 5 |

* SV40hp cells were dispersed with trypsin, counted, and diluted into 0.25% soft agarose so as to plate $10^2$, $10^3$, and $10^4$ cells in each of six petri dishes (60-mm). Clones were macroscopically visible.

TABLE II

*Growth at Different Temperatures of Hepatocytes Transformed with Mutant SV40tsA1609*

| Incubation temperature* | Average number of clones per 100 cells plated‡ | | |
|---|---|---|---|
| | SV40hpts1 | SV40ts4 | SV40hpts5 |
| 33°C | 11.4 ± 0.9 | 27.0 ± 10.0 | 3.6 ± 0.10 |
| 37°C | 8.2 ± 1.7 | 28.8 ± 3.8 | 0.27 ± 0.11 |
| 40.5°C | 6.0 ± 0.5 | 0.70 ± 0.20 | 0.62 ± 0.25 |

* Cells grown at 37°C were subcultured and placed at stated temperatures immediately after dilution.

‡ Values were obtained from seeding cells in 12 plates, four at each of three dilutions: $10^2$, $10^3$, and $10^4$ cells per 60-mm dish.

We first measured albumin synthesis in hepatocytes plated in monolayer for 1 h after isolation (Table III). To maximize the amount of intracellular albumin, cells were labeled for 10 min with [$^3$H]leucine (50 $\mu$Ci/ml). Cultures were then harvested, washed twice in PBS, and the pelleted cells were lysed in 1% Triton X-100 and sodium deoxycholate. The extract was sonicated, centrifuged at 16,000 *g* for 5 min, and the supernatant fluid was assayed for radioactivity in total protein and albumin. Under these conditions, normal hepatocytes in monolayer contained 10.2% of the total intracellular protein as albumin. After incubation for 3 h, up to 20% of the total secreted protein in the medium was albumin. When normal hepatocytes were plated on meshes for 72 h, incorporation of [$^3$H]leucine into total protein remained linear over a 3-h period and was at the same rate as at 24 and 48 h. However, the amount of secreted albumin had diminished 80% by 72 h. This observation is consistent with the findings of Sirica et al. (21).

Of the SV40hp cell lines tested for albumin synthesis, SV40hp3, SV40hp5, and SV40hpI secreted significant amounts of albumin, whereas SV40hpJ and SV40hpts2 secreted only low levels. These measurements were taken at various times after transformation, representing a minimum of 50 d postinfection (Table III). Of the transformed cell lines tested, SV40hp5 cells produced the greatest quantity of secreted albumin; the intracellular albumin concentration was 1.3% of total protein. The other transformed cells contained much lower levels of intracellular albumin. Three cells lines, SV40hp3, SV40hp5, and SV40hpI were also tested for the ability to secrete $\alpha$-fetoprotein, the fetal analog of albumin, and all three were found to be negative for this property (data not shown). The $\alpha$-fetoprotein secretion was measured by immunoprecipitation of radioactively labeled media from the cell lines with specific $\alpha$-fetoprotein antibodies.

TABLE III

*Albumin Secretion in Isolated Hepatocytes and SV40-transformed Hepatocytes*

| Cells | Conditions | Secreted albumin |
|---|---|---|
| | | *% of total protein secreted* |
| Hepatocytes | Freshly plated | 20.0 |
| | Mesh—24 h | 12.8 |
| | Mesh—48 h | 19.1 |
| | Mesh—72 h | 4.1 |
| SV40hp3 | p16* | 5.7 |
| SV40hp5 | p9 | 24.0 |
| SV40hpD | p30 | 0 |
| SV40hpI | p9 | 4.2 |
| SV40hpJ | p9 | 0.8 |
| SV40hpO | p22 | 0 |
| SV40hpts1 | p9 | 0 |
| SV40hpts2 | p6 | 0.52 |

* p denotes passage number. Transformed cells were subcultured at a ratio of 1:4 every 4–6 d.

TABLE IV

*TAT Activity of SV40-transformed Hepatocytes*

| Cell line | Dexamethasone ($10^{-5}$ M) | TAT activity |
|---|---|---|
| | | *nmol/mg/15 min* |
| HTC* | – | 1.62 |
| | + | 11.60 |
| SV40hpA | – | 1.10 |
| | + | 1.17 |
| SV40hpB | – | 0.48 |
| | + | 1.04 |
| SV40hpE | – | 1.31 |
| | + | 1.92 |
| SV40hpG | – | 0.78 |
| | + | 0.81 |
| SV40hpts1 | – | 1.58 |
| | + | 1.62 |

* The hepatoma tissue culture (HTC) cell line, a rat cell line derived from an ascites tumor (26), has been shown in numerous reports to be inducible by dexamethasone for TAT and was therefore used as a positive control.

SV40-transformed hepatocytes were also tested for two other functions, ability to synthesize TAT and TAT inducibility by growth of cells in the glucocorticoid, dexamethasone (Table IV). TAT activity of SV40-transformed hepatocytes was present at low levels and, in two cell lines, SV40hpB and SV40hpE, was inducible 2.2- and 1.5-fold, respectively, by dexamethasone treatment.

## DISCUSSION

This paper reports the transformation of adult, nonproliferating rat hepatocytes by SV40. Several properties of SV40 make it desirable for use as a transforming virus. SV40 is a small DNA virus that appears to code for only five proteins (9). Upon infection, the virus can stimulate host cell DNA synthesis (3, 18) and can transform rat cells (25), and well-characterized SV40 temperature-sensitive mutants are available (11). Transformation was satisfactorily accomplished many times with hepatocytes from two strains of rats and with

a temperature-sensitive (SV40tsA) mutant as well as wild-type virus. In each case, transformed cells arose from ~50% of the culture dishes containing hepatocytes on gels or meshes. Cells did not proliferate in mock-infected cultures or in cultures fed fresh medium. Because adult hepatocytes are nondividing cells, there was no background of proliferating cells in the transformation assay. Therefore, a satisfactory initial criterion for a transformed cell was its ability to attach to a plastic culture surface and to proliferate after removal from the artificial substratum. When necessary, transformed epithelial cells could subsequently be selected from transformed fibroblast or macrophage cells by cloning and/or growth beyond the 10th passage.

Our findings contrast with those previously reported for hamster liver cells (6). Diamandopoulos showed that hamster hepatic parenchymal cells from regenerating liver were incapable of in vivo or in vitro transformation by SV40. The discrepancy between these studies and ours may be the result of a difference in the species used (hamster vs. rat) or, more likely, of the methods used for liver cell preparation. Hamster liver cell cultures were prepared by killing the animal and trypsinizing the larger right dorsocaudal lobe of the liver, whereas rat liver cells in our study were isolated by collagenase perfusion of the entire liver and subsequent centrifugation to separate hepatocytes from other cell types. Furthermore, inasmuch as rat hepatocytes isolated by collagenase perfusion but plated on plastic petri dishes were incapable of transformation by SV40, it appears that the method of isolation and/or temporary maintenance in monolayer culture is critical in determining the competence of hepatocytes for SV40 transformation.

Physiologic conditions of the cell during a critical period after virus infection are a crucial parameter of the transformation event. For example, in rat embryo fibroblasts, it has previously been shown that transformation at 33°C with an early tsA mutant of polyoma virus yields transformants that are temperature dependent for expression of the transformed phenotype (N-type) and transformants that are temperature independent for the transformed phenotype (A-type) (19). N-type transformants were derived from cells grown in plastic dishes, whereas A-type transformants were originally selected from virus-infected colonies grown in soft agar (19). Subsequently, it was demonstrated that infection with multiplicities between 200 and 500 PFU per cell produces N-type transformants in growing cells and A-type transformants in nongrowing cells (15). In our study, intermediate multiplicities of 50–100 PFU per cell were used. Also, microscope examination of hepatocytes on collagen gel–nylon meshes indicated that cells were present both on and within the collagen gel. Moreover, using hepatocytes from Fischer rats, we confirmed the finding of Sirica et al. (21) that an increase in DNA synthesis occurred in hepatocytes 4–6 d after plating on collagen gel–nylon meshes, but that cell division did not occur (data not shown). This finding is significant because SV40 transformation may not occur until 4–5 d postinfection. Therefore, we did not know whether the artificial substratum used more closely resembled growth on plastic or growth in soft agar and whether the physiology of the cell more closely resembled a dividing or nondividing cell. As we have shown, transformation of rat hepatocytes with an early SV40tsA mutant produced transformants of both the N- and A-types with regard to cloning efficiency on plastic at 40.5, 37, and 33°C. The presence of N-types suggests that either dividing hepatocytes were present in low numbers within the population, or that within the hepatocyte system, DNA synthesis, in the absence of cell division, may provide sufficient conditions for the generation of N-types. Not enough SV40tsA-transformed hepatocytes have been isolated or characterized at this time to determine the frequency of N-type and A-type transformants.

Studies on albumin synthesis, TAT activity, and TAT inducibility by dexamethasone showed that the transformed cell lines, although 100% positive for T antigen expression, were diverse in their expression of liver genes. Secretion of albumin by normal hepatocyte monolayers on meshes diminished significantly by 3 d in culture. Because the transformation event may not occur until several days postinfection, one explanation for selection of transformants that do not secrete albumin or that secrete at diminished levels is that these hepatocytes lose this capacity by the time of transformation. However, the finding that at least one transformant produced nearly normal hepatocyte levels of albumin suggested that SV40 transformation may have significant effects on liver gene expression. The same situation may be true for TAT inducibility by dexamethasone, i.e., dexamethasone receptors may be lost in hepatocytes by the time they become transformed by SV40. In fact, hepatocyte monolayers were not cultured in the presence of dexamethasone, a factor that may be critical in the maintenance of hormone re-

sponse.

The transformation of adult rat hepatocytes with SV40 provides a system with multiple potential uses. Firstly, because transformation of inbred Fischer rat hepatocytes has been accomplished, the cells could be used to study tumor formation in syngeneic hosts and the subsequent gene expression of such tumors. Secondly, hepatocytes isolated by collagenase perfusion could be used to test the ability of other viruses to transform normal adult nonproliferating epithelial cells. Thirdly, inasmuch as transformation was apparent by 2–3 wk, and the background parental cell type did not proliferate, this system could be used to examine the effects of infection on the expression of proteins or enzymes that can be measured by immunofluorescence or by histochemical means such as albumin $\alpha$-fetoprotein, or $\gamma$-glutamyl transpeptidase. Fourthly, because hepatocytes can be transformed by temperature-sensitive SV40 mutants, and because temperature-dependent N-type transformants have been isolated, this system, like SV40-transformed human placental cells (2), permits, simply by temperature shift, analysis of loss and acquisition of proteins and enzymes particular to a specific cell type in parallel with altered cloning efficiency, growth properties, and SV40 T antigen expression.

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