Effect of Simian Virus 40 Infection on Albumin Production by Hepatocytes Cultured in Chemically Defined Medium and Plated on Collagen and Non-collagen Attachment Surfaces*

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Simian virus 40 (SV40)-infected rat hepatocytes were tested for albumin production (a marker of differentiated hepatocyte function) by growth in serum-free medium for 30-40 days. We observed a biphasic peak of albumin production, with the first peak at 4-6 and the second at 10-20 days after plating. A 2- to 3-fold increase in the second peak was observed when albumin production by SV40-infected cultures was compared with that by uninfected cultures. No increase in cell number in SV40-infected cultures was observed during this 20-day period. By 25-35 days, albumin production by uninfected cultures ceased but was detected in 50-60% of SV40-infected cultures. SV40-induced stimulation and persistence occurred regardless of the attachment surface and did not require collagen. Transformation occurred regardless of whether stimulation or persistence of albumin production was seen; however, stimulation and persistence were necessary to yield transformed cultures that produced albumin.

Interaction of differentiated epithelial cells with viruses is an underexplored area primarily because of the difficulty in defining appropriate culture conditions for maintenance of cellular differentiation during the time required to examine virus infection and malignant transformation. In our studies, we selected rat hepatocytes as a differentiated cell type (1-4). Isolation of functional hepatocytes became possible with the introduction of in situ enzyme perfusion of adult rats (5, 6). However, this procedure dissociates the hepatocytes from the tissue matrix and disrupts the cell interactions that most likely play a significant role in the regulation of hepatocyte gene expression (7). If the physical and chemical entities in intact liver can be simulated in culture by purified components, it will be possible to increase the functional life span of an isolated hepatocyte.

It is reasonably clear that two factors, medium composition and attachment surface, act synchronously in regulating proliferation and expression of differentiated properties of liver cells (8, 9). In the original studies with hepatocyte monolayers, medium supplemented with serum was used for culture maintenance (10, 11). Recently, the tendency has been to replace the serum in medium with hormones, growth factors, and trace elements (12-16). Attachment surfaces vary greatly and include plastic, collagen (17), floating collagen rafts (18), collagen gel/nylon meshes (1, 19, 20), fibronectin (21), purified liver basement membrane or biomatrix (22), and even cells from a rat liver epithelial cell line (23).

The effect virus infection has on the metabolism of the infected cell varies with the virus, the cell type, and the cell function analyzed. In addition, effects observed in culture during infection of a rapidly proliferating cell type of embryonic and fibroblast origin may be different from those observed during infection of a nonproliferating differentiated cell. Virus infection can have totally opposite effects on the expression of different sets of cellular genes, as has been shown for herpes simplex virus infection of erythroleukemia cells (24) or hepatoma cells (25). Similarly, SV40 large T antigen can cause a 100-fold increase in the levels of some cellular RNA species, but the effect is differential and is not the same on all cellular RNA species (26). The objective of this study was to determine the effect of SV40 infection on production of albumin by primary rat hepatocytes in culture from the time of infection until the outgrowth of transformed cells (approximately 40 days). Several different attachment surfaces and media were used because these factors not only affect the maintenance of expression of a differentiated protein such as albumin, but also may exert a considerably different influence on an SV40-infected hepatocyte compared with an uninfected counterpart.

MATERIALS AND METHODS

Preparation of Hepatocyte Cultures—Hepatocytes were isolated by in situ collagenase perfusion of male Fischer F344 rats (180-200 g) as previously described (6) and modified (1, 27), and plated on 60-mm collagen gel/nylon mesh (19), 60-mm plastic cell culture dishes coated with rat tail collagen (28), or on Primaria* T-25 flasks or 60-mm plates (Falcon, Cockeysville, MD) at a density of 10⁶ cells/culture. Primaria is a positively charged, stable, surface-modified plastic substrate designed to enhance the attachment of primary epithelial cells. Hepatocyte cultures were fed medium 6 h after plating. Cells were used only if the viability of the preparation was 90% or greater.

Medium.—Waymouth's formulation MAB87/3 (29) was prepared as previously described (30). Formulation of a second chemically defined medium, designated SF-N, was kindly provided by Drs. R. Shimah and L. S. Jefferson (The Pennsylvania State University

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1 The abbreviations used are: SV40, simian virus 40; AFP, alpha-foetoprotein; SF-N, serum-free medium; SF-N†, serum-free medium deficient in arginine and supplemented with ornithine; SF-N†*, SF-N† medium lacking insulin; pfu, plaque-forming units; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CGNM, collagen gel/nylon mesh(es); CC, collagen-coated; FCS, fetal calf serum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
SV40 infection of Hepatocytes

College of Medicine, Hershey, PA). SF-N medium was developed in their laboratories for the maintenance of differentiated properties of rat hepatocytes in monolayer culture. SF-N medium contained 0.7 mM arginine; medium deficient in arginine and supplemented with 0.4 mM ornithine was referred to as SF-Norn medium. (The amino acid composition of SF-N is that of 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's media supplemented with additional glutamine.) Chemically defined media were prepared fresh weekly. Additions or deletions of specific hormones or components from Waymouth's or SF-N medium in a particular experiment are stated in the text. L-15 medium consisted of Leibowitz basal medium supplemented with 10 mM Hepes, 0.2% BSA, 0.15% glucose, 5% FCS, penicillin (500 units/ml), streptomycin (500 µg/ml), and insulin (0.5 µg/ml).

**SV40 infection**—Hepatocyte monolayers were infected with 25 pfu of SV40/ml, 20 h after plating. After virus adsorption at 37°C, cells were fed fresh medium. The volume of virus or virus dilution (0.2–2.0 ml) and adsorption time (1.0–2.0 h) varied with the multiplicity of infection. SV40 strain VA45-54 was propagated and titred in African green monkey kidney TC-7 cells (31).

**Radiolabelling of Secreted Proteins**—Proteins secreted by hepatocyte monolayers were radioactively labelled as previously described (25) with the following modifications. Cells were washed twice in phosphate-buffered saline and incubated for 1 h at 37°C in serum- and methionine-free medium. The medium was removed and the cells were radio labelled for 24 h at 37°C with 1 ml of methionine-free medium containing 50 µCi of [35S]methionine (New England Nuclear) per ml of medium. At the end of the labeling interval, the medium was removed, supplemented with 330 µg of phenylmethylsulfonyl fluoride per ml and 1% aprotinin, and stored at −20°C.

**Immunoprecipitation of Albumin and AFP**—Antibody to AFP purified from amniotic fluid was prepared as previously described (25). Antibodies to purified rat albumin and rat IgG were purchased (Cappel Laboratories, West Chester, PA). Immunoprecipitation was performed by reacting radioactively labeled medium with antibodies to rat albumin, AFP, or IgG followed by adsorption to staphylococcal protein A (IgSORB; The Enzyme Center Inc., Boston, MA) as described (25). Proteins contained in the immune complexes eluted from the protein A were analyzed by SDS-PAGE (32) on 10–18% polyacrylamide gradient gels. Molecular weights of specific secreted polypeptides were determined with recombinant protein standards of known molecular weight (New England Nuclear).

**Rocket Immunoelectrophoresis**—The amount of rat albumin secreted into medium was measured by rocket immunoelectrophoresis as described (34). Rat serum albumin (Fraction V; Sigma) was diluted in the appropriate culture medium and used as a standard.

**RESULTS**

**Secretion of Albumin by Hepatocytes Maintained on CGNM**—To determine the effects of attachment surface on the production of secreted proteins by rat hepatocytes maintained in SF-N medium, cells grown on CGNM were labeled with [35S]methionine and the pattern of protein secretion with time was determined by SDS-PAGE and autoradiography (Fig. 1A). A rise and fall of secreted protein production was seen over a 17-day period, with peak levels produced 3–6 days after cell plating. After 6 days in culture, a continual decline in protein secretion was evident. The amount of radioactive incorporated into trichloroacetic acid precipitable material from medium samples gave similar results (Fig. 1B), showing that maximal levels of protein secretion occurred at day 6 in culture.

Two classes of secreted proteins were identified by this method: those induced or shutoff relative to the total secreted protein profile and those that paralleled overall protein secretion.

2 R. Shimam, D. McClure, and L. S. Jefferson, personal communication.

3 H. C. Ison, unpublished data.

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This text provides a detailed description of the culture conditions and experimental procedures used to study the effects of SV40 infection on hepatocyte secretion of proteins, focusing on the use of specific media, radiolabelling techniques, and immunoprecipitation methods to quantify and characterize secreted proteins.

**Fig. 1.** Protein secretion by hepatocytes plated on CGNM and maintained in SF-N medium. A, hepatocytes were labeled with [35S]methionine for 24-h intervals and culture medium was collected as described under “Materials and Methods.” For each culture, 106 cells were radioactively labeled with [35S]methionine or [14C]proline, and stored at −20°C. To determine incorporation into trichloroacetic acid (TCA)-precipitable material, duplicate 10-µl samples of medium were spotted on Whatman No. 540 filter papers and precipitated with 10% trichloroacetic acid, washed, digested with tissue solubilizer, and the counts/min incorporated were determined.

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**SV40 Infection of Hepatocytes**

Radioactively labeled medium from hepatocytes in culture for 2 (A), 4 (B), 9 (C), and 17 (D) days were immunoprecipitated with antibody to rat AFP (AFP), albumin (Alb), or immunoglobulin (IgG). Equal volumes of medium (100 μl) were used for each immunoprecipitation. Immunoprecipitates were analyzed on 10-18% polyacrylamide gradient gels. The three lanes for each panel were taken from the same film but were cut to eliminate lanes containing information not relevant to this study. Films used for all panels were exposed for equal lengths of time.

FIG. 2. Secretion of albumin by hepatocytes plated on CGNM and maintained in SF-N medium. Radioactively labeled medium from hepatocytes in culture for 2 (A), 4 (B), 9 (C), and 17 (D) days were immunoprecipitated with antibody to rat AFP (AFP), albumin (Alb), or immunoglobulin (IgG). Equal volumes of medium (100 μl) were used for each immunoprecipitation. Immunoprecipitates were analyzed on 10-18% polyacrylamide gradient gels. The three lanes for each panel were taken from the same film but were cut to eliminate lanes containing information not relevant to this study. Films used for all panels were exposed for equal lengths of time.

medium has been documented (2). To investigate the effect of SV40 infection on hepatocytes in SF-N medium, medium samples from infected cells on CGNM were analyzed by rocket immunoelectrophoresis for albumin (Fig. 3B). Infection of hepatocytes at day 4 with 25 pfu of SV40/cell stimulated albumin production beginning at day 15 after cell plating or 11 days postinfection. SF-N medium contains 10^-8 M insulin; after SV40 infection, SF-Nins- medium was fed to infected or control cultures. A third set of cultures was continuously fed SF-N medium. Increased albumin production in SV40-infected cultures was not seen in uninfected cultures fed medium containing insulin. At time points at which uninfected cultures no longer secreted albumin (27-34 days after plating), significant levels of albumin secretion by SV40-infected cells were detected. These effects were seen prior to the outgrowth of SV40-transformed cells.

Influence of CC Plating Surfaces on Albumin Production by SV40-infected Hepatocytes—Compared with CGNM as an attachment surface, CC plates have the following advantages: (i) ease of preparation, (ii) enhancement of the definition of culture conditions by deletion of the Hi/We/Ba medium used to make the CGNM, and (iii) elimination of artifacts introduced by trapping of precursors or secreted products into the gel of the CGNM. Hepatocytes cultured on CC dishes (21 cm²) were infected with SV40 and medium samples were analyzed for albumin (Fig. 4A). The data show that (i) the pattern of albumin production with time in culture for hepatocytes on CGNM was similar for hepatocytes on CC plates except that the amount of albumin produced by cells on a CC surface was 2-3 times greater than on a CGNM; (ii) stimulation of albumin beginning at day 15 for infected cells on CGNM was also seen for SV40-infected cells plated on CC plates; (iii) albumin production by SV40-infected hepatocytes on CC plates as on CGNM persisted when albumin was not detectable in medium from control cultures; and (iv) inclusion of insulin in the medium from day 4 instead of infection with SV40 at day 4 did not stimulate a major secondary peak of albumin production nor did it increase the length of time that significant levels of albumin were secreted by the hepatocytes. Stimulation was always observed in SV40-infected hepatocyte cultures, but persistence of albumin production only occurred in about 50-60% of SV40-infected cultures whether hepatocytes were plated on CGNM or on CC plates. An example of a culture in which persistence did not occur is shown (Fig. 4B).

SV40 can affect cell physiology in a manner similar to some hormones or growth factors (35); hence, our interest was in

*Fig. 3. Quantitation of albumin levels in culture medium by rocket immunoelectrophoresis. A, albumin production by hepatocytes on CGNM. Hepatocytes were plated on CGNM at 10⁵ cells/21-cm² mesh and fed SF-N medium. Culture medium was collected 24 h after cell feeding and analyzed by rocket immunoelectrophoresis for albumin (Fig. 3B). Infection of hepatocytes at day 4 with 25 pfu of SV40/cell stimulated albumin production beginning at day 15 after cell plating or 11 days postinfection. SF-N medium contains 10^-8 M insulin; after SV40 infection, SF-Nins- medium was fed to infected or control cultures. A third set of cultures was continuously fed SF-N medium. Increased albumin production in SV40-infected cultures was not seen in uninfected cultures fed medium containing insulin. At time points at which uninfected cultures no longer secreted albumin (27-34 days after plating), significant levels of albumin secretion by SV40-infected cells were detected. These effects were seen prior to the outgrowth of SV40-transformed cells.*
Fig. 4. Albumin Production by hepatocytes on CC dishes. A, SV40-induced stimulation and persistence of albumin production by hepatocytes on CC plates. Hepatocytes were plated on CC dishes and fed SF-N medium from time of plating (○). Hepatocytes were fed SF-N medium from time of plating until day 4, infected with SV40 at 25 pfu/cell, and subsequently fed SF-Nins- medium (▲). Hepatocytes were fed SF-N medium from time of plating until day 4 and then fed SF-Nins- medium (■). B, effect of FCS on albumin production by hepatocytes on CC plates. Conditions were the same as in A except hepatocytes were isolated from a different animal using a different lot number of collagenase (○, ▲, ■). SV40-induced persistence was not observed in this particular culture (▲). Hepatocytes were fed SF-N medium from time of plating until day 4 and then fed SF-N medium supplemented with 5% FCS (O. - O).

TABLE I

| Day* | Incorporation of [35S]methionine into trichloroacetic acid-precipitable material |
|------|---------------------------------------------------------------------------------|
|      | cpm × 10^6                                                                      |
| 2    | 41.5                                                                            |
| 4    | 38.2                                                                            |
| 6    | 46.2                                                                            |
| 9    | 48.9                                                                            |
| 13   | 17.0                                                                            |
| 17   | 5.10                                                                            |

*Hepatocytes were radioactively labeled for 24 h and medium was collected at the end of the time period. The day indicates day after plating and represents time of medium collection.

In all previously discussed experiments, SV40 infection was carried out at day 4 after plating because at this time hepatocytes had recovered from the isolation procedure and hepatocyte function, as measured by production of secreted proteins, was optimal (Fig. 1). Hepatocytes plated on CC plates and infected at day 1 or day 2 after plating demonstrated no significant differences in magnitude, stimulation, or persistence of albumin production (data not shown).

Effect of SV40 Infection on Albumin Production by Hepatocytes Plated on Primaria—To generate a totally defined system lacking collagen, we plated hepatocytes on Primaria flasks and maintained them in SF-N medium. Cells were labeled with [35S]methionine and the amount of radioactivity incorporated into trichloroacetic acid-precipitable material was determined (Table I). Maximum levels of protein secretion occurred at days 6 and 9. The amount of label incorporated was approximately 6-fold greater on Primaria than on CGNM (Fig. 1B) even though the cell number and medium were the same. This was due to the fact that the CGNM contained large amounts of nonradioactive methionine. As was seen previously for hepatocytes plated on CGNM (Fig. 2), immunoprecipitation and SDS-PAGE analysis of medium from day 17 cultures showed that albumin was present and that AFP was absent (Fig. 5). The pattern of albumin production by uninfected controls plated on Primaria and cultured in SF-N medium was similar to that for hepatocytes plated on CC plates except for the difference in magnitude of the first albumin peak (Fig. 6). This was particularly evident because the maximum levels achieved in the second peak remained equal to or greater than those seen on CC plates. When hepatocytes plated on Primaria were infected with SV40, stimulation of albumin production by infected cells began on day 15 and persisted at levels as high as 16–20 μg/culture/24 h (Fig. 6).

Increased Production of Albumin by SV40-infected Cultures Is Not Simply a Reflection of Increased Cell Number—Rat hepatocytes isolated by collagenase perfusion and placed in culture do not replicate and few cells synthesize DNA (36–38). SV40 infection stimulates cell DNA synthesis and also triggers cell division. We have previously reported that SV40 infection of hepatocytes plated on CGNM and maintained in serum-containing medium results in a marked increase in hepatocyte DNA synthesis (4). When hepatocytes were plated on CC coverslips, fed SF-N medium, infected with SV40, labeled for a 3-h interval with [3H]thymidine at 6 days
globulin and infected at 1 day after plating with SV40 at a multiplicity of 25 pfu/cell. Immuno-precipitated medium from hepatocytes in culture for 17 days was immunoprecipitated with antibody to rat AFP, albumin (Alb), or immunoglobulin (IgG). Equal volumes of medium (100 μl) were used for each immunoprecipitation. Immunoprecipitates were analyzed on a 10–18% polyacrylamide gradient gel.

Fig. 5. Secretion of albumin by hepatocytes plated on Primaria and maintained in SF-N medium. Radioactively labeled albumin secreted/ml of culture medium/24 h was determined by rocket immunoelectrophoresis. Hepatocytes were infected at 1 day after plating with SV40 at a multiplicity of 25 pfu/cell (△) or mock-infected (●). Culture medium was collected 24 h after cell feeding and analyzed by rocket immunoelectrophoresis. Hepatocytes were infected at 1 day after plating with SV40 at a multiplicity of 25 pfu/cell (△) or mock-infected (●). Culture medium was collected 24 h after cell feeding and analyzed by rocket immunoelectrophoresis.

Fig. 6. Quantitation of albumin production by hepatocytes plated on Primaria and infected with SV40. Hepatocytes were plated at 10⁶ cells per T-25 Primaria flask (25 cm²), fed SF-N medium, and infected at 1 day after plating with SV40 at a multiplicity of 25 pfu/cell (△) or mock-infected (●). Culture medium was collected 24 h after cell feeding and analyzed by rocket immunoelectrophoresis for rat albumin content.

postinfection, and analyzed by autoradiography, incorporation of label into the nucleus was observed in 20–25% of the infected cells, compared with <1% in uninfected cultures (data not shown).

We then wanted to determine whether the onset of DNA synthesis was directly followed by cell division. Observations of cultures by phase contrast light microscopy indicated no difference in cell number in infected compared with uninfected cultures before 40 days, when the outgrowth of transformed cell colonies occurred. Since alterations in albumin production in SV40-infected compared with control cultures were measured per culture, it was necessary to determine whether the rise in albumin production beginning at day 15 was caused by an increase in cell number. Hepatocytes plated on CC plates or Primaria flasks were maintained in SF-N medium and analyzed as described previously. A series of parallel cultures were generated so that duplicate cultures could be harvested at 2- to 5-day intervals and a viable cell count could be determined (Table II). No increase in cell number was observed in SV40-infected cultures up to and including day 20 in culture. A gradual decrease in cell number of almost a log was observed by day 20 for both infected and control cultures when Primaria flasks instead of CC plates were used as the attachment surface. These data also indicate that the marked increase in albumin production between days 4 and 6 in infected and control cultures was not caused by alteration in cell number but by changes in cell function.

Effect of SV40 Infection on Albumin Production by Hepatocytes Plated in SF-N Medium

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composition was consistently SF-N or SF-Nins−. The urea cycle enzymes necessary for arginine synthesis are found in liver (39). Although most cells in culture require arginine for maintenance and growth because they lack ornithine carbamoyltransferase activity (40), hepatocytes have ornithine carbamoyltransferase activity and can synthesize arginine from ornithine (41). We wanted to determine whether hepatocytes cultured under conditions in which the cells had to synthesize their own arginine would demonstrate the SV40-induced stimulation and persistence of albumin production. Hepatocytes were plated on CC plates, fed SF-Norn medium, and infected with SV40, and medium samples were analyzed for albumin (Fig. 7). Stimulation of albumin secretion began at day 11 and albumin production persisted at least through 40 days at levels as high as 20 μg/culture/24 h, whereas no albumin was detected after 34 days in the control cultures.

**Effect of Medium Composition on Retention of Albumin Production and on Transformation Using SV40-infected Hepatocytes**—Experiments were next initiated using the Primaria surface to determine the effect of altering medium composition on the ability of SV40 infection to stimulate albumin secretion and induce persistence of albumin production (Table III). In addition, transformation by SV40 and albumin production by transformed cultures were determined (Table III). Supplementation of SF-N medium with ornithine (with concomitant deletion of arginine) or ascorbic acid, or deletion of insulin did not prevent (i) SV40-mediated stimulation of albumin secretion and persistence of albumin production, (ii) SV40 transformation, or (iii) generation of transformed cultures containing cells that secreted albumin. Although stimulation of albumin production by SV40 was observed with Waymouth's medium or Waymouth's medium supplemented with BSA and hormones, persistence was not seen and transformation was not accomplished. When hepatocytes were fed L-15 medium supplemented with 5% FCS, stimulation and persistence of albumin production were absent. Transformation was accomplished in L-15 medium with FCS, as previously reported (2), but albumin was not detected in medium from transformed cultures.

**DISCUSSION**

SV40 infection stimulated albumin production by hepatocytes plated in serum-free medium beginning at approximately 11 days postinfection. Differences in the magnitude of albumin production were observed whether hepatocytes were plated on CGNM, CC plates, or Primaria flasks; however, SV40-induced stimulation and persistence occurred regardless of the attachment surface. In 50-60% of infected cultures, measurable amounts of albumin persisted through at least 40 days in culture and often until the outgrowth of epithelial cell colonies appeared. Eighty-five percent of transformed cultures continued to produce from 0.4-21 μg of albumin/culture/24 h; several of these transformed cultures maintained and subcultured over 4-5 months retained approximately the same levels of albumin production. The albumin-producing transformants can be purified but they grow slowly as colonies.

The results of experiments with Primaria flasks indicate that (i) SV40-induced albumin production (Fig. 5) and (ii) transformation yielding albumin-producing cultures (Table III) did not require the presence of a collagen attachment surface. Neither stimulation nor persistence was observed when SV40-infected hepatocytes were plated in serum-containing L-15 medium. Transformation of adult hepatocytes in serum-containing medium with a temperature-sensitive mutant of SV40 led to the derivation of an albumin-producing transformed cell line (42); however, transformation by wild type virus and reproducibility were not discussed by the authors. In our previous studies of SV40 transformation of hepatocytes on collagen gel/nylon meshes and in serum-containing medium (2), we were unable to reproducibly derive transformants that produced albumin. In the system described in this report, albumin-producing, wild type SV40-transformed cell colonies can be reproducibly generated.

Success in obtaining SV40-transformed hepatocytes that

| Medium          | Infection with SV40* | Stimulation of albumin secretion† | Persistence of albumin production‡ | Transformation | Albumin produced by transformed cultures |
|-----------------|----------------------|----------------------------------|-----------------------------------|----------------|------------------------------------------|
| SF-N            |                      | +                                | +                                 | -              | +                                        |
| SF-Norn         |                      | -                                | -                                 | -              | -                                        |
| SF-Naa*         | +                    | +                                | +                                 | +              | +                                        |
| SF-Nins−        | +                    | +                                | +                                 | +              | +                                        |
| Waymouth's      | -                    | +                                | +                                 | -              | +                                        |
| Waymouth's with |                      | +                                | -                                 | -              | -                                        |
| BSA and hormones|                      | +                                | -                                 | -              | -                                        |
| L-15 with FCS†  |                      | +                                | -                                 | -              | +                                        |

*Hepatocytes were infected with SV40 at 25 pfu/cell.
†Stimulation of albumin secretion beginning at approximately 15 days after plating (see Figs. 3-6).
‡Positive (+) indicates detectable levels of albumin present in medium from infected cells at 35-40 days after plating and at a time when albumin was no longer detected in control cultures.
§SF-N medium supplemented with ascorbic acid (0.1 mM).
*Waymouth's medium supplemented with 1% BSA, human transferrin (100 μg/ml), 10−5 M dexamethasone, 10−4 M glucagon, and 10−5 M insulin.
†L-15 medium with 5% FCS as described under "Materials and Methods."
continue to express a designated differentiated function (in this case, albumin) is dependent upon several factors. First, expression of SV40 genes or the presence of whatever agent is used to accomplish transformation or the complex array of changes that accompany cell transformation may turn off expression of albumin. The answer to this can be determined by following expression of the differentiated function from time of infection to transformation, as in this study. We conclude that SV40 infection not only failed to turn off, but actually stimulated albumin production by hepatocytes in serum-free medium. Stimulation of albumin secretion was not conclusive that SV40 infection not only failed to turn off, but that a fraction of the cell population is proliferating while another fraction is dying with no overall change in population size. Cells producing albumin at 20 days postinfection may represent the proliferating fraction, such that the overall increased albumin production results from an increased number of cells producing the protein. Alternatively, increased albumin production may result from increased transcription, such that albumin production per cell increases. Most likely, a combination of these two mechanisms is occurring. It is doubtful that this effect is specific for albumin but rather that albumin is one of a group of hepatocyte proteins simultaneously affected by SV40-infection; indeed, increased albumin production may be a side effect rather than a direct consequence of SV40 infection. It has previously been shown during lytic SV40 infection that synthesis of several groups of host proteins is stimulated and that a small fraction of host proteins is shut off (43). The purpose of this study was not to understand how albumin production increased in infected hepatocytes but rather to use one marker of differentiation (albumin) to define the events occurring from time of infection to transformation and to determine the dependence of these events on culture conditions.

It has been suggested that the mitogenic effects on host cell metabolism induced by SV40 are similar to those caused by lectins, proteolytic enzymes, serum factors, and polypeptide hormones (35). Insulin is a polypeptide hormone that has an anabolic effect on carbohydrate metabolism in liver (44). Injection of insulin into diabetic rats increases synthesis of plasma proteins secreted by liver (45). Insulin effects also have been noted using isolated hepatocytes instead of whole liver (46-52). In the system described in this study, neither FCS, as a source of insulin and other growth factors and polypeptides, nor insulin alone was able to replace SV40 in triggering stimulation or persistence of albumin production. Host cell effects caused by SV40 infection have been attributed to the SV40 large T or small t antigens. Large T antigen stimulates cellular DNA synthesis (53, 54), increases host transcriptional activity (55), binds to DNA (56), has an ATPase activity (57), and complexes with a $M_0 = 53,000-55,000$ host protein (58, 59). Small t antigen is responsible for loss of actin cables (60), synthesis of plasminogen activator (61), and stimulation of host centriolar antigen (62). Large T has been implicated in both the initiation and maintenance of the transformed state (63-66) and appears to be required for growth of transformed cells in low concentrations of serum (61). Small t antigen appears to be required for efficient transformation of growth-arrested cells (67, 68). We assume that large T and/or small t antigens are responsible for the observed stimulation and persistence of albumin production induced in SV40-infected hepatocytes. Rat hepatocytes in serum-free medium provide a unique environment to examine the effect of large and/or small T antigens on host metabolism during nonpermissive infection and on transformation. The isolated rat hepatocyte, a naturally growth-arrested cell type, is perhaps more stringently growth regulated than mouse, hamster or rat cells, which have the capacity to proliferate but are empirically prevented from doing so. Use of serum-free medium eliminates variability with regard to growth or promoting factors present in serum, which may be able to substitute for the small t antigen activity. Infection of hepatocytes in serum-free medium with mutants temperature-sensitive for large T antigen (69, 70), deleted in small t (68, 71, 72) or large T antigen (73), or double mutants temperature-sensitive for large T antigen and deleted in small t antigen (74) may enable us to establish the role of large T and/or small t antigen in the retention of albumin expression by SV40-infected hepatocytes and perhaps even in initiation and maintenance of transformation in hepatocytes.

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REFERENCES
1. Isom, H. C. (1980) Virology 103, 199-216
2. Isom, H. C., Tevethia, M. J., and Taylor, J. M. (1980) J. Cell Biol. 85, 651-659
3. Isom, H. C., Tevethia, M. J., and Kreider, J. W. (1981) Cancer Res. 41, 2126-2134
4. Isom, H. C. (1983) in Isolation, Characterization, and Use of Hepatocytes (Harris, R. A., and Cornell, N. W., eds) pp. 177-182, Elsevier, Amsterdam
5. Howard, R. B., Christensen, A. K., Gibbs, F. A., and Pesch, L. A. (1975) J. Cell Biol. 65, 675-684
6. Berry, M. N., and Friend, D. S. (1969) J. Cell Biol. 43, 506-502
7. Reid, L. (1970) Methods Enzymol. 53, 263-278
8. Reid, L. (1982) in From Gene to Protein: Translation into Biotechnology (Lam, J. W., ed.) pp. 53-73, Academic Press, New York
9. Gatmaitan, Z., Jefferson, D. M., Ruiz-Opa, N., Biempica, L., Arias, I. M., Dudas, G., Leinwand, L. A., and Reid, L. M. (1985) J. Cell Biol. 97, 1179-1190
10. Biassell, D. M., Hammaker, L. E., and Meyer, U. A. (1973) J. Cell Biol. 59, 722-734
11. Bonney, R., Becker, J. E., Walker, P. R., and Potter, V. R. (1974) In Vitro 9, 399-413
12. Gurr, J. A., and Potter, V. R. (1980) Exp. Cell Res. 126, 237-248
13. Hasegawa, K., Namai, K., and Koga, M. (1980) Biochem. Biophys. Res. Commun. 95, 243-249
14. Tanaka, K., Sato, M., Tomita, Y., and Ichihara, A. (1978) J. Biochem. 94, 937-946
15. McGowan, J. A., Strain, A. J., and Burcher, N. L. R. (1981) J. Cell Physiol. 108, 353-363
16. Enat, R., Jefferson, D. M., Ruiz-Opa, N., Gatmaitan, L., Leinwand, L. A., and Reid, L. M. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 141-145
17. Kletzen, R. F., Facetti, M. W., Becker, J. E., Potter, V. R., and Burcher, F. R. (1976) J. Biol. Chem. 251, 3014-3020
18. Michalopoulos, G., and Pitot, H. C. (1975) Exp. Cell Res. 94, 707-78
19. Sirica, A. E., Richards, W., Tsukada, Y., Sattler, C. A., and Pitot, H. C. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 283-287
