Transformation of Rat Hepatocytes by Transfection with Simian Virus 40 DNA to Yield Proliferating Differentiated Cells

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ABSTRACT

Cultured hepatocytes from adult Fischer 344 rats were transformed by virion or cloned simian virus 40 (SV40) DNA using the calcium phosphate method. Transformation by SV40 occurred in either serum-supplemented medium or chemically defined medium (CDM). The frequency was greatest in serum-supplemented medium but transformants did not remain differentiated. In contrast, SV40 transformants developed less frequently in CDM, but retained differentiated functions. The frequency of transformation was enhanced by treatments that stimulated cell proliferation, in particular supplementing CDM with epidermal growth factor. Hepatocytes transformed in CDM were epithelial in morphology, secreted albumin, transferrin, hemopexin, and expressed the enzyme glucose-6-phosphatase, all characteristics of normal liver. Transformants did not produce detectable levels of α-fetoprotein, a marker of fetal or abnormal liver. We conclude that (a) hepatocytes can be transformed by transfection with SV40 DNA; (b) the frequency of transformation is enhanced by stimulating DNA synthesis; and (c) the transformed cells retain specific functions of normal hepatocytes in situ. Using this system it will be possible to study transformation of hepatocytes by viral and cellular oncogenes and to determine their effects on hepatocellular differentiation.

INTRODUCTION

DNA transfection has been used to investigate the ability of virus DNA, virus DNA fragments, and viral and cellular oncogene sequences to transform cells in culture (1–11). Several cell types have been used in these assays but NIH3T3, rat-1, rat embryo fibroblast, and baby rat kidney cells have been used most frequently (1, 2, 5, 8). These cells are predominantly of fibroblastic and embryonic origin, are derived from established cell lines, or are primary cells with the capacity to replicate for a limited number of generations. Because most human tumors are carcinomas that originate from epithelial cells, transformation of epithelial cells in culture by DNA transfection is of considerable importance. Studies to determine the nature and number of genes required for transfection and transformation of primary epithelial cells in culture have been limited (11). In addition, tumors vary greatly in their rate of growth and pattern of cellular differentiation, and many retain a differentiated phenotype. For this reason, we used differentiated epithelial cell lines, specifically adult rat hepatocytes, to develop a transfection assay in which transformation could be measured quantitatively and the resulting transformants could be assessed for expression of differentiated functions.

Hepatocytes isolated by perfusion of normal adult liver are differentiated epithelial cells that express a variety of well-characterized proteins, can be maintained in monolayer culture, and divide very slowly in culture without appropriate stimulation (12–18). We previously reported that hepatocytes infected with the DNA tumor virus, SV40,3 could be transformed to yield stable epithelial cell lines and that transformation did not occur in uninfected cultures (3, 19, 20). The absence of foci of replicating cells in control cultures indicated that hepatocytes were ideal for studying transformation of differentiated epithelial cells. Recently, we reported that hepatocytes maintained in defined medium and infected with SV40 could be transformed to yield replicating epithelial cells, that the transformation assay could be made quantitative, and that the number of transformed colonies containing cells that maintained the ability to express the differentiated function of albumin production could be identified and enumerated (20).

In this study, we used hepatocytes to test whether transformation could be mediated by DNA transfection. Transfection makes it possible to study transformation by DNA from viruses that cannot attach to and penetrate hepatocytes, by DNA from viruses that infect and kill hepatocytes, by virus DNA fragments, and by virus and cellular oncogenes. This study was initiated to (a) develop and optimize a transfection protocol for transforming rat hepatocytes with SV40 DNA; (b) determine whether the transformation frequency could be enhanced by stimulating cell proliferation; and (c) characterize transformants with regard to maintenance of normal differentiated functions.

MATERIALS AND METHODS

Preparation of Hepatocyte Cultures. Hepatocytes were isolated by in situ collagenase perfusion of male Fischer 344 rats (180–200 g) as described previously (21) and modified (22, 23). Partial hepatectomy was performed on selected rats (24) 24–72 h before perfusion. SSM was fetal calf serum-containing L-15 medium as previously described (23). CDM was SF-N (25) or RPMC medium. RPMC medium consisted of RPMI 1640 supplemented with 0.28% bicarbonate, 0.36% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, bovine serum albumin (0.08%), linolenic acid (2 mg/ml), 2-aminoethanol (4.1 mg/ml), insulin (0.06 µg/ml), glucagon (0.04 µg/ml), dexamethasone (0.4 µg/ml), transferrin (100 µg/ml), trace metals (26), penicillin (100 µg/ml), and streptomycin (100 µg/ml). Isolated hepatocytes were washed 3 times in SSM and plated at a density of 1 × 10⁶ cells/culture on 60-mm plastic cell culture dishes coated with rat tail collagen (27). Cultures were fed fresh SSM 5–8 h after plating and subsequently with SSM or CDM every 3 days. Selected cultures received CDM supplemented with culture grade EGF (Collaborative Research, Inc., Lexington, MA; 25 ng/ml of medium) and/or DMSO (Sigma Chemical Co., St. Louis, MO; 2%).

Transfection of Hepatocytes. Hepatocytes maintained for 18–24 h in vitro were transfected by the calcium phosphate procedure (2, 10). A solution containing specific amounts of rat hepatocyte carrier DNA, SV40 DNA, and 0.2 M CaCl₂ was added to an equal volume of another containing 0.27 M NaCl, 9.6 M KCl, 11 M dextrose, 2.8 M Na₂HPO₄, and 40 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.15). The two solutions were mixed slowly in the presence of bubbling air. One ml of the resulting calcium phosphate DNA coprecipitate was added directly to each culture and allowed to settle on the cell monolayer. At a specified time, the medium was removed and fresh SSM was added. When hepatocytes were maintained

1Supported in part by Grants CA 18450, CA 23931, and CA 09124 from the National Cancer Institute.
2To whom requests for reprints should be addressed. Recipient of Research Career Development Award CA 00759 from the National Cancer Institute.
3The abbreviations used are: SV40, simian virus 40; SSM, serum-supplemented medium; CDM, chemically defined medium; G6P, glucose-6-phosphatase; DMSO, dimethylsulfoxide; EGF, epidermal growth factor.
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in CDM, the transfection mixture was removed, cultures were fed SSM for 24 h, and then the medium was changed to CDM. In each experiment, several cultures were superinfected with SV40 (multiplicity of infection of 100 plaque-forming units per cell) to ascertain whether transfected cells remained competent for transformation. At 4–12 weeks after transfection, the transformed colonies were fixed with 10% formalin, stained with crystal violet, and counted. Statistically significant differences in transformation frequencies were determined by using the Student's t test.

The transfection protocol was modified in specific experiments. To examine the effect of osmotic shock, cultures were treated with SSM supplemented with 20 or 30% DMSO, or 5, 10, or 20% glycerol for 4 min immediately after transfection, rinsed 3 times with SSM, and re-fed with SSM. In independent experiments designed to define the optimum conditions for transfection, SSM was supplemented with 30, 100, or 300 mM chloroquine diphosphate (Sigma) during the application of the DNA precipitate (6). Hepatocytes also were transfected using Polybrene (Aldrich Chemical Co., Milwaukee, WI) or DEAE dextran according to previously published methods (4, 7). Transfection with DEAE dextran (10, 20, 100, or 200 μg/ml) or Polybrene (1, 3, 10, or 30 μg/ml) was followed by treatment of some cultures with DMSO and always by re-feeding with SSM.

Preparation of DNA. SV40 stocks were prepared by infecting TC7 cells, a continuous line of African green monkey kidney cells, at a multiplicity of infection of 0.01 plaque-forming unit per cell. Virus DNA was extracted from infected cells by the method of Hirt (28) and the supercoiled form was purified by cesium chloride ethidium bromide centrifugation (29). The plasmid pBRWT2, which contains the SV40 genome (strain VA 45–54) inserted at the EcoRl site of pBR322, was a gift from Dr. J. Tevethia (The Pennsylvania State University College of Medicine, Hershey, PA). Plasmid pBRWT2 was propagated in Escherichia coli strain HB101 and pBRWT2 DNA was isolated and purified by previously described methods (30). DNA concentrations were determined spectrophotometrically and 10% chloroform was added to sterilize each sample. The identity of virus and plasmid DNAs was verified by digestion with the restriction endonuclease EcoRl (Boehringer Mannheim, Indianapolis, IN) and electrophoresis on 0.8% agarose gels. In specific experiments, circular plasmid DNA was digested with EcoRl and the linear molecules were used in transfection assays.

Rocket Immunoelectrophoresis. The amount of rat albumin secreted into the culture medium was measured by rocket immunoelectrophoresis as described (31). Rat albumin (fraction V; Sigma) was diluted in the appropriate culture medium and used as standard.

Immunoooverlay Technique. The procedure for immunoooverlay of cultured cells has been described (20, 32). Briefly, cultures were covered with a mixture of 1% agarose in CDM supplemented with antibody to rat albumin (1:64 dilution). After 24 h the immunoooverlay was removed from the culture, washed 3 times with borate-buffered saline (24 h total), and incubated for 24 h with a 1:64 dilution of rabbit anti-goat IgG (Cooper Biomedical, Inc., Malvern, PA). The immunoooverlay was washed 3 times, placed on a glass slide, dried at 60°C, and stained with Coomassie blue to visualize specific reactive colonies.

Immunoprecipitation of Secreted Plasma Proteins. Proteins secreted by SV40-transformed hepatocytes were radioactively labeled as described previously (25), except that labeling was for 6 h. Immunoprecipitation was performed by reacting labeled medium with specific antibodies followed by adsorption to staphylococcal protein A (IgG sorb; The Enzyme Center, Boston, MA) as described (25, 33). Proteins contained in the immune complexes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (34) on 10–18% gradient gels followed by fluorography (35). Molecular weights of specific secreted polypeptides were determined with 14C-labeled protein standards (New England Nuclear, Boston, MA).

Immunoperoxidase and Histochemical Assays. Antibody directed against SV40 tumor (T) antigen was produced by s.c. injection of 1 × 106 TSV-5 cells (36) into 6-week-old Syrian hamsters. TSV-5 cells were kindly provided by S. Tevethia (The Pennsylvania State University College of Medicine). Serum was collected when tumors were larger than 3 cm3 (approximately 4–10 weeks). All normal sera and peroxidase-conjugated secondary antisera were purchased (Cooper Biomedical, Inc.). Cells from transformed colonies were harvested and sedimented onto glass slides by cytocentrifugation. In immunoperoxidase assays for SV40 T-antigen, cells were fixed with 100% ethanol for 30 min at −20°C. In immunoperoxidase assays for albumin and transferrin, cells were fixed essentially as described previously (37, 38). Primary and secondary antibodies were diluted in phosphate-buffered saline containing 4% bovine serum albumin (Miles Laboratories, Elkhart, IN) and 1% normal serum (from the same species in which the secondary antibody was obtained). Antisera were applied to fixed cells for 1–3 h at room temperature. Cells were thoroughly washed in phosphate-buffered saline and peroxidase activity was demonstrated by a 10-min incubation in 0.05% dianisidine containing 0.01% hydrogen peroxide. G6P activity was demonstrated using the technique of Wachstein et al. (39). Preparations of cytocentrifuged cells were stained without fixation and freshly isolated rat hepatocytes served as a positive control.

RESULTS

Transformation of Hepatocytes with SV40 DNA. Rat hepatocytes plated in SSM were transformed after transfection with DNA from either SV40 virions or pBRWT2, a plasmid that contains the entire SV40 genome (Fig. 1). Transformed colonies were apparent 3–4 weeks after transfection and the number of colonies was directly related to the amount of DNA used. Transformed colonies contained epithelial cells, but the morphology of individual colonies and the size, shape, and growth rate of cells within the colonies varied. No spontaneous transformation occurred after transfection with rat hepatocyte carrier DNA alone.

Optimization of Transformation Frequency. We examined specific experimental variables to optimize the efficiency of transformation using the calcium phosphate technique. First, we varied the duration over which hepatocytes were exposed to the calcium phosphate-DNA precipitate. The transformation frequency was optimal when cells were incubated with the precipitate for 4, 8, or 24 h (Fig. 2). Beyond 24 h of exposure, the transformation frequency dropped markedly and cytotoxicity was apparent. For subsequent experiments, exposure times of 4–8 h were used.

Second, we examined the effect on transformation frequency of the concentration of hepatocyte carrier DNA used to prepare the calcium phosphate-DNA precipitate. The amount of carrier DNA used had an effect; the greatest number of colonies was observed with 10 or 15 μg carrier DNA (Fig. 3). In subsequent experiments, 15 μg of carrier DNA was used.

![Fig. 1. Transformation frequency of cultured rat hepatocytes after transfection with SV40 DNA and maintenance in SSM. Hepatocytes were treated with specific amounts of either SV40 virus (O) or pBRWT2 (©) DNA. When pBRWT2 DNA was used, the concentrations in the figures represent the amount of SV40 DNA contained in the plasmid. Cultures were maintained in SSM for 4 weeks and transformed colonies were stained with crystal violet and counted. No transformation occurred when similar cultures were transfected with rat hepatocyte carrier DNA alone. Bars, SE from at least 3 independent experiments; points, average of 6–40 dishes.](attachment:image)
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Fig. 2. Effect of transfection time on transformation frequency. Hepatocytes were transfected with pBRWT2 (1.6 μg DNA/10⁶ cells), exposed to the calcium phosphate DNA precipitate for various times, and maintained in SSM. Transformation frequency was determined as described in Fig. 1 (4 independent experiments; 8–14 total dishes/point).

Fig. 3. Effect of varying the amount of carrier DNA on transformation frequency. Hepatocytes were transfected with pBRWT2 DNA (1.6 μg/10⁶ cells) plus various amounts of rat hepatocyte carrier DNA and maintained in SSM (3 independent experiments; 6 dishes/point).

Third, we examined the effect of osmotic shock on transfection and transformation. When hepatocytes in SSM were treated with 20 or 30% DMSO or 5, 10, or 20% glycerol immediately after transfection, no significant enhancement in the frequency of transformation occurred (data not shown). Treatment of cells with 30% DMSO caused marked cytotoxicity, whereas lower concentrations did not.

Fourth, we examined the effect of the lysosomotropic agent, chloroquine diphosphate, on transformation frequency. When the transfection mixture was supplemented with 30, 100, or 300 μM chloroquine diphosphate, the frequency of transformation was not enhanced (data not shown).

Finally, we compared the efficiency of transformation by circular versus linear DNA. The plasmid pBRWT2 was digested with the restriction endonuclease EcoRI to generate a linear molecule. The transformation frequencies obtained with circular and linear plasmid DNAs were comparable (data not shown).

We next tested whether hepatocytes could be transfected and transformed using polycations to facilitate the uptake of pBRWT2 DNA into the cells. When DEAE dextran or Polybrene was used, no transformed foci appeared (data not shown).

Transformation of Hepatocytes in CDM. All previously described results were obtained using hepatocytes maintained in SSM. We next determined whether hepatocytes maintained in CDM could be transformed by transfection with SV40 DNA. When rat hepatocytes maintained in CDM were transfected with pBRWT2 DNA, transformation occurred but the frequency was significantly lower than we had observed in SSM (Fig. 4). Colonies that arose in CDM could be visualized 4–6 weeks posttransfection, grew slowly, and were epithelial in morphology.

Fig. 4. Transformation frequency in SSM versus CDM. Hepatocytes were transfected with specific amounts of pBRWT2 DNA and maintained in SSM (□) or CDM (○). Transformation frequency was measured 6–8 weeks after transfection (at least 4 independent experiments; 8–40 dishes/point).

Fig. 5. Effect of stimulation of cell proliferation on transformation frequency. A, hepatocytes isolated from partially hepatectomized rats 24–72 h after surgery (○) or from normal rats (□) were transfected with pBRWT2 DNA and maintained in SSM. Differences between the 2 groups were not statistically significant (P = 0.05). B, hepatocytes isolated from normal rats were transfected with pBRWT2 DNA and maintained in either CDM (□) or CDM supplemented with EGF (25 ng/ml) (○). *, differences that were statistically significant (P = 0.05). Points, mean of at least 4 independent experiments; 6–36 dishes/point.

Effect of Hepatocyte Proliferation on Transformation Frequency. Since liver cells replicate in an animal subjected to partial hepatectomy (24), and hepatocytes in monolayer culture can be stimulated to replicate when treated with specific hormones (12, 13, 17) or growth factors (14, 15, 18), we tested the effect of using (a) hepatocytes from partially hepatectomized as opposed to normal animals; and (b) hepatocytes in monolayer culture fed CDM supplemented with EGF as opposed to unsupplemented CDM on transformation frequency.

When cells prepared from regenerating livers of partially hepatectomized rats were used, a slight increase in transformation frequency was observed, compared with cells derived from normal rats; however, the difference was not statistically significant (P = 0.05; Fig. 5A). In contrast, stimulation of proliferation of hepatocytes already in monolayer culture had a statistically significant effect on transformation frequency. When hepatocytes cultured in CDM supplemented with EGF were transfected with pBRWT2 DNA, a 10-fold enhancement in transformation frequency occurred compared with hepatocytes not exposed to EGF (Fig. 5B). In addition, foci appeared as early as 3–4 weeks posttransfection when hepatocytes were cultured in CDM supplemented with EGF.

Transformation of Hepatocytes in DMSO-supplemented Medium. We recently have shown that differentiation in adult rat hepatocytes, as measured at the morphological and biochemical level, can be maintained for extended periods of time in culture when DMSO is added to the medium (40). We tested whether
hepatocytes maintained in medium supplemented with DMSO could be transformed by transfection with SV40 DNA. Cultures maintained in CDM supplemented with DMSO and EGF were composed of closely associated polygonal cells that resembled hepatocytes and did not contain fibroblastic or mesenchymal cells. When hepatocytes fed CDM supplemented with 2% DMSO or CDM supplemented with 25 ng EGF/ml and 2% DMSO were transfected with pBRWT2 DNA, transformed foci of cells with an epithelial morphology developed. The transformation frequency was similar to that previously observed in CDM in the absence of DMSO (Fig. 6); however, the length of time required for the appearance of transformed foci was longer. Colonies were visualized first at 8–12 weeks posttransfection and the transformed cells grew more slowly.

Expression of SV40 T-Antigen by Transformed Cells. To establish that the transformed foci resulted from the introduction of SV40 genetic information, cells within the foci were tested for their ability to express SV40 T-antigen. Hepatocytes transformed by SV40 DNA were uniformly positive for expression of T-antigen (Fig. 7). SV40 T-antigen was observed not only in cells in the original foci but also was retained after they were subcultured.

Characterization of Transformed Foci for Production of Albumin. We next examined whether cells present in transformed foci resembled normal hepatocytes. Medium was removed from culture plates and analyzed to establish whether any of the foci within an individual culture contained transformed cells that secreted albumin, a plasma protein produced by normal liver. When medium from cultures fed SSM was examined by rocket immunoelectrophoresis, albumin was not detected (0 of 20 cultures). In contrast, when hepatocytes were maintained in CDM, CDM supplemented with EGF, or CDM supplemented with EGF and DMSO, albumin was detected in some cultures. More specifically, when medium from cultures containing foci which arose from hepatocytes fed CDM or CDM supplemented with EGF was analyzed, the percentage of cultures containing albumin-producing cells was 38 and 48%, respectively.

Cultures that contained albumin-producing cells, based on rocket immunoelectrophoretic analysis of the culture medium, were further examined by immunoblot (Fig. 8). This technique enabled us to determine the number of colonies in each culture that produced albumin and their location in the culture dish. Not all of the colonies in cultures positive for albumin production secreted albumin. The percentage of albumin-producing colonies relative to the total number of transformed foci varied with the experiment, ranging from approximately 6 to 26% when CDM was used (Table 1). Because supplementation of CDM with EGF increased the transformation frequency, it was particularly important to determine the effect of EGF on
Fig. 8. Transformed colonies stained with crystal violet (A) and corresponding immunooverlay (B) demonstrating albumin-producing colonies. The 3 circles represent the reference mark used to orient the positions of the immunoprecipitates and the stained colonies. Note that the large immunoprecipitates often correspond to small colonies (arrow).

Table 1 Effect of EGF and EGF plus DMSO on the percentage of albumin-producing SV40-transformed colonies

<table>
<thead>
<tr>
<th>Medium</th>
<th>No. of cultures examined</th>
<th>No. of albumin-producing colonies/no. of transformed colonies*</th>
<th>% of colonies producing albumin</th>
<th>Average</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDM</td>
<td>11 (2)*</td>
<td>6/35</td>
<td>17</td>
<td>6–26</td>
<td></td>
</tr>
<tr>
<td>CDM + EGF*</td>
<td>27 (5)</td>
<td>51/242</td>
<td>21</td>
<td>4–36</td>
<td></td>
</tr>
<tr>
<td>CDM + EGF + DSMO*</td>
<td>10 (2)</td>
<td>31/62</td>
<td>50</td>
<td>45–59</td>
<td></td>
</tr>
</tbody>
</table>

* Number of albumin-producing colonies was determined by immunooverlay.
* Numbers in parentheses, number of independent experiments.
* EGF concentration was 25 ng/ml.
* DMSO concentration was 2%.

The percentage of transformed colonies containing cells that retained the ability to secrete albumin. When transformation occurred in CDM supplemented with EGF, the percentage of albumin-producing colonies ranged from approximately 4–36%, indicating that the addition of EGF did not reduce the probability of obtaining SV40-transformed hepatocytes that retained the property of albumin production. When hepatocytes were maintained during transformation in medium containing EGF and DMSO, the number of albumin-producing transformed colonies increased to 45–59%.

The immunooverlay technique also made it possible to locate and examine the morphology of albumin-producing colonies and the cells within these colonies. Cells in albumin-producing colonies were small, epithelial in appearance, and closely associated with each other within the colony (Fig. 9A), whereas cells in colonies negative for albumin production were larger, more irregular in shape, and grew as less well-defined colonies (Fig. 9B).

Fig. 9. Epithelial morphology of hepatocytes transformed in CDM. A, transformed hepatocytes formed colonies consisting of small, tightly associated polygonal cells that often grew on top of one another. Cells in colonies with this morphology often secreted albumin. Although SV40-transformed hepatocytes grew as dense foci when first isolated, after subculture these cells grew as monolayers and were similar in morphology to established hepatoma cell lines. Phase contrast, × 380. B, transformed hepatocytes also formed colonies that were composed of large, rapidly growing cells that were not tightly associated. These rapidly growing transformants did not secrete albumin. Phase contrast, × 380.
Fig. 10. Immunocytochemical localization of albumin and transferrin in SV40-transformed hepatocytes using the indirect immunoperoxidase method. A, albumin was detected in all cells, although the intensity of staining varied. Note the perinuclear localization of albumin. B, control in which the primary anti-albumin antiserum was replaced by the same dilution of normal goat serum. C, transferrin was localized to the perinuclear region of cells and the intensity of staining varied from cell to cell. D, control in which the primary anti-transferrin antibody was replaced by the same dilution of normal rabbit serum. All x 1300.

The intensity of staining varied (Fig. 10). These results indicate that albumin and transferrin were produced by the transformed cells and not by a low level of normal hepatocytes that might have survived within a transformed focus. G6P also was detected in all the cells and the staining was uniform among individual cells (Fig. 11).

Plasma Proteins Produced by Transformed Hepatocytes. Transformed hepatocytes from an albumin-producing colony that had been subcultured were radioactively labeled at passage 3, and the medium was analyzed by immunoprecipitation and gel electrophoresis for various plasma proteins. Albumin, transferrin, and hemopexin were produced, but α-fetoprotein was not detectable. (Fig. 12).

DISCUSSION

We have developed, optimized, and characterized a system for transforming adult rat hepatocytes by transfection with SV40 DNA. Transformation was accomplished whether hepatocytes were transfected with SV40 DNA prepared from virions or contained within a plasmid vector. Transformation occurred when hepatocytes were maintained in SSM, but the transformed cells did not retain differentiated functions. In contrast, the transformation frequency was lower when hepatocytes were fed CDM, but a percentage of the transformants maintained specific properties of normal hepatocytes in situ, in particular, albumin secretion. Although transformation was delayed in CDM supplemented with DMSO, the frequency was unaltered and the percentage of albumin-producing colonies was enhanced. Individual colonies of transformed hepatocytes contained cells that varied in morphology, growth rate, and in the quantity of plasma proteins produced. We conclude from these studies that (a) rat hepatocytes can be transfected and transformed by SV40 DNA; (b) transformation frequency is increased when hepatocytes are stimulated to synthesize DNA, in particular, when stimulated in vitro by the addition of EGF; and (c) transformed cells can be isolated that produce albumin at levels comparable to those of primary hepatocytes and that secrete several other plasma proteins produced by normal adult hepatocytes.
Fig. 11. Histochemical localization of G6P activity in SV40-transformed hepatocytes. A, G6P activity was evident throughout the cytoplasm of transformed hepatocytes and the intensity of the reaction product was relatively uniform from cell to cell. B, control for G6P activity in which the substrate, glucose-6-phosphate, was removed from the reaction. A and B, x 1300.

Fig. 12. Profile of plasma proteins secreted by SV40-transformed hepatocytes. Hepatocytes transformed with SV40 DNA, maintained in CDM, and subcultured were radioactively labeled at passage 3. Labeled proteins in the medium were analyzed by immunoprecipitation (ALB, albumin; PP, plasma proteins; P, plasminogen; H, hemopexin; T, transferrin; AFP, α-fetoprotein; and I, immunoglobulin control) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Ordinate: K, molecular weight in thousands.

Although we have referred throughout this manuscript to SV40 transformation of hepatocytes, the use of the term hepatocyte requires clarification. Colonies containing cells which no longer produce significant quantities of albumin may have arisen from nonparenchymal liver cells, from undifferentiated hepatocyte stem cells, or from hepatocytes that lost their ability to express the albumin gene prior to transformation. Since there is no single accepted marker for hepatocytes, we cannot rule out the possibility that even the albumin-producing transformants are not hepatocytes. We know of no nonhepatocyte-like cell in normal liver that secretes large quantities of albumin, produces plasma proteins, does not secrete α-fetoprotein, and expresses G6P; at least some of the SV40-transformed cells derived by the technique described in this paper retain all of these characteristics. It has been previously reported that immortalized nonparenchymal, clear epithelial cells can arise spontaneously from liver cell cultures (41). In the system we describe in this report, cell lines did not arise spontaneously. Clear epithelial cells express G6P activity and secrete low levels of albumin; the SV40-transformed cells express G6P activity but produce albumin at levels comparable to that of freshly isolated hepatocytes, and therefore, several orders of magnitude higher than those previously reported for clear epithelial cells. It is difficult to visualize the morphology of the SV40-transformed cells present in freshly isolated albumin-producing colonies because the cells are packed so closely. After the cells have been subcultured and begin to become established as cell lines, the morphology of the cells can be ascertained. The albumin-producing cells that we derived to date were epithelial and were morphologically more similar to established hepatoma cell lines than to primary hepatocytes in culture.*

The system described in this report makes it possible to reproducibly isolate SV40-transformed hepatocyte cell lines that maintain some properties associated with hepatocyte differentiation. Albumin-producing colonies identified by the techniques of immunoelectrophoresis and immunooxidase can be selected and transferred. Subcultured colonies can be recharacterized by immunolectrophoresis and immunooxidase to purify albumin-producing cells from contaminating nonalbumin-producing cells. Using this procedure, we have developed SV40-transformed cell lines containing cells that continue to secrete albumin (15-47 pg/cell/24 h) and other plasma proteins produced by normal adult liver. The characterization of one subcultured colony that subsequently was used to derive a cell line is described in this report. Further characterization of this and other cell lines with passage is in progress.* Because these cells replicate in CDM, they are particularly appropriate for biochemical studies examining the effects of growth factors or hormones on hepatocyte function in vitro.

The importance of cell replication in transformation and, in particular, in the development of experimental liver cancer has been well documented (41). Adult hepatocytes in culture have only a limited capacity to divide but can be stimulated to

* C. Woodworth and H. C. Isom, unpublished data.
synthesize DNA by exposure to hormones, in particular EGF, insulin, and glucagon (12, 13, 17), serum factors (14, 15, 18), and virus infection (3, 23). Our results suggest that the frequency of transformation after transfection with SV40 DNA was enhanced by stimulating hepatocyte DNA synthesis. A statistically significant increase in transformation frequency was seen for hepatocytes treated with EGF compared with untreated cells. In the studies reported in this paper EGF was present in the medium for the duration of the experiment; however, in similar studies, we found that removal of EGF from the medium 2 weeks after transfection did not decrease the transformation frequency. In addition, cells transformed in the presence of EGF did not require EGF for growth. One possible explanation for our findings could be that hepatocytes synthesizing DNA have a higher probability of transforming than hepatocytes in the G0-G1 phase of the cell cycle. This hypothesis requires further evaluation, but our results are consistent with other observations that the state of the cell with regard to DNA synthesis plays a critical role in transformation (42-45).

The pathological and biochemical changes that occur during the sequential development of liver cancer have been well characterized (46); however, the genetic alterations responsible for these changes are not known. Recently, transfection has made it possible to identify viral (2, 3, 8, 11) and cellular (1, 5, 8, 9) DNA sequences that transform cells in culture. Transformation of many types of primary cells in vitro requires introduction of specific combinations of viral and cellular oncogenes (5, 8), but also can be achieved by a single oncogene modified such that it is expressed at high levels in the transfected cells (1, 9). The primary cell types used for these studies are usually of fibroblastic origin and have the ability to replicate for a limited number of passages in culture. Because most neoplasms in humans are carcinomas that develop from differentiated adult epithelium, systems for studying the role of oncogenes in transformation of differentiated epithelial cells are particularly important. Epithelial cells have been transformed by oncogenes via virus infection (20, 47-55) or DNA transfection (3, 11), but the cells used in these assays were either dividing primary cultures of cells (11, 20, 47-52) or were derived from cell lines (53-55). In the system described in this paper, the primary cell type, the hepatocyte, is not only of epithelial origin but also has an extremely limited capacity to divide in culture. The advantages of this system are that it can measure quantitatively (a) the frequency of transformation of an epithelial cell with limited capacity to divide; and (b) the percentage of transformants that continue to express functions characteristic of an adult differentiated cell type. Our initial studies have been performed with SV40 DNA because SV40 rapidly transforms a variety of cell types including differentiated cells (47-52). Currently, we are testing other viral and cellular oncogenes in combination with specific growth factors for their ability to transform hepatocytes in vitro. We are interested in the transforming activity of these oncogenes and also in their effect on normal hepatocyte differentiation.

ACKNOWLEDGMENTS

We thank L. Mengel and T. Miller for excellent technical assistance; T. Grierson for help with photography; M. Clement for editorial assistance; and Dr. F. Rapp for his continued interest.

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