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Quantitative assay for albumin-producing liver cells after simian virus 40 transformation of rat hepatocytes maintained in chemically defined medium

(epithelial cell transformation/maintenance of differentiated function)

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ABSTRACT **Transformation of rat hepatocytes by simian virus 40 in chemically defined medium was examined. When hepatocytes plated on collagen-coated plates were infected with simian virus 40, colonies of replicating cells appeared as early as 40 days after infection, whereas no colonies appeared in control cultures. Medium from 85% of the transformed cultures contained albumin. When collagen was eliminated and hepatocytes were plated on Primaria plastic cell culture dishes, transformation occurred; medium from 86% of the transformed cultures contained albumin but the maximum albumin level secreted per culture was only 62% of that produced by cultures on collagen-coated plates. Quantitative assays for transformation were established. Transformation was linear after infection with 2–50 plaque-forming units of virus per hepatocyte, and the transformation frequency was the same on the two plating surfaces. An immuno-overlay technique made it possible to identify, purify, and determine the morphology of the albumin-producing cells. When ornithine was substituted for arginine in the medium, the transformation frequency decreased markedly while the percentage of colonies producing albumin increased from 30% to 100%. We conclude that we have defined an assay for quantifying transformation of a normal hepatocyte population and for identifying and enumerating epithelial liver cell transformants that produce albumin.**

If differentiated cells can be transformed without loss of expression of differentiated properties, these cells can be used to study gene regulation. Of equal or greater importance is the need to develop a quantitative transformation assay in which the target cell is a nondividing differentiated epithelial cell, the cell type from which most human cancers arise. Hepatocytes are ideal for such an assay because (*i*) they are epithelial cells that do not proliferate in culture (1–3), (*ii*) numerous differentiated and undifferentiated functions have been documented and can be measured, and (*iii*) information on hepatocarcinogenesis *in vivo* is available.

Conditions for maintaining hepatocyte function *in vitro* for more than a few days have been difficult to establish (1–3). It has been reported that hepatocytes plated on a collagen gel/nylon mesh surface and fed L-15 medium supplemented with fetal calf serum can be transformed by simian virus 40 (SV40) to generate stable epithelial cell lines (4) that can produce carcinomas in test animals (5); however, this assay was not quantitative and none of the transformed or tumor cell lines produced significant amounts of albumin. One possible explanation for the absence of albumin secretion was that the liver cells had lost expression of this differentiated property prior to transformation because of inadequate culture conditions. In a recent study (6), we have shown that, if SV40-infected hepatocytes are cultured on collagen-coated plates or Primaria flasks and fed serum-free medium, albumin production is stimulated and persists through at least 40 days after plating and often until SV40-transformed epithelial cell colonies appear. Stimulation and persistence are not seen in mock-infected cultures or in SV40-infected cultures fed serum containing L-15 medium.

This study was initiated to determine whether SV40-transformation of hepatocytes in chemically defined medium could be made quantitative. In this report, we describe a procedure for quantifying the transformation of a normal cell population and for identifying and enumerating the transformants that maintain a differentiated function, synthesis of albumin.

MATERIALS AND METHODS

Preparation of Hepatocyte Cultures. Hepatocytes were isolated by collagenase perfusion of male Fischer F344 rats (180–200 g) as described (7) and plated on 60-mm plastic cell culture dishes coated with rat tail collagen or on Primaria T-25 flasks or 60-mm plates (Falcon) at a density of 10^6 cells per culture. Primaria is a positively charged, stable, surface-modified plastic substrate designed to enhance the attachment of primary epithelial cells. The chemically defined medium (designated HCD medium) consisted of a 1:1 ratio of Williams' medium E and Ham's F12 medium supplemented with 0.25% bicarbonate, 15 mM Hepes, penicillin (500 units/ml), streptomycin (500 μg/ml), 65.5 μM ethanolamine (8), transferrin (100 μg/ml), insulin (0.6 μg/ml), 1 μM dexamethasone, 10 nM glucagon, trace elements (9), 7.18 μM linoleic acid linked to 0.08% fatty acid-free bovine serum albumin (10, 11), 7.0 mM glucose, 0.4 mM sodium pyruvate, and 0.1 mM ascorbic acid. HCD medium contains 0.6 mM arginine; medium deficient in arginine and supplemented with 0.4 mM ornithine is referred to as HCDorn medium. HCD medium was prepared fresh weekly. For all experiments using HCD medium, hepatocytes were fed HCD medium 6 hr after plating. No differences were observed whether hepatocytes were washed and plated in L-15 medium containing 5% fetal calf serum (7) or HCD medium.

SV40 Transformation. Hepatocyte monolayers were infected with SV40 as described (6). To measure transformation frequency, cultures were washed, fixed for 2 hr in 5% formaldehyde, and stained with crystal violet.

Rocket Immunoelectrophoresis. The amount of rat albumin or α-fetoprotein (AFP) secreted into medium was measured by rocket immunoelectrophoresis as described (12). Rat serum albumin (fraction V; Sigma) or AFP was diluted in the appropriate culture medium and used as a standard. Goat anti-rat albumin antibody was purchased from Cappel Labo-

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Abbreviations: SV40, simian virus 40; pfu, plaque-forming units; HCD medium, chemically defined medium; HCDorn medium, chemically defined medium deficient in arginine and supplemented with ornithine; AFP, α-fetoprotein.

ratories (Westchester, PA). Purified rat AFP and goat anti-rat AFP were provided by W. Liao (Stanford University School of Medicine, Stanford, CA) and J. Taylor (Gladstone Foundation Laboratories, San Francisco). The anti-albumin and anti-AFP antibodies immunoprecipitated single bands migrating at M_r 68,000 and M_r 72,000, respectively, in 10–18% polyacrylamide gradient gels. Medium from freshly isolated hepatocytes and from McA-RH7777 hepatoma cells (13) served as positive control media for albumin and AFP, respectively.

Immuno-Overlay Technique. Immuno-overlay was carried out as described (14). A 1% agarose solution containing goat anti-rat albumin was prepared by mixing equal parts of 2% agarose (42°C) with 2× HCD medium supplemented with goat anti-rat albumin. The final dilution of anti-rat albumin was 1:64. A 1:64 dilution of rabbit anti-goat IgG (Cappel Laboratories) was used as the second antibody.

RESULTS

Transformation in HCD Medium. Hepatocytes were plated on collagen-coated dishes and fed HCD medium. At 20 hr after plating, cells were infected with 50–100 plaque-forming units (pfu) of SV40 per cell. Colonies of epithelial cells appeared as early as 38–40 days after infection. No colonies appeared in uninfected cultures even after 160 days. Transformants derived in HCD medium were not ready to be subcultured until 12 weeks after infection. Analysis of HCD medium from transformants showed that 85% of the cultures secreted 0.4–21.2 μg of albumin per culture per 24 hr (Table 1).

The transformation assay of hepatocytes on collagen-coated plates was not ideal because (*i*) low-level fibroblast contamination occurs, (*ii*) the crudely purified collagen is not chemically defined, and (*iii*) the collagen coating interferes when cells are studied by immunochemical or histochemical techniques. Therefore, we investigated whether hepatocytes plated on Primaria flasks could be transformed by SV40. Hepatocytes attached by 2–3 hr and spread over the surface of the flask 24–36 hr after plating (Fig. 1*A*). By 6 days after plating, both mock-infected and SV40-infected hepatocytes pulled together, appeared smaller, and oriented in cords in a fashion similar to hepatocytes in liver (Fig. 1*B*). The edges of these cell groups were distinct and fibroblasts were not present. No differences were observed in mock- or SV40-infected cultures until after 5 weeks after infection, at which time outgrowth of epithelial colonies was observed in infected cultures. At least six morphologically different cell types were observed whether hepatocytes were plated on collagen-coated plates or on Primaria. Other unusual changes in morphology were noted. For example, when one colony type grew and met another colony type, no overlap occurred and a line of morphologically distinct cells was observed where the two colonies interfaced (Fig. 1*C*). Cells often oriented in long chains or circles as though surrounding a vessel (Fig. 1*D*). Analysis of HCD medium from transformants on Primaria showed that 86% of cultures secreted 0.8–13.2 μg of albumin per culture per 24 hr (Table 1), an amount slightly less than that secreted by transformants on collagen-coated plates.

Quantitative Assay for Hepatocyte Transformation. One advantage of using hepatocytes for a transformation assay is the absence of spontaneous transformation. In previous studies (13), cultures were harvested from the collagen gel/nylon mesh 2–4 weeks after infection and then seeded on plastic dishes. The number of colonies that grew out was not a measure of the number of transformed cells in the original culture because all the colonies on the transfer plate could have been derived from a single colony on the original mesh.

To determine whether SV40 transformation in HCD medium could be made quantitative, hepatocytes were infected with SV40 at different input multiplicities of infection. Transformation was linear from 2 to 50 pfu (Fig. 2*A*). The transformation frequency of hepatocytes on collagen-coated plates (Fig. 2*B*) was not significantly different from that of hepatocytes in Primaria flasks (Fig. 2*C*). It was not possible to quantitate transformation reproducibly in L-15 medium containing fetal calf serum on either collagen-coated or Primaria plates because of fibroblast contamination.

Quantitative Assay for Albumin-Producing SV40-Transformed Colonies. Cultures containing transformed colonies were screened (6 weeks after infection) for albumin production by assaying culture medium. Positive cultures were analyzed by the immuno-overlay technique and the number of transformed colonies secreting albumin was determined by counting the number of Coomassie blue-stained circles (Fig. 3*A*). In this particular example, 9 of 34 colonies (26%) contained albumin-secreting cells (Fig. 3 *A* and *B*). The number of albumin-producing colonies per number of transformed

Table 1. Transformation frequency and number of albumin-producing colonies that arose when hepatocytes in chemically defined medium were transformed with SV40

	HCD medium						HCDorn medium			
	Primaria			Collagen coated			Primaria		Collagen coated	
No. of cultures transformed/no. of cultures infected										
	4/4	10/10	8/8	5/5	10/12	4/4	1/5	2/16	2/8	3/4
Average number of colonies/transformed cultures										
	ND	38	ND	32	32	31	1	1	1	1
Transforming frequency, no. of colonies per 10^6 cells										
	ND	38	ND	32	26.7	31	0.2	0.3	0.25	0.75
No. of albumin-producing cultures/no. of cultures transformed										
	2/4	10/10	7/8	5/5	7/10	4/4	1/1	2/2	2/2	3/3
Average albumin production,* μg per ml of culture medium per 24 hr										
	1.0	1.7	0.7	3.2	1.1	2.1	1.0	0.7	0.7	2.1
	(0.8–1.2)	(0.2–3.3)	(0.1–2.0)	(0.7–5.3)	(0.5–2.7)	(2.0–3.1)		(0.5–0.8)	(0.2–1.0)	(1.0–3.3)
No. of albumin-producing colonies/no. of transformed colonies†										
	ND	ND	ND	ND	ND	38/125 (4)	ND	2/2 (2)	2/2 (2)	1/1 (1)
Transformation frequency of albumin-producing colonies, no. of colonies per 10^6 cells										
	ND	ND	ND	ND	ND	9.5	ND	0.33	0.25	ND

Hepatocytes were infected with 50 pfu of SV40 per cell. ND, not determined.
*Each culture of 1×10^6 cells was fed 4 ml. Numbers in parentheses indicate range of values.
†Numbers in parentheses indicate numbers of transformed cultures examined by the immuno-overlay technique.

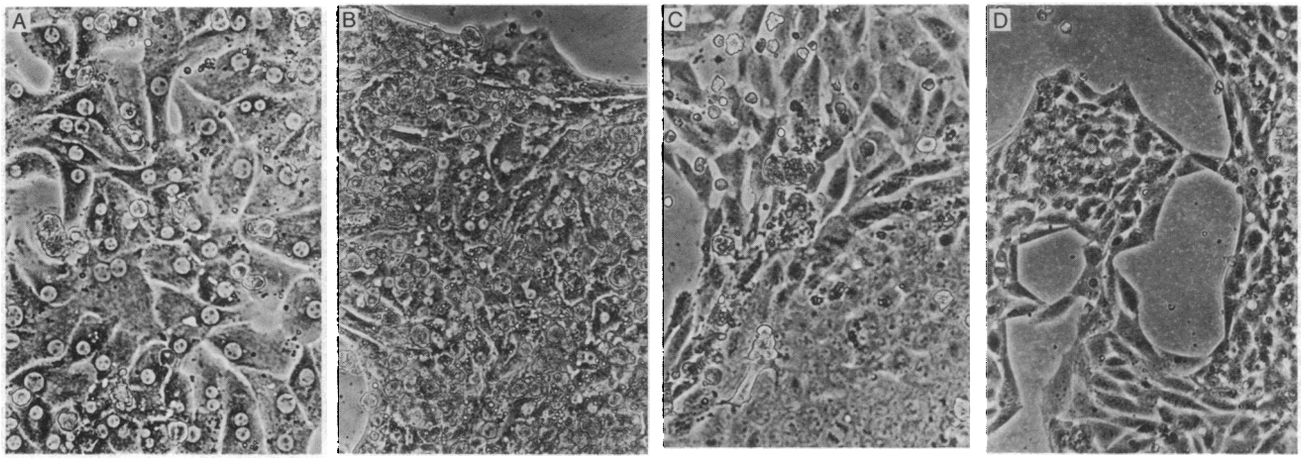

FIG. 1. SV40 infection and transformation of hepatocytes plated on Primaria flasks and fed HCD medium. (*A*) Uninfected hepatocytes 24 hr after plating. (*B*) Hepatocytes infected with SV40 (50 pfu per cell) 6 days after plating and 5 days after infection. (*C*) Two morphologically distinct SV40-transformed cell colonies. (*D*) SV40-transformed rat liver cells growing in long chains or circles.

colonies averaged 30% (Table 1) whether hepatocytes were plated on collagen-coated plates or on Primaria. No albumin-positive colonies were detected by immuno-overlay in cultures designated negative by screening the culture medium (Fig. 3*C*). When a control antibody was used, no stained circles were present (Fig. 3*D*). The immuno-overlay technique also provided an approximation of the amount of albumin produced by any one colony. In some cases the immunoprecipitin ring was not significantly larger than the cell colony, while in others the diameter of the precipitin ring was 2–3 times the colony size.

The immuno-overlay technique also provided a method for purifying albumin-producing cells. After removal of the gel, cultures were refed and allowed to recover. Success in subcloning colonies was the highest with collagen-coated plates (50–60%) but was also achieved on Primaria (10–20%). Colonies could not be transferred to plastic dishes. When the transferred colonies achieved a size of 0.2–0.5 cm in diameter, medium was screened and the new culture was analyzed by the immuno-overlay technique to determine the purity of the culture. For example, in retransferring a clone designated H8, four colonies developed in the subculture and cells in all four colonies produced albumin (Fig. 3*E*), indicating that a pure culture had been obtained. H8 cells produced 10 μg of albumin per 3.5×10^5 cells per 24 hr and did not produce measurable levels of AFP (<1.0 μg of AFP per 3.5×10^5 cells per 24 hr). The large, round, regular nuclei in the H8 cells (Fig. 4*A*) were the same size as those in normal hepatocytes (Fig. 1*A*), but the overall H8 cell size was significantly smaller than a normal hepatocyte. Colonies containing cells with the H8 type morphology were observed in almost every albumin-positive culture. Rounded cells (Fig. 4*B*), which appeared at the periphery of these colonies attached easily when transferred, underwent several rounds of division and then died. In initial cultures it was not possible to determine whether the rounded cells produced albu-

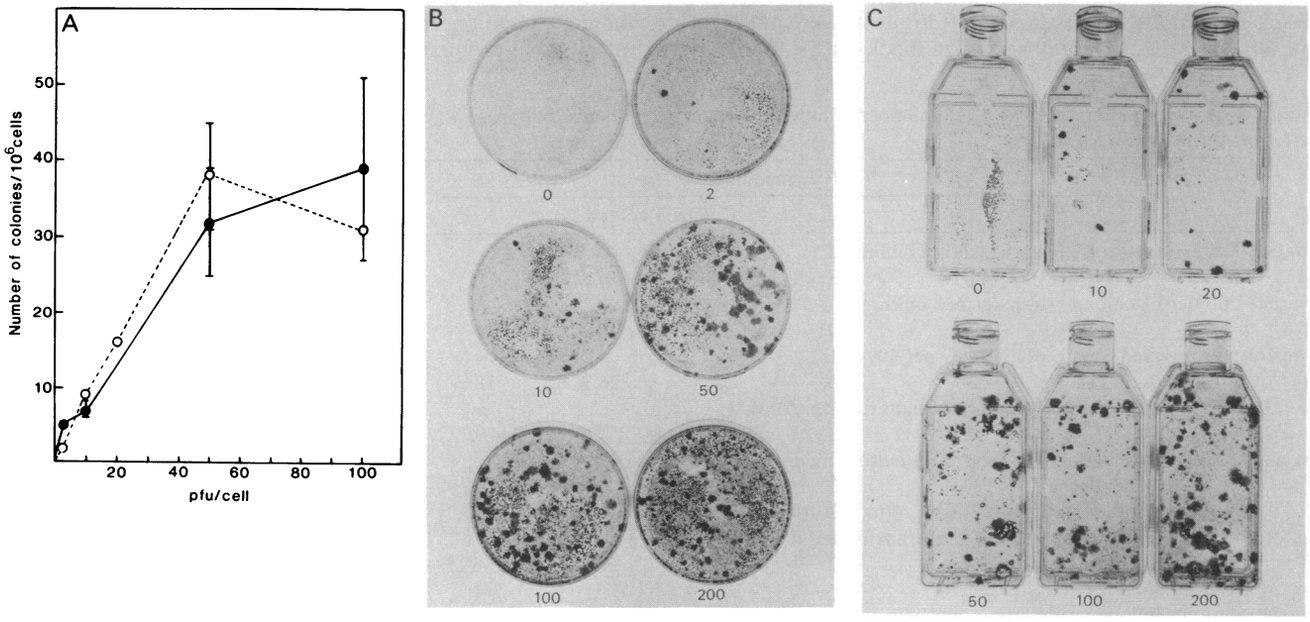

FIG. 2. Transformation frequency. (*A*) Hepatocytes were plated on collagen-coated plates (●) or in Primaria flasks (○) and infected with SV40 at various multiplicities of infection. At 55 days after infection, monolayers were washed, fixed, and stained and the number of colonies was counted. (*B*) Hepatocytes on collagen-coated plates were infected with 0, 2, 10, 50, 100, or 200 pfu per cell. (*C*) Hepatocytes in Primaria flasks were infected with 0, 10, 20, 50, 100 or 200 pfu per cell.

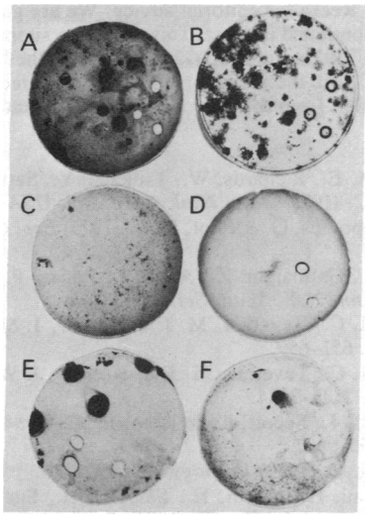

FIG. 3. Quantitative assay for albumin-producing cell colonies. (*A*) Immuno-overlay of albumin-producing culture. Dark-staining circles indicate immunoprecipitate from cells secreting albumin. The three open circles represent holes punched in the agarose gel so that the gel and culture could be aligned to identify colonies containing albumin-producing cells. (*B*) Fixed and stained culture from which the immuno-overlay shown in *A* was derived. The plastic culture dish was scored (open circles) at the positions where holes were punched in the agarose gel. (*C*) Immuno-overlay of SV40-transformed rat liver cells that did not demonstrate albumin secretion when tested by immuno-overlay. The background observed in the immuno-overlay represents stained cells or cell debris that sometimes sticks to the gel. (*D*) Immuno-overlay of albumin-producing culture using a gel prepared with anti-IgG. (*E*) Immuno-overlay of H8 subclones. A single colony designated clone H8 was identified by immuno-overlay as an albumin producer and picked from the original culture. The cells were allowed to grow and were retransferred two more times. The culture used for this immuno-overlay was the third transfer. (*F*) Immuno-overlay of albumin-producing culture resulting from SV40 transformation of hepatocytes on Primaria in HCDorn medium. The only colony present secreted albumin.

min; however, when transferred they did not. In contrast, the cells in the interior of the colony were difficult to transfer but, if they attached, they survived, grew slowly, and produced albumin at levels similar to those produced by colonies in the initial culture (0.5–2.0 μg of albumin per colony per 24 hr). A photomicrograph of cells that did not produce albumin or AFP, grew more rapidly than albumin-producing cells, and formed colonies with ragged edges is shown in Fig. 4*C*.

SV40 Transformation of Hepatocytes in HCDorn Medium. Hepatocytes cultured on Primaria, infected with SV40, and maintained in HCDorn medium synthesized albumin for at least 40 days (6). When hepatocytes in HCDorn medium were infected with SV40, transformation occurred in 20–75% of the cultures (Table 1) compared with 83–100% when HCD medium was used. In addition, 1 instead of 31–38 transformed colonies was present per culture. An average of 0.8–13.2 μg of albumin per culture per 24 hr was found in 100% of the transformed cultures. This was a surprisingly high figure because the number of cells in HCDorn-transformed cultures averaged only 3% of those in HCD cultures. Every colony contained albumin-producing cells (Fig. 3*F*) and the cells in all colonies were similar in morphology to each other and to cells from clone H8 (Fig. 4*D*).

DISCUSSION

Growth of rat embryo fibroblast cells (previously transformed by SV40 in serum-containing medium) in serum-free medium has been demonstrated previously (15). In the present study, not only was growth of SV40-transformed cells observed in HCD medium, but the process of transformation also was accomplished. Transformation of liver epithelial cells by virus (16) and by chemical carcinogens (17, 18) has been reported. Previously (4), it was shown in our laboratory that epithelial liver cells present in a population of adult hepatocytes can be transformed by SV40, but the assay was not quantitative and albumin-producing cells were not obtained reproducibly. Transformation of adult hepatocytes in serum-containing medium with a temperature-sensitive mutant of SV40 has led to the derivation of a cell line that synthesizes and secretes albumin; however, transformation by wild-type virus, quantitation of transformation, and reproducibility were not discussed (19). The assay system described here differs from the previously reported L-15 medium/fetal calf serum system in that in HCD medium (*i*) transformed colonies were detected 5 instead of 3 weeks after infection, (*ii*) transformants could not be subcultured before 12 instead of 4 weeks after transformants first appeared, (*iii*) transformation could be quantitated on both collagen

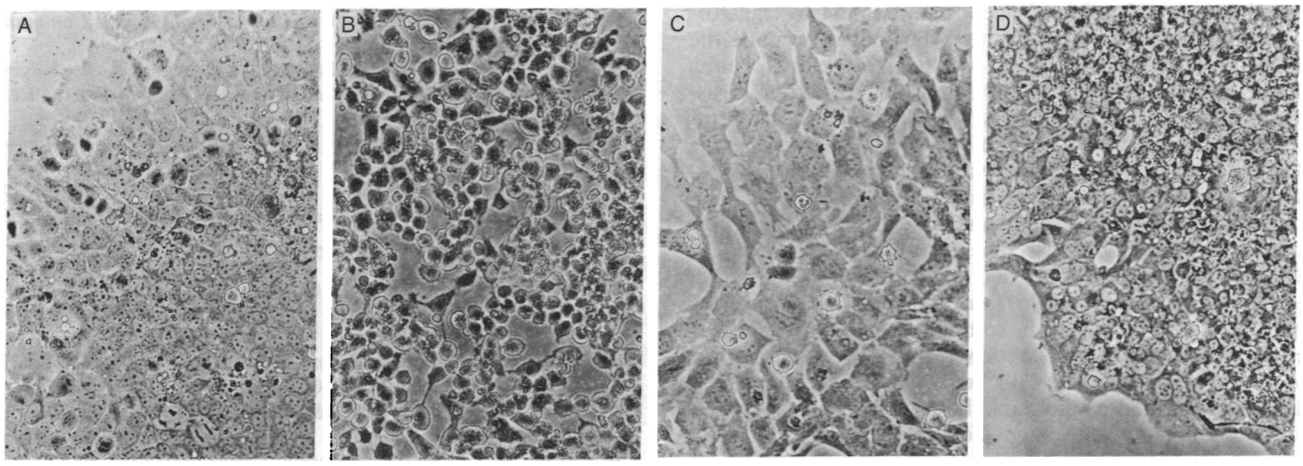

FIG. 4. Phase-contrast photomicrographs of SV40-transformed liver cells that do or do not produce albumin. (*A*) Morphology of albumin-producing cells from the H8 culture used for the immuno-overlay shown in Fig. 3*E*. (*B*) Rounded cells developed at the periphery of albumin-producing colonies after colonies were grown for 15–20 days. Cells shown in this photomicrograph were located at the periphery of a colony containing cells that had the same morphology as those shown in *A*. (*C*) Morphology of SV40-transformed liver cells in a colony that did not produce albumin. (*D*) Morphology of albumin-producing cells at the periphery of the colony in the culture used for the immuno-overlay shown in Fig. 3*F*.

and noncollagen surfaces, and (*iv*) albumin-producing transformed cell colonies were reproducibly derived at a frequency of 9 or 10 colonies per 10^6 cells. After transformation in either medium, the cell types that arose were not homogeneous with regard to size, shape, organization within the cell colony, or growth rate. Although a spectrum of epithelial cell types has been observed after SV40 transformation, none of the transformants has the morphology of freshly cultured hepatocytes in liver. The small slow-growing cells described in this report are well organized in colonies with defined smooth borders, are differentiated, and produce albumin but not AFP; however, further biochemical characterizations are required before we can determine whether these cells more closely resemble normal adult hepatocytes, oval cells (20, 21), or perhaps more primitive hepatic epithelial cells.

We predict that a specific function of a differentiated cell will not be lost when it is transformed by SV40 as long as expression of that function can be maintained by SV40-infected cells in culture for the time required to observe outgrowth of transformed cells (6). Culture conditions play a critical role in this process. For example, it has recently been found that albumin production increases when SV40-transformed fetal liver cells are cultured in the presence of retinoic acid (22). Although albumin is considered an important marker of differentiation in adult liver, it may not be the most sensitive one. There may be other normal adult plasma proteins or enzymes that are not expressed by cultured hepatocytes retaining the ability to secrete albumin. A different attachment surface or medium composition may have to be employed if expression of such properties is to be retained by infected hepatocytes during the time in culture required for SV40 transformation. To have a simplified quantitative assay we applied only the single criterion of albumin production as a marker of differentiation.

Adult hepatocytes in culture are nondividing cells. Recent reports suggest that growth regulation of mature hepatocytes is density dependent (23–26). At low density hepatocytes replicate, while at high cell densities hepatocyte-specific characteristics are induced and cells do not proliferate. The effect of high cell density can be mimicked by addition of purified plasma membranes to hepatocytes cultured at low density. After SV40 infection of hepatocyte cultures there is a period of 35–40 days before replicating epithelial cells appear. In mock-infected cultures and at early time points in infected cultures, hepatocytes may elaborate a surface protein (similar to that found in rat liver plasma membranes) that inhibits proliferation. Once a commitment to transformation has occurred, the cells may no longer produce the inhibitor but instead begin to synthesize a transforming growth factor similar to those elaborated by other virus-transformed and neoplastic cells (27, 28). The system reported here could be used to test this hypothesis by examining HCD medium from cultures at various times after SV40 infection for the presence of inhibitor and/or TGF.

Introduction of DNA fragments into cells has made it possible to identify oncogenes. Whereas a single oncogene can transform an NIH/3T3 cell, two oncogenes are required for transformation of rat embryo fibroblast cells (29). It has been reported that hepatocytes in serum-containing medium can be transformed after transfection with SV40 DNA and that the assay is quantitative (30) but whether hepatocytes in chemically defined medium can be transformed after transfection with SV40 DNA is not known.

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