MOLECULAR AND CELLULAR BIOLOGY, Oct. 1987, p. 3740–3748
0270-7306/87/103740-09$02.00/0

Vol. 7, No. 10

Regulation of Albumin Gene Expression in a Series of Rat Hepatocyte Cell Lines Immortalized by Simian Virus 40 and Maintained in Chemically Defined Medium

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Received 9 March 1987/Accepted 17 July 1987

A series of simian virus 40 (SV40)-immortalized hepatocyte cell lines were characterized for albumin production, the regulation of albumin production, and the expression of other liver-specific genes. This series of cell lines is particularly useful for studying the regulation of hepatocyte gene expression because the cell lines express liverlike levels of a number of liver-specific functions and do so while growing in a chemically defined medium. SV40-immortalized hepatocyte cell lines were derived from colonies of albumin-producing epithelial cells that arose after primary hepatocytes maintained in chemically defined medium were transfected with SV40 DNA. Some cell lines secreted albumin at levels equal to or greater than those secreted by freshly plated primary hepatocytes, and all but one line continued to produce albumin for more than 20 passages. The variation in albumin secretion among cell lines reflected differences in the amount of albumin produced per cell and not in the percentage of albumin-producing cells in each line. The characterization of selected cell lines showed that albumin production was regulated by cell density during the growth cycle. Albumin production in most cell lines was also regulated by dexamethasone; however, one cell line continued to produce high levels of albumin when the cells were grown in medium lacking dexamethasone, demonstrating that although glucocorticoid can induce albumin production in some cell lines, it is not required for high levels of albumin production by all cells in culture. Regulation of albumin production measured at the level of protein secretion was paralleled by changes in steady-state levels of a 2.3-kilobase albumin RNA. Albumin-producing SV40-immortalized hepatocytes secreted a variety of other plasma proteins, including transferrin, hemopexin, and the third component of complement. These cells also expressed tyrosine aminotransferase activity that was inducible by dexamethasone. Alpha-fetoprotein production was not detected in any of the cell lines examined.

Tissues in the adult animal are functionally specialized and contain fully differentiated cells that express specific proteins. To understand the molecular mechanisms involved in regulating the expression of genes that code for these differentiated functions, it is necessary to select a differentiated cell type that can be studied at the physiological, biochemical, and molecular levels. Liver tissue is particularly appropriate for studying the expression of differentiated functions because it is composed predominantly of a single type of differentiated cell, the hepatocyte, and hepatocytes express a large number of well-characterized tissue-specific proteins.

Systems available for studying the regulation of liver-specific genes include liver from the intact animal, liver slices (10, 39), primary hepatocytes in culture (9, 15, 17, 21, 22, 43), and cocultures of primary hepatocytes with epithelial liver cells (12). Although all of these systems are useful for studying the expression of liver-specific proteins, many have limitations. For example, it is necessary to use liver from the intact animal to identify and measure quantitatively liver-specific protein levels, to demonstrate how these proteins are regulated in vivo, and to determine liver-specific mRNA levels and rates of transcription of liver-specific genes. However, it is not possible to measure the direct effects of specific agents on the regulation of liver-specific genes in intact liver because of the physiological influences present in the whole animal. The problem of direct administration of agents can be overcome by using liver slices. Another advantage of liver slices is that their rates of transcription of liver-specific mRNAs approach those in the intact animal (10). The disadvantage of liver slices is that they have a limited life-span. Liver slices also contain a mixture of liver cell types, but like liver, they are composed predominantly of hepatocytes. Primary hepatocytes in culture are useful for these studies because they can be derived from normal adult liver, and when maintained under the appropriate conditions, they produce levels of albumin mRNA similar to that produced by the liver. The disadvantage of primary hepatocytes is that unlike a cell line, hepatocytes do not replicate, making it difficult to readily obtain large quantities of cells. Culture of differentiated primary hepatocytes also requires the use of collagen or matrix. Cocultivation of primary hepatocytes with an epithelial cell line eliminates the need for collagen, matrices, or chemically defined medium (CDM), but a serious difficulty with the cocultivation system is that all studies have to be carried out and interpreted taking into account that the cultures contain two distinct cell types.

Liver-derived cell lines, including hepatoma cells (1, 5, 6, 11, 13, 23, 24, 31, 33, 35–37, 40, 41) and immortalized or transformed hepatocytes (8, 20, 42), have also been used to study the expression of liver-specific proteins. Hepatoma cell lines replicate continuously in culture in the absence of collagen or matrices, making it reasonably easy to acquire large numbers of cells. Although hepatoma cell lines are derived from tumor and not normal tissue, the H4IIEC3 rat hepatoma (35, 37) and the HepG2 human hepatoma (24) cell lines have been used successfully to study liver-specific proteins. One limitation in using many hepatoma cell lines is that they are grown in serum, which contains undefined substances that can alter gene expression. In general, a

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disadvantage of most hepatoma cell lines is that they express liver-specific genes at levels much lower than those expressed by intact liver. A simian virus 40 (SV40)-immortalized cell line has been derived previously from normal adult rat hepatocytes (8), but like many hepatoma cell lines, it produces the liver-specific protein albumin at low levels compared with those produced by intact liver and it is grown in a serum-supplemented medium.

An ideal liver system would be a replicating cell line that (i) is derived from normal adult liver, (ii) grows in the absence of collagen or matrices, (iii) has liverlike expression of liver-specific proteins, and (iv) grows in CDM. We have derived a series of immortalized hepatocyte cell lines that fit these four criteria. In so doing, we have established the first hepatocyte cell lines that produce albumin and albumin mRNA at levels found in normal liver and freshly isolated hepatocytes. These cell lines are also the first cell lines derived from primary adult hepatocytes to grow in CDM.

Previous work in our laboratory has shown that adult rat hepatocytes can be immortalized by infection with SV40 (20) or by transfection with SV40 DNA (42) and that a percentage of the cells retain differentiated functions, including the ability to produce albumin. Colonies containing albumin-producing cells were used to derive differentiated hepatocyte cell lines because albumin is a plasma protein produced in large quantities by normal hepatocytes and it was technically possible to identify and purify albumin-secreting cells. To date, we have established 11 albumin-producing SV40-immortalized hepatocyte cell lines that grow in CDM. In this study, several of these SV40-immortalized hepatocyte cell lines were examined in detail at the physiological, biochemical, and molecular levels. We concentrated on characterizing these cells for albumin production and the regulation of albumin gene expression. In addition, the cell lines were examined for the expression of several other liver-specific genes.

MATERIALS AND METHODS

Cell lines and primary hepatocytes. The methods used for the immortalization of adult rat hepatocytes by transfection with SV40 DNA and the selection of albumin-secreting colonies of cells as well as the composition of the CDM (designated RPCD) have been described previously (42). Individual albumin-producing colonies were picked, dispersed, and transferred to fresh culture dishes. Colonies in secondary cultures were analyzed by immuno-overlay for purity with regard to albumin production. This procedure was repeated three to five times until pure albumin-producing cell lines were established. SV40-immortalized hepatocyte cell lines were plated in 100-mm plastic tissue culture dishes and fed RPCD medium with changes every 3 days. When cultures became confluent, the cells were trypsinized and plated at a dilution of 1/10 in fresh dishes by using RPCD supplemented with 5% fetal calf serum to aid cell attachment. After attachment (1 to 4 h after plating), fresh RPCD was added. The cell lines McA-RH7777, derived from Morris hepatoma 7777 (1), and H4IIEC3, isolated from Reuber H35 hepatoma (37), were obtained from V. R. Potter, McArdle Laboratory for Cancer Research, University of Wisconsin College of Medicine, Madison, Wis. Both cell lines were maintained in Swim S77 medium supplemented with 20% horse serum, 5% calf serum, 1.5 mM glutamine, 1.3 mM $CaCl_2$, 36 μM L-cystine, penicillin (250 U/ml), and streptomycin (250 μg/ml). Primary cultures of adult rat hepatocytes were isolated by collagenase perfusion as described previously (2) and modified (16, 19).

Immunoprecipitation of secreted plasma proteins. Proteins secreted by cell lines were radioactively labeled as described previously (17), except that labeling was for 6 h. Cells were labeled at approximately 50 to 80% confluence. At the end of the labeling period, the medium was collected. Immunoprecipitation was performed by reacting labeled medium with specific antibodies, followed by adsorption to staphylococcal protein A (IgSORB; The Enzyme Center, Boston, Mass.) as previously described (17). Specific antisera were directed against albumin, the third component of complement, transferrin, a mixture of purified plasma proteins (all from Cooper Biomedical, Inc., Malvern, Pa.), hemopexin (John Taylor, Gladstone Foundation Laboratories for Cardiovascular Disease, San Francisco, Calif.), and alpha-fetoprotein (AFP) (Warren Liao, M. D. Anderson Hospital and Tumor Institute, Houston, Tex.). Proteins contained in the immune complexes were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (25) on 10 to 18% gradient gels, followed by fluorography (3). ^{14}C-labeled protein standards were used as molecular weight markers (New England Nuclear Corp., Boston, Mass.).

Rocket immunoelectrophoresis. The amount of rat albumin secreted into the culture medium was measured by rocket immunoelectrophoresis as previously described (27). Rat albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.) was diluted in the appropriate culture medium and used as a standard.

TAT assay. Tyrosine aminotransferase (TAT) activity (EC 2.6.1.5) was assayed by the method of Diamondstone (14) and was induced by allowing cell lines to grow to confluence (4 to 6 days) in the presence of dexamethasone (0.4 μg/ml). TAT activity was induced in rat liver by intraperitoneal injection of hydrocortisone (50 mg/kg) 5 h prior to sacrifice (28). Protein was determined by the method of Lowry et al. (29), and enzyme activity was expressed as milliunits (mU) per milligram of protein per minute.

Intracellular localization of albumin by using flow cytometry. The CWSV1, CWSV2, CWSV3, CWSV5, and CWSV8 cells were trypsinized and then rinsed in RPCD containing 5% fetal calf serum to inactivate trypsin activity. The cell pellets were washed twice with phosphate-buffered saline to remove serum proteins, and the pellets were suspended in 2% paraformaldehyde for 30 min at 4°C. After fixation, the cells were centrifuged, suspended in phosphate-buffered saline to remove the paraformaldehyde, centrifuged again, and suspended in 0.1 M glycine for 10 min. To eliminate nonspecific staining, the cells were suspended in antibody diluent (antibody diluent is phosphate-buffered saline containing 1% bovine serum albumin, 1% normal goat serum, and 0.1% Triton X-100) supplemented with 10% normal goat serum and incubated for 30 min. The cells were then placed in antibody diluent containing various dilutions of fluorescein-conjugated goat anti-rat albumin (Cooper Biomedical) for 30 min at 4°C. The fluorescence profiles of the populations were determined by using an EPICS V flow cytometer (Coulter Electronics, Inc., Hialeah, Fla.). Excitation was at 500 mW from the 488-nm line of the argon ion laser. Green fluorescence from cell-size objects (a determination based on light scattering properties) was collected between 515 and 540 nm by using a 525-band pass filter backed up by a 560-band short-pass filter. The percentage of albumin-positive cells in each population relative to the non-albumin-producing cell line CWSV8 was determined by using an algorithm developed to subtract overlapping positive and negative distributions (IMMUNO; Coulter Electronics).

Recombinant plasmids. The probes pAlb576, a cDNA

encoding a portion of the rat albumin gene, and pAFP-3, a cDNA copy of the rat AFP gene (26), were kindly provided by John Taylor. The plasmid pBRWT2 contains the entire SV40 genome inserted into the *Eco*RI site of pBR322 and was provided by Mary J. Tevethia, The Pennsylvania State University College of Medicine, Hershey, Pa.

Northern and dot blot analyses. RNA was isolated from cell lines and from intact liver by lysis in guanidine thiocyanate (7), followed by centrifugation through cesium chloride. The concentration of RNA was determined spectrophotometrically. For Northern blot (RNA blot) hybridization, equal amounts of formamide-denatured total cellular RNA were electrophoretically separated on formaldehyde-agarose gels, and transferred to nitrocellulose filters. The filters were baked at 80°C and prehybridized for 48 h. For dot blot hybridization, RNA was serially diluted in 20× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) and immobilized onto nitrocellulose filters by using a Minifold apparatus (Schleicher & Schuell, Inc., Keene, N.H.). Plasmid DNA was nick translated (38) and hybridized to filters in 5× SSC–1× Denhardt solution–20 mM NaH_2PO_4 (pH 6.5)–0.1% SDS–1 mM EDTA–0.1 μg of salmon sperm DNA per ml–50% formamide–10% dextran sulfate. Hybridization was performed at 46°C for 36 to 42 h. Filters were washed twice in 2× SSC containing 0.1% SDS at room temperature and then washed three times in 0.1× SSC containing 0.1% SDS for 30 min each at 55°C.

Autoradiography and densitometry. Hybridized dot blots were exposed to preflashed Kodak XAR-5 film at −70°C. The relative signal intensities of the resulting autoradiographs were measured quantitatively by using a Transidyne 2955 scanning densitometer (Transidyne General Corp., Ann Arbor, Mich.). Comparisons of relative signal intensities were made only at RNA dilutions for which the signal intensity was directly proportional to the amount of RNA on the filter.

RESULTS

Albumin secretion per cell with cell passage. It has been a long-term goal to develop an immortalized hepatocyte cell line that expresses differentiated functions at liverlike levels. Such a cell line is important to better understand how the expression of differentiated functions are regulated at the molecular level. Immortalization or transformation of liver cells with SV40 information to yield replicating epithelial cells has been accomplished in our laboratory and in others, but the cell lines generated do not produce liverlike levels of albumin. The reason we have recently been able to isolate cell lines that more closely resemble hepatocytes is most likely due to the fact that these cell lines are derived from hepatocytes cultured in specialized medium during the process of immortalization. That is, because of the improved culture conditions, hepatocytes transfected with SV40 DNA retained the ability to express high levels of albumin and other liver-specific functions for a long time after transfection and often from the time of transfection until the appearance of epithelial cell colonies.

Each SV40-immortalized hepatocyte cell line was initially characterized based on the quantity of albumin secreted per cell as measured by rocket immunoelectrophoresis. The amount of albumin secreted varied among the cell lines and ranged from 1 to 40 pg per cell per 24 h (Fig. 1). The amounts of albumin secreted by cell lines producing large amounts of albumin (high-albumin-producing cell lines) were equal to or greater than those produced by primary hepatocytes 1 to 4 days after plating in CDM (8 to 20 pg of albumin per cell per 24 h). Each cell line was passaged at least 20 times. A certain amount of fluctuation in albumin production was observed for each cell line over the first 20 passages in culture and has continued to be observed for those cell lines that have been analyzed beyond 20 passages. The variability appears to be an inherent property of the cells and is also partially caused by the fact that albumin production by some of the cell lines is dependent upon cell density (see Fig. 4). The CWSV1 and CWSV2 cell lines were identified as high-albumin-producing cell lines, and CWSV4 and CWSV5 were identified as low-albumin-producing cell lines. Although the CWSV8 cell line initially secreted albumin, the amount of albumin secreted at passage 11 was below the limits of detection, and at passages beyond 11, CWSV8 was used as a negative control.

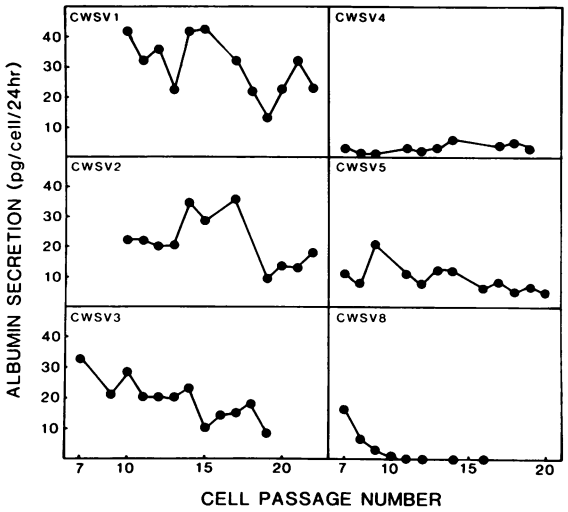

FIG. 1. Quantitation of albumin secretion per cell during continuous passage of six SV40-immortalized hepatocyte cell lines in CDM. The concentration of rat albumin in the culture medium was determined by rocket immunoelectrophoresis. The amount of albumin secreted per cell was then determined by dividing the amount of albumin secreted by a culture by the number of cells in the culture.

Quantitation of the number of albumin-producing cells by flow cytometric analysis. The values for albumin secretion reported in Fig. 1 were obtained by measuring the amount of albumin secreted into the medium by all cells in a culture. The value for albumin secretion per cell was determined by dividing the amount of albumin secreted into the medium by the number of cells in the culture. To measure quantitatively the percentage of individual cells from different cell lines that synthesized albumin, we examined four SV40-immortalized hepatocyte cell lines that had been identified by rocket immunoelectrophoresis as low- or high-albumin-producing cell lines (Fig. 2). A large proportion (78 to 95%) of individual cells in both high- and low-albumin-producing cell lines stained positively for the presence of albumin. The examination of stained cells under a microscope prior to flow cytometric analysis showed that albumin was localized in the perinuclear area of the cytoplasm and that the intensity of staining varied among cells from different cell lines and also among cells within the same cell line. We conclude that the large differences in the amount of albumin produced by the four cell lines tested reflected differences in the amount of albumin secreted per cell and were not due to differences in the number of albumin-producing cells in each cell line.

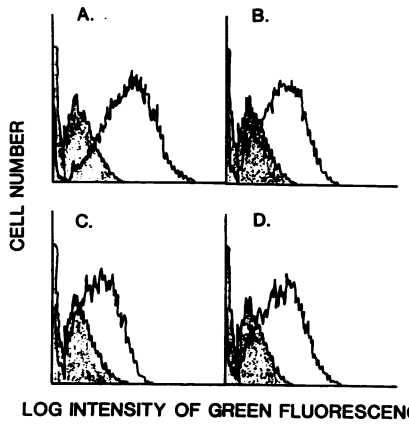

FIG. 2. Percentage of albumin-positive cells in different cell lines as determined by flow cytometry. Data are displayed as histograms of cell number in the population as a function of the log of the integrated green fluorescence signal per cell, a function proportional to the amount of albumin per cell. The percentage of albumin-positive cells in each population relative to the non-albumin-producing cell line (CWSV8) shown in gray was as follows: (A) CWSV1, 96%; (B) CWSV2, 85%; (C) CWSV3, 78%; and (D) CWSV5, 87%. Specific values of albumin secretion per cell in this experiment were as follows: passage 25 of CWSV1, 21 pg per cell per 24 h; passage 21 of CWSV2, 18.6 pg per cell per 24 h; passage 27 of CWSV3, 4.5 pg per cell per 24 h; and passage 14 of CWSV5, 8.1 pg per cell per 24 h.

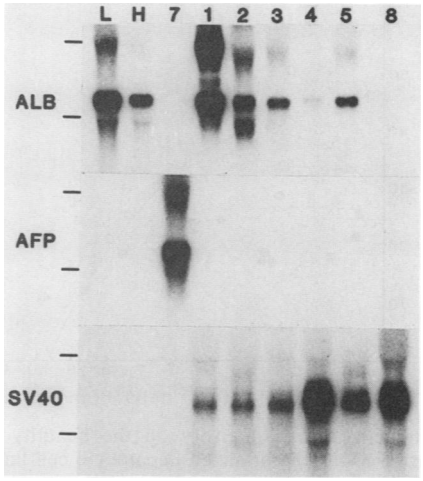

FIG. 3. Northern blot analysis of albumin, AFP, and SV40 RNA in SV40-immortalized hepatocyte cell lines. Total cellular RNA was extracted from liver (lane L), two hepatoma cell lines, McA-RH7777 (lane 7) and H4IIEC3 (lane H), and six SV40-immortalized cell lines, CWSV1 to CWSV8 (lanes 1 to 8), separated electrophoretically, blotted, and hybridized to ^{32}P-labeled probes. The six SV40-immortalized cell lines ranged in passage number from passage 12 to 18 at the time of RNA extraction. The probes were cDNA containing a portion of the rat albumin gene (ALB), a cDNA copy of the rat AFP gene (AFP), and a plasmid containing the entire SV40 genome (SV40). The markers indicate the positions of the 28S and 18S rRNA bands.

However, even within a cell line, the amount of albumin detected within each cell varied.

Analyses of albumin, AFP, and SV40 RNAs. Northern blot analyses of RNAs from the SV40-immortalized hepatocyte cell lines were carried out to determine whether (i) the albumin message expressed is the same size as that found in intact rat liver and (ii) the relative amounts of albumin RNA in each cell line are related to the amounts of albumin secreted by these cell lines. The 2.3-kilobase (kb) albumin message was expressed by the albumin-secreting hepatoma cell line H4IIEC3 but not by the non-albumin-producing hepatoma cell line McA-RH7777 (Fig. 3). The CWSV8 cell line at passage 15 contained no detectable albumin RNA, indicating that the inability to secrete albumin was paralleled by the absence of albumin RNA in these cells. The remaining SV40-immortalized hepatocyte cell lines expressed the 2.3-kb albumin RNA present in normal liver. Higher levels of albumin RNA were found in cell lines previously determined to secrete larger amounts of albumin than in cell lines that secreted low levels of albumin. A quantitative analysis of albumin RNA levels relative to albumin secretion was performed and is discussed below.

AFP is a plasma protein which is not produced by normal liver but is produced by fetal hepatocytes, regenerating liver, some tumors of liver origin, and hepatoma cell lines. Therefore, it was of interest whether SV40-immortalized hepatocytes express the AFP gene. The 2.2-kb AFP RNA was expressed by the McA-RH7777 hepatoma cell line but not by adult liver, the H4IIEC3 hepatoma cell line, or any of the SV40-immortalized cell lines (Fig. 3). Even when the gel films were overexposed, AFP RNA was only detected in RNA from McA-RH7777 cells.

Northern blot analyses were also carried out to ascertain whether the hepatocyte cell lines immortalized by transfection with SV40 DNA continue to express SV40 RNA. The SV40-immortalized cell lines expressed various amounts of SV40 RNA, whereas liver and the two control hepatoma cell lines did not (Fig. 3). Cell lines that contained no albumin RNA or that continuously expressed low levels of albumin expressed higher levels of SV40 RNA than the high-albumin-producing cell lines did.

The amount of albumin RNA expressed by the SV40-immortalized hepatocyte cell lines was measured quantitatively by using dot blot analysis to determine whether the variation in the levels of albumin secretion by the different cell lines is related to albumin RNA steady-state levels. Medium samples were collected from cultures immediately before RNA was extracted from the cells, and the amount of albumin in the samples was measured by rocket immunoelectrophoresis. The amount of albumin secreted by a

TABLE 1. Quantitative comparison of albumin RNA levels in different SV40-immortalized hepatocyte cell lines

Cell line	Albumin RNA (%)[a]	Albumin secretion (pg/cell per 24 h)[b]
CWSV1	110	21.5
CWSV2	80	17.5
CWSV3	50	13.2
CWSV4	6	1.6
CWSV5	23	7.6
CWSV8	0	0
H4IIEC3	40	2.9
McA-RH7777	0	0
Liver	100	ND

[a] Serial dilutions of total cellular RNA were immobilized on nitrocellulose and hybridized to the albumin probe, and the relative intensity of hybridization was measured by densitometric analysis of autoradiographs. The values are percentages of albumin RNA in normal intact liver.

[b] Secretion was determined on the same cultures used for the preparation of RNA immediately before the extraction of cellular RNA. ND, Not determined.

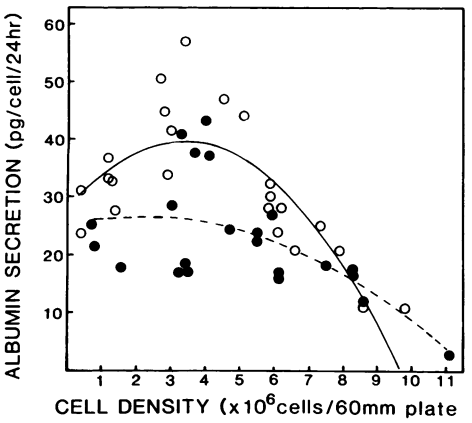

FIG. 4. Influence of cell density on the quantity of albumin secreted by two SV40-immortalized hepatocyte cell lines. CWSV1 (○) and CWSV2 cells (●) were plated at low density and allowed to grow to confluence. Medium from the cultures was collected daily and assayed for rat albumin by rocket immunoelectrophoresis. Cells were harvested daily, and the number of cells per culture was determined. Experiments were performed at 37°C. The values represent the results of two independent experiments, and the curves were drawn by using the least squares method, assuming a second-degree polynomial function.

particular cell line correlated directly with the steady-state level of albumin RNA in the cells (Table 1). For example, CWSV1 cells secreted the largest amounts of albumin and also contained the greatest amounts of albumin RNA. Cell lines that secreted smaller amounts of albumin expressed lower levels of albumin RNA. This quantitative analysis also made it possible to directly compare the amount of albumin RNA in the cells with the amount of RNA expressed by intact liver; such a comparison was not possible for albumin secretion that was measurable in cultured cells but not in intact liver. The levels of albumin RNA in CWSV1 cells were comparable with or greater than those of albumin RNA in intact liver. In interpreting these results, it is important to realize that not all the cells in liver are hepatocytes and not all the cells in liver produce albumin.

Effect of cell density on albumin secretion. The amount of a particular enzyme or protein produced by a cultured cell line often depends upon the cell density. It has also been reported that the expression of specific proteins considered to be markers of growth or differentiation in primary hepatocytes depends upon the density at which the cells are plated and maintained (32, 33). We used the two high-albumin-producing SV40-immortalized hepatocyte cell lines, CWSV1 and CWSV2, to measure whether albumin expression is regulated by cell density. Cells were plated at low density, and the cells and media from parallel cultures were harvested over a period of 10 days to determine the number of cells per culture and the amount of albumin secreted (Fig. 4). The amount of albumin secreted per cell was plotted against the cell number. Albumin secretion per cell for CWSV1 cells was maximal at slightly subconfluent cell density (2×10^6 to 4×10^6 cells per 60-mm dish) and declined when the cells reached a higher density. The viability of the CWSV1 cells 12 and 15 days after plating was greater than 90%, indicating that the decreased albumin secretion was not simply caused by cell death. Albumin secretion by CWSV2 cells was also density dependent but to a much lesser extent than that observed for CWSV1 cells.

Effect of temperature on albumin secretion. Since it was clear that albumin secretion was dependent upon cell density, the effect of temperature on albumin secretion per cell by CWSV1 and CWSV2 cells was measured at a series of densities rather than at a single density. Albumin secretion per cell was determined for cells cultured at 33, 37, and 40°C (Fig. 4 and 5). The effect of temperature on albumin secretion per cell for CWSV1 and CWSV2 cells was minimal. At all three temperatures, the amount of albumin secreted ranged from 10 to 40 pg per cell per 24 h for CWSV1 cells and from 5 to 30 pg per cell per 24 h for CWSV2 cells. Albumin secretion by CWSV1 cells at 33°C was maximal at a moderate density (2×10^6 to 4×10^6 cells per 60-mm dish), as observed at 37°C; however, at 40°C, albumin secretion by CWSV1 cells was maximal when cells were at a considerably lower density (0.5×10^6 to 1.0×10^6 cells per 60-mm dish). In addition, at 40°C, both cell lines failed to grow to as high a density as they did at 33 and 37°C. We conclude that hepatocyte cell lines immortalized by wild-type SV40 expressed albumin essentially in a temperature independent fashion when examined at 33, 37, and 40°C. This finding is an important control for examining the effect of a temperature shift on albumin production by hepatocytes immortalized by temperature-sensitive mutants of SV40.

Effect of dexamethasone on albumin secretion. The amount of albumin secreted by freshly isolated hepatocytes declines if glucocorticoids such as dexamethasone are omitted from the culture medium (34, 43). We examined whether the amount of albumin secreted by SV40-immortalized hepatocyte cell lines is dependent on dexamethasone. The cell lines were derived and maintained in a culture medium supplemented with dexamethasone at a concentration of 0.4 μg/ml. To test the effect of dexamethasone on albumin secretion, six cell lines were plated at low density and allowed to proliferate in medium with or without dexamethasone. Cells from five of the six SV40-immortalized cell lines cultured in dexamethasone secreted two- to fourfold higher levels of

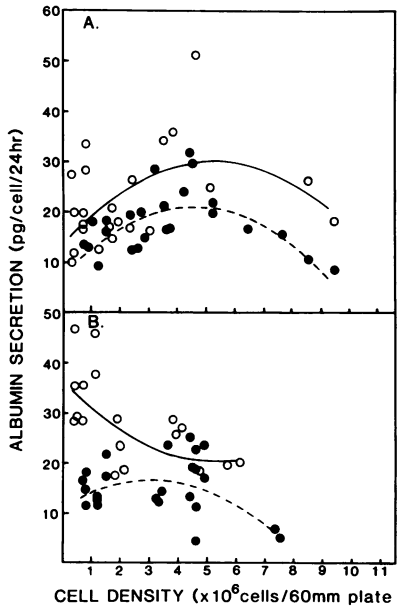

FIG. 5. Influence of temperature on the amount of albumin secreted per cell by two SV40-immortalized hepatocyte cell lines. Experiments were performed as described in the legend to Fig. 4, except that the CWSV1 (○) and CWSV2 (●) cells were grown at 33°C (A) or 40°C (B).

TABLE 2. Enhancement of albumin secretion in SV40-immortalized hepatocyte cell lines by dexamethasone[a]

Cell line	Albumin secretion (pg/cell per 24 h)[b]		
	Without dexamethasone	With dexamethasone (0.4 μg/ml)	Fold increase
CWSV1	14.1	44.8	3.2
CWSV2	11.7	12.1	1.0
CWSV3	1.1	3.9	3.5
CWSV5	3.3	13.0	3.9
CWSV14	1.6	3.6	2.3
CWSV15	5.7	21.6	3.8

[a] Cell lines were plated at low density and allowed to grow to confluency in CDM either in the absence or presence of dexamethasone.
[b] Albumin secretion was measured by rocket immunoelectrophoresis.

albumin than cells in parallel cultures grown in medium lacking dexamethasone (Table 2). The ability to secrete albumin remained at low but detectable levels even when these five cell lines were maintained for three to four passages in the absence of dexamethasone (data not shown). One cell line, CWSV2, secreted the same levels of albumin whether the cells were cultured in the presence or absence of dexamethasone, indicating that high levels of albumin secretion were not always dependent on dexamethasone. CWSV2 cells continued to secrete high levels of albumin even when maintained for several passages in the absence of dexamethasone (data not shown).

The effect of dexamethasone on albumin secretion by the CWSV1 cell line was examined in greater detail. Values for albumin secretion per cell were determined on a daily basis for cells cultured in the presence or absence of dexamethasone. The increase in albumin secretion that occurs with time after subculture and therefore as the density of the cells in the culture dish begins to increase (Fig. 4) was observed for cells grown in the presence of dexamethasone (Fig. 6). This increase, which was most apparent 6 to 8 days after plating, was not observed when cells were grown in the absence of dexamethasone. Albumin secretion did not decline measurably when dexamethasone was omitted from cultures that were already confluent (data not shown).

Regulation of albumin RNA levels by density and dexamethasone. RNA was extracted from cells maintained at subconfluent or high cell density or grown for 10 days in the presence or absence of dexamethasone and subjected to Northern blot analysis. Under all four culture conditions, the cells expressed the authentic 2.3-kb albumin RNA expressed by liver. A quantitative analysis showed that albumin RNA levels in CWSV1 cells decreased when the cells were confluent and also when they were grown in the absence of dexamethasone (Table 3). To directly compare the levels of albumin RNA and the amounts of albumin secreted, medium samples were collected from cultures immediately before RNA was extracted. The alterations in albumin RNA levels in CWSV1 cells paralleled the changes observed at the level of albumin secretion. The levels of albumin RNA in CWSV2 cells were not altered by culture density as markedly as were those observed for CWSV1 cells. It was reproducibly shown that the levels of albumin RNA in CWSV2 cells grown in the presence of dexamethasone were equal to or slightly lower than those in CWSV2 cells grown in the absence of dexamethasone.

TAT induction by dexamethasone. Another protein produced by liver that is of particular interest is the inducible enzyme TAT. In normal liver, TAT is induced 2- to 10-fold

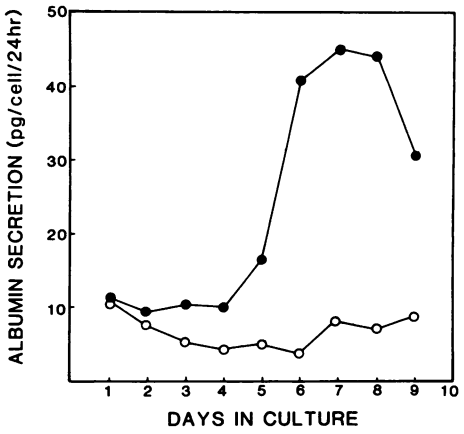

FIG. 6. Effect of dexamethasone on albumin secretion by the CWSV1 cell line. Cells were plated at low density and maintained in the presence (●) or absence (○) of dexamethasone for 10 days. Albumin secretion per cell was determined as described in the legend to Fig. 1.

by the administration of glucocorticoid hormones (28). To determine whether SV40-immortalized hepatocyte cell lines have TAT activity and whether the enzyme activity is inducible by the glucocorticoid dexamethasone, three albumin-producing cell lines and one control hepatocyte cell line were grown to confluence (4 to 6 days) in either the absence or presence of dexamethasone and TAT activity was measured (Table 4). TAT activity was increased by dexamethasone by more than 6-fold in CWSV2 cells and by more than 37-fold in CWSV1 cells. The finding that TAT activity was induced by dexamethasone in CWSV2 cells shows that these cells had the ability to respond to dexamethasone and indicates that the inability of dexamethasone to regulate albumin secretion must occur by another mechanism. The induced TAT activity in CWSV1 and CWSV2 cells was actually greater than that in induced intact rat liver.

Secretion of plasma proteins. The purpose of the work described above was to characterize albumin production and the regulation of albumin production in a series of SV40-immortalized cell lines. We also were interested in whether these cell lines express other liver-specific functions, including the abilty to secrete plasma proteins other than albumin.

TABLE 3. Effect of culture density and dexamethasone on levels of albumin RNA in SV40-immortalized hepatocyte cell lines

Cell line	Culture condition	Albumin RNA (%)[a]	Albumin secretion (pg/cell per 24 h)[b]
CWSV1	Subconfluent	91	23
	Confluent	44	8
	With dexamethasone	85	17
	Without dexamethasone	36	8
CWSV2	Subconfluent	74	14
	Confluent	73	10
	With dexamethasone	39	8
	Without dexamethasone	56	9

[a] Serial dilutions of total cellular RNA were immobilized on nitrocellulose and hybridized to the albumin probe, and the relative intensity of hybridization was measured by densitometric analysis of autoradiographs. The values are percentages of albumin RNA in normal intact liver.
[b] Secretion was determined on the same cultures used for the preparation of RNA immediately before the extraction of cellular RNA.

TABLE 4. Induction of TAT activity by glucocorticoid

Cell line[a]	TAT activity (mU/min per mg of protein)		
	Without glucocorticoid	With glucocorticoid[b]	Fold increase
CWSV1	2.4	89.7	37.4
CWSV2	7.4	46.9	6.3
CWSV5	2.6	3.5	1.4
CWSV8	0.5	0.3	0
Liver[c]	4.2	21.3[d]	5.1

[a] Cells were plated at low density and allowed to grow to confluence (4 to 6 days) in CDM either with or without dexamethasone.
[b] The dexamethasone concentration was 0.4 μg/ml.
[c] Liver from adult rats.
[d] Total liver from rats injected intraperitoneally with hydrocortisone (50 mg/kg).

A series of 11 SV40-immortalized hepatocyte cell lines that produced various amounts of albumin were labeled with [^{35}S]methionine, and the radioactively labeled proteins in the culture medium were immunoprecipitated with specific antibodies and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 7). Freshly isolated adult rat hepatocytes and the hepatoma cell lines H4IIEC3 (which secretes albumin) and McA-RH7777 (which secretes AFP) were included for comparison. The SV40-immortalized cell line that had lost the ability to secrete albumin, CWSV8, was used as a negative control. Each of the SV40-immortalized cell lines that produced albumin also secreted other plasma proteins into the culture medium. These secreted proteins included transferrin, hemopexin, and the third component of complement. Each plasma protein immunoprecipitated from medium from SV40-immortalized hepatocyte cell lines migrated to the same position as its counterpart from freshly isolated adult rat hepatocytes. Not all plasma proteins were secreted by each cell line, and the quantity of albumin produced by an individual cell line was not necessarily related to its ability to secrete detectable levels of other plasma proteins. For example, the cell line CWSV3 secreted lower levels of albumin but produced higher levels of the third component of complement than CWSV1 did. None of the SV40-immortalized cell lines examined secreted detectable amounts of AFP.

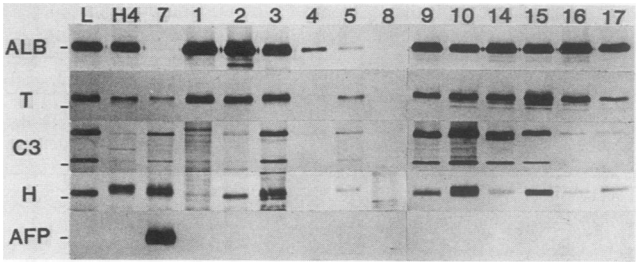

FIG. 7. Detection of plasma proteins secreted by SV40-immortalized hepatocyte cell lines. Primary hepatocytes, two rat hepatoma cell lines (McA-RH7777 and H4IIEC3), and 12 SV40-immortalized hepatocyte cell lines were radioactively labeled with [^{35}S]methionine. Plasma proteins were immunoprecipitated from the labeled culture media by using antibodies to albumin (ALB), transferrin (T), the third component of complement (C3), hemopexin (H), and AFP. Immunoprecipitates for each antibody were analyzed on individual gels, and an autoradiograph was prepared from each; this figure is a composite of a series of these autoradiographs. Lanes: L, primary hepatocytes; 7, McA-RH7777; H4, H4IIEC3; 1 to 17, SV40-immortalized hepatocyte cell lines. As stated in the text, 12 SV40-immortalized hepatocyte cell lines were isolated; 11 of these were albumin-producing cell lines. Cell lines CWSV1 through CWSV8 have been characterized previously. Cell lines CWSV9 through CWSV17 have been characterized by rocket immunoelectrophoresis for albumin production with passage and, like the CWSV1 cell line, are high-albumin-producing cell lines. The marks at the left of the gels indicate the position of the 69-kilodalton molecular weight marker.

DISCUSSION

Several of the cell lines described in this work produced liverlike levels of albumin RNA and, as such, represent the first cell lines ever derived with this property. For example, albumin RNA levels in CWSV1 cells were 110% of those of liver. The amount of albumin RNA in liver was an underestimation of the amount of albumin RNA in the hepatocytes, since RNA was isolated from the whole liver and approximately 90% of the mass of the liver is hepatocytes (18). If the albumin RNA value for liver is corrected to take into account the hepatocyte content of liver, the amounts of albumin RNA in CWSV1 cells and hepatocytes from intact liver are approximately equal. In a previous report, infection of normal adult rat hepatocytes maintained in a serum-supplemented medium with a temperature-sensitive mutant of SV40 yielded an immortalized rat liver cell line designated RALA255-10G (8). The RALA255-10G cells secreted albumin, but the amount secreted per cell was more than 40-fold less than that secreted by CWSV1 cells. The rat hepatoma H4IIEC3 cell line is a high-albumin producer, but even in this cell line, the albumin RNA levels are only about 40% of those in normal liver (Table 1). In comparing albumin RNA levels with the amount of albumin secreted by CWSV cell lines, we anticipated that H4IIEC3 cells would secrete higher levels of albumin. This finding suggests that the intracellular processes that regulate albumin synthesis and secretion are probably similar within the series of CWSV cells but differ when compared with those in H4IIEC3 cells. Under our most ideal culture conditions, we have been able to achieve albumin mRNA levels in primary hepatocytes in long-term culture that are 45% of those in normal liver (unpublished data) or about half the value found for CWSV1 cells.

It has been reported that albumin production by fetal hepatocytes, primary hepatocytes, hepatoma cells, and RALA255-10G cells declines in the absence of glucocorticoid (4, 8, 30, 34, 43). Both the amount of albumin secreted and the level of albumin mRNA decrease when hepatocytes derived from 15- and 19-day-gestation rats are placed in culture medium lacking dexamethasone (43). When RALA255-10G cells are shifted during growth to culture medium containing no cortisol, the amount of albumin secreted by the cells rapidly declines, and when the cells are subcultured in medium lacking cortisol for 3 consecutive weeks, they no longer produce detectable levels of albumin (8). A similar dependence on glucocorticoid has been shown for albumin production by primary adult hepatocytes, and it was recently demonstrated that the rate of albumin gene transcription, measured as the incorporation of [α-^{32}P]UTP into mRNA in isolated nuclei from adult rat hepatocytes in culture, declines rapidly in cells cultured in the absence of dexamethasone and is restored when dexamethasone is added back to the culture medium (34). We also found that in five of six albumin-producing SV40-immortalized hepatocyte cell lines, the removal of dexamethasone from the medium led to a decline in albumin secretion and in the levels of albumin RNA. However, the CWSV2 cell line secreted large

amounts of albumin and produced high levels of albumin RNA regardless of whether dexamethasone was in the medium, indicating that glucocorticoid is not always required for albumin production by hepatocyte cell lines. Since TAT activity in CWSV2 cells was induced by dexamethasone, there is no reason to believe that CWSV2 cells are generally unable to respond to dexamethasone.

We have derived a series of albumin-producing hepatocyte cell lines, and several were characterized in this study. The generation of this series has made apparent several points. First, the procedure we used for deriving an immortalized albumin-producing cell line is reproducible. Second, if a cell line is selected based on its ability to secrete albumin, it will most likely express several other liver-specific functions. Third, there is some degree of diversity among the cell lines in the amount of albumin produced per cell, in the manner in which albumin expression is regulated, and in terms of which other liver-specific proteins are produced. This variation among the cell lines may arise during the process of immortalization or it may indicate that diversity in gene expression and regulation is already present in the original population of hepatocytes. This diversity essentially provides a series of natural variants, which will be useful for understanding the mechanisms of liver-specific gene expression at the molecular level. For example, it will be of interest to compare the rate of transcription of albumin RNA in the CWSV1 cell line (which is a high-albumin producer) with that of the CWSV5 cell line (which is a low-albumin producer). Similarly, had we only derived and characterized one cell line, we would not have realized that although dexamethasone is generally needed by hepatocyte cell lines for the induction of albumin synthesis, it is not required, as evidenced by the fact that CWSV2 cells produced high levels of albumin when grown in the absence of dexamethasone.

We have referred to the hepatocyte cell lines characterized in this study as immortalized as opposed to transformed because they are stable cell lines; at least through passage 22, neither the CWSV1 nor the CWSV2 cell lines produced tumors when inoculated subcutaneously into newborn syngeneic rats (unpublished data). Although some of the cell lines express albumin at levels even higher than those expressed by primary hepatocytes, it must be remembered that the cell lines differ from primary hepatocytes in culture and hepatocytes in normal liver in that they replicate; hence the term immortalized. Although the SV40-immortalized hepatocytes replicate, none of the cell lines expressed AFP or AFP RNA. AFP is a plasma protein secreted by replicating fetal hepatocytes, regenerating liver, and some liver tumor cells. That is, differentiated hepatocytes were immortalized by the introduction of DNA containing SV40 genetic information without the induction of AFP, which is associated with growth, loss of differentiation, and tumorigenicity. Similarly, the expression of SV40 information by these cells does not prevent the expression of differentiated functions, as evidenced by the fact that SV40 RNA was detected in the same CWSV1 RNA sample used to demonstrate that CWSV1 cells express high levels of albumin RNA. The expression of SV40 information may not be totally unrelated to the expression of albumin RNA. We observed that cell lines that contained no albumin RNA or continuously expressed low levels of albumin RNA expressed higher levels of SV40 RNA than did the high-albumin-producing cell lines (Fig. 3). At this point, this is only an observation and requires further investigation.

One of the advantages of the SV40-immortalized hepatocyte cell lines described in this study is that they grow in CDM. This fact will make it possible to (i) use these cells to test systematically the effects of pharmacologic agents on the expression of liver-specific genes without interference from serum and (ii) identify and quantitate substances secreted by the cells. An advantage of the SV40-immortalized hepatocyte cell lines over primary hepatocytes is that they grow on plastic without the addition of collagen or more complex matrix material. This makes the SV40-immortalized cell lines easier and more economical to use, and perhaps more important, it means that the effects on the cells produced by changes in the environment can be attributed directly to the cells, thereby eliminating the need to interpret the role played by substances present in attachment factors or provided by cocultivated cells.

In this study, the SV40-immortalized hepatocyte cell lines were characterized for the ability to produce the plasma proteins albumin, AFP, transferrin, hemopexin, and the third component of complement and to express TAT activity, which is inducible by dexamethasone. In future studies, these cells will be analyzed for the expression of other liver-specific proteins. It is equally important to test the ability of these cells to respond to changes in their environment. We have measured the ability of several of the cell lines to respond to density, temperature, and dexamethasone. In future studies, these cells will be tested for the ability to respond to hormones, growth factors, biological substances known to interact with hepatocytes, and to drugs and chemicals metabolized by cells of the liver. Because these cells are established cell lines, it will be possible to study the regulation of the expression of liver-specific proteins not only at the level of the final product but also at the underlying molecular level. For example, in this study, we looked at the regulation of albumin production at the level of protein secretion and expression of albumin RNA. We established that in each condition studied, the amount of albumin produced corresponded directly to changes in the amount of albumin RNA detected. Experiments are in progress with these cells to measure the rates of transcription of albumin RNA, to establish values for albumin RNA half-life, and to identify tissue-specific factors that affect transcription of the albumin gene.

ACKNOWLEDGMENTS

We thank L. Mengel, T. Miller, and J. Cochrane for excellent technical assistance; T. Grierson for help with photography; M. Clement for editorial assistance; and B. M. Ohlsson-Wilhelm and E. D. Kloszewski for assistance with flow cytometry.

The investigation was supported in part by Public Health Service grants CA 23931 and CA 09124 from the National Cancer Institute. We also acknowledge support from a developmental grant provided by the Flow Cytometry Facility.

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