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# Tumorigenicity of Simian Virus 40-Hepatocyte Cell Lines: Effect of In Vitro and In Vivo Passage on Expression of Liver-Specific Genes and Oncogenes

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**Five simian virus 40 (SV40)-hepatocyte cell lines were examined for tumorigenicity and the effect of in vitro passage on the expression of four liver-specific genes (albumin, transferrin, $\alpha_1$-antitrypsin, and phosphoenolpyruvate carboxykinase), two oncogenes (c-Ha-*ras* and c-*raf*), and two genes associated with hepatocarcinogenesis (α-fetoprotein and placental-type glutathione-*S*-transferase). At low passage (12 to 22), all five cell lines expressed the four liver-specific genes at levels similar to those in the liver and were not tumorigenic or were weakly tumorigenic. At high passage (33 to 61), the cell lines formed carcinomas, and four out of five cell lines produced primary tumors that metastasized. At least two cell lines produced well-differentiated hepatocellular carcinomas that expressed liver-specific RNAs. Levels of expression of liver-specific genes changed with time in culture. Some of the changes in liver-specific gene expression in the tumor tissue (such as for the phosphoenolpyruvate carboxykinase gene) paralleled those that occurred with in vitro passage, while other changes (such as for the albumin gene) did not parallel those that occurred with in vitro passage. Correlations between enhanced expression of c-Ha-*ras* and tumorigenic potential and between the process of SV40 immortalization and induced expression of c-*raf* and glutathione-*S*-transferase-P were observed. Induction of α-fetoprotein was detected with in vitro and in vivo passage only in the CWSV14 cell line and was paralleled by diminished albumin expression. In conclusion, we developed a model system with five SV40-hepatocyte cell lines, tumors induced by them, and tumor cell lines to examine changes in gene expression that accompany the progression from a normal cell to a hepatocellular carcinoma. Because the SV40-hepatocyte cell lines and tumor cell lines remain highly differentiated and vary in the magnitude of expression of specific genes, they can be used to study the molecular mechanisms regulating gene expression, in particular those regulating specific genes associated with differentiation.**

Because most human neoplasms are carcinomas that develop from differentiated adult epithelium, in vitro model systems for studying transformation of differentiated epithelial cells are particularly important. In many in vitro transformation studies, a primary cell type is used that has the ability to replicate for a limited number of passages in culture, is of fibroblastic origin, and produces an undifferentiated sarcoma in a test animal. Although studies to examine in vitro transformation of primary epithelial cells with defined genetic information have been performed (8–10, 32, 34, 37, 58, 61), the emphasis has been on determining what genes are required for transformation. Our interest was to determine (i) whether transformation of a nonreplicating differentiated epithelial cell could be accomplished, (ii) whether the transformed cells would produce a well-differentiated carcinoma, and (iii) how transformation altered expression of differentiated functions by the epithelial cells in culture and in the tumor.

Primary rat hepatocytes are particularly appropriate epithelial cells for studying transformation of differentiated cells because they have a limited capacity to replicate and express a variety of well-characterized differentiated functions. We have demonstrated previously that primary hepatocytes can be immortalized by simian virus 40 (SV40) genetic information to yield colonies of replicating epithelial cells that retain the capacity to produce high levels of albumin (24, 59). Eleven albumin-producing hepatocyte cell lines have been derived from these albumin-producing colonies (57). Characterization of these cell lines has shown that they express at least some functions associated with differentiation in hepatocytes in normal adult liver. Several of the cell lines produce levels of albumin RNA similar to those found in the liver, and many also secrete the plasma proteins transferrin, the third component of complement (C3), and hemopexin.

In the previous study (57), we examined several of the low-passage SV40-hepatocyte cell lines for albumin production and regulation of albumin expression. In this study, we characterized five albumin-producing SV40-hepatocyte cell lines for tumorigenicity and the effects of in vitro and in vivo passage on gene expression. The five cell lines at low and high passage numbers and the tumors produced by these cell lines were examined at the molecular level for expression of four genes that are hallmarks of normal hepatocyte differentiation (albumin, transferrin, $\alpha_1$-antitrypsin [$A_1AT$], and phosphoenolpyruvate carboxykinase [PepCK]); two oncogenes (c-Ha-*ras* and c-*raf*); and two genes associated with

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hepatocarcinogenesis (α-fetoprotein [AFP] and placental type glutathione-*S*-transferase [GST-P]).

## MATERIALS AND METHODS

**Cell lines.** The SV40-hepatocyte cell lines CWSV1, CWSV2, CWSV14, CWSV16, and CWSV17 (57) were maintained in 100-mm plastic tissue culture dishes and fed fresh RPCD medium (59) every 3 days. When cultures became confluent, the cells were trypsinized and subcultured at a dilution of 1:10 in RPCD medium supplemented with 5% fetal calf serum to aid cell attachment. After attachment (1 to 4 h after plating), fresh RPCD medium without serum was added. Cell lines McA-RH7777, derived from Morris hepatoma 7777 (4), and H4IIEC3, isolated from Reuber H35 hepatoma (35), were obtained from V. R. Potter (McArdle Laboratory for Cancer Research, University of Wisconsin College of Medicine, Madison). Both hepatoma cell lines were maintained in Swim S77 medium as previously described (57).

**Establishment of SV40 tumor cell lines.** Tumor cell lines were derived by minicing tissue from the primary tumor into small fragments (1 $mm^3$), digesting the fragments with trypsin for 15 min, and plating the disaggregated cells on plastic tissue culture dishes containing RPCD medium plus 5% fetal calf serum. After attachment, fresh RPCD medium without serum was added, and the tumor cell lines were maintained as described for the SV40-hepatocyte cell lines.

**Tumorigenicity of SV40-hepatocyte or tumor cell lines.** Cells were removed from culture dishes by trypsinization and rinsed once with Hanks balanced salt solution, and the number of viable cells was counted. Cells were resuspended at a concentration of $10^8$ cells per ml, and 0.1 ml was inoculated subcutaneously over the scapular region of neonatal, syngeneic rats within 24 h after birth. Animals were observed for tumor formation at weekly intervals for at least 6 months. At the time of sacrifice, animals were examined for metastases.

**Recombinant plasmids.** The plasmids used in these experiments were pAFP-3 (rat AFP [27]); plivS-3 (mouse $A_1AT$ [13]); plivS-6 (mouse transferrin [13]); pPCK-2 (rat PepCK [63]); and pGP5 (rat GST-P [46]). The plasmid pALb576 (rat albumin) was kindly provided by John Taylor (Gladstone Foundation Laboratories for Cardiovascular Disease, San Francisco, Calif.) and pBRWT2 (SV40) was kindly provided by Mary J. Tevethia (The Pennsylvania State University College of Medicine, Hershey). The plasmids pT24C3 (c-Ha-*ras*-1) and p627 (c-*raf*-1) were purchased from the American Type Culture Collection, Rockville, Md. Plasmid DNAs were isolated and purified by previously described methods (29). The identity of plasmid DNAs was verified by digestion with the appropriate restriction endonuclease followed by electrophoresis on 1.0% agarose gels.

**Northern (RNA) blot analysis.** RNA was isolated from cell lines, intact liver, and tumors by lysis in guanidine thiocyanate (11), followed by centrifugation through cesium chloride (29) as described previously (25). The concentration of RNA was determined spectrophotometrically. The rRNA concentration on each lane of ethidium bromide-stained gels was estimated visually to confirm that the lanes contained equal concentrations of total RNA. For northern blot hybridization, equal amounts of formamide-denatured total cellular RNA were electrophoretically separated on 1.4% agarose gels. RNA was transferred to nitrocellulose filters, and the filters were baked at 80°C. Prehybridization, hybridization, and autoradiography were carried out as described previously (25).

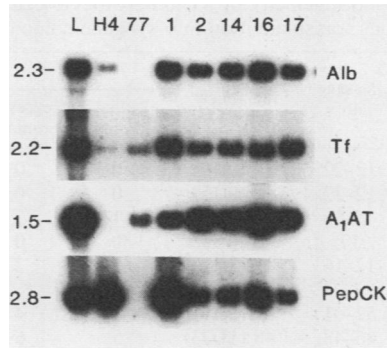


FIG. 1. Northern blot analysis of liver-specific RNAs expressed by low-passage SV40-hepatocyte cell lines. Total cellular RNA was extracted from liver (L), the two hepatoma cell lines, H4IIEC3 (H4) and McA-RH7777 (77), and five SV40-hepatocyte CWSV cell lines: 1, CWSV1; 2, CWSV2; 14, CWSV14; 16, CWSV16; and 17, CWSV17. The five SV40-hepatocyte cell lines ranged in passage number from 12 to 22. The samples of RNA were separated electrophoretically, blotted to nitrocellulose, and hybridized to $^{32}P$-labeled probes. The probes detected albumin (Alb), transferrin (Tf), $A_1AT$, and PepCK RNAs. In this and subsequent figures, the numbers at the left refer to the size (kilobases) of each specific message.

## RESULTS

**Liver-specific gene expression in SV40-hepatocyte cell lines.** Five SV40-hepatocyte cell lines (CWSV1, CWSV2, CWSV 14, CWSV16, and CWSV17 [57]) were examined for several RNAs that are expressed only in liver or more abundantly in liver than in other tissues. These included RNAs for albumin, transferrin, $A_1AT$, and PepCK. Expression of these four liver-specific genes was also measured in liver and two rat hepatoma cell lines.

We previously reported that CWSV1 and CWSV2 produced albumin RNA at high levels (57). In this study, we confirmed our previous finding and showed that CWSV14, CWSV16, and CWSV17 also produced high levels of albumin RNA (Fig. 1). All five cell lines were examined for the expression of three other liver-specific genes. We conclude from the northern blot analyses that (i) the transferrin, $A_1AT$, and PepCK RNAs in the CWSV cell lines were the same size as those found in liver and (ii) at low passage all five cell lines expressed the three RNAs at levels similar to those in the liver. The amount of each liver-specific RNA produced varied from one cell line to another and did not follow a consistent pattern. For example, the amount of PepCK RNA produced by CWSV1 was higher than that produced by CWSV2, while the amount of $A_1AT$ produced by CWSV2 was higher than that produced by CWSV1. It is interesting that the five CWSV cell lines produced higher levels of the four liver-specific RNAs than the two hepatoma cell lines. Also, each of the five CWSV cell lines produced levels of all four liver-specific RNAs similar to those found in the liver, whereas the H4IIEC3 cell line produced detectable levels of only albumin, transferrin, and PepCK and the McA-RH7777 hepatoma cell line produced detectable levels of only transferrin and $A_1AT$.

**Tumorigenicity of SV40-hepatocyte cell lines.** The five SV40-hepatocyte cell lines described above were tested for their ability to form tumors in syngeneic hosts. The cell lines at in vitro passage levels ranging from 12 to 61 were inoculated subcutaneously into newborn F344 rats (Table 1).

TABLE 1. Tumorigenicity of SV40-hepatocyte cell lines after subcutaneous transplantation into syngeneic neonatal rats

| Cell line | Passage no. | Tumor incidence[a] (%) | No. of: | | Metastases[b] |
|---|---|---|---|---|---|
| | | | Lethal tumors | Regressing tumors | |
| CWSV1 | 12–22 | 0 (0/16) | 0 | 0 | – |
| CWSV2 | 12–22 | 0 (0/15) | 0 | 0 | – |
| CWSV14 | 17 | 40 (4/10) | 1 | 3 | – |
| CWSV16 | 17 | 0 (0/9) | 0 | 0 | – |
| CWSV17 | 12–16 | 18 (3/17) | 1 | 2 | – |
| CWSV1 | 52–61 | 32 (6/19) | 2 | 4 | + |
| CWSV2 | 46–48 | 48 (11/23) | 5 | 6 | + |
| CWSV14 | 33–47 | 60 (6/10) | 4 | 2 | + |
| CWSV16 | 33 | 78 (7/9) | 4 | 3 | + |
| CWSV17 | 37 | 50 (6/12) | 4 | 2 | – |
| CWSV2T1[c] | 2–13 | 82 (23/28) | 15 | 8 | + |
| CWSV14T1[c] | 3 | 83 (10/12) | 10 | 0 | + |
| CWSV17T1[c] | 12 | 100 (9/9) | 7 | 2 | – |

[a] In parentheses is shown the number of tumors/number of animals injected.

[b] Metastases of primary tumor to lung, kidney, or liver.

[c] Tumor cell lines derived from tumors induced by SV40-hepatocyte cell lines.

Only two of the five cell lines (CWSV14 and CWSV17) at low passage (12 to 22) produced tumors. Less than 50% of the animals injected (40% for CWSV14 and 18% for CWSV17) developed tumors, and these were observed 1 to 2 months after inoculation. Most tumors grew to sizes ranging from 5 to 18 $cm^3$ and subsequently regressed. The tumors that regressed did not reappear in animals maintained for 4 months after tumor regression. No metastases developed in animals inoculated with low-passage cells.

All five of the SV40-hepatocyte cell lines at higher passage levels (33 to 61) were tumorigenic. Approximately 50% of the tumors grew progressively and killed the hosts. The remaining tumors grew to a size of approximately 23 $cm^3$ and regressed. Four of the five cell lines produced metastases.

**Morphology of tumors induced by SV40-hepatocyte cell lines.** The histologic appearance of the tumors induced by SV40-hepatocyte cell lines varied depending on the cell line. A comparison of the morphologies of tumors that arose after inoculation of three different CWSV cell lines showed that CWSV1 cells produced an undifferentiated tumor (Fig. 2A), whereas CWSV2 (Fig. 2B, C, and D) and CWSV14 (Fig. 3A and B) cells yielded well-differentiated hepatocellular carcinomas. The tumor cells in the hepatocellular carcinomas resembled hepatocytes in normal liver in that they were cuboidal and were arranged in some areas of the tumors in well-developed cords (Fig. 2B and C, 3A and B). The cells in the tumors differed from those in normal liver in that (i) the nuclei in the tumor cells were more eccentric and (ii) the cords were multilayered. In other areas of the same tumors, the cells were organized into well-differentiated ducts composed of cuboidal epithelium (Fig. 2D). In an animal inoculated subcutaneously with CWSV2, a lung metastasis arose that morphologically resembled the primary tumor (i.e., a hepatocellular carcinoma, Fig. 3C). Most tumors contained areas of necrosis, particularly in the central region of the tumor.

**Liver-specific gene expression in tumors induced by SV40-hepatocyte cell lines.** RNA extracted from tumors induced by the SV40-hepatocyte cell lines was analyzed by the northern blot technique for expression of the albumin, transferrin, $A_1AT$, and PepCK genes (Fig. 4). It was possible to determine qualitatively whether a specific gene was expressed in the tumor by examining the autoradiograms, but quantitative analyses were approximate since the tumor tissue contained variable numbers of other cell types (lymphocytes, macrophages, fibroblasts) and necrotic tumor cells. In general, the level of expression of the liver-specific genes in the tumors was diminished relative to that in the low-passage cell lines. The tumor induced by CWSV14 cells expressed all four liver-specific genes at the highest level. All four liver-specific genes also were expressed by tumors induced by the CWSV16 and CWSV17 cell lines, but expression of $A_1AT$ RNA was markedly diminished in both tumors. Albumin, transferrin, and $A_1AT$ RNAs were expressed by the CWSV2 tumor, but PepCK RNA was not detectable. The tumor induced by the CWSV1 cells was morphologically undifferentiated and expressed low levels of albumin, transferrin, and PepCK RNAs, but $A_1AT$ RNA was not detectable.

**Effect of in vitro passage on liver-specific gene expression in SV40-hepatocyte cell lines.** We wanted to determine whether the alterations in expression of the liver-specific genes detected in the tumors relative to that in low-passage cells in culture were a reflection of changes that had occurred during in vitro passage. The RNA levels for albumin, transferrin, $A_1AT$, and PepCK reported above (Fig. 1) were obtained by using RNA extracted from low-passage cells. RNA was extracted from higher-passage cells and analyzed by the northern blot technique; RNA extracted from liver was used as a control (Fig. 5). A comparison of the northern blot analyses for the low- and higher-passage cell lines indicated that (i) albumin expression diminished only slightly, if at all, in higher-passage CWSV1, CWSV2, and CWSV17 cell lines but diminished markedly in higher-passage CWSV14 and CWSV16 cell lines; (ii) transferrin expression was only slightly decreased with passage; (iii) $A_1AT$ RNA levels diminished in all the cell lines except CWSV1; and (iv) PepCK RNA continued to be expressed at high levels only in the CWSV14 cell line.

A comparison of liver-specific gene expression in the higher-passage CWSV cells with the tumors induced by these cells led to several interesting observations. Although albumin and $A_1AT$ RNAs continued to be expressed at high levels in high-passage CWSV1 cells, albumin RNA was barely detectable and $A_1AT$ RNA was not detectable in the CWSV1 tumor. In contrast, PepCK was not detectable in either higher-passage CWSV1 cells or the CWSV1 tumor. Very different results were obtained with CWSV14 cells. Albumin RNA levels were low in higher-passage CWSV14 cells, but the highest albumin expression in the tumors produced by the five CWSV cell lines was in the CWSV14 tumor. $A_1AT$ RNA was barely detectable in higher-passage CWSV14 cells but was expressed at almost liver levels in the CWSV14 tumor. PepCK was expressed at high levels in higher-passage CWSV14 cells and in the CWSV14 tumor. We conclude that (i) changes in liver-specific gene expression during cell passage varied depending on the specific cell line studied, (ii) some changes in liver-specific gene expression in the tumors, such as those for the PepCK gene, paralleled the changes that occurred with in vitro passage; and (iii) some changes in liver-specific gene expression in the tumors, such as those for the albumin gene, did not parallel the changes that occurred with in vitro passage.

**Expression of c-Ha-*ras*, c-*raf*, and GST-P in SV40-hepatocyte cell lines.** Since the tumorigenic potential of the CWSV cell lines increased with in vitro passage, the cell lines were examined at low and high passage for expression of two

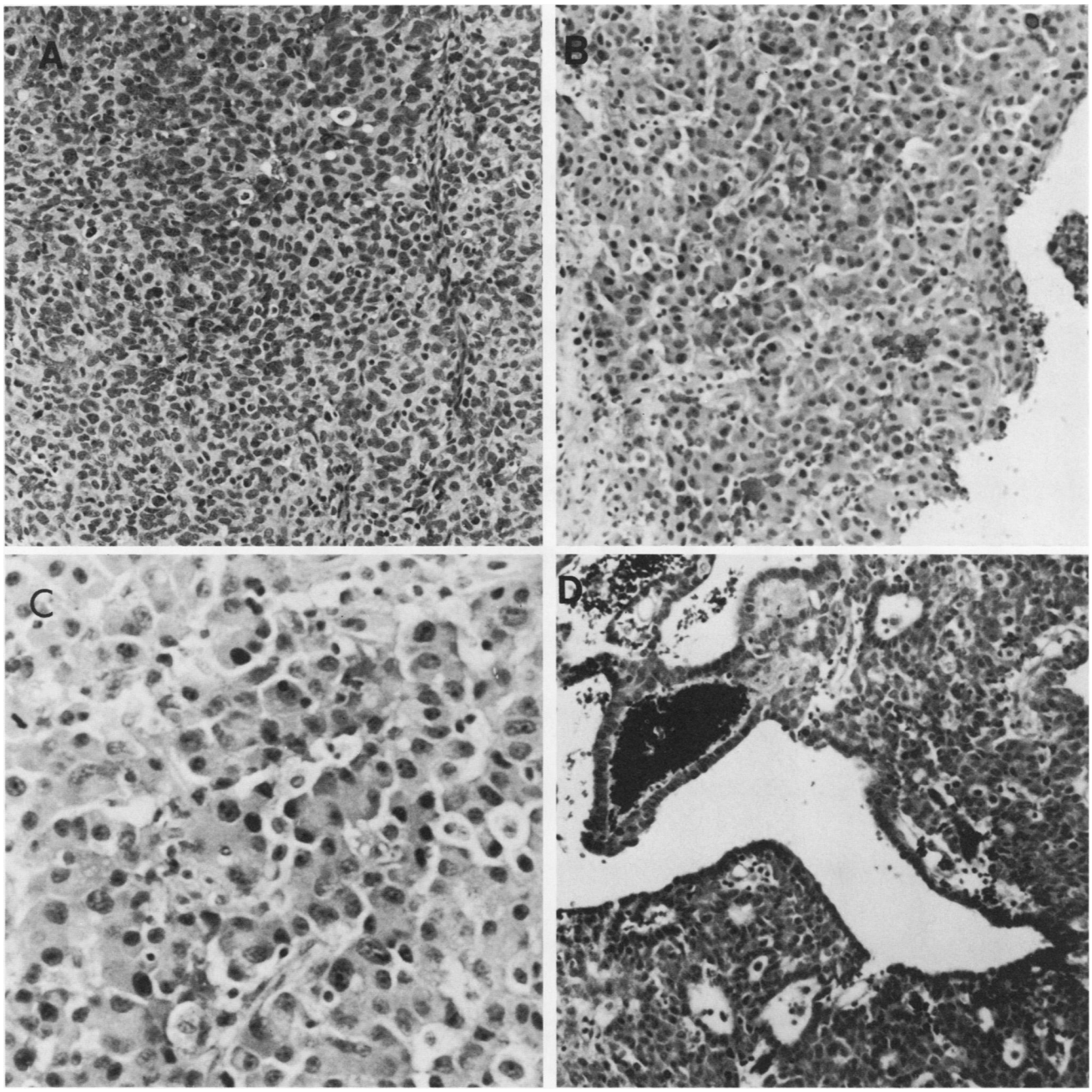


FIG. 2. Comparison of the morphologies of primary tumors that arose in newborn syngeneic rats injected with CWSV1 and CWSV2 cells. (A) Photomicrograph of a fixed section of tumor induced by CWSV1 cells. The tumor was predominantly composed of undifferentiated epithelial cells. (B) Photomicrograph of a tumor induced by CWSV2 cells, illustrating that the tumor was a well-differentiated hepatocellular carcinoma. (C) Higher magnification of the CWSV2 tumor, showing the cells aligned in well-developed cords and resembling the organization of hepatocytes in normal liver. (D) Photomicrograph showing a section of the CWSV2 tumor in which the cells were organized into well-differentiated ducts composed of cuboidal epithelium.

oncogenes and a gene associated with hepatocarcinogenesis. RNA was extracted from low- and high-passage cells and examined for expression of c-Ha-*ras*, c-*raf*, and GST-P. Increased expression of activated c-Ha-*ras* has been observed in many tumors and has been associated with a more malignant phenotype and increased metastatic potential. In liver, elevated expression of c-Ha-*ras* RNA has been observed during liver development (60), during liver regeneration (15, 18), in carcinogen-treated liver (28), in rat liver tumors induced by carcinogens (20), and in chemically induced and spontaneously occurring mouse liver tumors (14). Activation of c-*raf* has been observed in a human gastric cancer (43), a glioblastoma (16), and chemically induced and spontaneously occurring mouse liver tumors (36), as determined by DNA transfection into NIH 3T3 cells. The P isoenzyme of GST is barely detectable in normal liver but is induced early in the development of preneoplastic liver nodules during chemically induced carcinogenesis and is expressed in hepatocellular carcinomas (39, 40, 45).

c-Ha-*ras* RNA was not detected in low-passage CWSV1

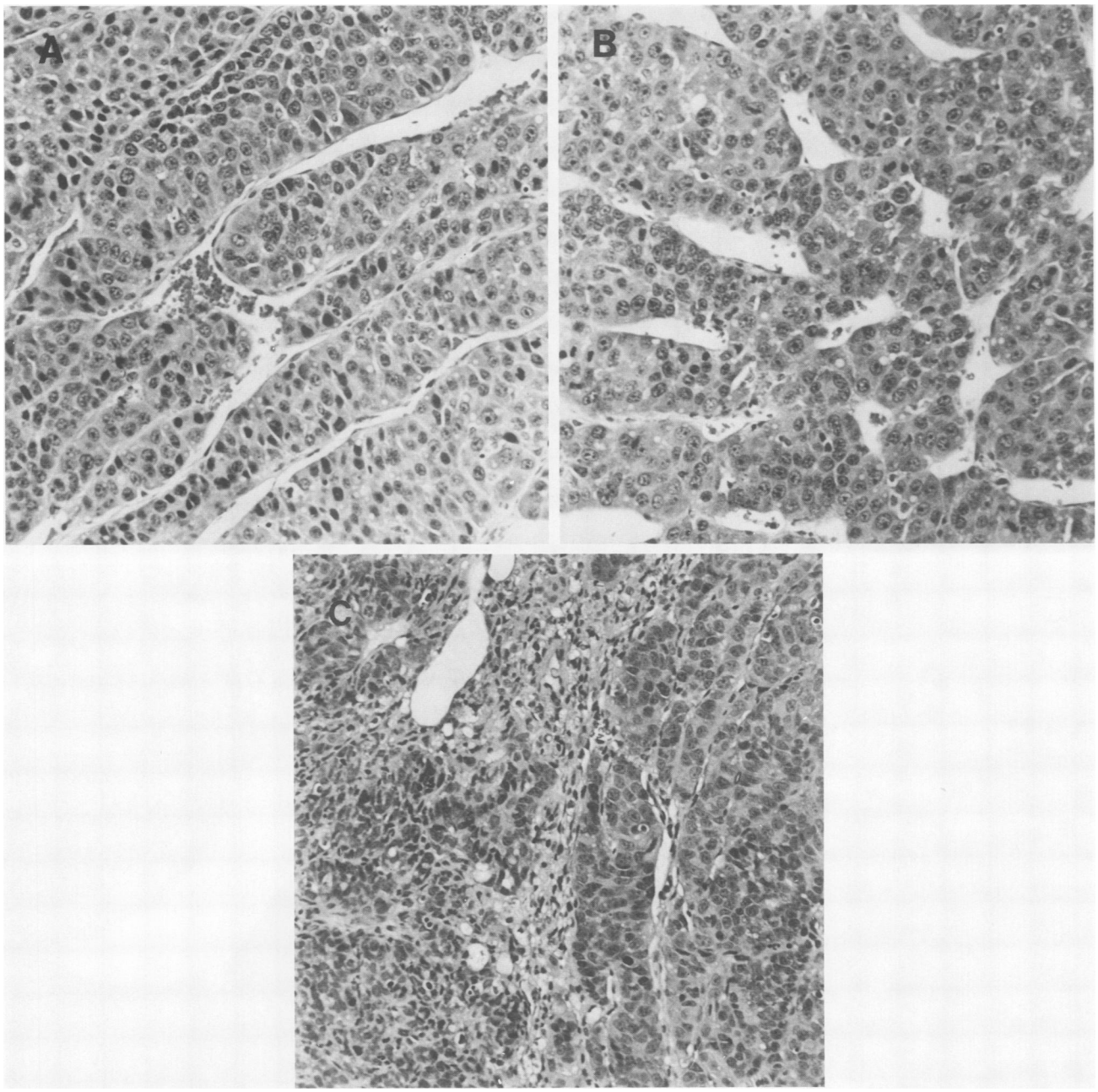


FIG. 3. Morphology of a primary tumor and metastatic tumor that arose in newborn syngeneic rats injected with SV40-hepatocyte cell lines. (A) Photomicrograph of a tumor induced by CWSV14 cells, illustrating that the tumor was a well-differentiated hepatocellular carcinoma. (B) Higher magnification of CWSV14 tumor. (C) Photomicrograph of a lung metastasis from an animal injected with CWSV2 cells. Hemotoxylin and eosin stain. Magnification, ×200.

or CWSV2 cells (Fig. 6); however, at higher passages, c-Ha-*ras* RNA was expressed. c-Ha-*ras* RNA was expressed in CWSV14, CWSV16, and CWSV17 cells at both low and high passage. It is of interest that low-passage CWSV1 and CWSV2 cells were not tumorigenic, whereas CWSV14 and CWSV17 cells, even at low passage, were weakly tumorigenic. In contrast, c-Ha-*ras* was also induced in early-passage CWSV16 cells, which were not even weakly tumorigenic at low passage. With regard to the c-*raf* oncogene, expression was higher in the CWSV cell lines than in liver, was approximately the same in the CWSV cell lines as in the two hepatoma cell lines, and did not change with in vitro passage of the CWSV cell lines. The level of GST-P RNA was higher in the CWSV cell lines than in liver and increased with passage only in the CWSV1 cell line. It was not surprising that GST-P was already expressed by low-passage CWSV1 cells, since GST-P-positive cells are apparent early after initiation and continue to be expressed throughout the carcinogenic process in animal models of hepatocarcinogenesis.

**Expression of SV40 RNA in tumors induced by SV40-hepatocyte cell lines.** SV40 expression was readily measurable in the five tumors and was not detected in liver or the hepatoma cell lines (data not shown). Detection of SV40 information in RNA extracted from the tumors showed that

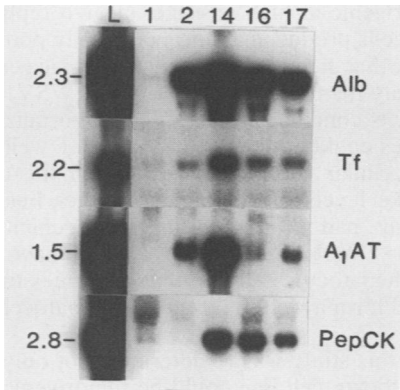


FIG. 4. Northern blot analysis of liver-specific RNAs in tumor tissue. Total cellular RNA was extracted from normal rat liver (L) and five primary tumors removed from rats inoculated with five SV40-hepatocyte cell lines: 1, CWSV1; 2, CWSV2; 14, CWSV14; 16, CWSV16; and 17, CWSV17. Northern blot analysis was carried out as described in the legend to Fig. 1 with the previously mentioned probes.

the tumors were induced by inoculation of the SV40-hepatocyte cell lines and did not arise spontaneously. SV40 RNA was also detected in both low- and high-passage cell lines (data not shown).

**Expression of AFP in SV40-hepatocyte cell lines and tumors.** As determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitated radioactively labeled medium, at low passage, 11 of the 12 SV40-hepatocyte cell lines produce albumin and none of the 12 cell lines produce AFP (57). Similarly, CWSV1 and CWSV2 produce albumin RNA but do not produce AFP RNA. When RNA extracted from the tumors induced by each of the five different SV40-hepatocyte cell lines was subjected to northern blot analysis, we found that AFP RNA was expressed only by the CWSV14 tumor (data not shown). We wanted to determine (i) whether the expression of AFP RNA was only detectable after in vivo passage of the CWSV14 cell line or whether AFP was detectable in the higher-passage CWSV14 cells inoculated into the animal to produce the tumor and (ii) whether the one CWSV14 tumor examined was unique in its ability to express AFP RNA or

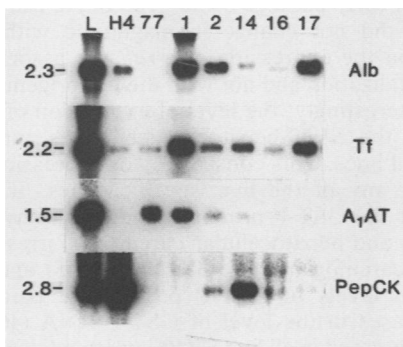


FIG. 5. Northern blot analysis of liver-specific RNAs expressed by high-passage SV40-hepatocyte cell lines. Total cellular RNA was extracted from liver (L), the two hepatoma cell lines, H4IIEC3 (H4) and McA-RH7777 (77), and five SV40 hepatocyte cell lines: 1, CWSV1; 2, CWSV2; 14, CWSV14; 16, CWSV16; and 17, CWSV17. The five SV40-hepatocyte cell lines ranges in passage number from 33 to 64. Northern blot analysis was carried out as described in the legend to Fig. 1 with the previously mentioned probes.

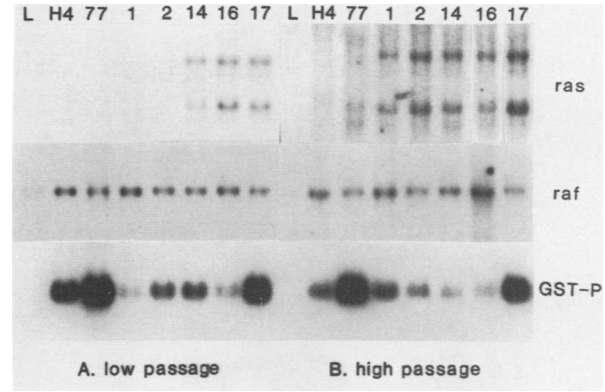


FIG. 6. Expression of c-Ha-*ras*, c-*raf*, and GST-P RNAs in (A) low- and (B) high-passage SV40-hepatocyte cell lines. Total cellular RNA was extracted from liver (L), the two hepatoma cell lines, H4IIEC3 (H4) and McA-RH7777 (77), and five SV40 hepatocyte cell lines: 1, CWSV1; 2, CWSV2; 14, CWSV14; 16, CWSV16; and 17, CWSV17. Low-passage SV40-hepatocyte cell lines ranged from passage 14 to 18, and high-passage SV40-hepatocyte cell lines ranged from passage 33 to 64. Northern blot analysis was carried out as described in the legend to Fig. 1. The probes detected c-Ha-*ras* (*ras*), c-*raf* (*raf*), and GST-P RNAs.

whether this result was reproducibly seen in other tumors induced by the CWSV14 cell line. Because the CWSV2 cell line produced tumors similar to those produced by CWSV14 cells, in that they were morphologically well-differentiated hepatocellular carcinomas, but dissimilar, in that the CWSV2 tumor did not express AFP RNA, parallel studies were carried out with CWSV2 cells.

RNA was extracted from CWSV14 and CWSV2 cell lines at several in vitro passage levels, from two or three tumors induced by these cell lines, and from tumor cell lines. Analysis of the northern blots (Fig. 7) showed that (i) AFP RNA was expressed by high-passage CWSV14 cells, (ii) AFP RNA expression was considerably higher in CWSV14 tumors than in high-passage CWSV14 cells, (iii) three independently prepared CWSV14 tumors expressed high levels of AFP RNA, (iv) albumin RNA expression diminished in CWSV14 cells at higher passage, (v) AFP RNA was not expressed in two independently generated CWSV2 tumors or in high-passage CWSV2 cells, and (vi) the level of albumin RNA expressed in CWSV2 cells did not diminish with passage.

## DISCUSSION

We demonstrated in this report that SV40-hepatocyte cell lines produce well-differentiated hepatocellular carcinomas when inoculated into syngeneic rats, and as such, these represent the only cell lines derived from primary rat hepatocyte cultures that have this property. The liver has been used to study the development of cancer because a great body of knowledge exists about its pathology, biochemistry, and molecular biology. The procedures frequently used to experimentally induce carcinogenesis (44, 62) are complex, and it is difficult to assess the direct effects on liver cells because the treatments are administered to the whole animal and in many cases include a combination of different treatments. In the SV40-hepatocyte system, the viral genetic information is introduced into hepatocytes in vitro, thereby eliminating extraneous influences present in the whole animal. In vitro passage of the cells is required to transform the immortalized cells to a tumorigenic phenotype. Although the

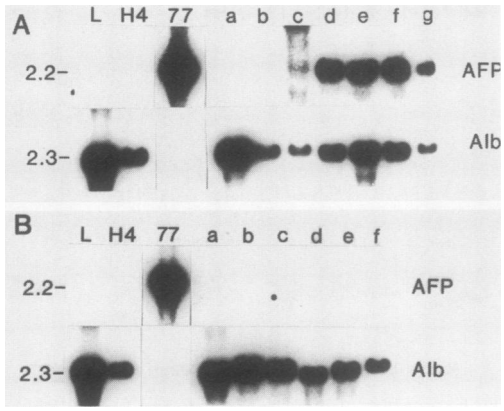


FIG. 7. Expression of albumin and AFP in SV40-hepatocyte cell lines, tumors, and tumor cell lines. (A) Expression of albumin and AFP in CWSV14 cells, tumors induced by CWSV14 cells, and CWSV14 tumor cell lines. Total cellular RNA was extracted from liver (L), the two hepatoma cell lines, H4IIEC3 (H4) and McA-RH7777 (77), CWSV14 passage 16 (a), CWSV14 passage 35 (b), CWSV14 passage 54 (c), CWSV14 tumor 1 (d), CWSV14 tumor 2 (e), CWSV14 tumor 3 (f), and CWSV14 tumor cell line (g). Northern blot analysis was carried out as described in the legend to Fig. 1. The probes detected albumin (Alb) and AFP RNAs. (B) Expression of albumin and AFP in CWSV2 cells, tumors induced by CWSV2 cells, and CWSV2 tumor cell lines. Total cellular RNA was extracted from liver (L), the two hepatoma cell lines, H4IIEC3 (H4) and McA-RH7777 (77), CWSV2 passage 12 (a), CWSV2 passage 19 (b), CWSV2 passage 46 (c), CWSV2 tumor 1 (d), CWSV2 tumor 2 (e), and CWSV2 tumor cell line (f). Northern blot analysis was carried out as described in the legend to Fig. 1. The probes detected albumin (Alb) and AFP RNAs.

use of SV40 genetic information to induce hepatocarcinogenesis appears to be a multistep phenomenon, the process that eventually results in the development of a hepatocellular carcinoma can be initiated by the single step of introducing a defined piece of SV40 DNA into a liver cell in culture.

SV40 transforms hamster, mouse, and rat cells in vitro. Transformed hamster cells do not need to be passaged in vitro to be tumorigenic (7); in contrast, SV40-transformed mouse cells will induce tumors in test animals only after prolonged culture in vitro (26, 47, 48, 53). In this study, the number of in vitro passages required for differentiated SV40-rat hepatocyte cell lines to become tumorigenic varied, but in general, a minimum of 30 to 40 passages was required before the cells became strongly tumorigenic. The fact that some SV40-hepatocyte cell lines become tumorigenic with in vitro passage provides a system in which the cells can be examined for alterations in gene expression that occur in parallel with conversion to tumorigenicity.

We previously noted diversity among the SV40-cell lines with regard to the amount of albumin produced per cell, the manner in which albumin expression was regulated, and the other liver-specific proteins produced (57). As we characterized the cell lines further, the diversity continued to be observed; the cell lines differed in (i) the level of expression of liver-specific genes, oncogenes, and genes associated with hepatocarcinogenesis in low-passage cells, (ii) the effect of in vitro passage on the expression of these genes, and (iii) the passage number at which the cell lines became tumorigenic. The diversity in the properties of the cell lines pointed to the need to characterize more than one line. For example, although the CWSV1 cells continued to produce levels of albumin similar to those found in the liver when the cells became tumorigenic at high passage, in vivo replication of high-passage cells produced a morphologically poorly differentiated tumor that did not express liver-specific genes. If we had studied only the tumorigenicity of the CWSV1 cell line, we would have concluded that SV40-immortalized hepatocyte cell lines could not be used to induce a well-differentiated hepatocellular carcinoma even when the cells produced liver-like levels of albumin and other liver-specific genes. Similarly, had we studied the tumorigenicity of only the CWSV2 or CWSV14 cell line, we would have assumed that an SV40-hepatocyte cell line that continues to produce albumin would form a well-differentiated hepatocellular carcinoma.

The goal of this study was to determine not only whether an SV40-hepatocyte cell line could be tumorigenic but also whether the expression of specific genes was altered when the cells became tumorigenic. Certain properties of the SV40 hepatocyte cell lines not directly related to their tumorigenicity are critical to their usefulness in studying expression of liver-specific genes. (i) CWSV1 cells previously shown to produce liver-like levels of albumin RNA through passage 18 continued to do so through at least passage 64. (ii) Not all of the cell lines were stable in albumin production with time in culture. (iii) The levels of albumin and transferrin RNA measured for the five cell lines paralleled those reported previously for secreted albumin and transferrin protein. (iv) All five cell lines previously shown to produce albumin, transferrin, C3, and hemopexin also produced AAT and PepCK.

We conclude that when SV40-hepatocyte cell lines that express high levels of albumin and other liver-specific genes acquired a tumorigenic phenotype, they did not necessarily undergo any specific changes associated with expression of the four liver-specific genes analyzed. For example, transferrin RNA levels did not change markedly in any of the cell lines, and albumin RNA expression did not change in three of the cell lines but diminished in two of the cell lines. The changes in liver-specific gene expression observed in the cell lines as they became tumorigenic were unique to each cell line and are consistent with the general concept that each cell line has its own biological and molecular properties.

We did not observe any correlation between the expression of the c-*raf* and GST-P RNAs and tumorigenicity of the SV40-hepatocyte cell lines. The oncogene c-*raf* was expressed at low levels in normal liver. The expression of c-*raf* was higher in early-passage SV40 hepatocyte cell lines than in liver but did not change in magnitude with passage, indicating that the expression of c-*raf* was associated with SV40 immortalization and not with the tumorigenicity of the cell lines. Interestingly, the level of expression of c-*raf* was the same in the SV40-hepatocyte cell lines and the two hepatoma cell lines. This consistency of expression was not observed for any of the liver-specific genes. It has been reported that the GST-P protein is induced in hyperplastic liver nodules and hepatocellular carcinomas irrespective of the kind of carcinogen used (33, 39, 40, 45) and that the alterations in levels of GST-P protein are paralleled by similar increases in the level of GST-P RNA (46). GST-P RNA was expressed in all five SV40-hepatocyte cell lines but not in liver, suggesting that GST-P RNA was induced directly by SV40 genetic information or secondarily to SV40-induced proliferation of the cells. The expression of GST-P in the SV40-hepatocyte cell lines more closely resembled that of the liver-specific genes than the oncogene c-*raf* in that (i) the cell lines varied in the amount of GST-P RNA expressed and (ii) the changes in GST-P RNA levels with

passage were specific to each cell line. In the CWSV1 cells, expression of GST-P increased slightly with passage, whereas in the CWSV14 cell line, the GST-P RNA levels decreased in high-passage cells. All of the SV40-hepatocyte cell lines except CWSV17 expressed lower levels of GST-P RNA than the hepatoma cell lines.

The expression of c-Ha-*ras* paralleled tumorigenic potential in four of the five cell lines. Increased expression of c-Ha-*ras* RNA has been observed in experimentally induced primary rat liver tumors (12, 23, 60), in four rat hepatoma cell lines (28), in hepatocellular carcinomas that arose spontaneously in C3Hf mice (14), and in tumors induced in B6C3 $F_1$ mice treated with diethylnitrosamine (14). It has been suggested that c-Ha-*ras* activation is an early event in B6C3 $F_1$ mouse hepatocarcinogenesis, because activated c-Ha-*ras* oncogenes have been identified in spontaneous liver tumors and chemically induced tumors in B6C3 $F_1$ mice with the NIH 3T3 cell transfection assay (56). In contrast, data suggest that induction of c-Ha-*ras* is not an early event in rat liver hepatocarcinogenesis and may represent a variable secondary alteration that occurs during the multistage process of hepatocarcinogenesis (5). In one study (5), the level of c-Ha-*ras* RNA in gamma-glutamyltranspeptidase-positive foci in rats treated with diethylnitrosamine after dietary exposure to phenobarbital was no greater than in surrounding normal hepatocytes, and in a separate study (60), the expression of c-Ha-*ras* RNA was not elevated in the isolated oval cells and bile duct cells during hepatocarcinogenesis induced by a choline-deficient diet. At least two groups have also reported that induced expression of c-Ha-*ras* in rat liver tumors is variable (5, 20). Similarly, c-Ha-*ras* expression has not been detected in the human hepatoma cell line $HLD_2$-6 (derived from HepG2) or in tumors induced in nude mice by this cell line (21).

In the SV40-hepatocyte system, we did not observe a correlation between immortalization and c-Ha-*ras* expression, but we did observe a correlation between the ability of SV40-hepatocyte cell lines to proliferate in vivo and c-Ha-*ras* expression. c-Ha-*ras* RNA was not induced in low-passage CWSV1 or CWSV2 cells, but was induced in high-passage CWSV1 and CWSV2 cells, correlating directly with the finding that these lines were not tumorigenic at low passage but were tumorigenic at high passage. Similarly, c-Ha-*ras* RNA was elevated in both low- and high-passage CWSV14 and CWSV17 cells, which at low or high passage produced tumors in test animals. The ability of the CWSV14 and CWSV17 cell lines to consistently produce progressive tumors was enhanced with repeated passage, and this change was not accompanied by a further increase in the expression of c-Ha-*ras*. No correlation between c-Ha-*ras* expression and tumorigenicity was observed for the CWSV16 cell line.

It was recently demonstrated that c-Ha-*ras* transcription is induced by SV40 large T antigen in a cell line containing the gene for SV40 T antigen under hormone-dependent control (41). This finding contrasts with a published report showing that c-Ha-*ras* RNA is expressed equally in normal and SV40-transformed cells (55). It is of interest that we detected two c-Ha-*ras* RNA species in the SV40-hepatocyte cell lines. The RNA species at 1.4 kilobases was the size usually reported when a c-Ha-*ras* DNA probe is used. The larger species was previously observed in cells expressing SV40 RNA (41).

Although the CWSV1 cells produced high levels of albumin, the CWSV1 tumor was morphologically undifferentiated and expressed minimal levels of liver-specific RNAs but no AFP RNA. In contrast, the AFP mRNA was expressed at high levels in the CWSV14 tumor, which was morphologically well differentiated and expressed high levels of albumin, transferrin, $A_1AT$, and PepCK RNAs. From the number of tumors we examined, there appears to be a relationship between tumor morphology and expression of adult liver-specific genes, but the expression of AFP RNA is not necessarily associated with an undifferentiated tumor morphology.

The albumin and AFP genes are important for studying gene regulation during normal development of mammalian liver and oncogenesis. AFP is the major serum protein of the mammalian fetus (2), and AFP RNA constitutes 20% of the total RNA in the visceral endoderm of the yolk sac, 5% in fetal liver, and less than 0.1% in the fetal gut (3, 6, 49). The levels of AFP RNA decline dramatically just prior to birth (38, 50), but the AFP protein can reappear in liver neoplasia and in germinal tumors (1, 42). Albumin is the major serum protein in the adult, and the plasma concentration of albumin increases at the same time that the concentration of AFP declines. The relationship that exists between the albumin and AFP genes at the biological level has also been observed at the molecular level. A comparison of the structure of the AFP and albumin genes suggests that they evolved by intragenic duplication of a common ancestral gene followed by intergenomic duplication and mutational events (17, 19, 22, 30, 31, 51, 52, 54). We have shown in this study that AFP production and albumin RNA production are coordinately regulated in CWSV14 cells; that is, the levels of albumin RNA diminished and AFP RNA was induced with repeated passage of CWSV14 cells in culture. Because the expression of AFP RNA was amplified in the CWSV14 tumor and the CWSV14T1 tumor cell line expressed high levels of both AFP and albumin RNA, the CWSV14 cell line series (including the CWSV14T1 tumor cell line) can be used to define *cis*-acting regulatory elements for the AFP and albumin genes and the transcriptional factors that interact with these elements.

In this study, we further characterized the SV40-hepatocyte system. We originally showed that rat hepatocytes can be transfected with SV40 DNA to yield colonies of proliferating epithelial cells and that approximately 20% of these colonies contain cells that produce albumin (59). From this original finding, we have developed a series of SV40-hepatocyte cell lines (57). We refer to these cells as hepatocyte cell lines because they express many gene products of normal adult hepatocytes in intact liver or in long-term culture (25) and do not express AFP. We have no way of knowing whether the cells immortalized were normal adult hepatocytes or other liver cell types present in the primary hepatocyte culture. From this study, we do know that the cell type immortalized is capable of developing into a cell that can produce a well-differentiated hepatocellular carcinoma. From the beginning of the process, we studied only the cells that continued to produce reasonably high levels of albumin. We discarded 80% of the immortalized colonies because they did not produce high levels of albumin, and in so doing we may have discarded cell types that are important in hepatocellular carcinogenesis.

We expanded our knowledge of the properties of five SV40-hepatocyte cell lines and generated several tumor cell lines. Each cell line is valuable for different purposes. We have already described the significance of using the CWSV14 cell line and tumor cell line to study coordinate regulation of AFP and albumin production. To study regulation of the PepCK gene, the CWSV14 cell line would be the

most likely choice because it continues to express high levels of PepCK at high passage. Similarly, to study regulation of the $A_1AT$ gene, the CWSV1 cell line would be the best choice because it continues to express high levels of $A_1AT$ after many passages. Studies on the regulation of transcription of the albumin gene could be accomplished with almost any of the cell lines. The CWSV1 cell line is of value because it maintains constant expression of albumin RNA for numerous in vitro passages, and the CWSV14 and CWSV16 cell lines, which lose expression of albumin RNA with time in culture, are of value for studying *trans*-acting factors and the transcriptional mechanisms that regulate albumin expression.

In conclusion, we developed a series of cells progressing from normal hepatocytes to immortalized cells to tumor cell lines that can be used at the biological or molecular level to investigate (i) changes in the cell that accompany tumorigenesis and (ii) regulation of expression of specific genes, including those associated with differentiation.

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