Tumorigenicity and Transcriptional Modulation of c-myc and N-ras Oncogenes in a Human Hepatoma Cell Line

Brian E. Huber, Kerry L. Dearfield, Jerry R. Williams, Carole A. Heilman, and Snorri S. Thorgeirsson

ABSTRACT

Tumorigenicity and oncogene expression were examined in HepG2 derived cells, a human hepatoma cell line. HepG2 cells and a single cell clonal HepG2 line, HLD2-6, were equally tumorigenic when injected s.c. into athymic nude mice. Cyclophosphamide pretreatment of both cell lines (500 µg cyclophosphamide/ml/two cell cycles) had no effect on tumor incidence or latency (P > 0.05). Tumors were nonencapsulated, highly invasive adenocarcinomas and were positive for γ-glutamyltranspeptidase activity and bile production. Plasma from tumor-bearing mice was positive for human α-fetoprotein and negative for hepatitis B virus surface antigen as measured by radioimmunoassay. Two cell lines reestablished into tissue culture from HLD2-6 derived tumors had unaltered cell cycle times. Detailed in vitro translation analysis of RNA isolated from HLD2-6 derived cells and tumors were extremely similar to the translation products of RNA isolated from a normal human liver sample except for a M, 53,000 polypeptide with an apparent charge shift. c-myc specific transcripts, when compared to a normal human liver sample, were increased in all HLD2-6 cell lines and tumors derived from HLD2-6 cells. This increase in c-myc expression could not be explained by gene amplification or hepatitis B virus integration. N-ras specific transcripts were not elevated in HLD2-6 cells grown in tissue culture but there was a selective increase of the 5.5-kilobase N-ras transcript in HLD2-6 derived tumors grown in nude mice. This increased 5.5-kilobase transcript did not remain elevated if the tumors were reestablished into tissue culture, suggesting some interaction with the host animal. c-Ha-ras expression could not be detected in any HLD2-6 derived tumor or cell line.

INTRODUCTION

Recent experiments using chemically induced primary rodent liver tumors or established rodent hepatoma cell lines have implicated the role of cellular protooncogenes in HCC. Protooncogenes, homologous to part of the retroviral genomes (v-oncogenes), have been shown to be highly conserved genetic elements in nonviroically infected cells (for a review, see Refs. 1 and 2). Quantitative or qualitative alterations in expression of certain protooncogenes have been implicated in neoplastic growth (for a review, see Refs. 3 to 5) as well as nonneoplastic processes such as embryonic development (6, 7) and liver regeneration (8, 9). Makino et al. (10), using v-oncogene probes, have shown that c-Ha-ras and c-myc are elevated in 3’-methyl-4-dimethylamino-azobenzene induced primary rat liver tumors as well as in four rat hepatoma cell lines. They suggest that quantitative changes in the expression of c-Ha-ras and c-myc in rat hepatocytes are associated with proliferation and hepatocarcinogenesis, respectively (10). Additional studies of c-myc expression have shown that in three Morris hepatoma cell lines (11) c-myc expression is increased, with one cell line having the gene amplified 5- to 10-fold (12).

The sequence of cellular events leading to human HCC are not yet understood, although epidemiological studies suggest that HBV and aflatoxin could be etiological factors (13, 14). However, the possible role of protooncogenes and/or v-oncogenes on both the formation and the maintenance of human HCC is at present unclear. It has been suggested that HBV may increase the expression of one or more protooncogenes by inserting itself in a critical regulatory position controlling the transcription of those protooncogenes (15).

The present work was undertaken to examine the relationship between tumorigenicity and expression of c-myc, N-ras, and c-Ha-ras in the human hepatoma cell line HepG2 (16). Furthermore we have compared the data from the HepG2 cells with corresponding results obtained from normal human liver.

MATERIALS AND METHODS

Cell Lines. HepG2 is a human cell line derived from a hepatoblastoma (16). This cell line retains some biosynthetic capabilities of normal liver parenchymal cells (16, 17) as well as metabolic activating capacity for a variety of chemicals such as CY (18), benzo(a)pyrene (19), and diethylstilbestrol (20). The growth conditions for these cells have been described elsewhere (18).

HLD2-6 (HepG2 Low Density passage 2, clone 6) is a cell line derived from HepG2 cells after undergoing two successive low density passages. After the second low density passage, 96-well plates were inoculated with an average of 0.9 cell/well. Clones originating from one cell were expanded and frozen. Clone 6 was maintained in continuous culture for experimentation.

HLD2-6 CY and HLD2-6 CO are cell lines reestablished in tissue culture from tumors grown in athymic nude mice from s.c. injected HLD2-6 cells (see below for inoculation procedures).

These three low density cell lines were grown in the same medium as HepG2, but with antibiotics added (penicillin, 50 units/ml; streptomycin, 50 µg/ml).

In Vitro Drug Treatment. CY (Cytosar; Mead Johnson, Evansville, IN) dissolved in MEM (GIBCO) and was immediately added to cell...
cultures at a final concentration of 500 µg CY/ml. It had previously been established that this dose of CY does not affect cell viability or replication kinetics (18).

Transplantation into Athymic Nude Mice. HepG2 and HLD2-6 control and treated cells (two cell cycles with 500 µg CY/ml) were harvested in 0.3 ml serum free MEM and inoculated s.c. into the flank of male nude mice (atheymic NCR-NU strain; National Institutes of Health Animal Supply). When tumors were greater than 2 cm, the animals were sacrificed and sections of the tumors were removed for histological evaluation (see below). Blood was collected from the descending aorta using a heparinized syringe and plasma obtained after centrifugation. The plasma was below). Blood was collected from the descending aorta using a heparinized syringe and plasma obtained after centrifugation. The plasma was

Histology. Sections of tumors were fixed in Bouin’s solution, processed for paraffin embedding, and stained with hematoxylin and eosin or H&H’s stain for bile (normal rat liver sections were used as a positive bile control). Additional tumor sections were prepared as frozen sections for histochemical staining for GGT activity as described previously (22) (preneoplastic rat liver nodules produced by the Solt-Farber technique (23, 24) were used as a positive GGT control).

Establishment of Cell Lines Derived from Tumors. Tumors were washed and trimmed of excess tissue in phosphate-buffered saline (Ca²⁺ free, containing gentamicin, 100 units/100 ml phosphate-buffered saline). The tumor was then incubated in 20 ml MEM with 0.05% collagenase, 0.10% hyaluronidase, and Pen/Strep at 37°C with 95% O₂-5% CO₂ for 60 min. After incubation the tissue was passed successively through 265 and 65-μm sterile nylon filters and centrifuged at 350 rpm for 3 min. The pellet was resuspended in MEM-10% fetal bovine serum with Pen/ Strep. HLD-6 colonies were further isolated using cloning rings.

RNA Isolation and Northern Blot Analysis. RNA was isolated from cell cultures and tumors which were extensively trimmed to assure the removal of all mouse tissue. In addition RNA was isolated from a normal human liver sample obtained from a kidney transplant donor who was maintained on life support systems until kidney removal and lobectomy (supplied by D. Davies and A. Boobis, Department of Clinical Pharmacology, Royal Postgraduate Medical School, London, England). Histological evaluation of this human liver sample showed no premalignant changes.

Approximately 6 x 10⁶ log phase cells or 2 g of tissue were used for each isolate. RNA was isolated with guanidine thiocyanate by the method of Schweizer and Goerttler (25) and enriched for poly(A)RNA by oligo-decorhymidylate cellulose chromatography (26). Electrophoresis of poly(A)RNA samples on horizontal denaturing formaldehyde agarose gels with subsequent transfer to nitrocellulose membranes was performed as described previously (27). Membranes were hybridized to ³²P-nick-translated DNA probes (28) (specific activity > 5 x 10⁶ cpm/µg DNA) for 17 h at 42°C in 50% deionized formamide, 5 x SSC, 10 x 0.02% Ficoll 400-0.02% bovine serum albumin-0.02% polyvinylpyrrolidine-360, 5 x 0.01 sodium phosphate buffer (pH 7.4), and 0.1% SDS with yeast tRNA carrier (0.5 mg/ml) (29). Slot blot analysis was performed with the apparatus and procedures provided by Schleicher and Schuell (Keene, NH). Unless otherwise stated blots were washed at high stringency (2 x SSC-0.1% SDS at room temperature for 30 min with a total of three changes and 0.1 x SSC-0.1% SDS at 50°C for 30 min with a total of two changes), exposed to Kodak XAR-5 X-ray film with intensifying screens. The autoradiograms were analyzed using a Beckman DU-8 spectrophotometer.

DNA Probes. The recombinant plasmids used to analyze RNA or DNA sequences included the 1.5-kilobase EcoRI/ClaI human c-myc fragment (30), a 7-kilobase EcoRI N-ras insert from which a 1.5-kilobase PvuII/PvuII fragment was purified (31), and a 6.6-kilobase BamH1/BamH1 c-Ha-ras fragment (32). Hepatitis virus integration was analyzed on Southern blots by utilizing two BPV-1/pBR322 constructs containing the inserts of the DNA coding for the hepatitis B surface antigen. The kilobases, construct 63-2) or the hepatitis B core antigen (1.85 kilobases, construct 211-3) (33), supplied by N. Sarver.⁶

Sister Chromatid Exchange Studies. All cell lines were cultured and dosed for cytogenetic analysis according to procedures described previously (18). Briefly cell cultures were plated at 4.5 x 10⁶ cells/25-cm² flask and 24 h later BrdUrd (10 µg/ml; Sigma Chemical Co., St. Louis, MO) and CY (500 µg/ml), when indicated, were added in a final volume of 10 ml for approximately two cell cycles. Cultures with BrdUrd were shielded from exposure to white light. Colcemid (GIBCO) was added to a final concentration of 0.2 µg/ml for 3–4 h before culture termination. Cell harvest, slide preparation, and examination have been described previously (18). All slides were coded and SCE determinations were performed on 20–30 second replication mitoses. The replication index was determined from the percentage of mitotic cells that divided once, twice, and three or more times in the presence of BrdUrd (18). All SCE values were transformed by half-power to obtain an acceptable Gaussian distribution (34), thus allowing analysis by the two-tailed Z statistic for the transformed data.

In Vitro Translation Analysis. Poly(A)RNAs were translated in a cell free rabbit reticulocyte lysate system containing [³²P]methionine (New England Nuclear). Translation products were analyzed by two-dimensional polyacrylamide gel electrophoresis as described by O’Farrell (35). The gels were subjected to fluorography using ENHANCE (New England Nuclear) and Kodak XAR-5 X-ray film.

RESULTS

Tumorigenicity of HepG2 and HLD2-6 Cells. HepG2 cells, isolated from a well differentiated human hepatocellular carcinoma (17), were originally described as nontumorigenic when injected under the kidney capsule of athymic nude mice (16). Since this cell line can metabolize CY to reactive intermediates which interact with DNA (18), we proposed to treat cells with CY, monitor the effects on DNA by SCE induction, and examine whether this treatment protocol enhanced the tumorigenicity of these cells. Both the parent cell line HepG2 and the low density single cell clonal line HLD2-6 were used in this experiment. Contrary to earlier reports both untreated HepG2 cells and HLD2-6 were found to be tumorigenic when injected s.c. into athymic nude mice, with the tumorigenicity being dependent on the number of injected cells (Table 1; since no differences were found between the HepG2 and HLD2-6 cell lines these data were combined). CY had no statistically significant effect on the incidence or latency of tumor formation (P > 0.05, x² test) despite the interaction with DNA, as measured by SCE induction. Tumor-bearing mice had elevated human AFP plasma levels. No hepatitis B virus surface antigen was detected in the plasma of any animal. Gross examination of the liver, spleen, and lung detected no metastases in any tumor-bearing mouse at the time of sacrifice.

Tumor Morphology and Cell Cycle Characteristics of Tumor Derived Cells. After an initial latency period tumors grew slowly as a solid brown mass and had invasive properties penetrating into muscle and vasculature. No gross differences were seen between tumors derived from HepG2 cells or those derived from HLD2-6 cells. Histological examination of the tumors by light microscopy indicated that the tumors were an adenocarcinoma type with many glandular-like structures toward the periphery of the tumor (Fig. 1, A and B). Nucleoli were enlarged, darkly staining, and irregular. Cells were characterized by nuclear atypia

⁶ N. Sarver, unpublished data.
and multinucleated giant cells were commonly seen. Mitotic figures were numerous and evenly dispersed. Tumors were not encapsulated and were highly invasive into surrounding mouse tissue (Fig. 1C). Staining with Hall's stain indicated the presence of bilirubin and collagen (Fig. 1D). The most pronounced positive staining for bile pigments was found in the lumen of the glandular-like structures. Cryostat sections histochemically stained for GGT showed extensive positive staining evenly dispersed throughout the section (Fig. 1E). Staining was confined to round or oval aggregates and was not seen in regions of the plasma membrane.

Two cell lines were reestablished in culture from tumors derived from HLD2-6 cells. Cell cycle characteristics between these newly established cell lines (HLD2-6-CO, HLD2-6-CY) and the parent cell line (HLD2-6) were determined by measuring the replication index subsequent to BrdUrd incorporation as described previously (18). Cell cycle times, as determined by replication index values, appeared to be very similar for all three cell lines (HLD2-6-CO, HLD2-6-CY, and HLD2-6). The major difference in all fluorograms of human liver RNA translation products compared to all HLD2-6 derived RNA translation products (Fig. 2, C to E) was an apparent charge shift on

Table 1
Tumorigenicity in nude mice of control and cyclophosphamide treated HLD2-6 and HepG2 cells

<table>
<thead>
<tr>
<th>Cell inoculum</th>
<th>Cell treatment</th>
<th>SCEa</th>
<th>% of tumor bearing mice (total tumors/total injections)</th>
<th>Latency (wk)</th>
<th>Human AFPb</th>
<th>HBsAgc</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 × 10⁶</td>
<td>Control</td>
<td>11.1 ± 0.3</td>
<td>70 (16/23)</td>
<td>4 (2-6)</td>
<td>120</td>
<td>NEGd</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>34.9 ± 0.8⁵</td>
<td>88 (21/24)</td>
<td>4 (2-7)</td>
<td>159</td>
<td>NEGd</td>
</tr>
<tr>
<td>7 × 10⁶</td>
<td>Control</td>
<td>11.1 ± 0.3</td>
<td>8 (1/12)</td>
<td>8</td>
<td>ND</td>
<td>NEG</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>34.9 ± 0.8⁵</td>
<td>8 (1/12)</td>
<td>8</td>
<td>ND</td>
<td>NEG</td>
</tr>
</tbody>
</table>

a CY treatment was 500 μG CY/ml for 2 cell cycles prior to injection.

b Values are SCE/cell ± SE, with a minimum of 40 cells counted; numbers were standardized to 52 chromosomes/cell.

c Data were obtained at time of sacrifice. Mean negative control value for this assay was 0.55 μG/ml serum in three uninjected age matched nude mice.

d Data were determined at time of sacrifice.

* NEG, negative; ND, not determined.

Numbers in parentheses are range.

* Significantly different from control cells (Z statistic, P ≤ 0.001).

Protooncogene Expression in HLD2-6 Cells. c-myc expression was analyzed by hybridizing Northern blots with a recombinant plasmid containing a 1.8-kilobase 3'-fragment of the human c-myc gene. Ethidium bromide staining properties of the denaturing agarose gels before and after nitrocellulose transfer confirmed that equal amounts of RNA were electrophoresed on each lane with subsequent complete transfer to the nitrocellulose membrane filters. Normal human liver contains just detectable amounts of a 2.7-kilobase transcript that hybridizes to the c-myc probe (Fig. 3, Lane 5) which is the size of the transcript reported for the human promyelocytic HL60 cell line (36). c-myc specific transcripts were elevated in all HLD2-6 derived RNA samples and in tumors produced by HLD2-6 cells injected into nude mice (Fig. 3, Lanes 1 to 4). Densitometric analysis of the autoradiogram using a Beckman DU-88 densitometer indicated that Lanes 1 to 4 had comparable intensities of hybridization while Lane 5 was below the detection threshold of the machine. This blot was washed (boiled for 10 min) and rehybridized to 32P-labeled total HLD2-6 DNA which indicated that all lanes had equal amounts of hybridizable RNA. In addition no c-myc specific gene amplification could be detected in any HLD2-6 derived DNA sample by Southern blot analysis. These data suggest that transcription of the c-myc gene is elevated in both cultured cells and transplantable tumors of HLD2-6 origin. However, other possibilities such as specific stabilization of c-myc mRNA cannot be excluded.

DNA transfection experiments in rat embryo fibroblasts suggests that two genes from two distinct complementation groups of oncogenes or viral elements are required for cell transformation (37). We therefore examined the expression of an oncogene family, RAS, which is complimentary to the myc gene for cell transformation. N-ras expression was investigated using a 1.5-kilobase N-ras specific fragment. In the human liver sample and the three cell lines (HLD2-6, HLD2-6-CY, and HLD2-6-CO) two weakly hybridizing 5.5- and 2.5-kilobase transcripts were seen (Fig. 4, Lanes 1 to 4). However, RNA isolated directly from the tumors produced by HLD2-6 cells had increased 5.5-kilobase transcripts which hybridize to the N-ras probe (Fig. 4, Lane 5).
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Fig. 2. In vitro translation products of HLDz-6 cells and tumors. Poly(A)RNAs (1 µg) were translated in vitro using a rabbit reticulocyte lysate system and analyzed by two-dimensional gel electrophoresis with subsequent fluorography. A, minus RNA control showing translation artifact (TA); B, normal human liver translation products; C, HLDz-6 cell translation products; D, HLDz-6 derived tumor translation products; E, HLDz-6-CO cell translation products (cells reestablished in culture from HLDz-6 derived tumors). An apparent charge shift was noted in polypeptide labeled A in B, and polypeptide labeled A' in C, D, and E.

Densitometric analysis of the autoradiogram indicated that the level of hybridization of the 5.5- and 2.5-kilobase transcripts to the N-ras probe were approximately 5 and 1.3 times higher, respectively, in Lane 5 compared to Lanes 1 to 4. All lanes were determined to contain equal amounts of hybridizable RNA by the methods described above. This selective increase in the 5.5-kilobase N-ras transcript was reproducibly elevated in three tumors that were tested.

To obtain additional quantitation on N-ras expression, slot blot analysis was performed. Poly(A)RNA from the five different sources were applied at 1/5 dilutions and hybridized to the 32P-N-ras probe. After hybridization, washing, and exposure to film the individual slots of the blot were cut out and assayed for radioactivity by liquid scintillation counting (Fig. 5). Defining N-ras specific expression in the normal human liver sample as 100% (Fig. 5, Row 1), band intensities and radioactivity indicated that expressions in HLD2-6 (Fig. 5, Row 2), HLD2-6-CO (Fig. 5, Row 3), and HLD2-6-CY (Fig. 5, Row 4) cell lines are approximately 115, 130, and 120%, respectively. However, expression in the HLD2-6 derived tumors is approximately 245% of the human liver sample. No N-ras specific gene amplification could be detected in any HLD2-6 DNA sample by Southern blot analysis.

We have also examined for the presence of HBV sequences in the genomes of HLD2-6 cells by probing Southern blots with two DNA sequences coding for the hepatitis B surface antigen and hepatitis B core antigen. No HBV homologous sequences could be detected.

DISCUSSION

The results of these series of experiments indicate that HepG2 cells and a cell line derived from low density passage and single cell cloning of the HepG2 cells, called HLDz-6 cell line, are tumorigenic in nude mice. The tumors are highly invasive hepatocellular carcinomas yet demonstrated some differentiated characteristics as evidenced by morphology and bile formation. HumanAFP, an oncofetal protein, and GGT, a useful marker for preneoplastic liver (38, 39) as well as hepatocellular carcinomas (40), are both positive in the tumors. The staining patterns for GGT in the tumors are identical to the pattern reported previously for cultured HepG2 cells (41). GGT activity is predominantly confined to round or oval aggregates and is not found in regions of the plasma membrane. CY pretreatment of both cell lines has no effect on the tumor incidence or latency period despite inducing SCE formation in both cell lines.

Despite the fact that HLDz-6 cells are rapidly proliferating and tumorigenic in nude mice, the in vitro translation products are remarkably similar to those found in normal human liver. These data indicate that N-ras specific transcripts, specifically the 5.5-kilobase species, is elevated between 2.5- and 5-fold if HLDz-6 cells are grown as an organized tumor in athymic nude mice when compared to normal human liver and HLDz-6 cell cultures. RNA from the five samples seen in Figs. 2, 3, and 4 were also probed with a 32P-labeled c-Ha-ras probe. No H-ras transcripts could be detected.

We have also examined for the presence of HBV sequences in the genomes of HLDz-6 cells by probing Southern blots with two DNA sequences coding for the hepatitis B surface antigen and hepatitis B core antigen. No HBV homologous sequences could be detected.
Fig. 3. Relative levels of c-myc transcripts. Poly(A)RNA (5 µg) from a tumor derived from s.c. injection of HLD-66 cells into a nude mouse (Lane 1); from the cell lines HLD-66-CY (Lane 2), HLD-66-CO (Lane 3), and HLD-66 (Lane 4); and from a human liver sample (Lane 5) were fractionated on a denaturing formaldehyde gel, transferred to nitrocellulose, and hybridized to a 32P-labeled c-myc probe as described in Materials and Methods. rRNAs (28S, 18S, 23S, and 16S) were used as standards to establish base size, kb, kilobase.

but could reflect an alteration to actin which has been reported in other systems (43). It must be emphasized, however, that at most 150 to 200 abundant RNA species are detected by this technique and consequently other potential changes in less abundant or rare RNA species would go undetected.

Similar to reports on rodent hepatocarcinogenesis (10, 12) c-myc expression is increased equally in all HLD-66 cell lines and in tumors produced by those cells when compared to a normal liver sample. This increase in c-myc expression could not be explained by gene amplification or HBV integration. The role of c-myc in tumor formation is not yet understood although recent experiments suggest that the myc gene may play a critical role in cell proliferation (44, 45) and/or cell differentiation (46). Normal adult hepatocytes, in which transcripts of c-myc are just detectable (Fig. 3), are well differentiated, and presumably at G0 in the cell cycle. However, in HLD-66 derived cells which display less differentiated characteristics, such as AFP secretion, and have reentered the cell cycle, c-myc transcripts are greatly elevated. Whether myc expression is casually associated with the differentiation state and/or the immortalization properties of these cells is presently under investigation.

N-ras specific transcripts in HLD-66 derived cells are increased only in the tumor state and not in cultured cells (Fig. 4). The increase in N-ras specific transcripts is selective for the 5.5-
of specific interactions between the injected HLD2-6 cells and the host animal, such as with a serum growth factor or hormone, since N-ras transcription did not remain elevated when the tumors were reestablished into tissue culture.

It is important to note, however, that an increase in N-ras specific transcripts may not be critical in tumor formation if the gene has been activated by a point mutation at the 61st codon (47, 48). Recent experiments have shown that the N-ras gene of HepG2 derived cells is capable of transforming NIH/3T3 cells in a DNA transfection assay (49). This indicates that the gene is activated since unmutated N-ras genes derived from normal human cells are not capable of transforming NIH/3T3 cells in this assay system (47, 48). Hence low transcriptional levels of the activated N-ras gene may compliment c-myc expression in the tumorigenic properties of HLD2-6 cells.

H-ras expression could not be detected in any HLD2-6 derived tumor or cell line. This finding is different from the report of Makino et al. (10) where c-H-ras was elevated in livers treated with 3’-methyl-4-dimethyl-aminobenzene and in four rat hepatoma cell lines. However, in the context of the cooperating oncogene hypothesis (37), c-H-ras and N-ras belong to the same complementation group and both encode similar proteins. Hence it seems reasonable to propose that either gene can compliment c-myc expression in liver neoplasia.

In conclusion we have described a model system where oncogenic expression in human hepatoma cells can be examined in two distinct but well defined, environments, i.e., in tissue culture and in the nude mouse. The overall similarity of genetic expression, as determined by in vitro translation analysis, between these cells and normal human liver supports their use in human hepatocellular carcinogenesis studies. c-myc specific transcripts in the absence of HBV integration were elevated to the same extent in HepG2 derived cells whether transplanted into the nude mouse or grown in tissue culture. The activated HepG2 N-ras gene, although not transcriptionally elevated, may compliment c-myc expression in the tumorigenic properties of these cells. The data also suggest that the selective increase of the 5.5-kilobase transcript of N-ras in the HLD2-6 derived tumors could be the result of some interaction with the host animal.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. David Jacobson-Kram for helpful discussions, Dr. Unnr Thorgersson for histological evaluations, and Frances Williams for her excellent secretarial assistance.

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1983.


Fig. 1. Morphology of a typical tumor derived from HLD−6 cells injected into a nude mouse. A, tumor in a nude mouse 14 days after inoculation of $3 \times 10^7$ HLD−6 cells. M, mitotic figure. H & E, $\times$ 330. B, same tumor seen in A, in this area showing glandular-like structures. H & E, $\times$ 330. C, same tumor seen in A, in this area showing the nonencapsulated invasive nature of the tumors. S, mouse striated muscle. H & E, $\times$ 170. D, same tumor seen in A, showing positive staining for bile pigments. Note predominant positive staining in glandular-like structures. B, bile. Hall’s stain, $\times$ 330. E, histochemical demonstration of GGT activity in cryostat section of tumor seen in A. Enzymatic activity was confined to round or oval aggregates. $\times$ 330.
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