Isolation and Characterization of Complementary DNA Clones for Genes Overexpressed in Chemically Induced Rat Hepatomas

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ABSTRACT

In order to characterize the genes overexpressed in an hepatoma cell line, the HTC cells, and in diethylnitrosamine induced solid hepatomas, we constructed a complementary DNA library from HTC cells and performed differential screening with probes from HTC cells, from malignant nodules obtained 70 weeks after the carcinogen treatment, and from hepatocytes from normal rat liver. Eight clones corresponding to messenger RNAs (mRNAs) much more expressed in hepatomas than in hepatocytes from normal liver were isolated. Three, clones pH T71, pH T13, and pH T26, were further analyzed by the study of their corresponding transcripts in hepatocytes from regenerating liver and in the hepatocytes from the nontumorous parts of the liver.

Clone pH T71 corresponds to a single 2.3-kilobase mRNA which is present in high levels in carcinoma nodules in hepatoma cell lines, in the nontumorous parts of the liver, and in hepatocytes isolated from regenerating liver 30 to partial hepatectomy.

Clone pH T13 hybridizes with three distinct transcripts 3.8, 2.6, and 1.6 kilobases long. High levels of the 3.8- and 1.6-kilobase mRNAs are present in carcinoma nodules, in hepatoma cell lines, and in the nontumorous parts of the liver. However, the levels of these RNAs are similar in hepatocytes from regenerating liver and in hepatocytes obtained from normal rat liver.

Clone pH T26 corresponds to a 0.6-kilobase mRNA which exists at a high level only in cancer nodules and in hepatoma cell lines.

We were unable to observe any cross-hybridization between these clones and the oncogenes which have been found to be expressed in hepatomas (c-fos, c-Ha-ras, c-Ki-ras, N-ras, and c-myc).

The mRNAs corresponding to the three clones have not been detected in various tissues from normal adult rats. Our study shows that a high level of these mRNAs might be associated with rat liver carcinogenesis.

INTRODUCTION

There is considerable evidence that malignant transformation proceeds along several steps, as shown by Farber (1) and by Land et al. (2). The transformed phenotype is inherited in a stable way indicating that transformation is very likely to result from a complex alteration of the genome (3, 4). A predominant feature of hepatocarcinogenesis is the appearance of foci of altered hepatocytes in liver long before a neoplasm becomes evident. The foci are hyperplastic nodules which can be identified by histological and biochemical methods (5–12). However, few of the foci express all the changes simultaneously (13).

Changes in the expression of specific genes, such as albumin and α-fetoprotein, have been described in various hepatomas (14–16). The amount of a M, 21,000 polypeptide has been found to be reproducibly increased in liver cytosol from five models of carcinogenesis (17). Of considerable interest would be the characterization of the genes and of their products involved in the alteration of the cellular phenotype associated with the process of malignant transformation of various cell types (18–20).

One approach is to isolate the transcripts which are present at abnormally high level in HTC cells, a hepatoma cell line, and in hepatocellular carcinoma induced by DENA. In this system the sequence of morphological and biochemical changes during the long time interval preceding the onset of plurifocal malignant growth has been well characterized (21–23). It includes the early appearance of hyperplastic nodules, the majority of which undergo remodeling and redifferentiation to normal or subnormal hepatocytes. Some of them persist and a further sequence of changes leads to malignant cell clones which develop into malignant nodules. The nontumorous parts of the livers are apparently normal, but some transformation markers such as c-Ha-ras, c-Ki-ras, N-ras (24), and c-fos (25) genes are overexpressed.

In this study DENA was administered to rats after a two-thirds hepatectomy (22). Malignant nodules and the hepatocytes from the nontumorous parts of the liver were isolated 70 weeks after the carcinogen administration.

In order to isolate and to characterize the gene products which are present at abnormally high levels in DENA induced hepatoma and in HTC cells we constructed a cDNA library of HTC cells (26) and we used the molecular hybridization technique which has been successful in isolating the genes specifically overexpressed in established tumor cell lines (19, 27, 28). We present here the characterization of three of the clones which correspond to mRNAs expressed at a much higher level in malignant nodules and in HTC cells than in normal hepatocytes. Furthermore the transcripts of two of these clones are also present at high levels in hepatocytes from the nontumorous parts of the livers suggesting that these cells present some alterations in gene expression as compared to hepatocytes from normal liver.

MATERIALS AND METHODS

Hepatocytes and Tumor Cells

HTC cells, an established cell line derived from Morris 7288 hepatoma, were grown in Swim's medium 77 supplemented with 10% newborn calf serum (29). Other hepatoma cell lines (Faza, Fu, and P4) are descendants of the Reuber H35 rat hepatoma.

Hepatocytes from normal and regenerating liver were obtained by collagenase perfusion of rat livers (30). A homogeneous suspension of single cells was obtained. Isolated hepatocytes were easily purified from the nonparenchymal cells by three differential centrifugations.

Malignant nodules and hepatocytes from the nontumorous parts of the liver were prepared as follows. Female Sprague-Dawley rats were given 3 doses of DENA (25 mg/kg) by stomach tube 24, 48, and 72 h after a two-thirds hepatectomy (22, 31, 32). The rats were sacrificed 70 weeks later and each liver was perfused via the portal vein with a

1 The abbreviations used are: DENA, diethylnitrosamine; cDNA, complementary DNA; HTC cells, hepatoma tissue cultured cells; poly(A)* RNA, polyadenylate-containing RNA; SSC, standard saline citrate.
collagenase solution. A mixture of isolated cells and packs of undissociated cells resulted from the perfusion. The undissociated cells, corresponding to malignant nodules, were collected separately by filtration of the suspension through a 150-μm nylon mesh. They were retained on the nylon whereas isolated hepatocytes arising from the well dissociated nontumorous zones of the liver were collected after passage through nylon and purified by three differential centrifugations.

The dissociated cells obtained from the nontumorous parts of the liver will be referred to as nonnodular hepatocytes; they present the characteristics of mature hepatocytes. Their hepatocyte nature was ascertained by (a) their size and their morphological characteristics, (b) their ability to reconstitute hepatic trabecular colonies and formations similar to bile canaliculi in culture, (c) their incapacity to grow in vitro, (d) their ability to secrete high amounts of albumin and transferrin, and (e) their high pyruvate kinase L and aldolase B activities and the inducibility of tyrosine aminotransferase by glucocorticoids.

These cells also presented alterations described in hyperplastic foci: deficiency in membrane bound ATPase; increased ferritin (32); increased γ-glutamyl transferase activity; increased pyruvate kinase M activity (12); and overexpression of the three ras genes (24) and of the for genes (25).

Histological studies of slices corresponding to the nonnodular zones show that the cell population is actually heterogeneous. Some of these cells, in a proportion varying from one slice to another and from one rat to another, appear as normal hepatocytes; some others presented the alterations described above.

**Nucleic Acid Preparation**

**Plasmid DNA.** Small scale preparation of plasmid DNA was performed by alkaline cesium chloride method.

**RNA Isolation.** Frozen tumors, various samples from normal rats, and hepatocytes were lysed in 5 mM guanidium thiocyanate, 0.1 M sodium acetate (pH 5.5), 1 mM EDTA, 5% (v/v) 2-mercaptoethanol, and 2% (v/v) N-lauroyl Sarcosyl. Total cellular RNAs were prepared by the method of Chirgwin et al. (34) as modified by Raymond Jean et al. (35). Poly(A)* RNAs were isolated by oligo(dT) cellulose chromatography as described by Aviv and Leder (36).

**Construction of the HTC cDNA Library**

Double stranded cDNA was synthesized from HTC poly(A)* RNAs, cloned in pBR322 using the deoxycytidine-deoxyguanosine tailing procedure (37), except that the second strand was synthesized immediately after the first strand without extraction (38). This gave a library of about 3000 ordered clones. Screening was performed in duplicate using either nitrocellulose filters (39) or Whatman No. 540 filters (40). In each case the filters were hybridized to [32P]cDNA synthesized from normal hepatocytes and from HTC cell poly(A)* RNAs. The same filters were used for differential screening using [32P]cDNA made from carcinoma nodules and from nonnodular hepatocyte poly(A)* RNAs.

**Blot Hybridization Analysis**

DNAs, 0.5 to 1.0 μg from each recombinant clone, were denatured by boiling for 3 min in 0.3 M NaOH and neutralized with HCl; 20 × SSC was added to a final concentration of 2 × SSC. DNA was spotted on strips of nitrocellulose filters, presoaked in 20 × SSC, and dried. Dot blot hybridizations were performed according to the method of Thomas (41). Since the filters were to be reused, they were never allowed to dry completely. For RNA dotting, RNAs treated with 5 mM methylmercury hydroxide were spotted on the nitrocellulose filters. Hybridizations were performed as described previously (25, 42). For Northern blotting RNAs were denatured by heating for 10 min at 60°C in 10 mM phosphate buffer (pH 7.0)-50% formamide-2.2 M formaldehyde-1 mM EDTA-5 mM sodium acetate and electrophoresed on 0.8% agarose gels according to the procedure of Gal et al. (43). rRNAs were used as size markers. After hybridization the filters were washed in 0.1 × SSC-0.1% sodium dodecyl sulfate at 55°C twice for 20 min and exposed for 2–4 days with Kodak X-AR X-ray films at -80°C.

**Probes**

Isolated clones pHT 13, pHT 26, and pHT 71 were used successively as 32P-nick-translation probes to determine the level of the corresponding mRNAs. They were used either as a complete clone or as a Pst1 insert isolated by agarose gel electrophoresis followed by electroelution. The c-Ha-ras probe was either the BR1 clone or the BamHI-EcoRI subclone of clone BS9 (44). The c-Ki-ras probe was clone KBE-2 (44). The N-ras probe was the human genomic 9.2-kilobase probe (45) and the for probe was the pFBJ-2 clone (46). The mvc probe was the third exon of human c-myc.

Single strand cDNA probes were synthesized from poly(A)* RNAs extracted from HTC cells, normal hepatocytes, malignant nodules, and nonnodular hepatocytes, using 5 μg of RNA under the same conditions as for the cloning procedure (37) except that the [α-32P]dCTP concentration was 20 μM (specific activity, 800 Ci/mmol; Amersham International, Amersham, United Kingdom).

**Liquid Hybridization Kinetic Study**

Hybridizations were carried out with [3H]cDNA from HTC cells and poly(A)* RNAs from HTC cells or from normal hepatocytes as described by Kitiz et al. (47). Aliquots of cDNA were mixed with poly(A)* RNA (0.5 to 1000 μg/ml) in 10 mM Tris-HCl (pH 7.4)-0.4 M NaCl-0.5% sodium dodecyl sulfate and incubated at 65°C to the desired Rot.

**RESULTS**

**Sequence Homology in Poly(A)* from HTC Cells and from Rat Hepatocytes.** We have compared the mRNA sequences expressed in hepatocytes from normal adult rats and in HTC cells. This was accomplished by hybridizing DNA complementary to poly(A)* RNA from HTC cells with homologous mRNA and with normal hepatocyte mRNA in excess. Fig. 1 shows that hybridization took place over an Rot range of at least 5 log units with 3 transitions corresponding to large heterogeneous populations of mRNAs with 3 ranges of abundance.

The maximum amount of hybridized sequences is not significantly different in the homologous and in the heterologous
reactions showing that almost all the sequences present in HTC cells are also present in normal hepatocytes. However, hybridization occurred in the homologous reaction at lower $R_d$ values showing that the common sequences, especially the abundant sequences and the sequences of intermediary abundance, are present at a 5–10 times higher concentration in HTC cells than in normal hepatocytes.

Screening of the Hepatoma cDNA Clone Library. In order to study the sequences present at a much higher level in tumor cells than in hepatocytes from normal rat liver, a cDNA library of HTC cell poly(A)$^+$ RNAs was constructed in the plasmid pBR322. With direct colony screening, 10% of the clones were positive with HTC cell $^{32}$P-cDNA as a probe and gave a weak signal with the hepatocyte $^{[32P]}$cDNA probe. These results are consistent with those of the liquid hybridization kinetic studies.

DNA was prepared from each of these clones as well as from some of the clones which gave no differential colony hybridization signal. Individual plasmid DNAs were applied to nitrocellulose filters and hybridized with the $^{[32P]}$cDNA probes from HTC cells and from hepatocytes.

Radioautography of the filters revealed that the previously isolated cDNA clones give a reproducibly much higher signal with the HTC cell probe than with the probe from normal hepatocytes. Only these clones were used for further analysis (Fig. 2).

In order to establish whether the transcripts corresponding to these cDNA clones are also present in high level in in vivo hepatomas, rats were treated with three doses of DENA 1, 2, and 3 days after partial hepatectomy and killed 70 weeks later. Poly(A)$^+$ RNAs were prepared from the malignant nodules and from the nonnodular hepatocytes. The previously used nitrocellulose filters were tested with the corresponding cDNAs taken as probes. Fig. 2 shows the results obtained with some of these clones. Most of the transcripts which have been found overexpressed in HTC cells were also found overexpressed in the malignant nodules. Some of these clones corresponded to mRNAs which were also present at high levels in the nonnodular hepatocytes of the same rat livers.

For further characterization we selected the clones corresponding to mRNA sequences present at high levels in HTC cells and in malignant nodules but appearing at low levels in hepatocytes from normal liver.

It is very likely that several clones corresponded to the same mRNA molecule. Cross-hybridizations between the clones allowed us to identify the clones corresponding to different mRNAs. The majority of the clones can be classified in 8 independent sets (Table 1).

No cross-hybridization has been found between these clones and the c-fos, c-myc, and $3_ras$ genes.

Analysis of the Transcripts Homologous to Three cDNA Clones. We have chosen to analyze 3 cDNA clones: clone pH 71, the prototype of set A; clone pH 13, the prototype of set B; and clone pH 26, the prototype of set C. We performed dot blot hybridizations of poly(A)$^+$ RNAs from carcinoma nodules and from nonnodular hepatocytes prepared from 6 rats which had been sacrificed 70 weeks after DENA treatment. For 5 of these rats malignant nodules were separated from the nonnodular hepatocytes. Fig. 3 shows the dot blot analysis of the RNAs from malignant nodules (Lanes 2, 3, 4, 5, and 10) and from nonnodular hepatocytes (Lanes 7, 8, and 9). For one rat we have not found large nodules but rather numerous small nodules which could not have been separated from the other hepatocytes (Lane 6). There was no doubt concerning the advanced malignant state of the liver of this rat.

Fig. 3 shows the steady state level of the mRNAs corresponding, respectively, to clones pH 71, pH 13, and pH 26 which is much higher in all the carcinoma nodules tested than in hepatocytes from normal, nontreated rats (Lane 1). These data clearly show that the overexpression of the corresponding genes is a common feature associated with DENA induced rat liver carcinogenesis.

As a control we used another clone, pH 33, which did not give a differential signal between malignant nodules and hepatocytes from normal rat liver in the first screening of the cDNA library. Fig. 3D confirmed that the level of the corresponding mRNA was not higher in malignant nodules than in hepatocytes from normal liver.

For further characterization of the mRNAs homologous to clones pH 71, pH 13, and pH 26, the poly(A)$^+$ RNAs were submitted to Northern blot analysis.

Clone pH 71, the prototype of set A, hybridized with a single size mRNA (2.3 kilobases) (Fig. 4A). The level of the mRNA was high in carcinoma nodules (Lanes 1 and 5) and in nonnodular hepatocytes (Lanes 2 and 4). It was also high in
Table 1 Eight independent sets of cDNA clones

The clones correspond to mRNA sequences present at high levels in HTC cells and in malignant nodules and absent or present at low levels in normal hepatocytes. Cross-hybridizations between the different clones allowed the separation of the corresponding cDNAs into 8 independent sets. For further characterization we used the clone of each set with the longest insert.

<table>
<thead>
<tr>
<th>Designation of the sets</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
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<tr>
<td>Prototype of each set</td>
<td>71</td>
<td>13</td>
<td>26</td>
<td>14</td>
<td>8</td>
<td>2</td>
<td>16</td>
<td>11</td>
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<tr>
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</tr>
<tr>
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<td>4</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>5</td>
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Fig. 3. Dot blot analysis of poly(A)* RNAs from normal and from carcinoma nodules of rats sacrificed 70 weeks after DENA treatment. Poly(A)* RNAs, 1 and 0.2 µg, were applied onto nitrocellulose filters and hybridized with 32P-nick translated cDNA from clones pHT 71 (A), pHT 13 (B), pHT 26 (C), and pHT 33 (D). Poly(A)* RNAs were prepared from: normal hepatocytes (Lane 1); carcinoma nodules from rats a, b, c, d, and e (Lanes 2, 3, 4, 5, and 10, respectively); nonnodular hepatocytes from rats a, b, and c (Lanes 7, 8, and 9, respectively); and from total hepatocytes from a rat with a collection of small nodules (Lane 6).

Liver containing collections of small nodules (Lane 3). The level of the pHT 71 mRNA was very low in normal hepatocytes isolated from normal rats (Lane 6).

Clone pHT 13, the prototype of set B, hybridized with at least two distinct mRNAs, 2.6 and 1.6 kilobases, respectively (Fig. 4B). These two mRNAs were present in carcinoma nodules (Lanes 1 and 5), in nonnodular hepatocytes (Lanes 2 and 4), and in the liver containing collections of small nodules (Lane 3). The 2.6-kilobase mRNA was also present in hepatocytes from normal liver although at a lower level (Lane 6), whereas the 1.6-kilobase mRNA has usually not been detected in hepatocytes from normal liver even after a long filter exposure time. A third mRNA (3.8 kilobases) was found in almost all the nodules and nonnodular hepatocytes, although at variable levels: in several cases its level was as high as that of the 2.6-kilobase mRNA (Lane 3); in other cases a longer filter exposure time was required to allow its detection (Lane 1). However, even after a long exposure time, the mRNA was not found in hepatocytes from normal liver. This mRNA could correspond either to a nonmature precursor of the 1.6-kilobase mRNA or to an unstable mRNA.

Clone pHT 26, the prototype of set C (Fig. 4C), hybridized with a single 0.6-kilobase mRNA. This mRNA was present at a high level in malignant nodules (Lanes 1 and 5) and could be detected neither in nonnodular hepatocytes nor in hepatocytes from normal rats (Lanes 2, 4, and 6).

These results confirm and extend the results obtained by dot blot analysis (Fig. 3).

The expression of the genes corresponding to clones pHT 71, pHT 13, and pHT 26 was also analyzed in three different cell lines derived from a Reuber hepatoma: Faza; Fu; and P4.
(Table 2). The level of mRNAs corresponding to these clones was found to be very high in the three cell lines.

Furthermore cell proliferation is one of the most striking events in cell transformation. In order to determine if the overexpressed genes are also implicated in cell growth, we analyzed their expression in hepatocytes from regenerating liver 30 h after hepatectomy, a time corresponding to an active cell proliferation. The mRNA homologous to clone pH 71 was present in high amounts and its level returned to the basal value within 7 days after hepatectomy. It can be concluded from this study that the overexpression of the gene corresponding to clones pH 13 and pH 26 might be restricted to the hepatoma cell lines whereas the gene corresponding to clone pH 71 is also overexpressed in proliferating hepatocytes and might be associated with cell growth.

We also measured the amounts of the mRNAs corresponding to the three clones in six tissues from normal rats. In all cases the amounts were very low and often not detectable.

**DISCUSSION**

Because of its susceptibility to cancer induction under a variety of conditions and because of a large body of knowledge about its cellular and molecular biology and pathology, liver has been one of the most utilized organs for the study of the development of cancer. We have used DENA as a carcinogen since this compound induces transformation of hepatocytes rather than that of other cell types (22) so that it is easy to obtain as a normal counterpart hepatocytes from normal liver by the use of collagenase perfusion.

In addition, hepatocytes from regenerating liver shortly after hepatectomy represent a good material for the study of the modifications of gene expression which occur during noncancerous cell proliferation.

We have recently found significant modifications in the in vitro translational pattern of RNAs from malignant nodules as compared to the pattern observed with mRNAs from normal hepatocytes (48) as has been observed during 3'-methyl-4-dimethylaminoazobenzene induced hepatocarcinogenesis (9).

In this work the changes in the mRNA populations during hepatocarcinogenesis were investigated using various hepatocyte populations.

The differential screening of the HTC cell cDNA library allowed us to isolate clones which correspond to genes overexpressed in HTC cells and in malignant nodules and, for some of them, in nonnodular hepatocytes, as compared to hepatocytes from normal rats. None of these clones correspond to the c-ras, c-fos, and c-myc genes which have been found to be overexpressed in these cells. In addition no expression of the oncoproteins myb, mos, sis, and abl was observed in hepatoma cells.

Cross-hybridization between various clones allowed identification of 8 independent sets of clones, three of which were chosen for further study.

Clone pH 71 corresponded to a single 2.3-kilobase mRNA which was barely detectable in hepatocytes from normal liver and is present at a high level in malignant nodules. It was also present at a high level in nontransformed proliferating hepatocytes and in nonnodular hepatocytes. The product of the mRNA corresponding to this clone might be implicated in cell growth.

Clone pH 13 hybridized with 3 distinct mRNAs; the 2.6-kilobase mRNA is expressed in hepatocytes from normal liver whereas the 3.8- and 1.6-kilobase mRNAs were found in carcinoma nodules and in nonnodular hepatocytes, but in neither hepatocytes from regenerating liver 30 h after hepatectomy nor hepatocytes from normal liver. At present it is unknown whether these different transcripts originate from different transcriptional units which share a common sequence or if they result from different processing of a same precursor.

Clone pH 26 corresponded to a single 0.6-kilobase mRNA which was found present only at a high level in carcinoma nodules. This cDNA sequence appears to be the complete copy of the mRNA molecule and it will be of interest to utilize it as a cancer marker in *in situ* hybridizations.

This method allowed the detection of several genes which are expressed at a higher level in malignant nodules and in various hepatoma cell lines. No or very little expression of these genes was observed in hepatocytes from normal liver and in various tissues from normal rats. Scholla et al. (49), using liquid hybridization, were unable to find any difference between poly(A)* RNAs from hepatoma 252 and normal liver.

Since nonnodular hepatocytes also expressed some of the sequences overexpressed in malignant nodules, it is necessary to consider the significance of these hepatocytes. These cell populations have the characteristics of normal mature hepatocytes and present several alterations: (a) foci of high γ-glutamyl transferase activity (50, 51); (b) overexpression of the c-fos and of the 3 ras genes; (c) increased level of the mRNAs related to clones pH 71 and pH 13. These hepatocytes could include normal hepatocytes, foci of early cancerous alteration, or altered cells undergoing remodeling and regression whereas some of their oncodevelopmental markers remained expressed (12). These cells could also exhibit changes in gene expression as a secondary effect of neoplasm in the surrounding tissue areas (52).

Histochemical studies of slices made in the nontumorous parts of the liver confirm the heterogeneity of this cell population, since only parts of the cell population present the alterations, especially the high γ-glutamyl transferase activity. *In situ* hybridization studies, now in progress, will help us to elucidate the extent of alterations in these cells and to determine the specificity of clones pH 13 and pH 71.

Further analysis of the sequences of these clones and the subsequent identification of these polypeptides products will

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<th>Table 2</th>
<th>Relative amounts of RNAs homologous to clones pH 71, pH 13, and pH 26 in hepatoma cells, regenerating liver, and normal rat tissues</th>
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<td>cDNA clones</td>
<td>Carcinoma nodules</td>
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<tr>
<td></td>
<td>HTC</td>
</tr>
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<td>pH 71</td>
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</tr>
<tr>
<td>pH 13</td>
<td>100</td>
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<td>pH 26</td>
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help to understand the transformation process. It will also be of interest to see if the overexpression of the corresponding genes is a process common to a wide range of hepatomas and of other chemically and virally induced cancers.

ACKNOWLEDGMENTS

We gratefully acknowledge Drs. C. Schwartz and A. Kitzis for critical reading of the manuscript. We gratefully acknowledge the gift of RNAs from hepatoma cell lines Faza, Fu, and P4 by Dr. Mary Weiss.

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