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Covalent modification and repressed transcription of a gene in hepatoma cells

(tumor gene structure/DNA methylation/DNA modification/restriction endonuclease/Southern blots)

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ABSTRACT **When liver cells undergo malignant transformation, certain genes cease being expressed. We have studied the structure of one such gene, whose protein product we have designated hepatic protein 22 (hp22), which is not expressed in the two Morris hepatomas studied. We have prepared a chimeric clone of pBR322 containing cDNA sequences complementary to mRNA coding for this protein. By using this cloned cDNA, we have examined changes in expression of this gene and changes in the restriction pattern of the DNA isolated from normal liver and these hepatomas. In both hepatomas, studies using the isoschizomeric pair of restriction enzymes *Msp* I and *Hpa* II have indicated hypermethylation of a cytosine residue within or proximal to the hp22 gene. Other differences in the restriction pattern between normal liver and hepatoma DNA were also detected with *Eco*RI and *Ava* I. Thus, in the nontranscribed form of this gene, the DNA has undergone covalent modification, distinguishing these two hepatomas from each other and from normal liver.**

When normal cells undergo neoplastic transformation, whether it be virally, chemically, or radiologically induced, alterations occur in the nature of the proteins synthesized in the transformed cell. Certain proteins appear, other tissue-specific proteins cease being synthesized, and the proportions of subunits and isozymic species of retained enzymes are often altered (1). Whether these altered enzyme levels are responsible for the neoplastic conversion remains uncertain. However, understanding the biochemical processes underlying this alteration in biochemical phenotype and the loss of control over neoplastic cell function by hormonal modulators is essential to understanding the nature of the neoplastic transformation.

We have shown that two hepatic proteins, the enzyme tryptophan dioxygenase (2, 3) and the urinary protein α_{2u}-globulin, are not synthesized by several rat hepatomas (4–6). In each instance we could not detect translationally active forms of the mRNAs coding for these proteins (4). Recently we have cloned the cDNA for a protein, hp22, which is specific to the liver, expressed to a greater extent in male than in female rats, and absent from the hepatomas examined. Although the identity and function of the hp22 protein are unknown, the protein is neither α_{2u}-globulin nor tryptophan dioxygenase. The availability of a probe for this gene provides an opportunity to test the hypothesis that impaired transcription is a consequence of structural alterations in the tumor genome.

This hypothesis was explored by isolation of high molecular weight DNA from normal and malignant tissues, digestion with various restriction endonucleases, electrophoresis through agarose gels, transfer onto nitrocellulose sheets, and hybridization to the labeled hp22 cDNA sequences cloned in pBR322 (p41). This study demonstrates that in the region of the hp22 gene there are differences in methylation and possibly base substitution between normal liver and these hepatomas.

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MATERIALS AND METHODS

Tumor Source and Animals. Male rats of either the Buffalo or Sprague–Dawley strain were used as sources of high molecular weight DNA. Male Buffalo rats bearing hepatomas 5123D and 7793 were kindly supplied by H. P. Morris (Dept. of Biochemistry, Howard University, College of Medicine, Washington, DC).

Preparation and Isolation of p41 Clone. The cDNA was prepared from male hepatic poly(A)-RNA and inserted into the *Pst* I site of pBR322 by the oligo(dA-dT) "tailing" method (7). After transformation of *Escherichia coli* χ1776 and culture under P2-EK1 containment conditions, clones that were both ampicillin sensitive and tetracycline resistant were screened with cDNA that was specific to livers of male rats (7–9). Of the clones that hybridized to this probe, the one containing the largest inserted sequence (p41) was selected for further study.*

Hybridization and Translation of mRNA Complementary to p41. The procedure used for isolating liver mRNA from male rats complementary to the cDNA insert in clone p41 was from the work of Ricciardi *et al.* (10) and Woolford and Rosbash (11). Briefly, plasmid DNA from a 100-ml culture was prepared by the method of Katz *et al.* (12), denatured by successive heat and alkali treatments, bound to nitrocellulose discs, and hybridized at 50°C in a medium consisting of 0.4 M NaCl, 30 mM 1,4-piperazinediethanesulfonic acid (Pipes; pH 6.5), 0.2% $NaDodSO_4$, 50 μg of yeast tRNA, and 25 μg of liver poly(A)-RNA from male rats. After 16 hr of hybridization, filters were removed, washed to remove nonspecifically bound RNA, and boiled in water to elute the specific mRNA. The RNA solution was then treated with phenol, washed, lyophilized, and finally dissolved in water. Half of this RNA solution was used to program a wheat germ-based translation system (13, 14). The L-[^{35}S]methionine-labeled translation products were displayed on a 12.5% polyacrylamide/$NaDodSO_4$ gel (15) supplemented with 6 M urea, after which the gel was fluorographed (16) and exposed to x-ray film at −70°C.

Hybridization of p41 DNA with Excess RNA. The hybridization mixture consisted of 0.4 M NaCl, 65% (vol/vol) formamide, 30 mM Pipes (pH 6.5), 0.2% $NaDodSO_4$, 10 μg of denatured *E. coli* DNA, ≈6000 cpm of ^{32}P-labeled *Ava* II fragment of clone p41 (≈160 pg), and various concentrations of total

Abbreviations: kbp, kilobase pair; Pipes, 1,4-piperazinediethanesulfonic acid.

* Feigelson, P., Chan, K.-M. C., Kurtz, D. T. & Monahan, J. (1980) *Proceedings of the VIth International Congress of Endocrinology*, Feb. 10–16, 1980, Melbourne, Australia, p. 369 (Abstr. 320).

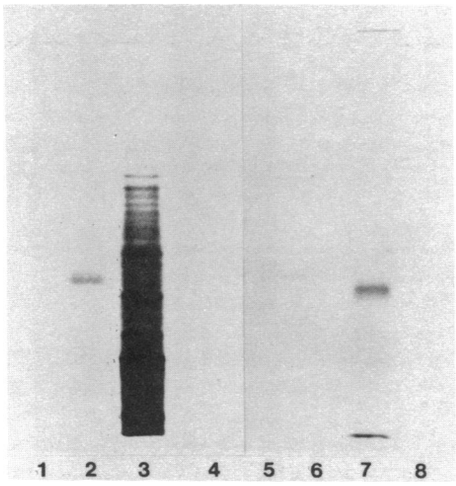

FIG. 1. Translation of RNA complementary to p41. This fluorogram depicts the translation product of the rat liver mRNA complementary to clone p41. Lane 1, control, showing the lack of translation products synthesized because no mRNA hybridized to the plasmid vector pBR322 lacking the cDNA insert. Lane 2, peptide synthesized by mRNA complementary to p41. Lane 3, peptides synthesized in response to total liver poly(A)-RNA from male rats (2 μg). Lane 4, endogenous activity of the wheat germ system. Lanes 5–8, translation products corresponding to lanes 1–4, but immunoprecipitated with monospecific antibodies against α_{2u}-globulin. Exposure time was 19 hr.

poly(A)-RNA in a final volume of 50 μl. The incubations were carried out for 20 hr at 50°C to obtain required R_0t values [R_0t is the initial concentration of RNA (mol of nucleotide per liter) multiplied by time (sec)]. After unhybridized cDNA was hydrolyzed with nuclease S1 (17), the hybridized cDNA was precipitated with an equal volume of cold 10% (wt/vol) trichloroacetic acid. Precipitates were collected by filtration, and radioactivity was measured in a scintillation spectrometer. Data from the hybridization experiments were expressed as percent hybridization plotted against log R_0t.

Preparation and Nick Translation of *Ava* II Fragment of p41. The probe used for these studies was prepared by digestion of p41 with the restriction endonuclease *Ava* II. This enzyme cleaves the plasmid outside the inserted sequence (≈110 base pairs on each side) (18). The digested plasmid was then electrophoresed on 7% polyacrylamide gel with Tris/borate/EDTA, pH 7.0 (19). Bands were visualized under short-wave UV radiation after they were stained with ethidium bromide (10 μg/ml). The gel band containing the insert was placed in a dialysis bag and electroeluted for 4 hr (20). This excised fragment (535 base pairs long) was then nick translated (21) to a specific activity of 1–2 × 10^8 cpm/μg.

Isolation of DNA and Restriction Endonuclease Digestion. High molecular weight DNA was isolated from the tissues (22, 23) and completely digested with various restriction endonucleases purchased from either Bethesda Research Laboratories (Rockville, MD) or New England BioLabs. From every restriction endonuclease reaction an aliquot was taken, which was mixed with 5 μg of unlabeled and 20,000 cpm of ^{32}P-labeled λ DNA and examined to ensure complete digestion. *Hin*dIII-digested [^{32}P]DNA was used as electrophoretic size markers. The digested DNA samples were then electrophoresed through 1% agarose gels with 40 mM Tris·HCl, pH 7.0/5 mM sodium acetate/1 mM EDTA at 30 mA for 14 hr.

DNA Transfer and Filter Hybridization. The DNA from the gel was then transferred onto nitrocellulose filters (Schleicher & Schuell BA 85) (24), hybridized overnight to the nick-translated *Ava* II fragment, and washed as described (25). The filters were dried and exposed to x-ray film at −70°C, with a Cronex Lightning-Plus image-intensifying screen.

RESULTS AND DISCUSSION

In order to identify the protein corresponding to p41 cloned cDNA, this cloned cDNA was used to select a single species of hepatic mRNA. This mRNA directed the synthesis of a 22,000-dalton protein in a wheat germ-based translation system. The protein designated hp22 is immunologically unrelated to α_{2u}-globulin (Fig. 1) and is also not tryptophan dioxygenase, the subunits of which are ≈40,000 daltons (6).

To determine the relative abundance of hp22 mRNA in livers of male and female rats, kidney of male rats, and hepatomas 5123D and 7793, cDNA was hybridized with excess mRNA. RNA sequences complementary to p41 were found in livers of male and female rats but were not detected in the RNA from kidney and hepatomas. However, the sequences complementary to p41 were only 1/10th as abundant in liver from female rats as in liver from male rats (Fig. 2). Thus, hp22 mRNA is more prevalent in liver from male rats, is hepatospecific, and cannot be detected in the two hepatomas examined.

To determine whether alteration in the structure of the gene coding for hp22 correlates with its expression, we performed comparative Southern blot analyses upon high molecular weight DNA digested with various restriction endonucleases. To explore whether differential base methylation is occurring in the hepatomas, we used the isoschizomeric pair of restriction enzymes *Hpa* II and *Msp* I. Each of these enzymes cleaves the same nucleotide sequence (5′-C-C-G-G-3′). *Hpa* II digests the DNA only if the penultimate cytosine residue is not methylated, whereas *Msp* I digests this site regardless of methylation (26, 27). Restriction endonuclease *Hin*fI generated a p41 complementary 0.8-kilobase-pair (kbp) DNA fragment from liver of male rats and both hepatomas (Fig. 3, lanes 2–4). When this *Hin*fI-generated fragment from each tissue was further digested by *Hpa* II, liver of male rats and hepatoma 7793 generated 0.7-kbp and 0.8-kbp complementary bands (Fig. 3, lanes 5 and 7), whereas DNA isolated from H5123D had a very faint hybridizable 0.7-kbp fragment (Fig. 3, lane 6). However, in other experiments, hepatoma 5123D completely lacked this 0.7-kbp fragment (Fig. 4, lane 8). An *Msp* I/*Hin*fI double digestion of

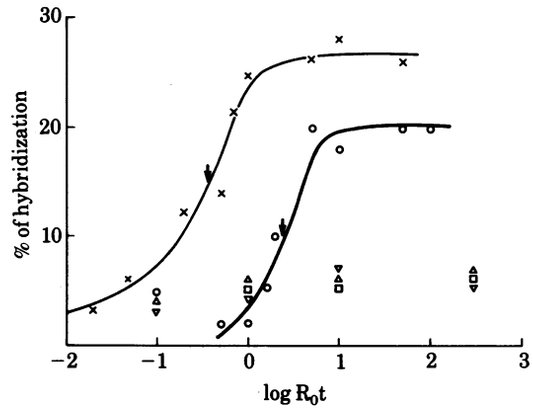

FIG. 2. Hybridization of total poly(A)-RNA from livers of male (×) and female (○) rats, kidney of male rats (△), hepatomas 5123D (□) and 7793 (▽) with the double-stranded *Ava* II fragment of p41. Arrows mark the $R_0t_{1/2}$ values. Maximal hybridization achieved was close to the theoretical 30%. This is a consequence of both DNA strands being labeled and the coding sequences constituting 59% of the *Ava* II fragment of p41.

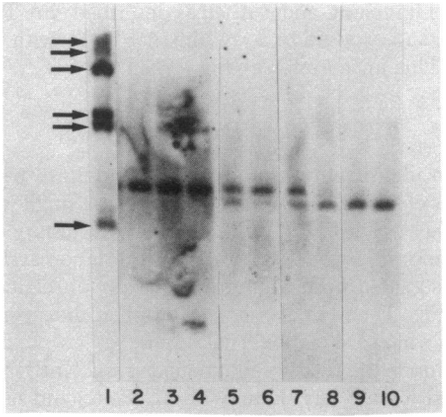

FIG. 3. Autoradiogram of a Southern blot of DNA from livers of normal Buffalo rats digested with *Hin*fI, *Hin*fI/*Hpa* II, and *Hin*fI/*Msp* I and DNA from hepatomas 7793 and 5123D. DNA (20 μg) was digested with the restriction endonucleases. In all cases, digestions were monitored with ^{32}P-labeled λ DNA. Lane 1, *Hin*dIII-digested ^{32}P-labeled λ DNA markers [23.7 and 9.46 kbp (unresolved), 6.67 kbp, 4.26 kbp, 2.25 kbp, 1.96 kbp, and 0.59 kbp.] Lanes 2–4, *Hin*fI-digested DNA from liver of normal male Buffalo rats, H5123D, and H7793, respectively. Lanes 5–7, *Hin*fI/*Hpa* II-digested DNA from liver of normal male Buffalo rats, H5123D, and H7793, respectively. Lanes 8–10, *Hin*fI/*Msp* I-digested DNA from liver of normal male Buffalo rats, H5123D, and H7793, respectively.

these three DNAs generated a single 0.7-kbp fragment complementary to p41 in each case (Fig. 3, lanes 8–10). Therefore, in liver and hepatoma 7793 there is a site that is methylated in only a fraction of the DNA molecules, whereas in hepatoma 5123D this DNA site is usually fully methylated.

No differences in the degree of methylation of this restriction site were detected when hepatic DNA from Sprague–Dawley and Buffalo rats were compared (Fig. 4).

Ava I and *Eco*RI are also restriction enzymes whose activity is inhibited by methylation of their recognition sequences. *Ava* I digestion of DNA from livers of male rats yielded two hybridizable fragments of 2.4 and 3.5 kbp. However, *Ava* I digestion of hepatomas 7793 and 5123D generated fragments of 5.9 and 5.5 kbp, respectively (Fig. 5). The existence of two *Ava* I-generated hybridizable fragments in liver DNA is consistent with the fact that the nonmethylated p41 cDNA clone contains an internal *Ava* I site.* This DNA site apparently becomes methylated in

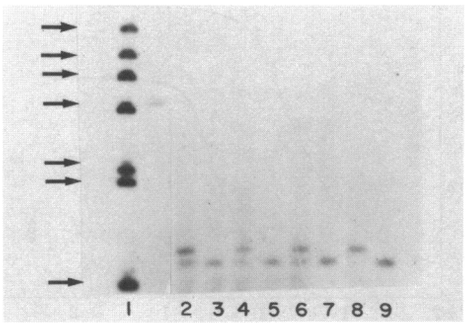

FIG. 4. Autoradiogram of a Southern blot of DNA digested with *Hin*fI/*Hpa* II and *Hin*fI/*Msp* I from livers of male Sprague–Dawley and Buffalo rats and from hepatomas 7793 and 5123D. Lane 1, *Hin*dIII-digested ^{32}P-labeled λ DNA markers (23.7, 9.46, 6.67, 4.26, 2.25, 1.96, and 0.59 kbp). Lanes 2, 4, 6, and 8, *Hin*fI/*Hpa* II-digested DNA from normal livers of Sprague–Dawley and Buffalo rats and of hepatomas 7793 and 5123D, respectively. Lanes 3, 5, 7, and 9, *Hin*fI/*Msp* I-digested DNA from normal livers of Sprague–Dawley and Buffalo rats and of hepatomas 7793 and 5123D, respectively.

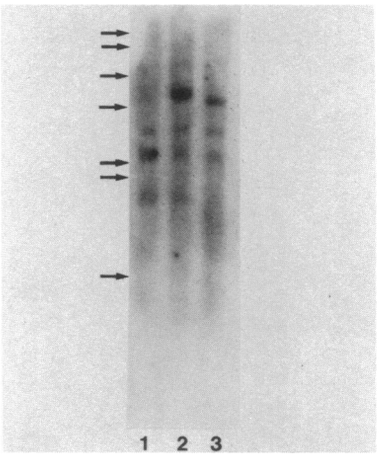

FIG. 5. Autoradiogram of a Southern blot of *Ava* I-digested DNA from livers of male Buffalo rats and hepatomas 7793 and 5123D. DNA (20 μg) was digested with *Ava* I. Lane 1, DNA from livers of male Buffalo rats; lane 2, hepatoma 7793 DNA; lane 3, hepatoma 5123D DNA. Arrows show the position of molecular weight markers of ^{32}P-labeled, *Hin*dIII-digested λ DNA markers.

hepatomas. Similarly, *Eco*RI digestion of DNA from liver of male rats and from hepatoma 5123D generated a single 6.6-kbp hybridizable fragment, whereas *Eco*RI digestion of hepatoma 7793 DNA generated an additional 9.4-kbp fragment (Fig. 6). These structural differences are also consistent with the hypermethylation of the hepatoma genome, but may be due to base substitution. Digestion of DNA from liver of male rats and hepatoma DNA with *Kpn* I, *Pst* I, and *Hin*dIII revealed no tissue-specific differences (data not shown). These data demonstrate that the lack of expression of the hp22 gene correlates with its hypermethylation and/or base substitution at several specific sites.

Numerous attempts have been made to correlate the expression of a specific gene with its extent of methylation. In the genome of *Xenopus laevis*, somatic DNA is more extensively methylated than amplified ribosomal DNA (26). McGhee and Ginder (28) have attempted to correlate methylation patterns

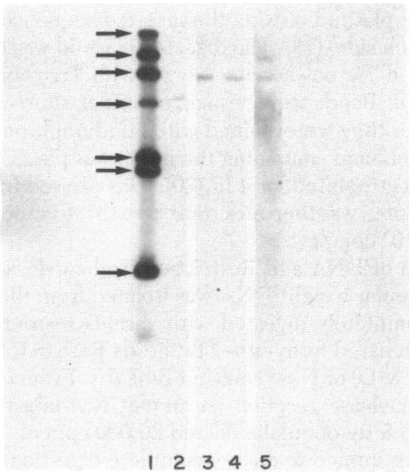

FIG. 6. Autoradiogram of a Southern blot of *Eco*RI-digested high molecular weight DNA from Buffalo rat liver and hepatomas 7793 and 5123D. DNA (20 μg) was digested with restriction endonuclease *Eco*RI. Lane 1, ^{32}P-labeled, *Hin*dIII-digested λ DNA as size markers (23.7, 9.46, 6.67, 4.26, 2.25, 1.96, and 0.59 kbp). Lane 2, p41 DNA; lane 3, DNA from liver of male Buffalo rats; lane 4, hepatoma 5123D DNA; lane 5, hepatoma 7793 DNA.

and chicken β-globin gene activity. In cells that are expressing or have expressed the adult β-globin gene (adult reticulocytes and erythrocytes), the C-C-G-G sites near the ends of the gene sequence seem to be completely unmethylated. However, in avian cells in which this gene is not expressed (oviduct, brain, and embryonic erythrocytes), these sites are partially methylated. In adenovirus type 12 DNA, Sutter and Doerfler (29) have shown an inverse relationship between the extent of methylation of specific DNA regions and the degree of their transcription. In contrast, Waalwijk and Flavell (30) showed that the presence or absence of a methyl group at a particular site within the rabbit β-globin gene does not correlate with its expression. Tissue-specific methylation of ovalbumin, conalbumin, and ovomucoid genes that influences their DNase I sensitivity has also been reported (31, 32).

Our studies indicate that several sites within or proximal to the gene coding for the hp22 protein are partially methylated in normal liver and are hypermethylated and possibly also base substituted in the DNA of hepatomas 7793 and 5123D. The question remains open as to whether these changes in gene structure are responsible for the lack of transcription of this gene in these hepatomas. What is evident from these studies is that covalent modification of the DNA has taken place. It seems reasonable to consider that alterations in gene structure of this nature may be of importance in mediating neoplastic transformation.

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