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Methylation of the alphafetoprotein gene in cell populations isolated from rat livers during carcinogenesis

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ABSTRACT

We examined the methylation pattern and organization of the AFP gene in whole livers and in isolated cell populations purified from livers of rats fed a carcinogenic diet which interferes with DNA methylation. Using restriction endonuclease digestion, we find no differences in methylation pattern and overall organization of the AFP gene in oval cells (AFP-producers) and hepatocytes (non-producers) isolated at the early stages of carcinogenesis. Our studies indicate that in cell populations which produce AFP as well as in cells which are not active in AFP synthesis, the majority of the CCGG sites of the AFP gene are extensively methylated. In addition, we describe the existence of polymorphism in the AFP and albumin genes of Sprague-Dawley rats.

INTRODUCTION

In mammals, large amounts of alphafetoprotein (AFP) are produced and secreted into the fetal circulation by the yolk sac and developing liver (1). Shortly after birth, hepatic AFP production falls sharply but it recurs in the adult during liver carcinogenesis and to a lesser extent in natural or experimental conditions which elicit cell proliferation in the liver (2). In humans, highly elevated serum AFP concentrations in the adult characterize hepatoma development or germ cell tumors (3).

Because of its oncodevelopmental regulation, the organization of the AFP gene and its expression during liver development, regeneration and carcinogenesis have been investigated extensively (3,4 for reviews; 5). Since in mammals the degree of DNA methylation of a gene may correlate with its transcriptional activity (5), it is logical to ask whether the changes in AFP gene expression which take place during development or carcinogenesis may be associated with modifications in the methylation level of the gene. However, an association between hypomethylation of a gene and its high transcriptional activity does not necessarily imply that the methylation changes play a primary role in controlling the activity of the gene (7,8).

Andrews et al. (9) reported that in mice the AFP gene is less methylated in fetal livers and AFP-producing hepatomas than in adult liver or non AFP-producing hepatomas and concluded that there was a positive correlation between hypomethylation of HpaII sites in the AFP gene and its expression. In contrast, Vedel et al. (10) and Kunnath and Locker (11) found only small changes in the methylation pattern of the AFP gene between fetal and adult rat liver. Moreover, the changes observed were indicative of hypomethylation of some HpaII sites in adult liver, a tissue in which AFP mRNA is barely detectable. In the rat hepatoma 7777 which produces large amounts of AFP as well as in the C8 cell line derived from this tumor the AFP gene is less methylated than in fetal and adult livers but no detailed information is available on changes in methylation of the gene during carcinogenesis (10,12).

One of the problems of studying gene methylation in complex organs such as the liver is that a particular gene under study might be actively transcribed in only a small proportion of cells. In this case, the analysis of methylation patterns of the whole tissue may obscure relationships which might exist between gene expression and the extent of methylation. One case in point is the "re-expression" of AFP which occurs in rat liver in response to most hepatocarcinogens. The carcinogen-induced early increase in AFP most often coincides with the proliferation in the liver of bile duct-like "oval cells". The nature of such cells is the subject of extensive studies but there is general agreement that at the early stages of carcinogenesis induced by most chemicals these cells (or transitional forms between oval cells and hepatocytes) contain AFP and AFP mRNA while mature hepatocytes do not (13-17).

With newly developed cell separation procedures (16,18) it is possible to isolate from preneoplastic rat liver, cell populations which produce AFP (oval cells) and compare them with cells (hepatocytes) which are not active in AFP synthesis at least in the early stages of carcinogenesis (17). In this study, we examine the methylation patterns of the AFP gene in oval cells and hepatocytes isolated from the livers of rats fed a carcinogenic diet which is choline-deficient and contains 0.1% ethionine. Since this diet induces the proliferation of large amounts of oval cells (13,16) and inhibits DNA methylation (19), we expected that an association between DNA methylation and the transcriptional activity of the AFP gene might become apparent from the analysis of the gene in purified oval cells and hepatocytes.

MATERIALS AND METHODS

Animals - Random-bred male Sprague-Dawley rats (Crl:CDSDBR) weighing 120-140 g were obtained from the Charles River Breeding Laboratory (Wilmington, MA). Rats were fed either a standard laboratory rat chow or a carcinogenic diet (CDE diet) which is deficient in choline and contains 0.1% of the hepatocarcinogen DL-ethionine (Teklad Test Diets; Madison, WI) as originally described by Shinozuka et al. (13).

Cell Separation - The procedures used for the isolation of oval and parenchymal cells from livers of rats fed the CDE diet have been described in detail (16,18). Briefly, livers are perfused in situ with calcium-free medium followed by collagenase digestion in calcium supplemented buffer. For isolation of oval cells the tissue that remains undissociated after combing the liver in calcium-free Hanks solution is digested in medium containing 0.1% collagenase, 0.1% Pronase and 0.004% DNase I. To prepare parenchymal cells from livers of rats fed the CDE diet the perfused livers were minced in medium containing 0.1% collagenase and 0.004% DNase I. The centrifugal elutriation procedures have been described previously and were used here without modification.

DNA Isolation - Excised livers were minced and homogenized at 1500 rpm in 4 volumes of cold 2% citric acid. Homogenates were centrifuged at 600 X g for 10 min at 4°C to pellet nuclei. Nuclear pellets and purified liver cells were resuspended in 3 volumes of 0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris (pH 7.4) and were disrupted by adding proteinase K and SDS to a final concentration of 100 ug/ml and 0.5% respectively. DNA was extracted twice by gentle swirling in the presence of an equal volume of buffer saturated phenol/chloroform. Following ethanol precipitation, DNA preparations were treated consecutively for 1 h each at 37°C with RNase A (100 ug/ml) and proteinase K (20 ug/ml). DNAs were then re-extracted with phenol/chloroform and precipitated twice with ethanol. Precipitated DNA was redissolved in water at approximately 0.5 mg/ml and stored at -20°C.

Restriction Endonuclease Digestion of DNA - DNAs were digested with either Pst I (Bethesda Research Laboratories), EcoRI, MspI or HpaII (New England Biolabs) under the conditions specified by the supplier. Reaction mixtures were incubated overnight at 37°C using 5-10 units of enzyme per ug of DNA and included lambda phage DNA to assure that digestions were complete. Reactions were terminated by bringing the concentration of the reaction mixture to 10 mM EDTA and heating to 65°C for 5 min. Digested DNA was

precipitated with ethanol at -70°C. DNA precipitates were collected by centrifugation and redissolved in electrophoresis loading buffer.

DNA Gel Electrophoresis - Fifteen ug samples of restriction endonuclease digested DNA were separated by electrophoresis in 1.0% agarose horizontal slab gels (9). Gels were run at 35 volts in the cold for not less than 20 h. HindIII digested lambda phage DNA and HaeIII digested ϕX 174 phage DNA (Bethesda Research Laboratories), were run in parallel lanes as size markers. Following electrophoresis, gels were stained with ethidium bromide and photographed under short wave U.V. light. Gels were then soaked at room temperature in 0.25 N HCl for 20 min, 0.5 M NaOH/1.5 M NaCl for 30 min and 3 M NaCl/0.5 M Tris (pH 7.0) for 30 min. DNA was transferred to nitrocellulose filters in 20 X SSC as described by Southern (20). Following transfer, filters were baked for 4 h at 75°C.

Filter Hybridization - The recombinant plasmids pRAF 87 and pRAF 65 (21) containing rat AFP cDNA and pRSA 57 and pRSA 13 containing (22) rat albumin cDNA were provided by Dr. T. Sargent. AFP DNA inserts were purified on glass beads (23) following digestion of plasmid or phage DNA with the appropriate restriction endonuclease(s) and separation of the digestion products by agarose gel electrophoresis. Purified inserts were nick translated to a specific activity of 1-5 X 10^8 cpm/ug (24). Methods for filter hybridization were those of Bostian et al., (25) with the following modifications: a) filters were prehybridized at 42°C for no less than 15 h in 5 ml of 50% formamide, 5 X SSC, 1 X Denhardt's solution, 50 mM sodium phosphate (pH 6.8), 100 ug/ml yeast tRNA; b) hybridization was at 42°C for 72 h in the same buffer diluted 4:1 with 50% dextran sulphate and included 5-10 X 10^6 cpm/ml of [^{32}P] labeled DNA.

RESULTS AND DISCUSSION

Oval Cell Proliferation and AFP mRNA in Livers of Rats Fed the CDE Diet - In rats fed the CDE diet, oval cell proliferation starts between 2-4 weeks. In rats fed the diet for 4-16 weeks, these cells comprise 30-50% of the cell population in the liver (13,26). The average numbers of oval cells recovered from the liver of a single rat are 5.1 X 10^6, 1.7 X 10^8, 4.7 X 10^8 and 5.1 X 10^8 at 2,4,9 and 16 weeks of diet feeding (16). In parallel with the change in cell population, the level of AFP mRNA in the liver changes from 0.006% of the polysomal poly(A)$^+$ RNA in normal liver to 0.015, 0.21, 0.18 and 0.27% at 2,4,9 and 16 weeks (27). Dot-blots of RNA isolated

from hepatocytes and oval cells from rats fed the CDE diet from 2-16 weeks shows that oval cells purified by centrifugal elutriation contain at least 50-fold more full length AFP mRNA than hepatocytes, but the level of AFP mRNA in hepatocytes (if any) is difficult to estimate (17). Immunocytochemical analysis of the isolated cell fractions showed that 25-35% of isolated oval cells contain AFP while very few, if any, hepatocytes contain AFP at 4-16 weeks of diet feeding (16). Rats which are fed the CDE diet generally develop tumors starting at 25-30 weeks. Most of the work reported here was done in rats fed the diet for 2-16 weeks and for simplicity we will refer to the livers of these animals as "preneoplastic" without wishing to imply that the changes observed in the early stages of carcinogenesis are irreversible or that they form a continiuum with the tumorigenesis stage.

Methylation of the AFP Gene in the Livers of Rats Fed the CDE Diet - Before examining the pattern of methylation of the AFP gene in isolated cell populations, we asked whether changes in the extent of methylation at CCGG sites within the coding region of the liver AFP could be detected in DNA extracted from whole livers during the early stages of carcinogenesis at a time when AFP mRNA levels increased by 35-50 fold (27). DNAs obtained from the livers of normal adult and carcinogen-fed rats were digested to completion with the restriction endonucleases MspI and HpaII. Although both enzymes recognize the DNA sequence CCGG, this site is refractory to HpaII digestion when the internal cytosine is methylated (28). An exception to this rule is the failure of MspI to cut the sequence GGC^{m}CGG(29). Digestion of rat liver DNA from normal or carcinogen-fed animals with MspI followed by hybridization with [^{32}P] labeled pRAF 87 and pRAF 65 AFP cDNA probes generated 7 fragments (Fig 1a). This pattern is in agreement with the work of Kunnath and Locker (11) and Vedel et al. (10). As reported by these authors, we find that the adult rat liver AFP gene is generally resistant to HpaII digestion (although small amounts of the 3.4, 2.75 and 0.5 kbp AFP gene fragments are generated). This is true for normal rats (not shown) and for animals fed the CDE diet for 4,11 and 30 weeks (Fig 2). These observations indicate that the AFP gene is heavily methylated in normal adult liver, that the methylation of certain sites in the gene is not complete and that the extent of methylation (as examined in DNA from whole livers) does not change at a time when these livers contain 30-50 fold more AFP mRNA than the normal liver.

Methylation of AFP Gene in Oval Cells and Hepatocytes Purified from Preneoplastic Livers - If changes in DNA methylation are associated with tran-

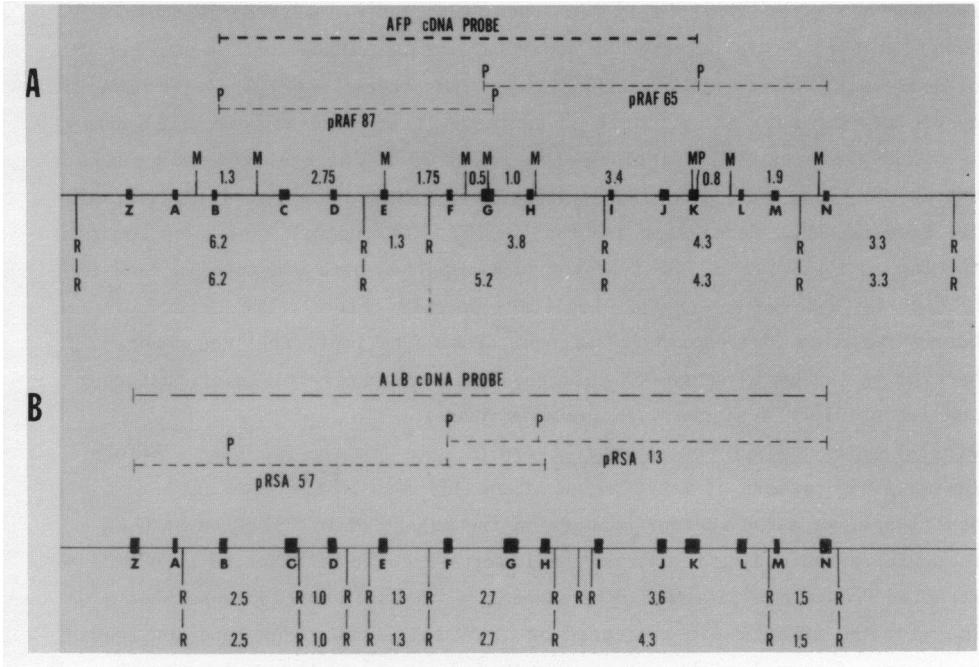

Figure 1. Map of Sprague-Dawley Rat AFP and Albumin Genes.
a). AFP Gene Map: The position of MspI sites (M), exons Z-N (shaded boxes) and introns (solid line) are from Kunnath and Locker (11). EcoRI sites (R) are positioned based on the restriction map of genomic clones lambda RAF 6 and RAF 7 (T. Sargent, Personal communication). A [^{32}P] labeled hybridization probe (heavy broken line) was prepared by excising AFP cDNA sequences from the recombinant plasmids pRAF 87 and pRAF 65 (light broken lines) by PstI (P) digestion as described in Methods.
b). Albumin Gene Map: The position of EcoRI sites (R), exons Z-N (shaded boxes) and introns (solid line) are published (10,22). A [^{32}P] labeled hybridization probe (heavy broken line) was prepared by excising albumin cDNA from the recombinant plasmids pRSA 57 and pRSA 13 (light broken lines) by PstI (P) digestion as described in Materials and Methods. In all figures, DNA fragment lengths are in kilobase pairs.

scriptional activity and only a proportion of the total cell population contains a transcriptionally active AFP gene, studies of AFP gene methylation in DNA obtained from whole livers may not provide a true picture of the extent of AFP gene methylation in a particular cell population. In the case of animals fed the CDE diet, it has been established that at the earlier stages of carcinogenesis (4-16 weeks) AFP is produced almost exclusively by oval cells and that such cells have levels of AFP mRNA which are at least 50 fold higher than those of hepatocytes. Since the methylation

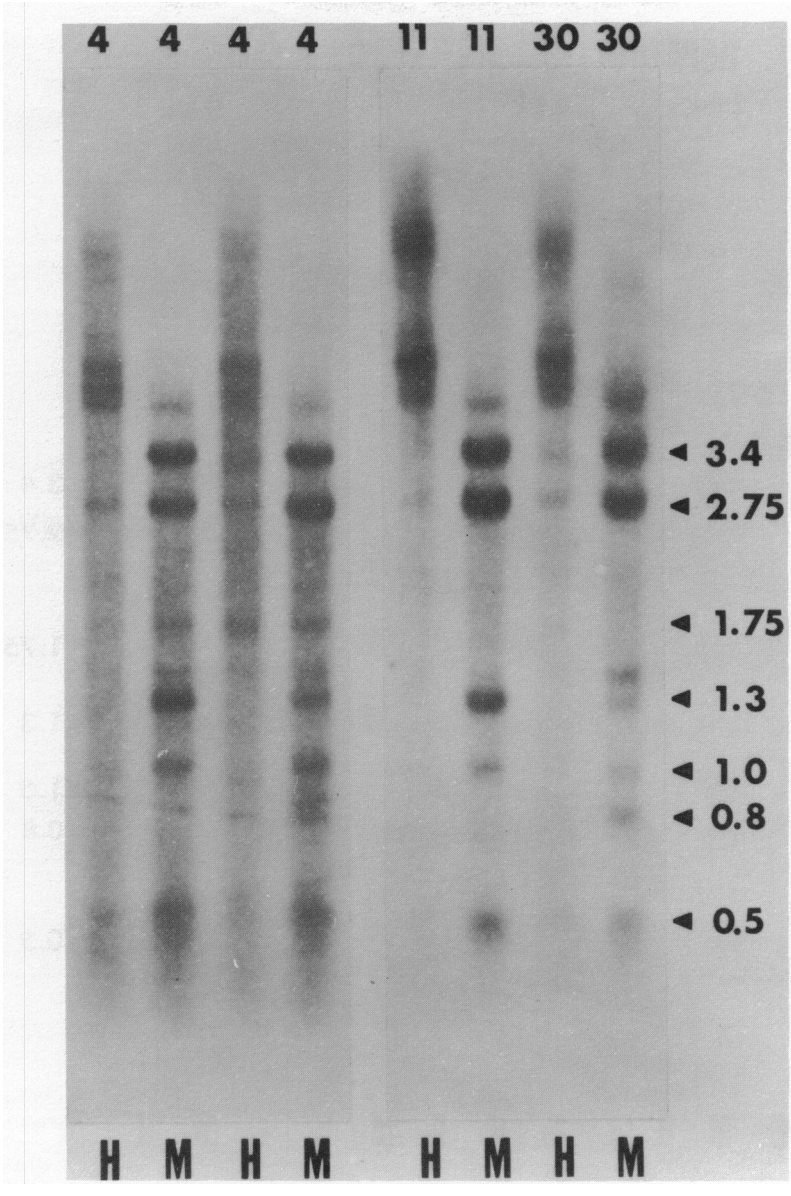

Figure 2. Methylation of the AFP Gene in Livers of Carcinogen-Fed Rats. Fifteen ug of DNA prepared from livers of rats fed the CDE diet for 4 (two individuals), 11 and 30 weeks were digested with the restriction endonuclease HpaII (H) or MspI (M). DNA fragments were separated by electrophoresis in 1% agarose gels, blotted onto nitrocellulose filters and the filters incubated with [^{32}P] labeled AFP cDNA (see Fig 1) as described in Materials and Methods. Autoradiograms were prepared by exposing Kodak XAR film to the filter at -70^{o}C using an intensifying screen.

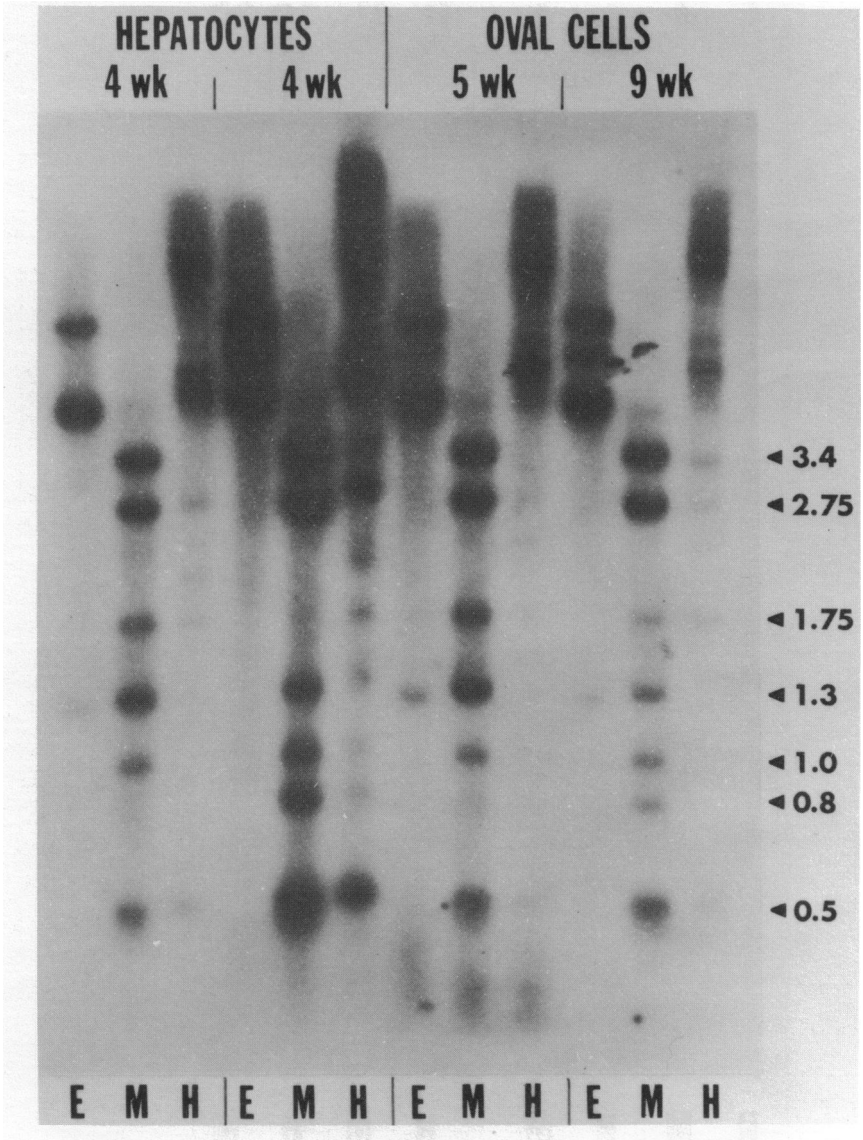

Figure 3. Methylation of the AFP Gene in Isolated Cells from Carcinogen-fed Rats. Oval cells and hepatocytes were isolated from livers of rats maintained on the CDE diet as described in Materials and Methods. DNAs prepared from 4 week CDE liver hepatocytes (two preparations) and 5 and 9 week CDE liver oval cells were digested with the restriction endonuclease EcoRI (E), MspI (M) or HpaII (H). Digestion products were analyzed with [^{32}P] labeled AFP cDNA as described in the legend for Fig 2. The hepatocyte preparation shown in lanes 4-6 contained 3 times more DNA than the fraction analyzed in lanes 1-3.

of the AFP gene in these two cell populations might be quite different and reflect the degree of transcriptional activity, we separated oval cells and hepatocytes from livers of rats fed the CDE diet using previously described methods (16,18). We prepared DNA from hepatocytes isolated after 4 weeks of carcinogen feeding and oval cells isolated at 5 and 9 weeks. The DNAs were digested with EcoRI, MspI and HpaII and hybridized with AFP cDNA probes (Fig 3). The EcoRI digestion patterns of hepatocytes and oval cells are similar revealing no striking differences in the organization of the AFP gene in these two cell types. Oval cell or hepatocyte DNAs are very resistant to digestion with Hpa II indicating a high level of methylation. The patterns shown in Fig 3 argue strongly that the preferential transcription of the AFP gene in oval cells is not correlated with hypomethylation of CCGG sites within the structural gene of these cells.

Using the standard MspI/HpaII digestion procedure Kunnath and Locker and Vedel et al. (10,11) have described two sites in the AFP gene of adult normal rat liver which are undermethylated. We investigated whether DNA from hepatocytes and oval cells contained hypomethylated sites. For this purpose we digested hepatocyte and oval cell DNA with PstI prior to incubation with MspI or HpaII. Following PstI digestion, [^{32}P] labeled AFP cDNA hybridizes to 4 DNA fragments which we estimate to be 4.3, 3.8, 2.25 and 1.4 kbp in size (Fig 4). When PstI digestion is followed by digestion with HpaII, the intensities of the hybridization signals generated by the 4.3 and 1.4 kbp PstI fragments are sharply reduced for both oval cell and hepatocyte DNAs. Because these two fragments (both in hepatocytes and oval cells) are very susceptible to HpaII digestion, we conclude that they must each contain at least one CCGG site which is not methylated to any great degree. Overall, the data in Figure 4 support the finding that there are specific sites within the AFP gene in oval cells and hepatocytes which lack extensive methylation. This type of analysis also strengthens our conclusion that methylation of CCGG sites within the AFP gene is not significantly different in the oval cells or hepatocytes obtained from rats maintained on the carcinogenic diet despite the large difference in AFP mRNA concentrations between these two cell populations.

Polymorphism of AFP and Albumin Genes in Sprague-Dawley Rats - Gal et al. (30) and Lucotte et al. (31) have described structural variants of the AFP and albumin genes in different strains of rats and indicated that Sprague-Dawley rats contain only one type of variant for the AFP in albumin genes. However, Boulter and Sell (32) reported that while Buffalo, Fischer and ACI

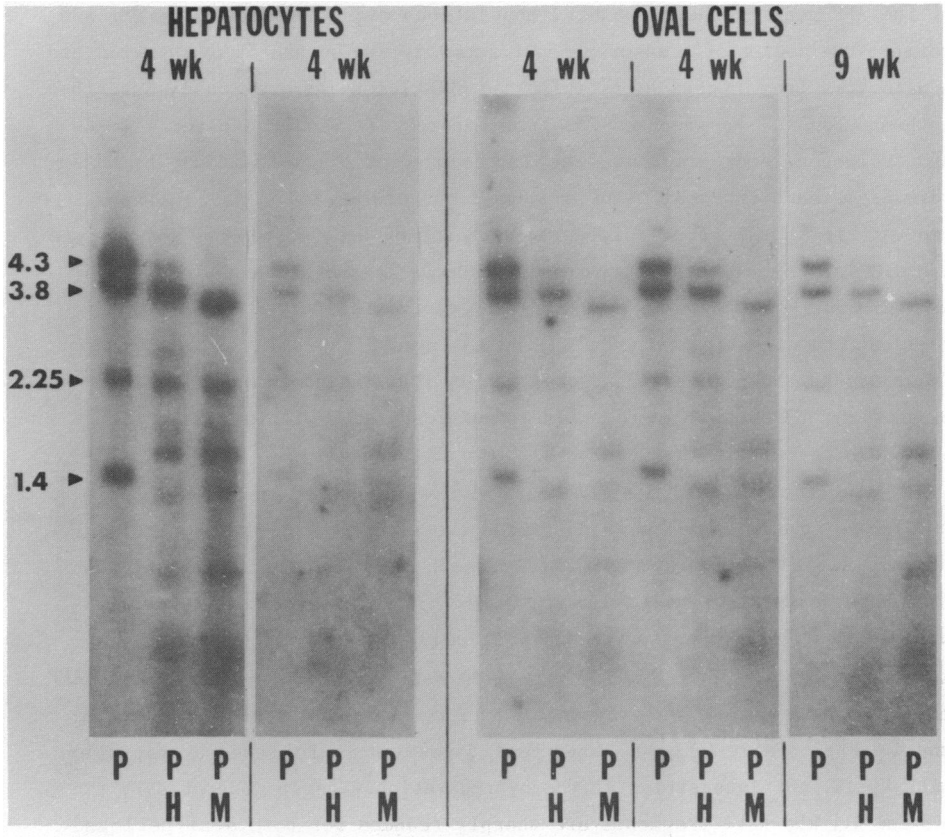

Figure 4. Detection of Hypomethylated Sites within the AFP Gene in Oval Cells and Hepatocytes. DNAs prepared from two 4 week CDE liver hepatocyte preparations, two 4 week CDE liver oval cell preparations, and a 9 week CDE liver oval cell preparation were digested with PstI alone (P), or with PstI followed by either HpaII (H) or MspI (M). Digestion products were analyzed with [^{32}P] labeled AFP cDNA as described in the legend for Fig 2.

rats have a characteristic banding pattern for the AFP gene upon digestion of DNA by HindIII and MspI, Sprague-Dawley rats might be heterozygous for variants of the AFP and albumin genes. To investigate the existence of intrastrain polymorphism of the AFP gene in Sprague-Dawley rats, we used the restriction endonucleases MspI, EcoRI and PstI and Southern blotting to analyze the AFP gene in 11 male Sprague-Dawley rats. The results are compiled in Table I. PstI digestion invariably generated 4 AFP gene fragments. In contrast, several different digestion patterns were observed after incubation of these same DNA preparations with either MspI or EcoRI.

Table 1. Digestion Patterns of the AFP Gene in Sprague-Dawley Rats

Animal #[a]	Restriction Fragments[b]															
	Msp I							Eco RI					Pst I			
	3.4	2.75	1.75	1.3	1.0	0.8	0.5	6.2	5.2	4.3	3.8	1.3	4.3	3.8	2.25	1.4
1	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
8	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+	+
9	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+	+
10	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+	+
11	+	+	-	+	+	-	+	+	+	+	-	-	+	+	+	+

a. DNA samples were prepared from 11 individual male Sprague-Dawley rats obtained from Charles River Breeding Laboratory (Wilmington, MA).

b. DNA samples were digested with Msp I, Eco RI, and Pst I and the digestion products separated on agarose gels before transfer to nitrocellulose filters. Filters were incubated with [^{32}P] labeled pRAF 87 and pRAF 65 insert DNA and each DNA sample was then scored for the presence (+) or absence (-) of specific restriction fragments. For detailed methods see Materials and Methods. Fragment lengths are in kilobase pairs.

Following MspI digestion, all DNA preparations generated fragments of 3.4, 2.75, 1.3, 1.0, and 0.5 kbp. Seven of 11 DNAs also contained an additional 1.75 kbp fragment and 6 of 11 produced a 0.8 kbp fragment. One DNA sample failed to generate both the 1.75 kbp and 0.8 kbp fragments (Table I). Alternate digestion products could not be detected in DNA preparations lacking the 1.75 and/or 0.8 kbp fragments. The two EcoRI digestion products common to all of the DNA preparations examined, were 6.2 and 4.3 kbp in length. All but one DNA preparation also produced a 5.2 kbp EcoRI fragment while a 3.8 kbp and a 1.3 kbp fragment could be detected in the DNA prepared from 7 of the 11 animals.

The presence or absence of the 5.2, 3.8 and 1.3 kbp EcoRI fragments in the Sprague-Dawley rat liver DNA used in this study probably reflects an allelic variation in the AFP gene which affects an EcoRI recognition site within the 5.2 kbp fragment (Fig 1a). If the EcoRI recognition site is present in both alleles (#1 in Table I), digestion with EcoRI produces 4 fragments that are 6.2, 4.3, 3.8 and 1.3 kbp in length. Alternatively, if the recognition site is absent in both alleles, EcoRI digestion produces 3 fragments of 6.2, 5.2 and 4.3 kbp (#8-11 in Table 1). In turn, the DNA

from animals heterozygous for the two AFP gene variants generates 5 EcoRI fragments 6.2, 5.2, 4.3, 3.8, and 1.3 kbp in length in a predicted molar ratio of 2:1:2:1:1 (#2-7 in Table 1).

To investigate whether or not more than one form of the albumin gene could also be detected in Sprague-Dawley rats, the EcoRI digestion products of rat liver DNA were analyzed by Southern blots using rat albumin cDNA from the recombinant plasmids pRSA 57 and pRSA 13 (22) as a hybridization probe (Fig 1b). Three distinct patterns are generated when the DNA from 10 individual animals are each incubated with EcoRI. Four DNA preparations generated fragments of 3.6, 2.7, 2.5, 1.5, and 1.3 kbp while another 5 preparations contained an additional 4.3 kbp fragment. These observations are consistent with the work of Lucotte et al. (31). However, one of the DNA samples produced fragments of 4.3, 2.7, 1.5, and 1.3 kbp but failed to generate the 3.6 and 2.5 kbp fragments. We therefore suggest that, as with the AFP gene, more than one form of the albumin gene is present in the Sprague-Dawley animals used in this study. We suggest that one variant generates 3.6, 2.7, 2.5, 1.5 and 1.3 kbp fragments while another produces fragments of 4.3, 2.7, 1.5, and 1.3 kbp (see Fig 1b) and that the presence of the 4.3, 3.6 and 2.5 kbp fragments within the same DNA preparation signifies that the animal was heterozygous for both albumin alleles.

In conclusion, we show that at least two forms of the AFP and albumin gene exist in Sprague-Dawley rats. From the analysis of the AFP gene in oval cells and hepatocytes purified by centrifugal elutriation from livers of rats fed the CDE diet, we find no evidence that the preferential transcriptional activity of the AFP gene in oval cells is due to major differences in the structure, copy number, or degree of methylation of the AFP gene in this cell type. However, we have not yet examined whether differences in cytosine methylation might exist between oval cells and hepatocytes in the DNA sequences immediately flanking the AFP structural gene.

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