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METHYLATION DIFFERENCES IN THE MURINE P_1450 AND P_3450 GENES IN WILD-TYPE AND MUTANT HEPATOMA CELL CULTURE

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Abstract—The murine P_1450 and P_3450 genes and flanking regions contain 14 and 15 *Msp* I sites (C-C-G-G-), respectively, designated M1 through M14 or M15. These two genes from mouse Hepa-1 wild-type (wt) parent and three mutant cell lines were studied for methylation differences with use of the isoschizomers *Msp* I and *Hpa* II The mutant lines included c1, having high constitutive P_1450 mRNA and believed to carry a mutation in the P_1450 structural gene, c2, having negligible levels of Ah receptor, and c4, having a defect in nuclear translocation of the inducer–receptor complex The P_3450 gene was not expressed constitutively or after treatment of these four cell lines with the P_1450 inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and, correspondingly, the P_3450 *Msp* I sites remained methylated Treatment of all four cell lines with TCDD did not alter the P_1450 methylation pattern, nor was there any evidence of P_1450 gene amplification Treatment of all four lines with 5-azacytidine caused demethylation of the P_1450 *Msp* I sites but did not change the usual P_1450 catalytic activity pattern found in each of the lines The only detectable difference in the P_1450 gene among the four lines was hypomethylation of the M9 site in c1 that was not seen in wt, c2 and c4 cells The M9 site is part of a 9-bp box (5′-C-C-G-G-G-A-C-A-T-3′), located near the beginning of exon 3 It is of interest that the same nine bases are found in intron 2 about 80 bp upstream from the 5′ end of exon 3 in the homologous P_1450 gene

The murine TCDD§-inducible P450 gene family comprises two genes, P_1450 and P_3450 [1], which are controlled by the cytosolic Ah receptor [reviewed in Refs 2 and 3] TCDD and other inducing foreign chemicals bind avidly to this receptor (apparent $K_d < 10^{-9}$). The inducer–receptor complex is translocated to the nucleus where P_1450 and P_3450 gene transcriptional activation occurs [reviewed in Ref. 4]. Developmental and tissue specificity in mouse P_1450 and P_3450 gene expression has also been determined [5]

The mouse hepatoma Hepa-1 cell line has inducible P_1450 expression but no constitutive or inducible P_3450 expression [6] The P_1450 induction process has been studied extensively by somatic cell hybridization [reviewed in Ref. 7]. These studies with benzo[*a*]pyrene-resistant mutant clones have shown that all recessive mutants belong to three complementation groups—A, B and C—indicating involvement of at least three genes in the P_1450 induction process [8] The c1 variant (Group A, subgroup IV) exhibits markedly elevated constitutive P_1450 mRNA [9] and is believed to carry a mutation in the P_1450 structural gene [10]. Groups B and C are regulatory mutants. The c2 variant (Group B) has Ah receptor levels that are less than 5% of those in wt cells [11]. The c4 variant (Group C) has a normal cytosolic Ah receptor concentration but impaired nuclear translocation of the inducer–receptor complex [11].

Changes in DNA methylation patterns may be a cause, or the result, of changes in eukaryotic gene expression [12–14]. In eukaryotes, the sites of methylation are almost invariably the C-5 position of cytosine and, in animals, CpG is the usual sequence which contains 5-methylcytosine The isoschizomers *Hpa* II and *Msp* I cleave the sequence C-C-G-G, methylation of the internal C residue renders this sequence resistant to *Hpa* II but not to *Msp* I. With use of these isoschizomers and the Southern blot technique, methylation at those CpG sites embedded in a C-C-G-G sequence can be assessed [15, 16]

In this report we examine DNA methylation patterns of the P_1450 and P_3450 genes among control and TCDD-treated wt cells at the c1, c2 and c4 variant cells. Differences in methylation pattern between the c1 variant and wt cells might contribute to the mechanism by which high constitutive P_1450 mRNA is expressed in the c1 variant cell line. The c2 and c4 regulatory mutants were used as controls in which no differences in methylation patterns between these variants and wt cells were expected

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§ Abbreviations TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, wt, wild-type, MEM, Eagle's Minimum Essential Medium, bp, base-pairs, and kb, kilobases

EXPERIMENTAL PROCEDURES

Materials

All tissue culture solutions and fetal calf serum were purchased from GIBCO (Grand Island, NY), tissue culture plastic ware was bought from Falcon Plastics (Oxnard, CA), nitrocellulose paper was obtained from the Millipore corp. (Bedford, MA), restriction endonucleases were purchased from BRL (Rockville, MD), 5-azacytidine was obtained from the Sigma Chemical Co. (St. Louis, MO); and [γ-^{32}P]ATP was bought from Amersham Radiochemicals (Cambridge, MA). TCDD was a gift from the Dow Chemical Co. (Midland, MI)

Cell cultures

The origin and development of the Hepa-1 wild-type line [17] and the c1, c2 and c4 mutants [8, 18, 19] have been described. Cultures were grown as monolayers at 37° in 95% air and 5% CO_2 in α-MEM medium supplemented with 10% fetal calf serum, penicillin (40 units/ml), streptomycin (40 μg/ml) and Mycostatin (25 μg/ml). Cells were plated at a density of 10^6 per 125-cm^2 T-flask and treated with TCDD (or control medium alone) during the second half of the logarithmic growth phase. Unless otherwise indicated, the TCDD concentration was 10 nM. In some experiments, the cultures were exposed to 5-azacytidine (3 μM) For experiments lasting more than 24 hr, the medium was replaced each day All experiments were repeated two to four times to ensure reproducibility

Southern hybridizations

DNA was isolated by the method of Blin and Stafford [20]. Restriction endonucleases were used according to the protocol of the vendor. Control DNA fragments having known sites were always included to ensure complete cutting by the restriction enzymes in each experiment The DNA fragments were separated by electrophoresis on agarose gels (0 7 to 1.4%), along with λ phage DNA molecular weight standards (BRL, Rockville, MD) The fragments were blotted onto nitrocellulose membranes [21] and hybridized to nick-translated ^{32}P-labeled probes [22] for 36 hr as described previously [23] The filters were then washed three times with 3X SSC (1X SSC = 150 mM NaCl and 15 mM sodium citrate) plus 0 5% sodium dodecyl sulfate for 1 hr at 68°, followed by one wash with 1X SSC at room temperature. Filters were exposed to Kodak RP-5 X-ray film at −70° for 2 days to 2 weeks with the use of intensifier screens (Lightning Plus, Dupont, Wilmington, DE). The probes included $P_1$450 and $P_3$450 full-length cDNA inserts, p$P_1$450FL and p$P_3$450FL, respectively [23], a 0 40-kb segment

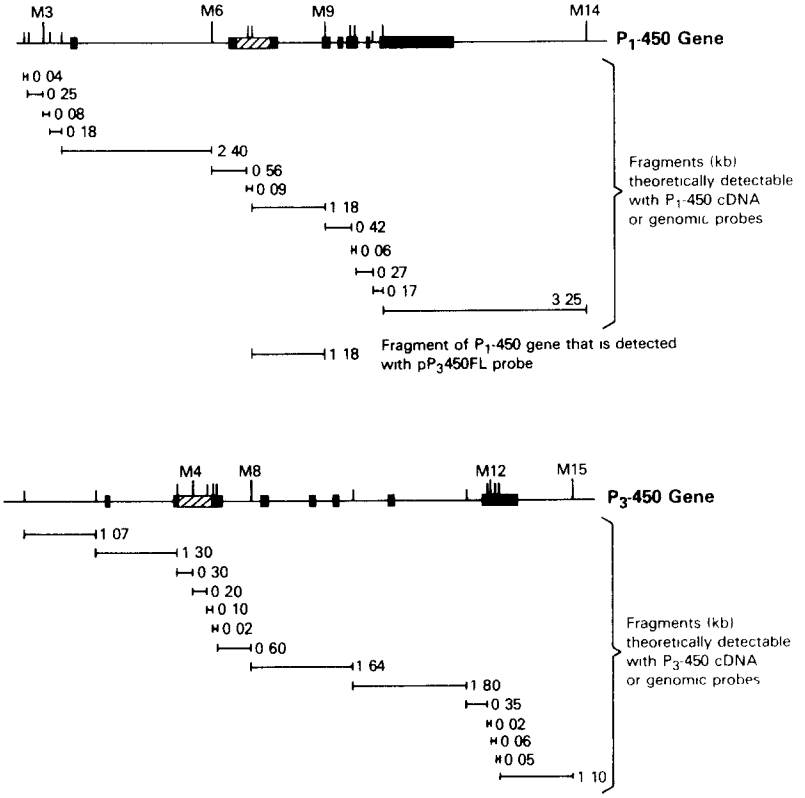

Fig 1 Exon–intron patterns of the $P_1$450 and $P_3$450 genes and flanking regions The seven exons of each gene are shown as *closed boxes* A 0 5-kb stretch of 96% similarity between the two homologous genes in exon 2 [24] is shown as a *striped area* Because of the strongly conserved sequence of the two cDNAs in the exon 2 region, the p$P_3$450FL probe cross-hybridizes with the $P_1$450 1 18 kb fragment *Msp* I sites (M1 through M14 or M15) on each gene are denoted by *vertical lines* Fragments theoretically detectable by either cDNA or genomic probes are illustrated, although fragments of less than 0 4 kb did not consistently transfer to the nitrocellulose membranes

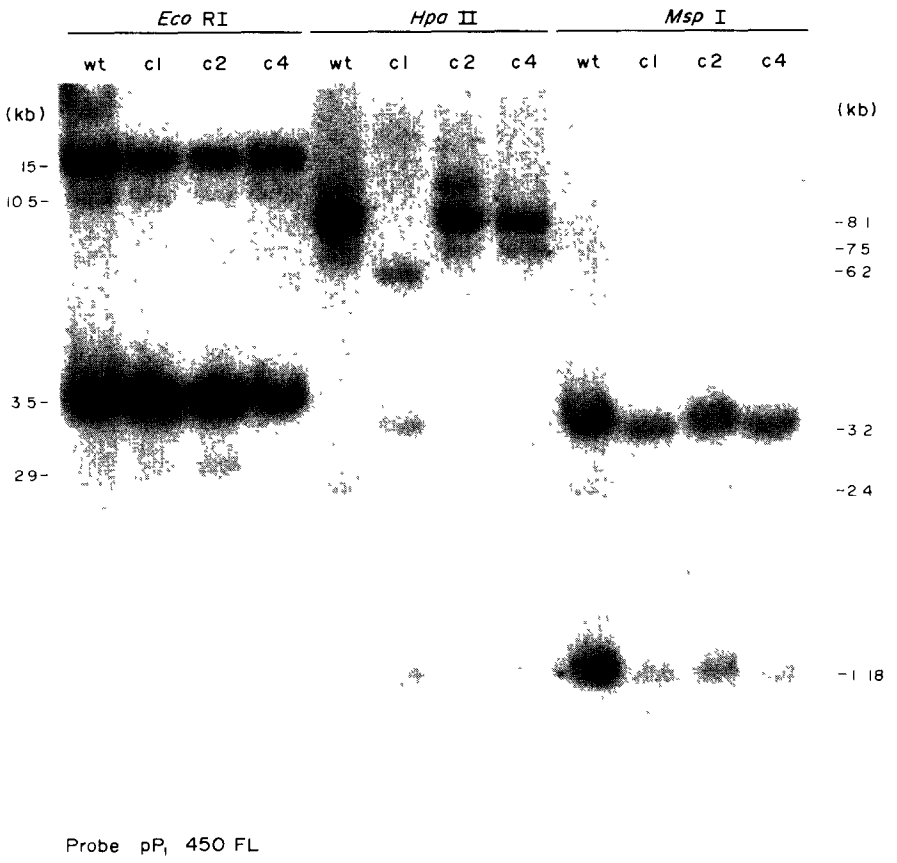

Fig 2 (A) Southern blot hybridization of the four cell lines' probed with DNA digested with each of three restriction enzymes and pP_1450FL

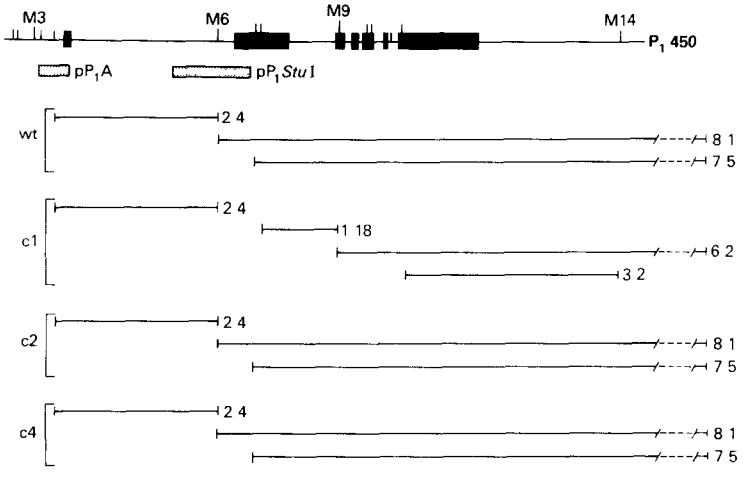

Fig 2 (B) The positions of the pP_1*StuI* and pP_1A genomic probes and the fragments detected by pP_1450FL The 14 *Msp* I sites and seven exons of the P_1450 gene are depicted as in Fig 1 The numbers at the right of each fragment are given in kb

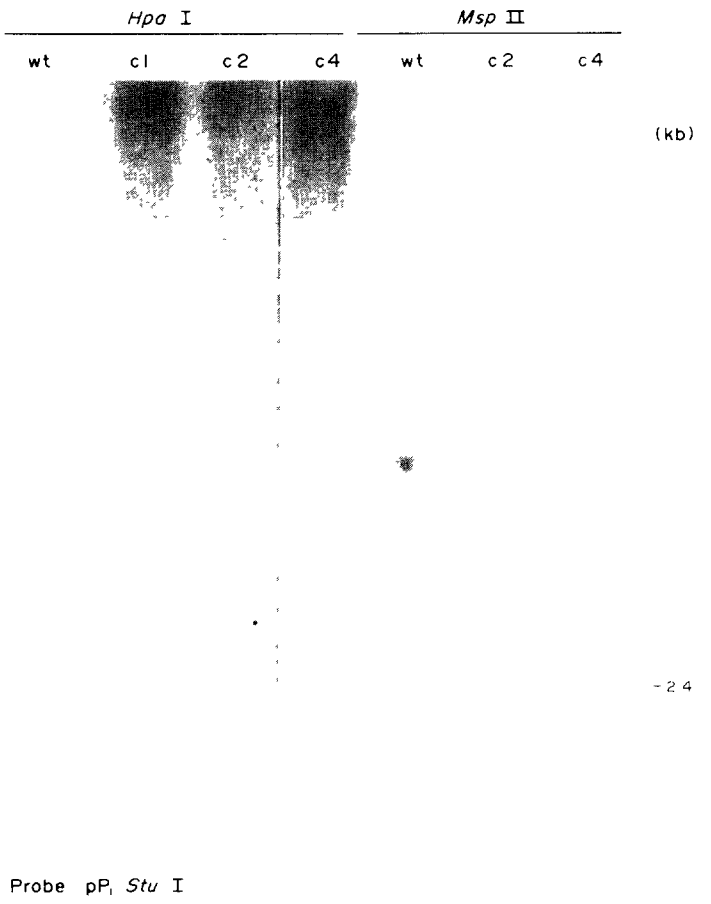

Fig 2 (C) Hybridization analysis of wt, c1, c2 and c4 DNA digested with *Hpa* II and *Msp* I and probed with pP_1*Stu*I The numbers at the margins of each Southern plot in panels A and C denote the fragment length in kb

(pP_1A) covering 380 bp upstream from the P_1450 cap site and a portion of the first exon [1], and a 1 2-kb *Stu* I fragment (pP_1*Stu*I) covering portions of the first intron and second exon of P_1450 [1]

RESULTS AND DISCUSSION

P_1450 genomic DNA studied with a single restriction endonuclease digestion

Sequence analysis of the mouse P_1450 gene and flanking regions [1] revealed 14 *Msp* I sites (Fig. 1, top), these sites are referred to as M1 through M14 The P_3-450 gene [1] has 15 *Msp* I sites (M1 through M15) (Fig 1, bottom). When the pP_1450FL probe was used (Fig 2A), no differences in the major *Msp* I fragments of P_1450 (3 2, 2 4 and 1 18 kb, [0 56 and 0 42 kb data not shown]) among wt and the c1, c2 and c4 variants were detected The 2 4-kb band is very weak because it hybridizes only to the 87 bases of exon 1 of P_1450 [1]

Eco RI-digested DNA probed with pP_1450FL yielded the same pattern in all four cell lines (Fig 2A), with major bands of 15 and 3 5 kb (minor bands of 10.5 and 2 9 kb represent cross-hybridization with the P_3450 gene) The results with *Msp* I and *Eco* RI (and *Bam* HI data, not included) thus indicate that any differences in the *Hpa* II pattern among the four cell lines would not be due to genomic rearrangement or translocation but rather to a methylation polymorphism

Hpa II-digested DNA probed with pP_1450FL (Fig 2A) gave identical patterns for wt and the two regulatory mutant clones c2 and c4, with fragment sizes of 8.1, 7.5 and 2.4 kb. It had been anticipated that the c2 and c4 regulatory mutants would exhibit patterns identical to the pattern of wt cells and thus could be used as control cell lines As compared with wt, c2 and c4 (Figs 2A and 2B), clone c1 exhibited a different pattern (6 2, 3 2, 2 4 and 1 18 kb) This pattern suggests a loss of methyl groups at sites M9, M13 and M14

The results in Fig 2A suggest that sites M5, M6 and M7 (and/or M8) are hypomethylated in all four lines As expected, *Hpa* II- and *Msp* I-digested DNA from all four lines probed with pP_1*Stu*I gave identical fragments of 2 4 kb (Fig 2C).

When *Hpa* II- and *Msp* I-digested DNA was probed with the 5′ genomic probe pP_1A no fragments of sufficient length were found to be transferred to the nitrocellulose membranes These data indicate that one or more of the sites M1 through M5 is(are) hypomethylated in all four lines tested As is commonly the case for any gene flanking regions in which

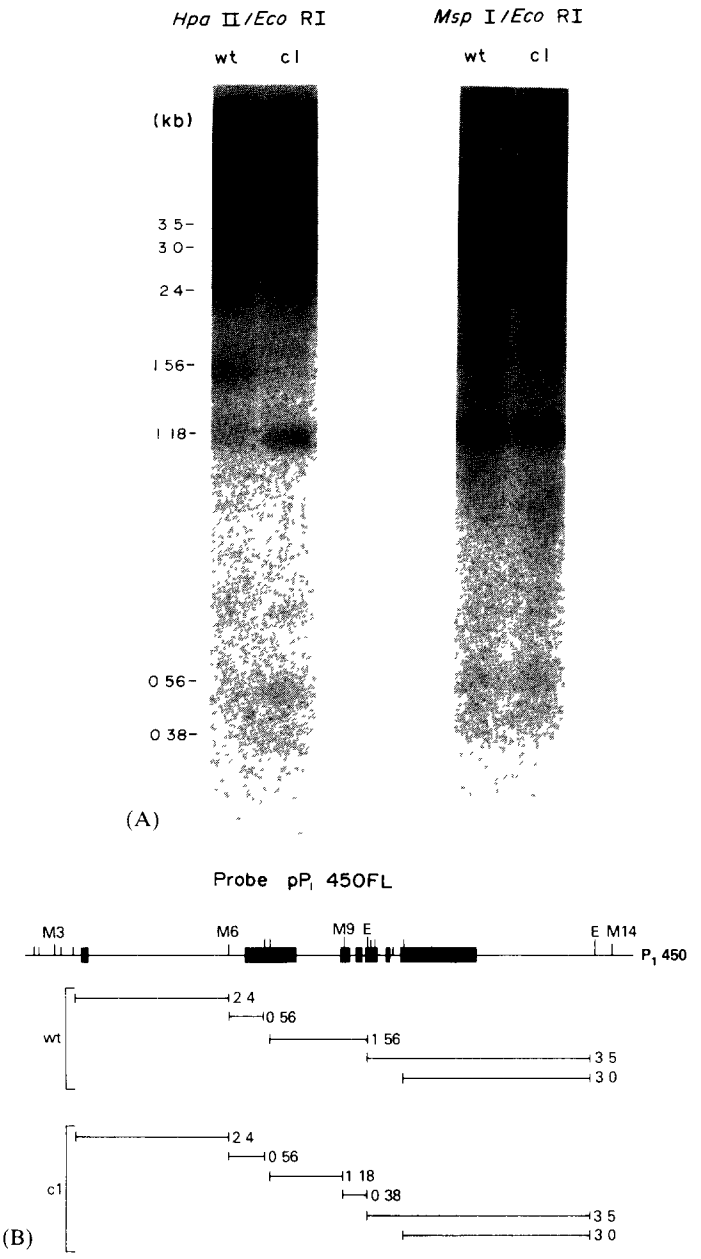

Fig 3 (A) Southern blot hybridization of wt and c1 DNA digested with *Eco* RI and either *Hpa* II or *Msp* I and probed with pP_1450FL (B) The *Hpa* II/*Eco* RI fragments (kb) detected with the pP_1450FL probe The 14 *Msp* I sites and seven exons of the P_1450 gene are illustrated as in Figs 1 and 2

Msp I sites are close to one another [13], DNA methylation of the P_1450 sites M1 through M5 cannot be adequately assessed due to limitations in the Southern blotting method

P_1450 genomic DNA studied with double restriction endonuclease digestions

An *Eco* RI site at the beginning of exon 5 [1] afforded us the opportunity to study in more detail the c1 mutant's methylation differences shown in Fig. 2. Double digestions of wt and c1 DNA with *Eco* RI and *Hpa* II gave similar patterns when probed with pP_1450FL, except for the 1 56-kb fragment present in wt but not c1 cells (Fig 3A) This finding strongly suggests a loss of cytosine methylation at site M9 in the c1 variant (Fig 3B)

P_3450 genomic DNA studied with Msp *I and* Hpa *II*

As predicted from Fig. 1, *Msp* I digestion of wt and c1 DNA probed with pP_3450FL yielded fragments of 1 8, 1 64 and 1 3 kb (Fig 4), and in other gels the smaller fragments (1.1 and 0 6) were found (data not shown). *Hpa* II-digested DNA probed with pP_3450FL gave fragments of 10 to 14 kb in wt and c1 cells (Fig 4), indicating that no significant hypomethylation of the P_3450 gene had taken place The

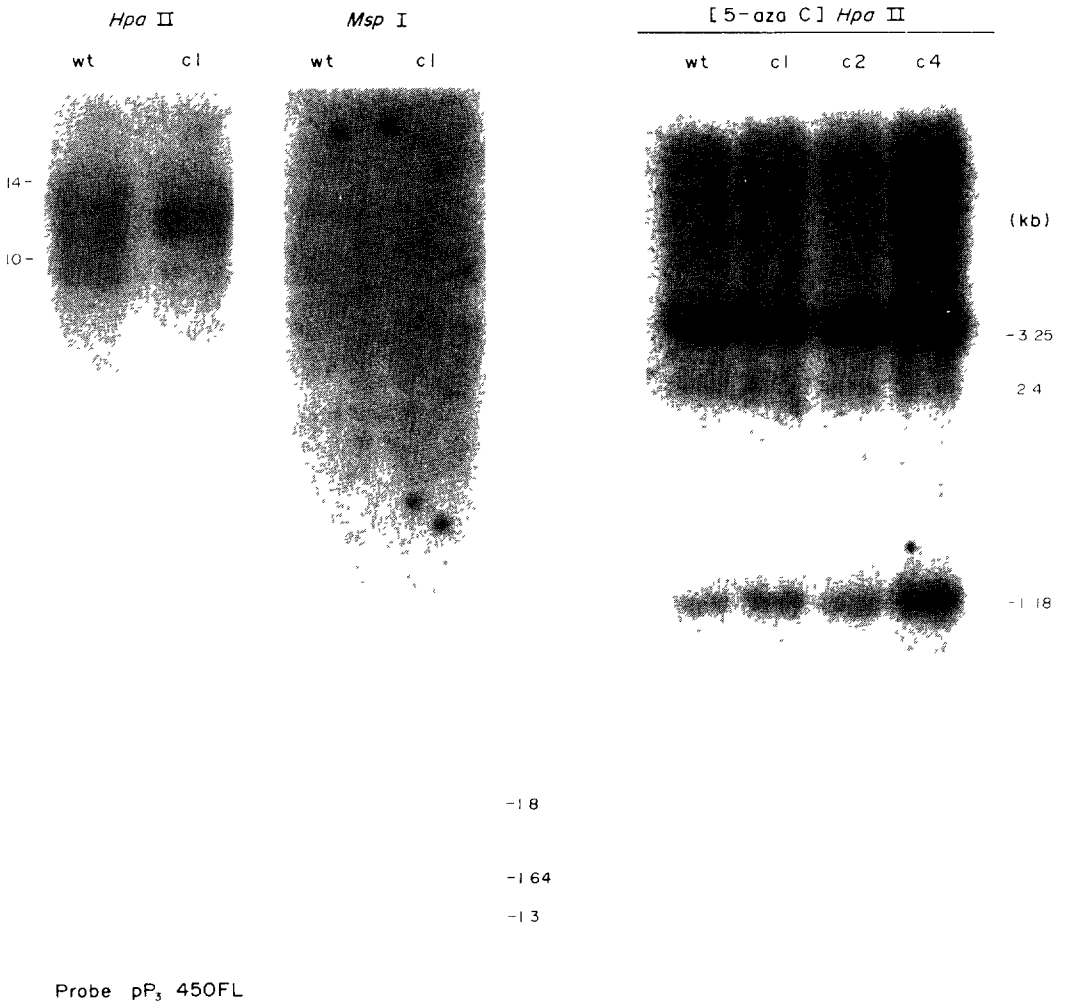

Fig 4 Southern blot hybridization of wt and c1 DNA digested with *Hpa* II or *Msp* I and probed with pP_3450FL See Fig 1 for P_3450 fragments that are theoretically detectable by this experiment

Fig 5 Southern blot hybridization of 5-azacytidine-treated wt, c1, c2 and c4 DNA digested with *Hpa* II or *Msp* I and probed with pP_1450FL See Fig 1 for P_1450 fragments that are theoretically detectable by this experiment

Southern blot patterns of *Msp* I- and *Hpa* II-digested DNA probed with pP_3450FL from c2 and c4 were similar to the corresponding patterns of wt and c1 cells (results not illustrated) The data in Fig. 4 are thus consistent with the observation that the Hepa-1 P_3450 gene is neither expressed constitutively nor inducible by TCDD [6]. The underlying reasons for this interesting lack of P_3450 gene expression in Hepa-1 cultures remain to be elucidated

No evidence for gene amplification or change in DNA methylation caused by the inducer

TCDD is known to induce the wt P_1450 mRNA, to have no effect on the already elevated P_1450 mRNA in c1, and not to induce P_1450 mRNA in the c2 and c4 lines [9]. TCDD exposure (1 and 10 nM) of all four cell lines for times ranging from 16 hr to 1 week did not alter the methylation status of either the P_1450 or P_3450 gene Moreover, when Southern blots of the wt line were compared with Southern blots of the three mutant lines, there was no evidence for P_1450 or P_3450 gene amplification as the result of such prolonged high doses of TCDD (data not shown)

5-Azacytidine treatment

Continuous exposure of all four cell lines to 5-azacytidine (3 μM) for 24 hr or 48 hr caused extensive hypomethylation in both genes (Fig 5) but did not change the previously characterized P_1450 or P_3450 catalytic activity status in wt or the three mutant lines These findings suggest that hypomethylation of the M9 site *per se* in wt cells was insufficient to mimick the situation in the c1 variant where high constitutive P_1450 mRNA levels were observed The results with 5-azacytidine also show that inducible genes do not necessarily respond to additional hypomethylation outside the control region It would be

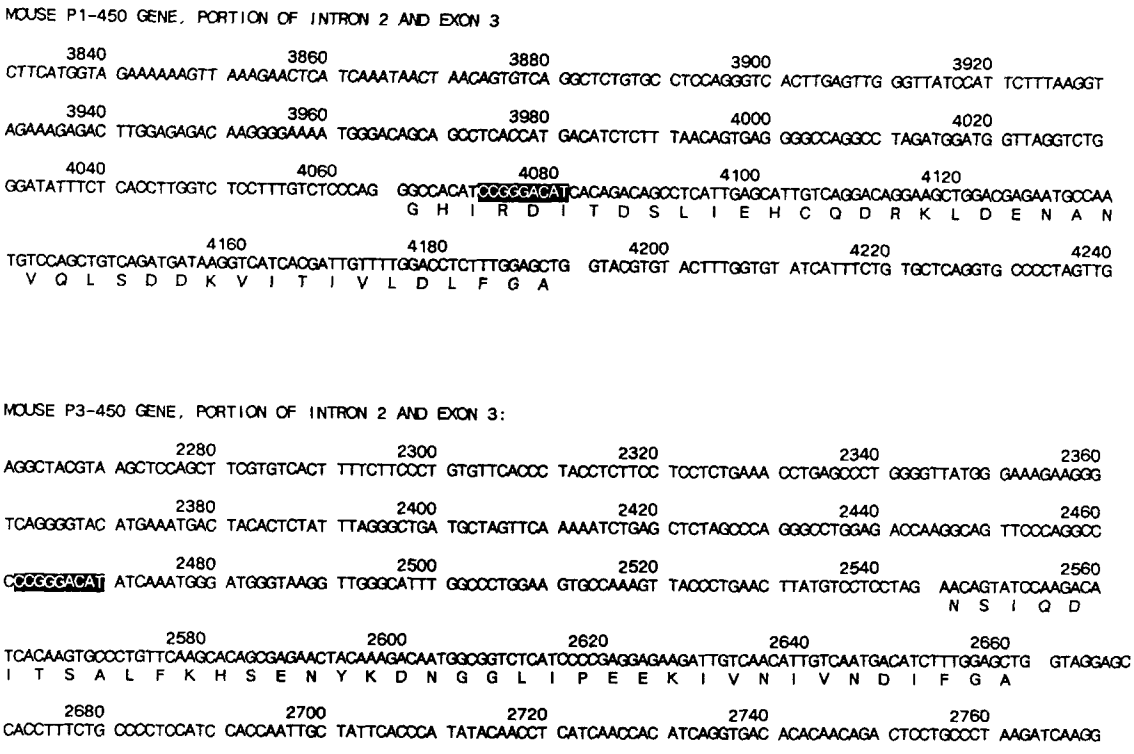

Fig 6 Portions of introns 2 and 3 and exon 3 of the $P_1$450 and $P_3$450 genes The exon and its corresponding encoded amino acids are illustrated without spacing between nucleotides, intron DNA nucleotides are placed in groups of ten Numbering of the nucleotides in either gene corresponds to +1 of the first exon, as determined by primer extension and S1 mapping [1] The *Msp* I sites M9 of $P_1$450 and M8 of $P_3$450 (C-C-G-G) are contained within the conserved 9-bp boxes, denoted as *closed boxes*

interesting to examine in greater detail the methylation pattern of the 5′-flanking region of $P_3$450 in these cell lines.

Speculation about Msp *I site M9 in the* $P_1$450 *gene*

In conclusion, the major methylation difference in the c1 mutant that is not seen in the wt or the regulatory mutants c2 and c4 involves hypomethylation of the M9 site, located near the 5′ end of exon 3 of the $P_1$450 gene (Fig 6) The reason for the hypomethylation difference in the $P_1$450 M9 site of the c1 variant is not known Despite the fact that 5-azacytidine treatment failed to convert the wt phenotype to the c1 phenotype, it is tempting to speculate that M9 represents a potential control site within the gene This control site may play a role in the c1 variant's expression of high constitutive P_1-450 mRNA levels

The M9 site of the $P_1$450 gene is part of a 9-bp box (5′-C-C-G-G-G-A-C-A-T-3′) identical to the M8 site of $P_3$450, located about 80 bp upstream from the beginning of exon 3 (Fig 6). The third exon of $P_1$450 contains 127 bp, whereas the third exon of $P_3$450 contains 118 bp The $P_1$450 and $P_3$450 genes most likely arose via duplication of an ancestral gene at least 65 million years ago [1, 24]. Following this gene duplication, it is possible that $P_3$450 underwent an internal duplication or an insertion of about 80 bp at the 5′ end of exon 3 The more upstream of the two 9-bp boxes in $P_3$450 may have been retained as the important regulatory site, while the other 9-bp box was lost because there was no longer any evolutionary pressure to retain this secondary site The regions surrounding the single conserved 9-bp box then diverged at the normal rate for intron DNA having no apparent function [1], and the resultant P_3-450 exon 3 has nine fewer bases. Consistent with this scenario is the presence of the conserved 9-bp box in exon 3 of the rat P450c gene which corresponds to mouse $P_1$450 [25], and the presence of a homologous region (5′-C-C-G-G-G-A-C-A-C-A-T-3′) 172 bases upstream from the start of exon 3 in the rat P450d gene which corresponds to mouse $P_3$450 [26] Furthermore, the 9-bp box is completely conserved at the beginning of exon 3 in the human $P_1$450 gene [27].

The $P_1$450 exon 3 has diverged 3% between mouse and rat [1, 25] and 11–13% between either rodent and human [27]. The $P_3$450 intron 2 has diverged 21% between mouse and rat [1, 26]. The fact that these two 9-bp boxes have been highly conserved, despite one existing in an exon and the other within a more rapidly diverging intron, provides further evidence for speculation that these 9-bp boxes might possess an important function Further studies on $P_1$450 and $P_3$450 gene transcriptional activation will be necessary to test the potential function of this conserved 9-bp box as a control site within the gene.

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