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Identification and functional characterization of a *cis*-acting positive DNA element regulating CYP 2B1/B2 gene transcription in rat liver

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ABSTRACT

A positive cis-acting DNA element in the near 5'-upstream region of the CYP2B1/B2 genes in rat liver was found to play an important role in the transcription of these genes. An oligonucleotide covering −69 to −98 nt mimicked the gel mobility shift pattern given by the fragment −179 to + 29 nt, which was earlier found adequate to confer the regulatory features of this gene. Two major complexes were seen, of which the slower and faster moving complexes became intense under uninduced and Phenobarbitone-induced conditions respectively. Minigene cloned DNA plasmid covering −179 to + 181 nt in pUC 19 and Bal 31 mutants derived from this parent were transcribed in whole nuclei and cell free transcription extracts and mutants containing only upto −75 nt of the upstream were poorly transcribed. Transcription extracts from phenobarbitone-injected rat liver nuclei were significantly more active than extracts from uninduced rats in transcribing the minigene constructs. Addition of the oligonucleotide (−69 to −98nt) specifically inhibited the transcription of the minigene construct (−179 to +181 nt) in the cell free transcription system. It is therefore, concluded that the region −69 to −98 nt acts as a positive cis-acting element in the transcription of the CYP2B1/B2 genes and in mediating the inductive effects of phenobarbitone.

INTRODUCTION

The cytochrome P-450 group of proteins constitutes a multi gene family and many of the subfamilies are inducible (1,2). For example, CYP1A1 subfamily in rat liver is induced by polycyclic aromatic hydrocarbons and a significant amount of information in terms of the cis-acting DNA elements and their organization is available. These elements include the xenobiotic responsive element (3,4) and the basal transcription element (5). An overall mechanism for the transcriptional activation of the CYP1A1 gene by the inducers involving the Ah receptor and its binding to the upstream elements has also been proposed (6,7). However, the mechanism of transcriptional activation of another cytochrome P-450 family, namely the CYP2B1/B2 genes, which are nearly 97% homologous in the coding and upstream regions (2), by the prototype drug, Phenobarbitone (PB) is not clear. Towards this objective, we have been involved in identifying the upstream cis-acting DNA elements of the CYP2B1/B2 genes in rat liver. We have earlier shown that the near 5' upstream region of the CYP2B1/B2 genes (upto −179nt) can mediate the different features of transcriptional regulation of this gene family and nuclear extracts from drug treated livers generate a foot-print between −54 and −89 nt (8). In the present study, we report the identification and characterization of a positive cis-acting element overlapping the foot-print region that is necessary for the expression of this gene.

MATERIALS AND METHODS

DNA fragments and probes

The plasmid pP450e179, same as pP450e4 reported earlier (8), containing 360 nt of the cytochrome P-450 (2B2) gene and covering positions −179 nt to + 181 nt was digested with Bam H1 (polylinker site at the 5' end) and digested with Bal31 to generate mutants containing −116 nt (pP450e116) and −75 nt (pP450e75) of the 5'-upstream sequence. The plasmid containing the promoter region of Phosphoenolpyruvate carboxy kinase (PEPCK) gene of rat liver (−490 nt to +73 nt) (9) was obtained as a gift from Dr.R.W.Hanson.

The following oligonucleotides and primers were synthesized using Gene Assembler Plus, Pharmacia: Oligonucleotide-1 (5'-GAGGAGTGAATAGCCAAAGCAGGAGGCGTG-3' covering −98 to −69 nt of the CYP2B2 gene and its complementary sequence); Oligonucleotide −2 (5'-TTTTGTC-CTGTGTATCTGTTTCGTGGTGTCCTTG-3' covering −160 to −127 nt of the CYP2B2 gene and its complementary sequence). Primer-1 (5'-CACGCCTCCT-3' complementary to oligonucleotide −1 at the 3' region); Primer-2 (5'-TGGTGTA-ACCACGGT-3') complementary to the CYP2B1/B2 RNA transcript from +11 to +26nt); Primer-3 (5'-GACTCAGAG-CGTCTCGCCGG-3' complementary to the PEPCK RNA transcript from +51 to +70 nt).

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Gel retardation assay

Nuclei were prepared using citric acid buffer homogenisation (10) and nuclear extracts were prepared using 0.45 M NaCl (in final concentration) and dialysis (8). The nuclear extract (1−5 μg protein) was incubated with 300 ng to 1 μg of Poly (dI). Poly (dC) (double stranded, average length varying from 250 to 350 nt in different commercial preparations) in a volume of 20 μl containing 25 mM HEPES.NaOH, pH7.5, 5mM $MgCl_2$, 2mM dithiothreitol and 50mM NaCl at 25°C for 10 min. Labeled NcoI-Bam HI fragment (−179 to +29 nt) of the CYP2B2 gene (10,000cpm) or oligonucleotide-1 was then added and incubation continued for another 30 min. The plasmid pP450e179 was cut with NcoI, labeled at the 3′ end with klenow polymerase and then the fragment (+29 to −179 nt) released using Bam HI (polylinker site). Chase experiments were carried out by adding 100 to 500 fold excess of non-radioactive DNA fragments. After incubation, 2 μl of loading buffer (0.05% Bromophenol blue in 50% glycerol) was added and the samples were analysed on low ionic strength 6% or 9% polyacrylamide gels (8).

U.V. Crosslinking experiments

This was carried out as described by Gallinari *et al* (9). Oligonucleotide-1 was annealed with primer-1 and then incubated in the presence of 50 μM each of 5-Br-dUTP, dATP, dGTP and 50 μCi of [α-^{32}P]dCTP (3000 Ci/mmole) for 1 hr at 37°C in a 25 μl reaction mixture containing 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM dithiothreitol and Klenow polymerase. The body labeled oligonucleotide was purified by electrophoresis on 16% polyacrylamide gels. Labeled DNA (2×10^5 cpm) was incubated with nuclear extract (10 μg protein) and 5 μg of poly (dI). poly (dC) in a total volume of 40 μl as described for gel retardation assay.

The complexes were electrophoresed on a low ionic strength 9% non-denaturing gel. The gel was then U.V.irradiated (1600 w, 260 nm) for 20 to 50 min at 4°C and autoradiographed overnight. Gel slices corresponding to both the complexes were soaked in 100 μl of a solution containing DNase I (10 μg), micrococcal nuclease (10 units), $CaCl_2$ (10 mM) and $MgCl_2$ (10 mM). The digestion was carried out at room temperature for 30 min and the reaction was terminated by the addition of EDTA and EGTA (25 mM). The crosslinked complexes were electroeluted in Tris-glycine-SDS buffer and precipitated with cold acetone. The pellet was dissolved in SDS-PAGE buffer and electrophoresed on 10% gels.

Transcription of Bal31 mutants in whole nuclei

The plasmids pP450e179, pP450e156, pP450e116 and pP450e75 were transcribed in frozen thawed rat nuclei as described earlier (8). The RNA transcripts were isolated and analysed by S1-nuclease protection assay using labeled NcoI-EcoRI (+29 to + 199nt) fragment isolated from pP450e179. This fragment includes 18 nt of the polylinker region and therefore can help to distinguish the transcripts generated from the externally added pP450e179 DNA and the endogenous CYP2B1/B2 transcripts. The former generates a 170 nt protected fragment, whereas the latter a 152 nt protected fragment by virtue of not having the 18 nt polylinker region. The protected fragments were analysed on urea-polyacrylamide (8%) gels and subjected to auto-radiography.

Transcription in cell-free extracts

Transcription extracts were prepared from liver nuclei from control and PB treated rats as described by Gorski *et al*(12), with minor modifications. The repeat layering of nuclei on sucrose gradient was omitted. Briefly, the nuclear pellets were extracted with buffer containing 0.1M KCl and other components, subjected to $(NH_4)_2So_4$ fractionation and then taken in buffer containing 25 mM HEPES, pH 7.6, 40 mM KCl, 0.1 mM EDTA, 1mM dithiothreiotol, 10% glycerol and dialysed twice against the same buffer. The aliquots (5−8 mg protein/ml) were quick frozen and stored at −80°C.

Transcription reactions were carried out in a volume of 20 μl containing 50μg/ml of supercoiled DNA template, 5 mg protein/ml of transcription extract, 25 mM HEPES, pH 7.0. 50 mM KCl, 6 mM $MgCl_2$, 0.6 mM each of ATP, GTP, CTP, UTP and glycerol (12% in final concentration) and 30 units of ribonuclease inhibitor (Rnasin). In some experiments 0.45M NaCl nuclear extract (crude nuclear extract) was also added. The mixture was incubated at 30°C for 45 min. The RNA transcripts

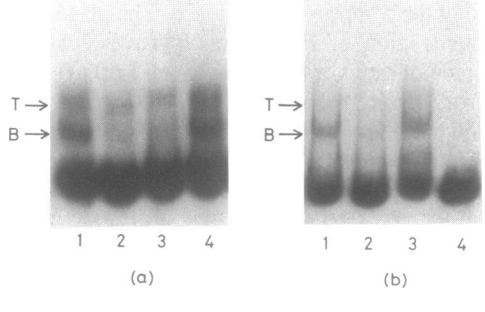

Figure. 1. Gel mobility shift analysis with the fragment −179 to +29 nt. pP450e179 was cut with NcoI at +29, end labeled and the fragment released with Hind III at the polylinker site. The labeled fragment was incubated with PB-nuclear extracts and analysed for the gel retarded complexes. The fragments used in chase experiments were generated from Bal31 mutants with NcoI and HindIII. (a) 1—No chase; 2,3 & 4—chased with 100 fold excess of non-radioactive fragments −179 to +29nt, −116 to +29 nt and −75 to +29 nt respectively. (b) 1—No chase; 2—chased with 200 fold excess of oligonucleotide-1; 3—chased with 200 fold excess of oligonucleotide-2; 4—free DNA. B—Bottom complex; T—Top complex.

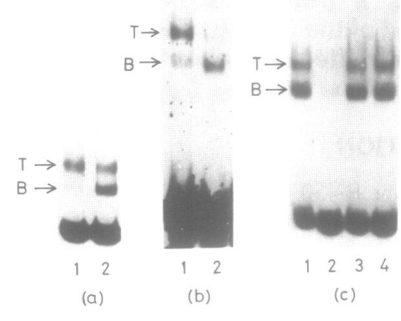

Figure 2. Gel mobility shift analysis with the fragment −179 to +29 nt and with oligonucleotide-1. (a) Labeled −179 to +29 nt was used. 1—Control nuclear extract; 2—PB-nuclear extract. Analysis was carried out on 6% gels. (b) Labeled oligonucleotide-1 was used. 1—control nuclear extract; 2—PB-nuclear extract. (c) Labeled oligonucleotide-1 was used with PB nuclear extract. 1—No chase; 2—Chased with 500 fold excess of non radioactive oligonucleotide-1; 3&4—Chased with 250 and 500 fold excess of oligonucleotide-2 respectively. The complexes in (b) and (c) were analysed using 9% gels.

were isolated after RNase-free DNase and proteinase K treatments followed by Phenol-chloroform extraction and ethanol precipitation. Supercoiled DNA preparations of the plasmids used in transcription studies were made by triton X-100 lysis of cells, phenol : chloroform extraction and sucrose density gradient centrifugation (13). The RNA transcripts were analysed by the primer extension method (14). The 5′ end labeled primers (primer 2 for CYP2B1/B2 and primer 3 for PEPCK gene transcripts) were co-precipitated with RNA transcripts and primer extension reaction carried out with MMLV-reverse transcriptase and other components. The extended products were analysed on urea (7M) polyacrylamide gels (12%) and subjected to autoradiography. The autoradiograms were scanned with laser densitometer and the relative intensities of the radioactive spots were assessed.

Treatment of animals

Male rats (80−90g) of the Institute strain were injected with saline (controls) or phenobarbitone (8 mg/100g i.p). The animals were killed 6 hr after drug treatment and livers processed for isolation of nuclei.

RESULTS

We have earlier shown that the near 5′-upstream region of CYP2B1/B2 gene (−179 to + 29 nt) generates a characteristic gel mobility shift pattern, when assayed with nuclear extracts from control and PB-treated rat liver (8,15). Although there is some quantitative variation in the pattern generated by different extract preparations, it has consistently been observed that the extracts give two major complexes, with the slower moving top band being predominant in control nuclear extracts and the faster moving bottom band being predominant in PB-nuclear extracts. We have now attempted to localise the DNA element within −179nt, responsible for generating this differential gel shift pattern between control and PB-nuclear extracts. The results presented in Fig 1a indicate that excess of non-radioactive homologous as well as −116 to +29 nt fragments are able to chase the gel shift pattern generated by the fragment −179 to +29 nt but the fragment −75 to +29 nt failed to do so. Next, several oligonucleotides were tested for their ability to chase the gel shift pattern generated by the fragment −179 to +29 nt and oligonucleotide −1 (−69 to −98nt) was found to be most effective. For example, oligonucleotide-2 (−127 to −160nt) was not effective in this regard (Fig.1b).

Oligonucleotide-1 is able to generate a gel shift pattern very similar to that of the pattern generated by the fragment −179 to +29 nt (Fig 2a and b). The complexes generated by oligonucleotide-1 are chased by excess non-radioactive oligonucleotide-1 but not by oligonucleotide-2 (Fig.2C).

Cross-linking experiments between the top and bottom complexes and oligonucleotide-1 were carried out and the results presented in Fig 3 indicate that two major protein species (Mr—42.0 and 39.0 Kd) are perhaps involved in the generation of the two complexes, since they were eluted together from gels after performing UV cross-linking analysis. A minor protein band was seen around 34 Kd. The protein species in control and PB nuclear extracts interacting with oligonucleotide-1 have the same electrophoretic mobilities (Fig.3). Further studies on the UV-crosslinking analysis of each individual complex are needed to comment on the quantitative aspects of the proteins involved in control and PB treatments.

In order to examine whether the 5′ upstream region covered by oligonucleotide-1 (−69 to −98nt) is functionally important, transcription experiments were carried out with whole nuclei as well as nuclear extracts. We have earlier shown that the plasmid pP450e179 can be transcribed in whole nuclei and nuclei from PB-treated rats (PB-nuclei) show a striking increase in the level of CYP2B1/B2 transcripts derived from exogenous DNA compared to control nuclei (8, 15). The results presented in Fig.4 indicate that when the upstream is shortened from −179 nt to −156 nt and then to −116nt, there is an increase by 2.5 to 3.0 fold in the level of the transcript derived from added DNA (170 nt protected fragment). However, when the upstream is further shortened to −88 and −75nt, there is a four fold decrease in transcription, indicating the presence of a positive cis-acting element between −116 and −75nt. The protected DNA derived from the endogenous gene transcripts (152 nt) shows a significant decrease, when exogenous DNA is added. As explained earlier (8, 15), this is perhaps due to the decreased transcript stability as a result of the depletion of specific protein factor(s) by the added DNA. The whole nuclear transcription experiments could not be carried out in control nuclei, since the CYP2B1/B2 transcripts were barely detectable.

In the second set of experiments, a cell-free transcription system (12) was used to transcribe Bal31 mutants and to study the effects of the addition of specific oligonucleotides on transcription. The

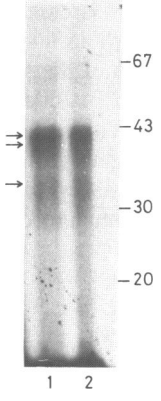

Figure 3. Cross-linking analysis of the complexes generated with oligonucleotide-1. The proteins after cross-linking were analysed on 10% SDS-PAGE. 1—PB-nuclear extract. 2—control nuclear extract. The sizes of marker proteins are indicated.

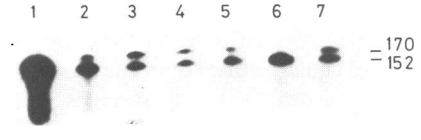

Figure 4. Transcription of Bal31 mutants in whole nuclei isolated from PB-treated rats. Supercoiled DNAs were used for transcription and the transcripts were analysed by S1 nuclease protection. The lower band (152 nt) corresponds to the the protected fragment from the endogenous transcript. The upper band (170 nt) corresponds to the protected fragment from the added DNA. 1 & 6—No DNA added (endogenous transcript. Lane 6 represents 1/8 the amount in Lane 1); 2—pP450e179 DNA; 3—pP450e116DNA; 4—pP450e88DNA; 5—pP450e75DNA; 7—pP450e156DNA.

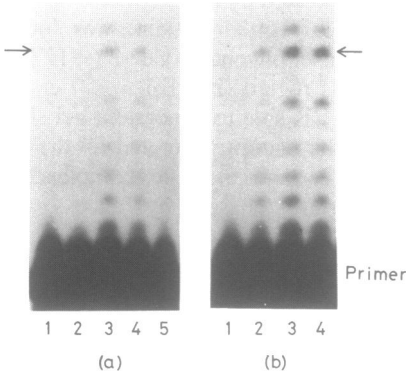

Figure 5. Cell-free transcription analysis. pP450e179 supercoiled DNA was transcribed using cell-free transcription extracts. In some experiments crude nuclear extracts were also added. The transcripts were analysed by primer extension and the arrow indicates position of transcript initiated at +1. (a) Control transcription extract was used. 1—No DNA; 2—pP450e179 DNA; 3—pP450e179DNA + control crude nuclear extract; 4—pP450e179 DNA+PB-crude nuclear extract; 5—Treatment 4 + RNase. (b) PB-transcription extract was used. 1—No DNA; 2—pP450e179 DNA; 3—pP450e179 DNA + control crude nuclear extract; 4—pP450e179 DNA + PB crude nuclear extract.

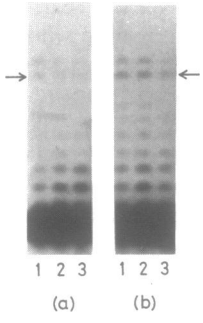

Figure 6. Transcription of Bal 31 mutants with control and PB-transcription extracts. (a) Control transcription extract was used (b) PB-transcription extract was used. 1—pP450e 179 DNA; 2—pP450e116 DNA; 3—pP450e75 DNA. Arrow indicates position of transcript initiated at +1.

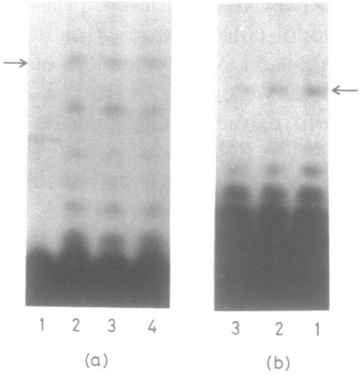

Figure 7. Effect of oligonucleotide-1 and 2 on cell-free transcription. pP450e 179 supercoiled DNA was used for transcription. (a) control transcription extract was used. 1—No.DNA; 2—pP450e179DNA; 3—pP450e179 DNA + 1 μg of oligonucleotide-1; 4—pP450e179 + 1 μg of oligonucleotide-2. (b) PB-transcription extract was used. 1—pP450e179 DNA; 2—pP450e179 DNA + 1 μg of oligonucleotide-2; 3—pP450e179 DNA + 1 μg of oligonucleotide-1. Arrow indicates position of transcript initiated at +1.

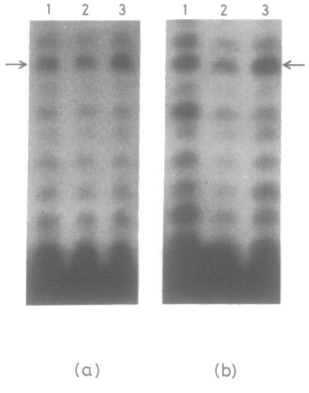

Figure 8. Effect of oligonucleotide-1 and 2 on cell-free transcription in presence of crude nuclear extracts. (a) Control transcription extract and control crude nuclear extract were used. (b) PB-transcription extract and PB-crude nuclear extract were used. 1—pP450e 179 DNA; 2—pP450e179 DNA + 1 μg of oligonucleotide-1; 3—pP450e179 DNA + 1.5 μg of oligonucleotide-2. Arrow indicates position of transcript initiated at + 1.

specific transcripts initiated at +1 nt of CYP2B2 gene were analysed by primer extension. The bands were quantified using a laser densitometer. The results presented in Fig.5 a and b indicate that there is a significant increase in transcription between control and PB-transcription extracts (nearly 8 fold). Addition of crude nuclear extracts (obtained with 0.4 M NaCl) stimulates transcription by both control and PB-transcription extracts. While the control and PB-transcription extracts show significant differences in transcription efficiency, the corresponding crude nuclear extracts are equally effective in stimulating transcription. The crude nuclear extracts by themselves have very low transcription activity.

The results presented in Fig.6 indicate that when Bal31 mutants are transcribed in control transcription extracts, there is some decrease in transcription when the upstream is shortened from −179 nt to −116 nt and then to −75 nt. When the mutants are transcribed in PB-transcription extracts there is 1.5 fold increase in transcription when the upstream is shortened from −179 nt to −116 nt, but there is a striking decrease in transcription (nearly 5 fold) when the upstream is further shortened to −75 nt. Thus, the results obtained in transcription extracts are in conformity with those obtained with whole nuclei.

The results on the effects of the addition of oligonucleotides 1 and 2 on transcription of pP450e179 in cell-free extracts is presented in Figs 7 and 8. With control transcription extract, the oligonucleotides have marginal effects. With PB-transcription extract, oligonucleotide-1 shows a significant inhibition of transcription, whereas oligonucleotide-2 does not have a significant effect (Fig.7). In presence of crude nuclear extracts, the results obtained are exaggerated. Oligonucleotide-1 shows inhibition in both control and PB-transcription systems,the effects obtained with the latter being more pronounced. Oligonucleotide-2 shows about 3 and 1.5 fold stimulation in the case of control and PB-transcription extracts respectively (Fig.8).

Finally, in all these cell-free transcription experiments, another liver gene construct namely that of phosphoenolpyruvate carboxykinase (−493 nt to + 73 nt) has been used as a control. However, the results show that there is no difference in the

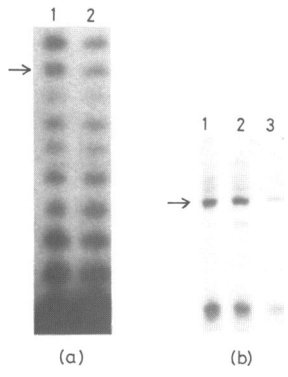

Figure 9. Effect of α-amanitin on pP450e179 and PEPCK DNAs transcription in cell-free extracts. (a) PB-transcription extract was used to transcribe pP450e179 DNA. 1 and 2—without and with α-amanitin (2μg/ml) respectively (b) PEPCK DNA was used 1—control transcription extract; 2— PB transcription extract; 3—PB transcription extract + α-amanitin (2μg/ml). Arrows indicate position of transcripts initiated at + 1nt. The bottom band seen in b is another primer extended product and not the primer (primer is not shown).

transcription of this plasmid between control and PB-transcription extracts. Interestingly while α-amanitin (2μg/ml) inhibits transcription of this DNA strikingly, the transcription of pP450e179 is only partially inhibited (Fig.9).

DISCUSSION

The results of the present investigation have identified the 5′-upstream region of the CYP2B1/B2 gene between −69 nt to −98nt, to mediate the effects of PB and to play a role in the basal level transcription of this gene in the uninduced state. This has been arrived at on the basis of gel retardation analysis, Bal31 mutant transcription in whole nuclei and cell-free extracts and the specific inhibitory effects of oligonucleotide-1 (−69 nt to −98nt) addition on pP450e179 transcription in cell-free extracts. Cross-linking experiments of the gel retarded complexes have indicated two proteins in the Mr range of 42.0 and 39.0 Kd binding to this region. It is interesting to point out that these proteins have a significantly lower Mr from the 85 Kd (8), subsequently revised to 95 Kd protein (15), binding to the entire upstream fragment of −179 nt in western analysis after SDS-PAGE. Since, oligonucleotide-1 mimics the gel mobility shift pattern of the 179 nt fragment, we suspect that the 95 Kd protein may have a low affinity for the DNA in the native state and as such may not be directly binding to DNA.

Although the results have clearly identified the region between −69 nt and −98 nt as a positive cis-acting element, there are indications of other regions within −179 nt being involved. For example, there is some increase in transcription when the upstream is shortened from −179 nt to−116 nt in PB-nuclei or PB-transcription extracts. However, this is not evident in control transcription extracts, although the results obtained with control transcription extract need careful evaluation in view of the low levels of basal transcription perhaps due to the limiting amounts of the positive trans acting factor(s) interacting with the oligonucleotide-1 region. However, oligonucleotide-2 addition shows stimulation of pP450e179 DNA transcription in cell-free extracts especially in the presence of crude nuclear extracts (Fig 8). There is thus the possibility of a negative transcription element being present between −179 nt and −116 nt and PB treatment may modulate interaction of specific factors with this element.

The cell-free transcription system is able to manifest a significant difference in the transcription of pP450e179 DNA between control and PB-treatment and, therefore, is quite suitable for the analysis of cis-acting elements and transacting factors. However, it should be pointed out that at the level of whole nuclei, the difference between uninduced and induced states is much more marked (8, 15). The transcripts in control nuclei are barely detectable. Thus, chromatin conformation and stability of transcripts may also contribute to the difference between control and PB-treated condition *in vivo* and at the level of intact nuclei.

The partial sensitivity of the cell-free transcription system to α-amanitin in the transcription of CYP2B2 gene 5′-upstream region is rather intriguing. In a different study, we have found that even under run on transcription conditions, transcription of the first exon of the CYP2B1/B2 gene is only partially sensitive to α-amanitin, but the transcription of the 9th exon is fully sensitive (unpublished data). The reasons for this result as well as the generation of multiple bands in primer extension analysis are under investigation. However, the transcripts initiated at +1 nt show up prominently and respond to treatment conditions appropriately. Multiple bands in addition to the transcripts initiated at +1 nt, in primer extension analysis have been observed in other systems as well (9, 17).

In a recent study, He and Fulco (16) have shown that a barbiturate-regulated protein binds to a common sequence in the cytochrome P-450 genes of *Bacillus megaterium* and rat cytochrome P-450 (barbiturate-inducible). They have identified a 17 nt sequence, which is part of the foot-print reported by us (8) and is within the oligonucleotide-1 reported in the present study. We have preliminary evidence (unpublished data) to suggest that difference in the levels of the two major complexes seen in gel mobility shift assays with oligonucleotide-1 and nuclear extracts obtained from control and PB-treated rat livers are governed by the phosphorylation status of the proteins involved. The cycloheximide-sensitivity of the PB-mediated effects reported earlier (8) appear to govern the phosphorylation machinery rather than the synthesis of the transcription factor (s) itself, thus influencing the phosphorylation status of the transcription factors and their binding to the DNA. Therefore, the phosphorylation status of the protein factors binding to the oligonucleotide-1 region appears to be atleast one of the important parameters governing the uninduced or induced state of the gene. It is thus a possiblity that phenobarbitone may be acting by modulating the phosphorylation status of the transcription factors involved rather than acting directly through a PB-responsive element in the target cytochrome P-450 gene.

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