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No Role of Protected Region B of Human Cytochrome P4501A2 Gene (*CYP1A2*) As an AP-1 Response Element

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Cytochrome P4501A2 (CYP1A2) is a member of the cytochrome P450 family of isozymes involved in the phase I drug metabolism of vertebrates. CYP1A2 is responsible for the activation of a number of aromatic amines to mutagenic and carcinogenic forms. Thus, the level of CYP1A2, which varies among different populations, may determine an individual's susceptibility to these chemicals. We have previously reported on the importance of a *cis* element named PRB (protected region B) in the regulation of human Cytochrome P4501A2 (CYP1A2) gene, which appeared to act as a positive regulatory element. Closer examination of the PRB sequence (-2218 to -2187 bp) revealed a putative AP-1 binding site, TGACTAA, at -2212 bp (Chung and Bresnick, 1997). To elucidate the role of AP-1 in *CYP1A2* regulation, we transiently overexpressed c-Jun and c-Fos transcription factors in human hepatoma HepG2 cells, and examined their influence on the CYP1A2 promoter activity by reporter gene assays. Cotransfection of the c-Jun and the c-Fos expression vectors increased the induced transactivation by five to six fold from the CYP1A2 promoter constructs. However, deletion of the PRB element did not affect the degree of activation by the c-Jun and the c-Fos. Therefore, it is unlikely that the c-Jun and the c-Fos activate the CYP1A2 promoter through this AP-1 consensus-like sequence in the PRB region.

Key words: Cytochrome P4501A2, CYP1A2, AP-1, Gene expression, c-Jun, c-Fos, PRB, HepG2, Hapatoma

INTRODUCTION

Cytochrome P450 (CYP) proteins form a superfamily of hemoproteins, which exert catalytic activities towards a variety of endogenous and exogenous chemicals, such as: fatty acids, steroids, drugs and environmental pollutants (Nelson *et al.*, 1996).

One of these hemoproteins, CYP1A2, is responsible for the metabolic activation of a number of aromatic amines and amides to mutagenic and carcinogenic moieties (Battula *et al.*, 1987; Aoyama *et al.*, 1989; Adamson *et al.*, 1990; Ioannides and Parke, 1993). Considerable variations in the level of *CYP1A2* expression in humans have been documented by the several groups (Sesardic *et al.*, 1988; Sesardoc *et al.*, 1990; Wrighton *et al.*, 1986; Kalow and Tang, 1991; Schweikl *et al.*, 1993; Nakajima *et al.*, 1994). Thus, the level of CYP1A2, which varies among different populations, may determine an individuals susceptibility to these chemicals.

In the process of learning how the human CYP1A2 gene is regulated, we have already reported the following results. Human hepatoma HepG2 cells are appropriate for the study of CYP1A2 regulation (Chung and Bresnick, 1994). An enhancer-like positive regulatory element exists within a 259 bp sequence, *i. e.*, -2352 to -2094 bp relative to the transcription start site, of the human *CYP1A2* gene. There are three protein binding sites in this region; protected region A, PRA, (-2283 to -2243 bp), PRB (-2218 to -2187 bp) and PRC (-2124 to -2098 bp) (Chung and Bresnick, 1995), which were detected by DNase I footprinting analyses. Functional examination of the nucleotide sequences, as indicated by PRA, PRB and PRC, in a human *CYP1A2* constitutive gene expression by several deletion mutants, indicated the B and C regions to be positive and negative regulating transcription factor binding sites, respectively. Moreover, PRC appears to be bound to a repressor that can be displaced by other factors, such as hepatonuclear factor-1 (HNF-1) (Chung and Bresnick, 1997).

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As described above, the 32 bp of the PRB DNA sequence (-2218 to -2187 bp) is involved in the constitutive CYP1A2 transcriptional regulation by serving as a positive transcription factor- binding site. The ultimate goal of this study, with regard to the PRB sequence, would be to characterize the binding of transcription factors in this sequence. In an effort to define the nucleotide sequence, containing information for positive regulation, we undertook reporter gene assays with constructs containing substitution mutations. Since a potential AP-1 binding site (TGACTAA) exists in the PRB, the nucleotide positions, which were to be mutated, were carefully selected with the aim of disrupting this site. The results of reporter gene assays with constructs containing various substitution mutations within the 32 bp PRB indicated a potential AP-1 binding site, and the rest of the 32 bp sequence to be important (Chung and Bresnick, 1997).

The AP-1 transcription factors are composed of Jun and Fos proteins that function as transcriptional regulators in a heterodimeric complex (Lee *et al.*, 1987; Angel and Karin, 1991). The members of this transcription factor family belong to the basic-leucine zipper class of proteins. Following cell activation, members of the Jun family (c-Jun, JunD, and JunB) form complexes with various partners of the Fos family (c-Fos, FosB, Fra-1 and Fra-2). The formation of heterodimers seems to increase their transcriptional activity (Suzuki *et al.*, 1991). The AP-1 consensus sequence, TGA(C/G)TCA, was originally identified as an activator element which binds transcription factor AP-1, a homodimer of the protooncogene *c-jun* product, or a heterodimer of the protooncogenes *c-jun* and *c-fos* products (Lamph *et al.*, 1988).

The present study was designed to test if AP-1 could work as a transcription factor in the PRB, in a sequence-dependent manner, to upregulate the human *CYP1A2* gene expression. To address the possible involvement of AP-1 in *CYP1A2* regulation, the transfection-reporter gene assay was carried out with the c-Jun and the c-Fos expression vectors.

MATERIALS AND METHODS

Cell culture Human hapatoma HepG2 cells were maintained in a humidified atmosphere of 5% CO_2, 95% O_2 at 37°C in DMEM (GIBCO BRL, Grand Island, NY) with 10% fetal bovine serum (GIBCO BRL) and 1 μ g/ml gentamycin.

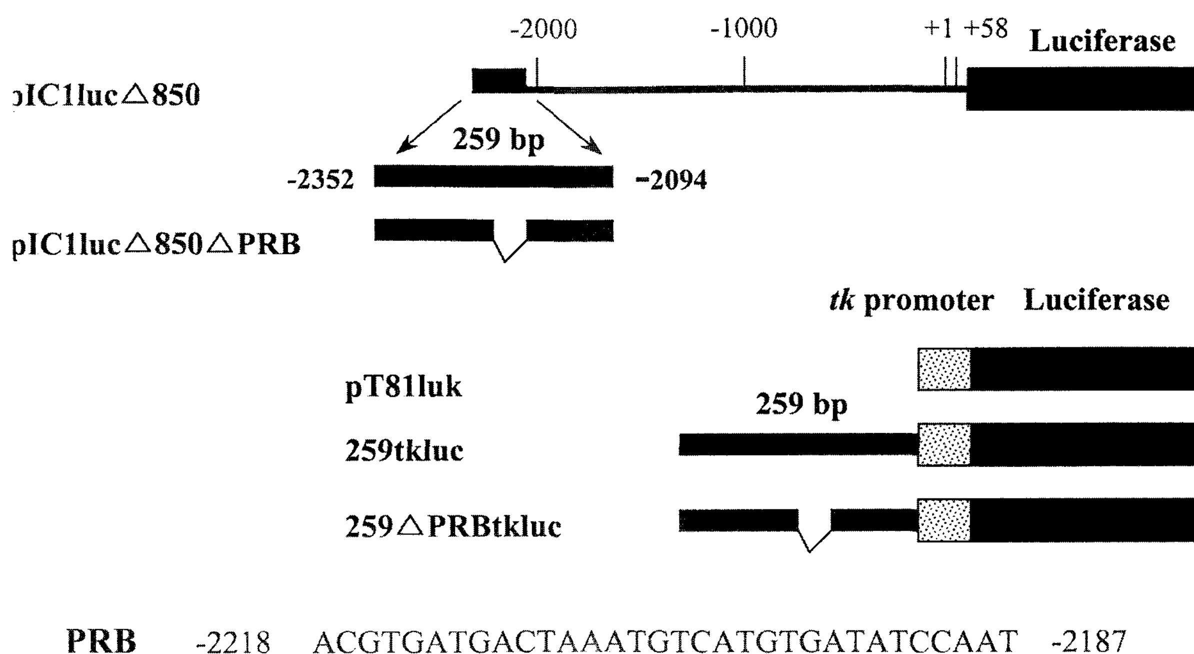
Transfection experiments. Transient DNA transfections were performed by the calcium phosphate coprecipitation method (Sambrook *et al.*, 1989), with modifications. Cells were plated onto 6-cm dishes, and the medium changed after 20 h, with the calcium phosphate coprecipitated DNA being added to the cells 4 h later. Each dish received 500 μl of the calcium phosphate/DNA precipitate containing 25mM Hepes, pH 7.05, 0.75mM Na_2HPO_4, 5mM KCl, 140mM NaCl, 6mM glucose, 125mM $CaCl_2$, and 10 μg of the test plasmid. In some experiments, c-Jun and c-Fos expression vectors or the control vector, RSVcat, were cotransfected. Twenty-four hours following DNA addition, the cells were subjected, for 2 min, to a 15% stock solution of glycerol, followed by the addition of complete medium. The cells were harvested 48 h later, and assayed for luciferase activity (Brasier *et al.*, 1989). All plasmids were prepared by sedimentation twice in CsCl gradients. All transfection experiments were performed a minimum of three times, using at least two different preparations of plasmid DNA. Within an experiment, each construct was studied in triplicate. Statistical analysis was performed using the Student' s *t* test.

Protein determination The cell lysate protein concentrations were determined by the method of Groves *et al.* (Groves *et al.*, 1968).

Plasmid constructs Schematic representations of the human *CYP1A2* constructs used in this study are shown in Fig. 1. The human *CYP1A2* promoter-reporter gene plasmids, pICluc△850, pICluc△850△PRB, 259tkluc, and 259△PRBtkluc have been described previously (Chung and Bresnick, 1995; Chung and Bresnick, 1997). The expression vectors, and their sources, were as follows: pCMV-fos (Sonnenberg *et al.*, 1989) and pRSV-cJ (Angel *et al.*, 1988), donated by Dr. Ken Sterling, University of Massachusetts Medical Center, Worcester, MA; and RSVcat, donated by Dr. Constance Brinckerhoff, Dartmouth Medical School, Hanover, NH.

RESULTS

In previous studies we carried out transfection experiments with a series of constructs bearing substitution mutations to pinpoint the nucleotide sequence responsible for the positive regulation of the human *CYP1A2* gene within the 32-bp PRB fragment. One of substitution mutants was designed specifically to contain altered bases in a putative AP-1 binding site, *i.e.*, TGACTAA, residing at the 5' of the PRB. All of the substitution mutants showed statistically significant diminution of 32-77% in their luciferase activity with either the CYP1A2 or the heterologous promoter system. These results suggested both the TGACTAA and the rest of sequence, within the 32-bp PRB fragment, could participate in the enhancement of the promoter activity (Chung and Bresnick, 1997).



PRB -2218 ACGTGATGACTAAATGTCATGTGATATCCAAT -2187

Fig. 1 Schematic representation of the plasmid constructs and the sequence of PRB. Constructs are presented with the sense orientation relative to the reporter genes. The CYP1A2 promoter is shown as a solid line while the TK promoter is presented as a dotted box. The numbers represent the position of the nucleotide relative to the transcription start site. The solid box of 259 above represents the 259-bp fragment from -2352 to -2094 bp. A deletion is indicated with a △ and a space within the solid box of 259 bp; the pICluc △850 △PRB contains the same DNA sequence as the pICluc △850 except it lacks the PRB.

Because of the similarities in the sequences described above, the elucidation of any biological activity associated with the AP-1 would be of interest. To test if the AP-1 transcription factor could activate *CYP1A2* promoter activity in a PRB dependent manner, the c-Jun and the c-Fos expression vectors were co-transfected into human hepatocarcinoma HepG2 cells together with constructs, with either the presence or the absence, of the PRB region. Wild type-PRB, and PRB-deleted, DNA fragment were incorporated into the 5'-upstream of the promoters in two different luciferase reporter genes: a thymidine kinase enhancerless promoter-luciferase vector, tkluc (resulting in the formation of 259tkluc and 259△PRBtkluc); and a *CYP1A2* promoter-driven luciferase vectors (i.e. pICluc△850 and pICluc△850△PRB). As a control, the CAT expression vector, RSVcat, was contransfected with the constructs under study. The plasmid constructs, containing the *CYP1A2* sequence, have been described previously (Chung and Bresnick, 1995; Chung and Bresnick, 1997), and are shown in Fig. 1.

The effects of *c-fos* and *c-jun* overexpression in HepG2 cells transfected with either the wild type or the deletion mutant PRB sequence containing-constructs are as follows.

The cotransfection of the *c-jun* and the *c-fos* expression vectors caused a 5 fold enhancement in the expression of the luciferase gene (promoter activity) in HepG2 cells that had been transiently-transfected with pICluc△850, as shown in Table I. However, a similar degree of enhancement by the c-Jun and the c-Fos was also observed in cells that had been transfected with pICluc△850△PRB, which was absent of a putative AP-1 binding site.

Consistent with a previous report (Chung and Bresnick, 1997), deletion of PRB (pICluc△850△PRB) resulted in a 40% reduction in the promoter activity. Conversely, using the control plasmid, RSVcat, no change in the luciferase expression was observed in the absence, or presence, of the PRB. The diminishing amount of basal promoter

TABLE I. Effect of *c-jun* and *c-fos* Expression upon Promoter Activity in HepG2 Cells

Construct	Relative luciferase activity (%)	
	+ RSVcat	+ pCMV-fos/pRSV-cJ
pICluc△850	100 ± 2.6	500 ± 38.9[a]
pICluc△850△PRB	58 ± 19.5	390 ± 7.8[a]

Note. The test plasmids were cotransfected with 0.5 μg each of pCMV-*fos* and pRSV-cJ or RSVcat. The luciferase activity is shown as the mean ± S.D. of three experiments. The values obtained after cotransfection with the PRB-containing constructs and RSVcat are set at 100%.

[a]$p < 0.0001$, compared with control expression vector, RSVcat.

pCMV-*fos* and pRSV-cJ indicate the expression vectors for c-Fos and c-Jun, respectively.

TABLE II. Effect of *c-jun* and *c-fos* Expression upon Promoter Activity in HepG2 Cells

Construct	Relative luciferase activity (%)	
	+ RSVcat	+ pCMV-fos/pRSV-cJ
259tkluc	100 ± 16.2	639 ± 140.8[a]
259△PRBtkluc	25 ± 7.0	141 ± 10.6[b]

Note. The test plasmids were cotransfected with 0.5 μg each of pCMV-*fos* and pRSV-cJ or RSVcat. The luciferase activity is shown as the mean ± S.D. of triplicate experiments. The values obtained after cotransfection with the PRB-containing constructs and RSVcat are set at 100%.

[a]$p<0.0001$, [b]$p<0.005$, compared with control expression vector, RSVcat. pCMV-*fos* and pRSV-cJ indicate the expression vectors for c-Fos and c-Jun, respectively.

activity, due to the deletion of the PRB sequence, subsequently caused the attenuation of the promoter activity in the cells where the c-Jun and the c-Fos expression vectors were cotransfected with pICluc△850△PRB, compared with pICluc△850.

In previous studies it was found that using the enhancerless heterologous promoter, as well as the CYP1A2 promoter, helped define the biological functions of the *cis* elements. Therefore, the biological activity of c-Jun/c-Fos in a PRB sequence environment was investigated in a system containing enhancerless thymidine kinase reporter genes (see Table II). In agreement with a previous report (Chung and Bresnick, 1997), the effect of the deletion from the B region is clearer in the heterologous promoter system, *i.e.*, 259△PRBtkluc, where a 75% reduction in the luciferase activity was observed. The control plasmid, RSVcat, had little effect on the promoter activities in either the presence, or absence, of the PRB- thymidine kinase systems. As shown in Table II, overexpression of the c-Jun and the c-Fos in the expression vectors increased the promoter activities 5 to 6 fold in HepG2 cells that had been transfected with constructs where the PRB sequence was either present or had been removed. No statistical differences were found in the extent of the enhancement of the promoter activity due to the c-Jun/c-Fos, between either the PRB present, or deleted, constructs. Cotransfection of the c-Jun and the c-Fos expression vectors did not affect the basal activity in the backbone vectors, such as; pXP1 or tkluc, where the *CYP1A2* DNA sequence had not been incorporated.

These results suggest the transient overexpression of c-Jun/c-Fos proteins enhances the human *CYP1A2* promoter activity, and the PRB sequence to be remote from the biological activity of these transcription factors.

DISCUSSION

In a previous study, we demonstrated a positive regulatory element within a 259-bp sequence (-2352 to -2094 bp) of the human *CYP1A2* gene in HepG2 cells. Subsequent DNase I footprinting analyses within the 259 bp sequence identified three regions where transcription factors could potentially interact. Those regions included the protected region A, PRA (-2283 to -2243 bp), PRB (-2218 to -2187 bp), and PRC (-2124 to -2098 bp) (Chung and Bresnick, 1995). Subsequent studies showed complex regulatory mechanisms direct the expression of this gene. PRB and PRC appear to operate in the positive and negative regulation of *CYP1A2*, respectively. Moreover, a hepatocyte transcription factor, HNF-1, activates the transcriptional activity of this gene *via* the PRC element.

The AP-1 promoter specific factor was identified as a trans-acting factor that binds to the SV40 virus enhancer element (Jones *et al.*, 1988). Subsequently, it was found that AP-1 binding sites could bind to a variety of transcriptional activators of the Fos/Jun cellular oncogenes family, as well as to the tumor promoter phorbol ester (TPA) binding factor, and to the glucocorticoid receptor. The AP-1 family of transcriptional activators form dimers *via* leucine zippers, and are encoded by a family of genes, including: *fos*, *fra-1*, *fra-2*, *fosB*, *c-jun*, *junB*, and *junD* (Hai *et al.*, 1991), which must be dimerize for there activation. The Fos/Jun heterodimers are the most active; the Jun-Jun homodimers are weakly active; and the Fos-Fos homodimers are difficult to form, and are not active. The activity of Fos and Jun appears to be regulated by their phosphorylation state (Abate *et al.*, 1993). The AP-1 binding proteins are induced by various stimuli, such as; mitogenic, differentiation-inducing, and neuronal-specific stimuli (Hai *et al.*, 1991). The PRB sequence (-2218 to -2187 bp), a protected region previously demonstrated by *in vitro* DNase I footprinting analysis, contains a putative AP-1 consensus sequence. Therefore, in this study, transient transfection experiments were conducted with PRB present, or absent, constructs, together with the c-Jun and the c-Fos expression vectors, to examine their involvement in the regulation of the *CYP1A2*. The results indicated that AP-1 is involved in the regulation of human CYP1A2, as shown by the enhanced expression of the reporter gene, which was under the control of the CYP1A2 regulatory sequence. The promoter activity of the 5'regulatory region, -2352 to +58 bp, was enhanced five fold upon cotransfection with the c-jun and the c-fos expression vectors (Table I) . Additionally, a similar degree of activation (six fold) by the c-Jun and the c-Fos transcription factors was observed when the 259 bp sequence (-2352 to -2094 bp) was tested in a heterologous thymidine kinase promoter environment. (Table II). We had no direct evidence, such as western blotting data, to show the production of the c-Jun and the

c-Fos had resulted from their transfected expression vectors, but the effects of the AP-1 on the promoter activities were always compared to those of the control vector (*e.g.* RSVcat)-transfected systems. Our results also suggest that a putative AP-1 sequence at -2212 bp in the PRB (-2218 to -2187 bp) may not serve as a functional site of the AP-1 factor since deletion of 32 bp of the PRB did not abolish the activation caused by the c-Jun and the c-Fos (Table I and II).

Quattrochi *et al.*, (1998) reported results on two AP-1 sites, named the 5'AP-1 and 3'AP-1sites, which reside at -2212 bp and -2029 bp of the human *CYP1A2* promoter, respectively. The 5'AP-1 was at the same site as the AP-1 sequence of the PRB of this study.

According to the report of Quattrochi *et al.*, (1998), protein bound at the 5'AP-1 site can occur in both the basal and the TPA-induced states, as demonstrated by the DNase I footprinting analysis. In cells treated with TPA the expressions of genes of AP-1 family are induced. Differences in the constitutive binding of the nuclear proteins to the 5'AP-1 and 3'AP-1 sites were shown by gel mobility shift assays. While equal amounts of nuclear proteins, which were prepared from either control or TPA-induced HepG2 cells, were bound with the 5'AP-1 sequence, more binding to the 3'AP-1 sequence was observed with the use of TPA treated nuclear extracts. With regard to the protein binding, it appeared that the c-Jun protein interacts with the 5'AP-1 when the cells were induced with TPA. Although very weak, binding of JunD and c-Fos, proteins of the TPA induced extracts to the 5'AP-1 sequence was also indicated. In contrast, either basal or TPA-enhanced, binding of the c-Jun, the JunD and the c-Fos to 3'AP-1 sequence were apparent. It was of interest that with the reporter gene assay, in the presence of the 5'AP-1 sequence, showed a reduced transactivation of the promoter activity by the TPA with the DNA sequence between -2293 and -1970 bp. These observations, coupled with our present results, indicate the possibility of a putative AP-1 site at -2212 bp of the human CYP1A2 gene, which could weakly interact with some of the AP-1 proteins. However, these interactions are not likely to lead to the activation of the promoter activity of the *CYP1A2* gene.

It is of interest to mention the existence of the potential functional AP-1 site(s) between -2352 to -2294 bp, which had not been detected previously in DNase I footprinting analyses, since the 259 bp (-2352 to -2094 bp) sequence used in this study did not contain the 3'AP-1 sequence, but still transactivated the heterologous promoter activity (Table II). A candidate as another AP-1 site would be the TGCCTGA at -2333 bp of the opposite strand of the *CYP1A2*. In regard to the putative AP-1 site at -2212 bp in the PRB or the 5'AP-1, a few of the following observations have been made; (1) reduced constitutive promoter activity by the mutation (Chung and Bresnick, 1997), (2) unresponsiveness to the c-Jun and the c-Fos, as determined by the reporter gene assay (present study), (3) reduced transactivation by TPA when this sequence was included in the reporter gene assay (Quattrochi *et al.*, 1998) and (4) abundant binding of nuclear factors rather than AP-1 family of proteins to this site (Quattrochi *et al.*, 1998). These results suggest that further characterization of the transcription factors for this *cis* element are required for a proper understanding of human *CYP1A2* regulation.

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FOOTNOTES

[1]The following abbreviations are used: AP-1, activator protein-1; CYP1A2, cytochrome P4501A2; *CYP1A2*, cytochrome P4501A2 gene; PRA, protected region A; PRB, protected region B; PRC, protected region C; TPA, phorbol 12-*O*-tetradecanoate 13-acetate.

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