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SHORT COMMUNICATION

Positive Regulation of the Rat *CYP2B2* Phenobarbital Response Unit by the Nuclear Receptor Hexamer Half-Site·Nuclear Factor 1 Complex

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ABSTRACT. A distal 163-bp fragment mediates phenobarbital responsiveness of the rat *CYP2B2* gene. Multiple *cis*-acting elements in this fragment cooperate to form a phenobarbital response unit (PBRU). A nuclear factor 1 binding site and an associated nuclear receptor hexamer half-site are present in both the rat *CYP2B2* PBRU and the homologous mouse *Cyp2b10* sequence. Based on mutational analyses, the hexamer half-site has been reported to act positively in *CYP2B2* and negatively in *Cyp2b10*. However, the specific mutations introduced into the rat and mouse hexamer half-sites were different, raising the possibility that the different roles attributed to the element may be a consequence of the different mutations used. We introduced into the rat *CYP2B2* hexamer half-site the specific mutational change previously introduced into the *Cyp2b10* sequence, where its effect was to increase the basal level of expression and to abolish phenobarbital responsiveness. In the rat context, this mutation reduced but did not abolish phenobarbital responsiveness and decreased, rather than increased, the basal level of expression. The residual phenobarbital responsiveness of the hexamer half-site mutant, as well as that of nuclear factor 1 mutants, indicates that these elements behave as positive accessory sites, suggesting that factors binding to them function as activators of phenobarbital-dependent transcription. BIOCHEM PHARMACOL **57**;9:1073–1076, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. *CYP2B2*; phenobarbital response unit; nuclear receptors; positive regulation; nuclear factor 1; accessory site

Many CYPs† are selectively inducible by xenobiotic compounds. For example, *CYP1A1* is induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and structurally similar aryl hydrocarbons [1], and the mechanism by which this occurs has been characterized in detail [2]. Progress has also been made toward understanding the molecular mechanism underlying inducibility of the homologous rat *CYP2B2* and mouse *Cyp2b10* genes in response to PB [3–8].

We identified a 163-bp *Sau*3AI fragment in the *CYP2B2* 5′-flanking region that confers PB inducibility on a *cat* reporter gene [3]. The *Sau*3AI fragment, at coordinates -2317/-2155, has the properties of a transcriptional enhancer [3]. Further analysis of the rat 163-bp *Sau*3AI fragment led us to conclude that it contains a PBRU [6, 9, ‡], whereas the homologous mouse *Cyp2b10* sequence has been termed a module [5, 10]. Our dissection of the *CYP2B2* 163-bp *Sau*3AI fragment revealed, notably, that an NF1 binding site and an associated nuclear receptor hexamer (HX) half-site, AGGTCA, are essential for maximal PB responsiveness, such that the mutation of either or both of the HX or NF1 sites decreases PB inducibility [6]. However, these mutations do not totally abolish the PB response, implying that, in the rat *CYP2B2* PBRU, the two components of the HX·NF1 complex play the role of accessory elements. In contrast, Honkakoski and Negishi [5] reported that in the mouse *Cyp2b10* sequence, mutation of the equivalent HX site (therein referred to as the Nr site, but hereinafter referred to as the HX site) abolished PB responsiveness. Furthermore, a 221% increase in the basal level of expression was observed, suggesting that in the mouse system a nuclear receptor-like factor that binds to the HX site acts as a repressor of PB responsiveness [5]. Results from other laboratories with both the rat *CYP2B2* PBRU [8] and the mouse *Cyp2b10* sequence [5] suggest that NF1 is an activator.

Hence, results obtained to date suggest that a protein(s) binding to the HX portion of the HX·NF1 complex acts as a PB-dependent activator in the rat *CYP2B2* gene and as a repressor in the homologous *Cyp2b10* gene. However, the specific mutations introduced into the rat and mouse HX sites were different [5, 6], which raises the possibility that

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† *Abbreviations:* CYP, cytochrome P450; PB, phenobarbital; PBRU, PB response unit; NF1, nuclear factor 1; oligo, oligodeoxyribonucleotide; CAT, chloramphenicol acetyl transferase; HNF-4, hepatocyte nuclear factor-4; and FTF, fetoprotein transcription factor.

‡ Trottier E, Dubois S, Vachon M-H, Stoltz C, Belzil A and Anderson A, Functional analysis of a multicomponent enhancer conferring phenobarbital responsiveness on the rat *CYP2B2* gene. In: *XIth International Symposium on Microsomes and Drug Oxidations*, p. 200. Los Angeles Organizing Committee, Los Angeles, CA, 1996.

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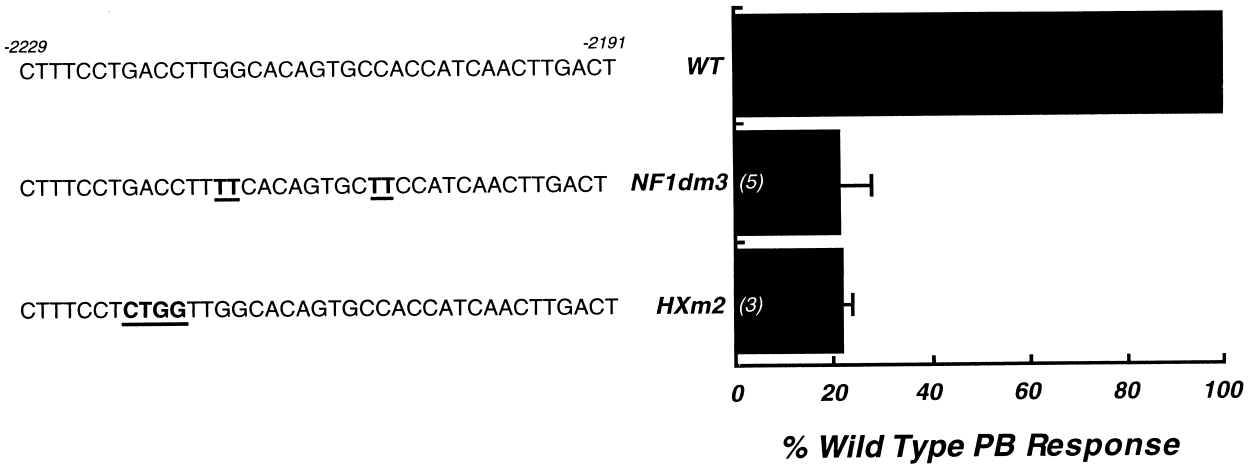

FIG. 1. Effects of mutation of NF1 or HX on PB responsiveness. Primary hepatocytes were transfected in parallel with the standard CAT reporter plasmid containing the wild type 163-bp *Sau*3AI fragment (-2317/-2155) as a positive control or with plasmids containing NF1dm3 or HXm2 mutants as shown, and then were incubated with or without 1 mM PB. All transfections were performed in duplicate. On the right, CAT assay results are expressed as normalized percent increase after PB treatment, with the percent increase obtained in a given transfection using the wild type 163-bp *Sau*3AI fragment set at 100. White numbers represent the number of different rats used for hepatocyte preparations, and the error bars are the standard error of the mean. DNA sequences at left represent the wild type F1′ protected region [6], denoted WT, and the NF1dm3 and HXm2 mutants as shown. For all sequences, the upper strand is shown and substitutions in the mutant sequences are underlined.

the different roles attributed to the element may simply be a consequence of the different mutations used, a possibility that must be taken seriously, given that the LS6 mutation of Liu *et al.* [8] changed two bases of the HX site, yet was without apparent effect on PB responsiveness. We report here results of experiments designed to test this possibility by introducing the same HX mutation into the *CYP2B2* PBRU as had been introduced previously into the homologous mouse *Cyp2b10* sequence, followed by transfection analysis of its effects on PB responsiveness.

MATERIALS AND METHODS

Rat hepatocytes were isolated, cultured, transfected, and treated with PB as previously described [6], except that Matrigel was omitted. CAT activity was assayed by the method of Gorman *et al.* [11]. The following oligos were synthesized by Life Technologies: NF1dm3, 5′-ACTTTC CTGACCTT**TT**CACAGTGCTTCCAT-3′; and HXm2, 5′ - ACTCTGTACTTTCCT**CTGG**TTGGCACAGTGC CACCA-3′. Substitutions in the wild-type sequence are indicated by bold characters. To generate the construct HXm2, the pSa-Sa163 plasmid [6] was mutated using the oligo HXm2 and the Altered Sites II In Vitro Mutagenesis System (Promega). To generate the double mutant NF1dm3, we used a mutant pSa-Sa163 plasmid which already carried a mutation in the distal motif of the NF1 consensus sequence (NF1 m1) [6] and the oligo NF1dm3. The fragments carrying the mutations were then subcloned into the non-PB responsive Ev construct, which contains 1681 bp of the *CYP2B2* 5′ flanking region cloned upstream of the CAT reporter of pBScat [3]. The normal orientation and integrity of the subcloned fragments in the reporter constructs were confirmed by DNA sequence analysis.

RESULTS AND DISCUSSION

As previously mentioned, based on mutational analyses, the HX half-site seems to act positively in rat *CYP2B2* and negatively in mouse *Cyp2b10*. To determine whether this apparent difference might be due to the different mutations introduced into the rat and mouse sequences, we generated an HXm2 mutant that carries the exact 4-bp substitution (AGGTCA → A**CCAG**A) introduced into the mouse half-site [5]. As we found previously with the HXm and HXm-NF1dm2 mutations [6], the HXm2 mutation reduced but did not abolish PB responsiveness (Fig. 1). Furthermore, there was no increase, but rather a decrease, in the basal level of CAT activity (Fig. 2), suggesting that the factor(s) binding to the HX site acts as an activator(s) in the rat PBRU.

The perfectly symmetrical binding site for NF1 (-2217 TG GCACAGTGCCA or TGG N_7CCA) in the 163-bp PBRU is predicted to be a high affinity NF1 recognition site [12]. In our previous work [6], we constructed a mutant, NF1 m1, with tandem base substitutions in the distal NF1 half-site (TGGCACAGTGCCA → TGGCACAGTGC**TT**), and from this derived a double mutant, NF1dm2, with an additional substitution adjacent to the proximal half-site (TGGCACAGTGC**TT** → TGG**T**ACAGTGC**TT**). Transfection analyses of the NF1 m1 and NF1dm2 mutants revealed that PB responsiveness is reduced but not abolished [6]. To test the possibility that the residual PB responsiveness conferred by the NF1dm2 mutant sequence is due to the intact 5′ TGG half-site, we generated NF1dm3 (TGGCACAGTGC**TT** → **TTT**CACAGTGC**TT**). Hence, in NF1dm3, both the 5′ NF1 half-site, which is known to be of major importance for the binding affinity of NF1 [13], and the 3′ half-site were modified. Transfection

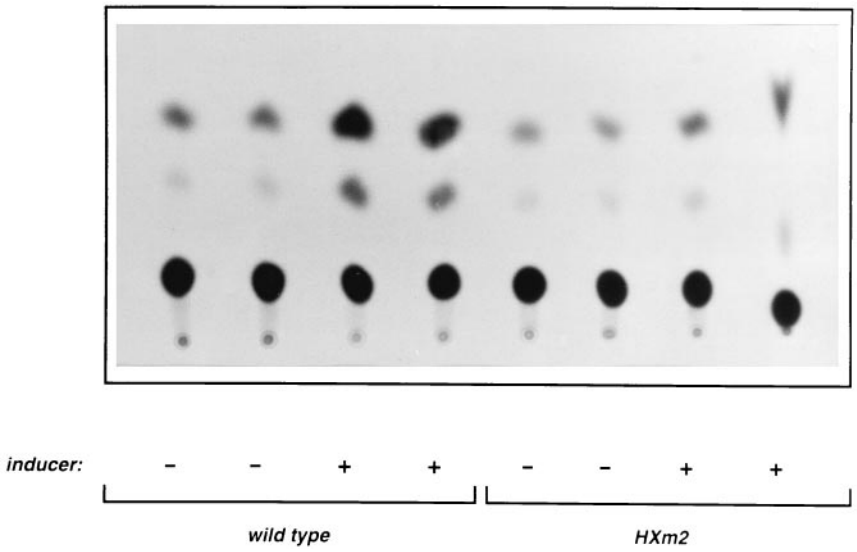

FIG. 2. Effect of the HXm2 mutation on basal levels of CAT activity. Results of a representative series of CAT assays with a single preparation of hepatocytes for the HXm2 mutant are shown. In these assays, the average CAT activity levels (counts per minute per microgram per hour) without and with PB were 52.4 and 346.4, respectively, for the wild type and 37.2 and 76.3, respectively, for the HXm2 mutant.

analysis of NF1dm3 revealed that, as for the NF1 m1 and NF1dm2 mutants tested previously [6], PB responsiveness was reduced but not abolished (Fig. 1). Taken together, these results indicate that NF1 is an accessory factor in the PB induction of the rat *CYP2B2* gene.

The results presented here, in accord with those presented previously, indicate that NF1 acts positively to assure PB-dependent transcription of the rat *CYP2B2* [6, 8] and mouse *Cyp2b10* [5] genes. The situation with respect to the role of the HX nuclear receptor half-site is less clear. According to the model of Honkakoski and Negishi [5], a protein binding to or in the region of the HX site acts as a transcriptional repressor. This model was based on the observation that in the *Cyp2b10* system, the precise equivalent of the HXm2 mutation led to an increase in basal level accompanied by an abolition of PB responsiveness. According to the results presented here, however, the HXm2 mutation reduces the basal level and diminishes but does not abolish PB responsiveness. Hence, in the rat *CYP2B2* gene, protein(s) binding in the region of the HX site appears to act as a transcriptional activator(s) rather than a repressor(s). The reasons for the apparent discrepancy in the role of the HX sequence between the otherwise very similar *CYP2B2* and *Cyp2b10* genes are not entirely clear, but they may be related to differences in the conditions of hepatocyte culture, to subtle species differences in the sequence outside the HX·NF1 complex, or to use of the homologous promoter for the analyses of *CYP2B2* and of a heterologous *tk* promoter for the analyses of *Cyp2b10*. It is noteworthy, however, that the replacement of six nucleotides, including one in the HX site, in *Cyp2b10* by corresponding nucleotides of *Cyp2b9* (which is not PB inducible) results in loss of PB responsiveness, but no increase in basal level [5]. This suggests that even in the mouse *Cyp2b10* gene, the role of HX as the site of action of a repressor is not unambiguous.

Multiple sequence elements cooperate to form the *CYP2B2* PBRU, and one such element, AF1, has been identified and localized [6]. According to the results presented here, both elements of the HX·NF1 complex act as accessory sites necessary for maximal PB responsiveness of the rat *CYP2B2* gene, and therefore factors binding to them [6] should function as activators of PB-dependent transcription.

The nature of the proteins that interact functionally with the HX site to elicit maximal PB responsiveness remains to be determined, although both the orphan nuclear receptor, FTF [14, 15], and HNF-4 bind to a double-stranded oligo containing the HX sequence [6]. When primary cultures of rat hepatocytes were cotransfected with the wild type CAT reporter plasmid and expression vectors for FTF and HNF-4, no increase in PB responsiveness was observed with the FTF expression vector, and a modest 2-fold increase was observed with the HNF-4 expression vector (data not shown). Further experiments will be required to clarify the role of FTF, HNF-4, NF1, and other proteins binding to the HX·NF1 complex in the activation of PB-dependent transcription of *CYP2B2*.

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