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Rat *CYP1A1* Negative Regulatory Element: Biological Activity and Interaction with a Protein from Liver and Hepatoma Cells

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SUMMARY

Rat *CYP1A1* promoter activity was suppressed by the presence of a *cis* negative regulatory element (NRE) at position −843 to −746 in transiently transfected rat H4IIE and human HepG2 hepatoma cells. Removal of the NRE from the promoter-fusion gene constructs caused an increase in the basal promoter activity of 2–6-fold. Co-transfection of the NRE-containing or non-NRE-containing *CYP1A1* promoter-fusion gene constructs with a cloned rat NRE, i.e., pNRE, into HepG2 cells caused a 2-fold or greater reduction in constitutive and induced promoter activities. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced expression of the endogenous human *CYPA1* was also inhibited by transfection of pNRE into HepG2 cells. Deletion of the sequence from base pairs (bp) −658 to −269 in the NRE-containing construct caused a dramatic decrease of constitutive expression in transiently transfected HepG2 cells, compared with an identical construct that lacked the NRE. Deletion of the sequences between bp −658 and −158 in the *CYP1A1* promoter did not affect reporter gene activity, indicating a second site of interaction. At least three different rat liver nuclear proteins bound to the rat NRE, as determined by gel mobility shift and DNase I footprinting assays. A 32-bp sequence within the rat NRE, with significant sequence identity to the 26-bp c-*myc*, *fos*/*jun*-octamer-binding, NRE, was protected from DNase I cleavage by rat liver nuclear extracts. These data suggested a role for this region in the negative regulation of rat *CYP1A1*.

P450 represents a family of proteins involved in the biotransformation of a number of endogenous and exogenous substances, including steroids, certain fatty acids, chemotherapeutic agents, pesticides, and environmental contaminants (1, 2). The P450 superfamily consists of more than one dozen gene subgroups (3), one of which is *CYP1* (P450I). This subfamily includes two genes, *CYP1A1* and *CYP1A2*, both of which are inducible by exposure to polycyclic hydrocarbons and dioxins (4, 5). *CYP1A1* is also responsible for the conversion of the polycyclic hydrocarbon B(*a*)P to proximal carcinogenic species (1, 2). Increased expression of *CYP1A1* has been implicated as a causative factor in the development of cancer (6).

The expression of *CYP1A1* is under both positive and negative control (4, 5, 7). Our interest in the regulation of *CYP1A1* has been focused on the nature and function of a purported NRE that is located in the 5′-upstream region of the gene (8–10); in the rat, this region occurs betwen bp −843 and −746. The NRE sequence of *CYP1A1* has apparently been conserved in the rat, mouse, and human (10). Two highly conserved subregions have been identified within the NRE, i.e., nre_1, from bp −833 to −814, and nre_2, from bp −778 to −760 of rat *CYP1A1* (10). The conservation of nre_1 and nre_2 in the rat, mouse, and human is suggestive of an important function for the NRE in the suppression of *CYP1A1* promoter activity.

In transiently transfected mouse and human hepatoma cells, removal of the NRE from the *CYP1A1-cat* fusion constructs resulted in significantly increased *cat* expression (8, 10). Mouse *CYP1A1-cat* constructs in stably transformed mouse hepatoma cells gave similar results upon deletion of the NRE (9).

The aim of the current experiments was to study the involvement of the NRE in the regulation of rat *CYP1A1* in more detail. In these studies, gel mobility shift and DNase I footprinting assays with extracts from human HepG2, rat H4IIE hepatoma, and rat liver cells were carried out. A fragment of rat *CYP1A1* from bp −881 to −707 was used as the source of the NRE. Contained within the NRE was a 32-bp sequence that had >80% sequence identity to the 26-bp, *fos*/*jun*-octamer-binding NRE of the c-*myc* gene (11, 12).

The NRE was removed from rat *CYP1A1* fusion constructs to further define its function under basal and induced conditions in transiently transfected hepatoma cells. In addition, a

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ABBREVIATIONS: P450, cytochrome P450; B(*a*)P, benzo(*a*)pyrene; NRE, negative regulatory element; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; *cat*, chloramphenicol acetyltransferase gene; NRE-200, the polymerase chain reaction-derived DNA fragment that contains the negative regulatory element; DRE, dioxin response element; *luc*, luciferase gene; *hGH*, human growth hormone gene; bp, base pair(s); RSV, Rous sarcoma virus.

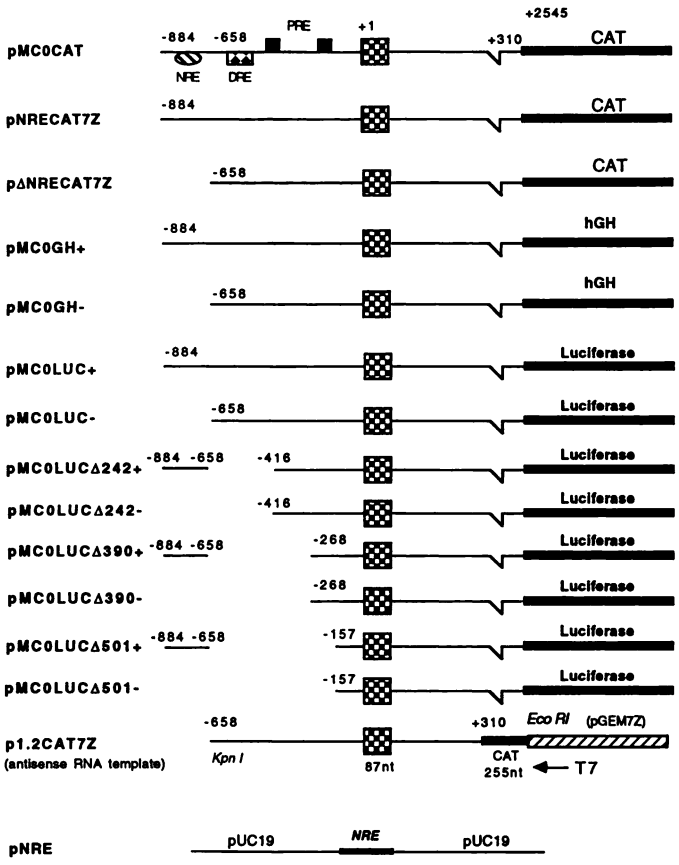

Fig. 1. Schematic representation of the plasmid constructs. *NRE*, rat *CYP1A1* NRE; *PRE*, polycyclic hydrocarbon response element. *Numbers*, position (bp) in the rat *CYP1A1*.

plasmid bearing a cloned NRE was co-transfected into these cells and the effects upon reporter gene and endogenous *CYP1A1* activities were determined. Our results are consistent with an NRE-protein complex acting at a second, proximal, transcriptional regulatory site in controlling expression of the rat *CYP1A1*. This second site of interaction appeared to be located between bp −268 and −157 of the rat *CYP1A1*, a region wherein a 4-S polycyclic hydrocarbon-binding protein site (13), a DRE (14), and a $(GT)_{22}$ sequence are contained.

Materials and Methods

The following materials were obtained from the indicated sources: restriction endonucleases, T4 DNA polymerase, the Klenow fragment, and T4 DNA ligase from either New England Biolabs (Beverly, MA) or GIBCO BRL (Gaithersburg, MD); the plasmids pUC19 and pGEM7Z from GIBCO BRL and Promega (Madison, WI), respectively; pXGH5 and p0GH from the Nichols Institute (San Juan Capistrano, CA) as a component of their *hGH* transient gene expression assay system; minimal essential medium, gentamycin, penicillin/streptomycin, trypsin, and phosphate-buffered saline from GIBCO BRL; fetal calf serum and type I collagen (Vitrogen 100) from ICN Biomedicals (Costa Mesa, CA) and Collagen Corp. (Palo Alto, CA), respectively; Riboprobe Gemini II system and calf intestinal alkaline phosphatase from Promega; [^{3}H] acetyl-CoA, [α-^{32}P]dCTP, [α-^{35}S]dATP, and [α-^{32}P]UTP from DuPont-NEN (Boston, MA); B(*a*)P and TCDD from Aldrich (Milwaukee, WI) and Chemsyn Laboratories (Lenexa, KS), respectively; and luciferin from Sigma Chemical Co. (St. Louis, MO). DNA sequencing analysis was performed by the dideoxy method using Sequenase obtained from United States Biochemicals (Cleveland, OH).

Plasmid construction. Schematic representations of the constructs used in this study are indicated in Fig. 1. The plasmid pMC0CAT contains a 3.4-kilobase fragment that spans nucleotides −881 to +2545 of rat *CYP1A1* fused to *cat* (13). This construct includes the NRE, which is located at position −843 to −746 (10). The non-NRE-containing plasmid pΔNRECAT7Z was constructed by cloning the 4.8-kilobase *Kpn*I/*Bam*HI fragment of pMC0CAT into the *Kpn*I/*Bam*HI sites of pGem7Z. This construct contains nucleotides −658 to +2545 of rat *CYP1A1* fused to the *cat* reporter.

A 200-bp, *Eco*RI linker/*Kpn*I linker-containing fragment (bp −881 to −707 of the rat *CYP1AI* promoter) was synthesized by polymerase chain reaction (15) using appropriate primers and pMC0CAT as a template; this fragment is referred to as NRE-200. NRE-200 was digested with *Eco*RI and *Kpn*I and cloned into *Eco*RI/*Kpn*I-digested pUC19 to create pNRE. The plasmid pNRECAT7Z was constructed by ligation of *Kpn*I-digested NRE-200 to *Apa*I/*Kpn*I-digested

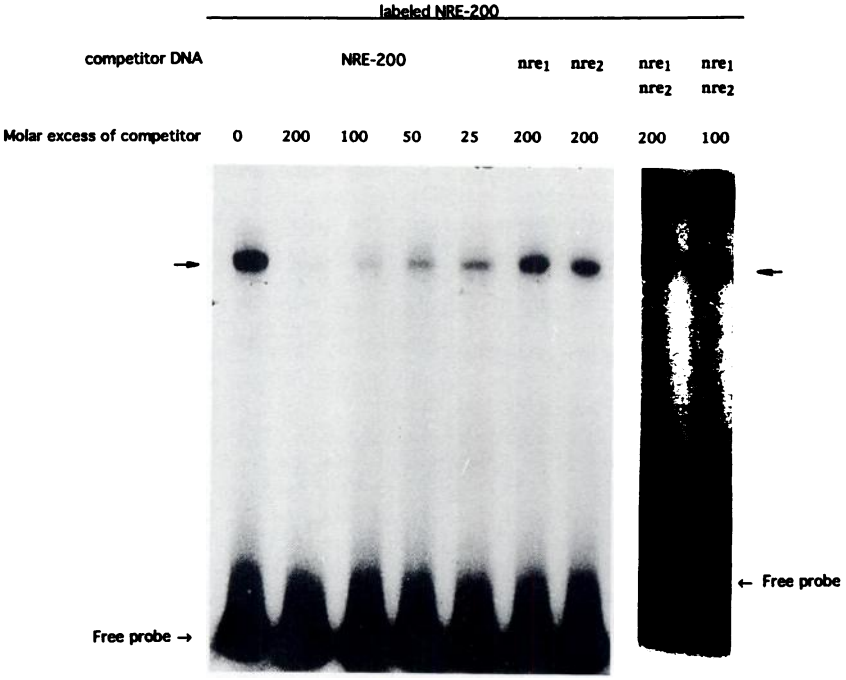

Fig. 2. NRE-binding proteins in a rat liver extract. Labeled NRE-200 was incubated with 16 μg of rat liver nuclear extract protein in the presence of a 20,000-fold excess of poly(dI·dC) and no specific competitor (*lane 1*, from left to right). *Lanes 2-5*, same as *lane 1*, except for the inclusion of a 200-, 100-, 50-, or 25-fold excess of unlabeled NRE-200 in the reaction mixture, respectively. *Lanes 6-9*, same as *lane 1*, except for the addition of a 200-fold excess of nre_1 (*lane 6*), a 200-fold excess of nre_2 (*lane 7*), a 200-fold excess of both nre_1 and nre_2 (*lane 8*), or a 100-fold excess of both nre_1 and nre_2 (*lane 9*). The position of the probe is indicated. *Arrows*, migration of the probe associated with protein.

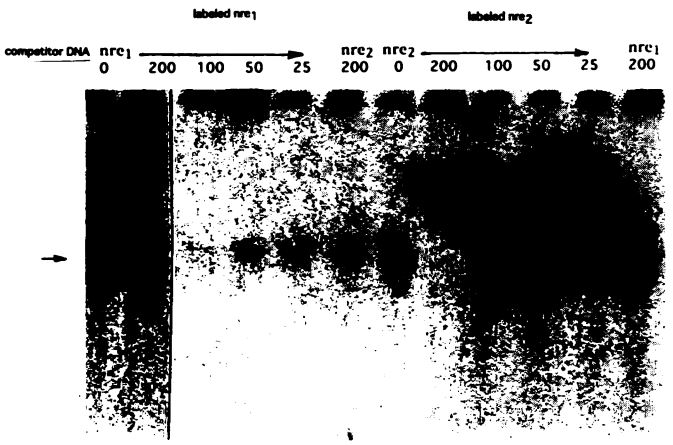

Fig. 3. Presence of nre_1- and nre_2-binding proteins in rat liver. The labeled, double-stranded nre_1 (*lane 1*, from left to right) and nre_2 (*lane 7*) oligodeoxynucleotides were incubated with 16 μg of rat liver nuclear extract protein in the presence of a 20,000-fold excess of poly(dI·dC) and no specific competitor. *Lanes 2-5*, the same as *lane 1*, except for the addition of a 200-, 100-, 50-, or 25-fold excess of unlabeled nre_1, respectively. *Lane 6*, the same as *lane 1*, except for the addition of a 200-fold excess of unlabeled nre_2. *Lanes 8-11*, the same as *lane 7*, except for the addition of a 200-, 100-, 50-, or 25-fold excess of unlabled nre_2, respectively. *Lane 12*, the same as *lane 7*, except for the addition of a 200-fold excess of unlabeled nre_1. Free probe was run off the gel. *Arrows*, positions of the shifted probe.

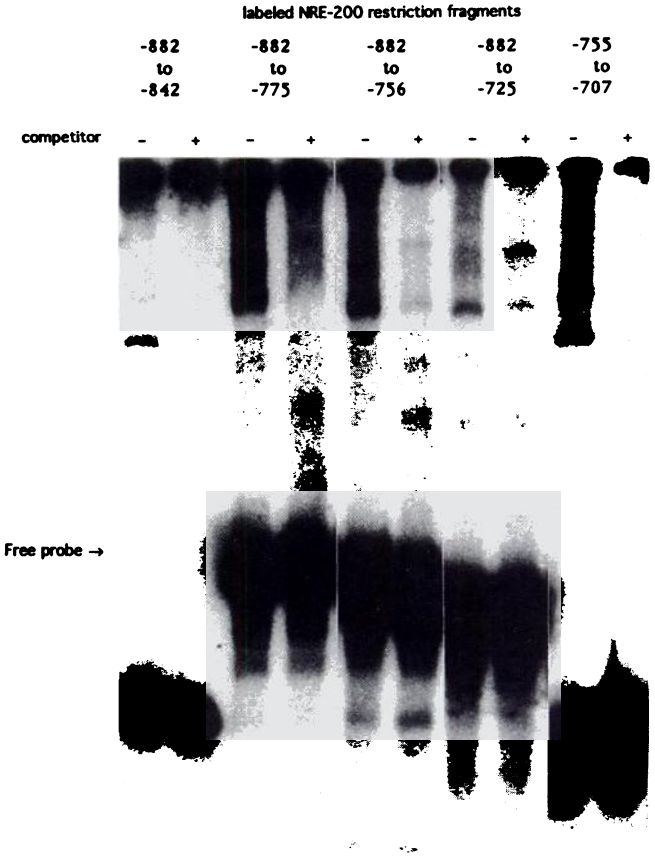

Fig. 4. Gel mobility shift assay of restriction fragments of NRE-200. Labeled restriction fragments of the rat NRE corresponding to bp −882 to −842 (*lanes 1* and *2*, from left to right), bp −882 to −775 (*lanes 3* and *4*), bp −882 to −756 (*lanes 5* and *6*), bp −882 to −725 (*lanes 7* and *8*), and bp −755 to −707 (*lanes 9* and *10*) were incubated with 24 μg of rat liver nuclear extract in the presence of a 10,000-fold excess of poly(dI·dC), as described in the text. −, Absence of unlabeled specific competitor; +, presence of a 200-fold excess of unlabeled specific restriction fragment from NRE-200.

pΔNRECAT7Z. T4 DNA polymerase was used to blunt the *Apa*I site before a second ligation reaction. Successful cloning of NRE-200 was indicated by digestion with *Eco*RI and *Kpn*I, which released the appropriately sized fragment. The presence of NRE-200 in its correct orientation was verified by sequence analysis. The plasmids pNRECAT7Z and pΔNRECAT7Z are identical except for the presence of NRE-200. A pair of NRE-containing and NRE-lacking rat *CYP1A1* promoter-*hGH* fusion plasmids were constructed using p0GH (16) and were called $pMC0GH^+$ and $pMC0GH^-$, respectively. A similar pair of rat *CYP1A1* promoter-*luc* fusion plasmids ($pMC0LUC^+$ and $pMC0LUC^-$) were constructed with pXP2 as the parent vector (17). Other plasmids used in this study were pRSVCAT, a RSV long terminal repeat-*cat* fusion gene; pSV0CAT, a promoterless *cat* construct (18); pA8, a rat *CYP1A1* genomic clone that contains approximately 80% of rat *CYP1A1* (19); and pHMC6b, the comparable human *CYP1A1* genomic clone (20). The plasmids pMC0CATΔ242, pMC0CATΔ390, and pMC0CATΔ501 were deletion mutants in which 242, 340, and 501 bp, respectively, had been removed from pMC0CAT, starting at and proceeding downstream from the *Kpn*I site (bp −658). These deletions had been prepared by timed exonuclease III digestion after cleavage of pMC0CAT with *Kpn*I and *Nhe*I. These deletion mutants were modified by replacement of *cat* by *luc*, creating pMC0LUCΔ242, pMC0LUCΔ390, and pMC0LUCΔ501. The former constructs were further modified to include (superscript +) the NRE or not (superscript −).

Cell culture. Rat hepatoma H4IIE and human hepatoma HepG2 cells were maintained in minimal essential medium supplemented with 10% fetal calf serum and gentamycin or penicillin/streptomycin.

Transfection and reporter gene assays. Transfection was performed using the calcium phosphate-DNA co-precipitation method, followed by glycerol shock (21). One million cells were seeded onto type I collagen-coated (6 mg/cm²) 100-mm dishes. Twenty hours after seeding, the medium was changed. Four hours later, plasmid DNA was co-precipitated with carrier DNA and calcium phosphate and added to the cells. Twenty-four hours after the addition of DNA, the cells were glycerol-shocked and then treated with either B(*a*)P in acetone, TCDD in toluene, or solvent vehicle alone. The solvent never exceeded 0.1% in volume. Forty-eight hours later, the cells were assayed for reporter activity (22). Expression of the *hGH* reporter gene was assayed as secreted growth hormone in the cell culture medium, according to the manufacturer's instructions (Nichols Institute). The luciferase assay was performed as described (23).

RNA isolation and hybridization analysis. Total cellular RNA was isolated from cell cultures (24) and was incubated with RNase-free DNase to remove co-purified plasmid DNA (25). The RNA (30 μg) was separated on a denaturing formaldehyde-agarose gel (1%) and RNA blot hybridization analysis was performed (21).

Protein determination. The protein concentration of the cell lysates was determined by the method of Groves *et al.* (26).

RNase protection assay. The RNase protection assay was conducted as described (25), using an antisense probe. p1.2CAT7Z (Fig. 1) was linearized with *Kpn*I (at bp −658 of rat *CYP1A1*) and used as a template for the synthesis of ^{32}P-labeled antisense RNA with T7 RNA polymerase. The synthesized antisense RNA probe was 1273 nucleotides in length.

The labeled antisense RNA probe (5×10^5 cpm) was hybridized to 50 μg of H4IIE RNA (control RNA for the 87-nucleotide protected fragment) isolated from cells that had been treated with either B(*a*)P, TCDD, or solvent alone. The same amount of labeled antisense RNA was hybridized to 50 μg of total RNA from HepG2 cells that had been transfected with pRSVCAT (control for the 255-nucleotide protected fragment) or 100 μg of RNA from HepG2 cells that had been co-transfected with 2.5 μg of pMC0CAT, 2.5 μg of $pMC0GH^+$, and either 10 μg of pUC19 or 10 μg of pNRE. Hybridization was carried out at

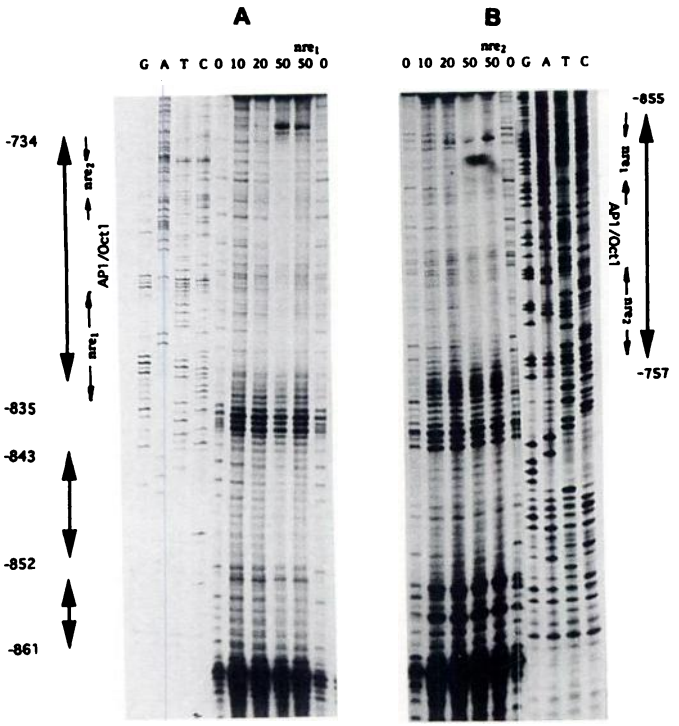

Fig. 5. DNase I footprinting analysis of NRE-200. The footprint analysis was conducted with rat liver nuclear extract. Results with rat liver nuclear extract for the sense (A) and antisense (B) strands of NRE-200 (20,000 cpm/reaction) are presented. *Lanes G, A, T,* and *C*, dideoxy sequencing reactions using either the forward or reverse primer and pNRE as the template. NRE-200 was digested with DNase I in the absence or presence of a rat liver nuclear lysate (0, 10, 20, or 50 μg), as indicated in the figure. All reactions contained bovine serum albumin. The nre_1 or nre_2 double-stranded oligonucleotide was added as a competitor for nre_1- or nre_2-binding protein(s), respectively, at a 3000-fold excess, as indicated in the figure. *Numbers*, position (bp) in the rat *CYP1A1* 5′-flanking sequence. *AP1/Oct1*, sequence with identity to the c-*myc* NRE. *Double-headed arrows*, protected sequences.

55° for 12–16 hr. RNase digestion was conducted at 37° for 1 hr and the samples were electrophoresed on an 8 M urea-6% polyacrylamide gel.

Preparation of rat liver nuclear extracts for gel mobility and footprinting assays. Rat liver nuclear extracts were prepared according to the method of Gorski *et al.* (27).

Specific binding and gel mobility shift assays. The specific binding of proteins to various oligodeoxynucleotides was determined by gel mobility shift assays (28). ^{32}P-Labeled DNA (0.01–0.05 ng, representing approximately 10,000 cpm), extract (16 μg of protein), poly(dI·dC) [at a molar ratio of labeled DNA/poly(dI·dC) of 1:10,000 or 1:20,000], and buffer consisting of 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 5 mM phenylmethylsulfonyl fluoride, and 5% glycerol, in a total volume of 12.5 μl, were incubated at room temperature for 20 min. The specificity of the binding was established by the addition of competing nonradioactive oligodeoxynucleotides (2–10 ng). The reaction was stopped by the addition of 1.5 μl of 6× gel-loading buffer (40 mM Tris, pH 8.0, 20 mM sodium acetate, 6 mM EDTA, 15% Ficoll type 400, 0.24% bromphenol blue), and the reaction mixture was loaded onto a pre-electrophoresed vertical 6% polyacrylamide gel and electrophoresed at 17 V/cm at 4°. The gel was dried under vacuum and autoradiographed.

DNase I footprinting. DNase I footprinting was carried out as described (29).

Results

Gel mobility shift studies. The gel mobility shift assays demonstrated that the rat liver nuclear extracts contained a protein that bound with high specificity to NRE-200, restriction fragments of NRE-200 that contained NRE motifs, and double-stranded nre_1 and nre_2 oligonucleotides (Figs. 2-4). HepG2 and H4IIE cell extracts also contained proteins that bound with high specificity to NRE-200 in gel mobility shift assays (data not shown). The shift in the migration of the NRE-200 DNA fragment with rat liver nuclear extract can be seen in Fig. 2 (*lane 1*). The major shifted band (Fig. 2, *arrow*) was competed with by a 200-fold excess of unlabeled NRE-200 (Fig. 2, *lane 2*). Two faint bands just below the major one were also competed with by unlabeled NRE-200 (Fig. 2). Unlabeled double-stranded nre_1 and nre_2 oligodeoxynucleotides at a 200-fold excess, alone or in combination, failed to compete with the major shifted band of NRE-200 (Fig. 2). The rat liver extract also contained a protein that bound specifically to double-stranded nre_1 or nre_2 oligodeoxynucleotides (Fig. 3, *lanes 1* and *7*). Unlabeled nre_1 and nre_2 at a 200-fold excess proved to be effective competitors (Fig. 3, *lanes 2* and *8*). However, a 200-fold excess of unlabeled nre_2 did not compete with labeled nre_1, nor did a 200-fold excess of unlabeled nre_1 compete with nre_2, indicating the interaction of specific liver proteins with nre_1 and nre_2 (Fig. 3, *lanes 6* and *12*). Restriction fragments of NRE-200 corresponding to bp −882 to −842 contained a c-*myc* NRE CT motif and a 3-bp indirect repeat; bp −882 to −775 contained nre_1 and a 32-bp *fos/jun*-octamer sequence (11, 12); bp −882 to −756 contained nre_1, the 32-bp *fos/jun* octamer, and nre_2; bp −882 to −725 contained nre_1, the 32-bp *fos/jun* octamer, and nre_2; and bp −755 to −707 contained a 6-bp inverted repeat. Each of these DNA fragments bound a protein from rat liver (Fig. 4). A 200-fold excess of the unlabeled restriction fragments of NRE-200 abolished the shifted band(s) for each of the labeled substrates (Fig. 4). The gel mobility shift assays indicated that distinct proteins bound to nre_1 and nre_2 and that other sequences were involved in the binding of specific protein factors to the entire NRE.

DNase I footprinting studies. The DNA sequences that interacted with specific proteins in nuclear extracts of rat liver were defined by DNase I footprinting analysis. The extracts gave protection patterns that included the nre_1 and nre_2 sequences as well as the *fos/jun*-octamer-binding sequence (11, 12) that was located between nre_1 and nre_2 (Figs. 5 and 6). The addition of a 3000-fold molar excess of unlabeled double-stranded nre_1 produced little or no competition for the nre_1-binding protein(s) (Fig. 5A). Similarly, a 3000-fold molar excess of unlabeled double-stranded nre_2 did not compete for the nre_2-binding protein (Fig. 5B). These results, in conjunction with the gel mobility shift assays described above, indicated that the proteins that bound to the nre_1 and nre_2 sequences apparently overlapped with adjacent sequences of the NRE. The gel mobility shift studies and DNase I footprinting results were consistent with the existence of three or more proteins that were capable of binding with high affinity both to the conserved nre_1 and nre_2 sequences and to a sequence of the rat *CYP1A1* NRE with significant identity to a c-*myc* NRE (11, 12). The DNase I footprints also indicated the overlap of the individually bound factors. HepG2 and H4IIE extracts showed a similar pattern of protection from DNase I digestion (data not shown).

Transient gene expression assays. To show the biological activity of the NRE, HepG2 cells were transfected with rat *CYP1A1* promoter-reporter fusion gene constructs that did or did not include the NRE. After transfection of HepG2 cells

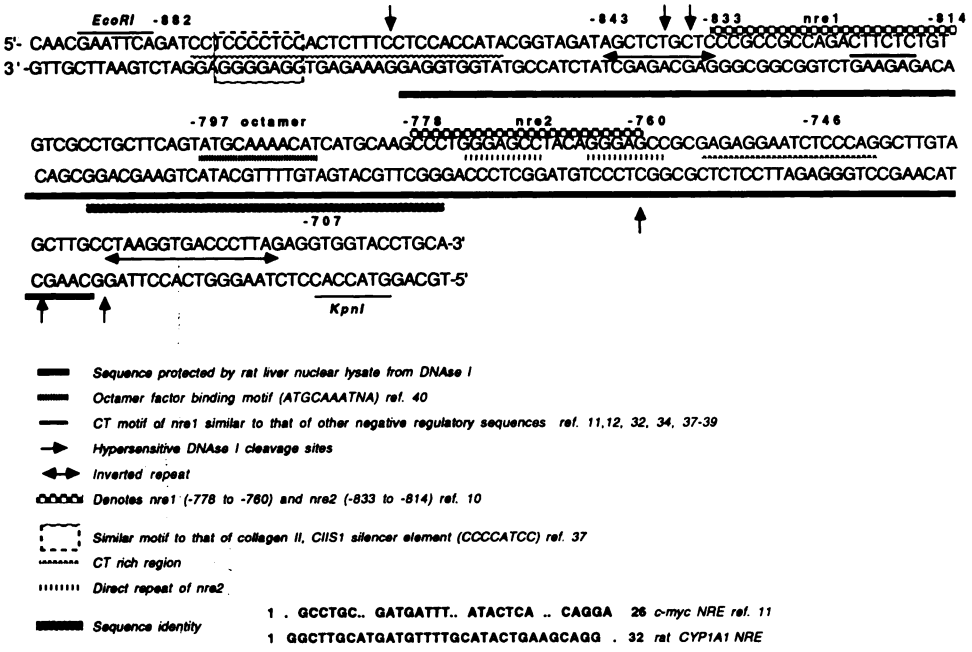

Fig. 6. Nucleotide sequence of NRE-200 protected from DNase I digestion by rat liver nuclear extract. H4IIE and HepG2 extracts gave similar footprints (data not shown). NRE motifs and inverted and direct repeats are also shown. *Numbering*, location of 5′-flanking sequences with respect to the start site of transcription (+1).

TABLE 1

Constitutive promoter activity of NRE⁺ and NRE− fusion genes in HepG2 cells

HepG2 cells were transfected with 5 μg of plasmid DNA as described in Materials and Methods. Promoter activity was determined by the expression of *cat*, *luc*, or *hGH*. Constitutive promoter activity of the NRE-containing constructs was given a value of 100%, for direct comparison of promoter activity-containing between the three different promoter-reporter gene constructs. In A and C, cells were co-transfected with a construct that contained the metallothionein promoter linked to *hGH* (pXGH5); in B, a construct that contained the RSV long terminal repeat linked to the *cat* gene (pRSVCAT) was used as a control for transfection efficiency.

		cat activity	
A.	Expt.	pNRECAT7Z	pΔNRECAT7Z
		%	
	1	100	280
	2	100	640
	3	100	180
	4	100	470
	Mean ± SE	100 ± 0	393 ± 102
		Secreted human growth hormone	
B.	Expt.	pMC0GH⁺	pMC0GH⁻
		%	
	1	100	250
	2	100	160
	3	100	210
	4	100	150
	Mean ± SE	100 ± 0	193 ± 22
		Relative luciferase activity	
C.	Expt.	pMC0LUC⁺	pMC0LUC⁻
		%	
	1	100	438
	2	100	464
	3	100	526
	Mean ± SE	100 ± 0	476 ± 25

with a NRE-lacking (pΔNRECAT7Z) or a NRE-containing (pNRECAT7Z) reporter gene construct, the mean increase of constitutive reporter activities was 4-fold (range, 2–8-fold), as shown in Table 1. Constitutive activity was also assessed with NRE-containing (pMC0GH⁺) or NRE-lacking (pMC0GH⁻) constructs in which *hGH* served as the reporter gene. Approximately 2-fold higher constitutive *hGH* activity was observed upon transfection of HepG2 cells with the construct that lacked the NRE. Finally, HepG2 cells transfected with the NRE-lacking (pMC0LUC⁻) construct showed 5-fold greater constitutive luciferase activity, compared with cells that were transfected with the NRE-containing construct (pMC0LUC⁺) (Table 1). Similar results were obtained with H4IIE cells that were transfected with these pairs of reporter fusion gene constructs (data not shown). These results demonstrated a biological function of negative regulation for the rat NRE when included in rat *CYP1A1* promoter-reporter gene fusion constructs.

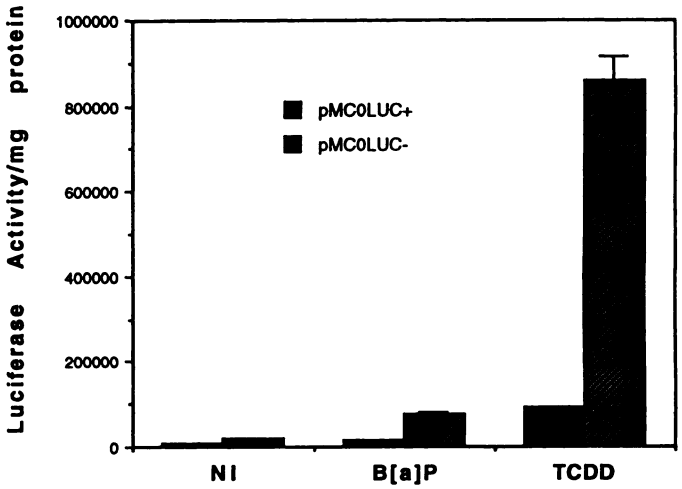

Fig. 7. Relative induction of pMC0LUC⁺ and pMC0LUC⁻. Luciferase activity is indicated as relative luminescence/mg of protein (*ordinate*). *NI*, vehicle control. The data represent the mean ± standard error of three results.

Effect of B(*a*)P and TCDD on reporter gene expression. The level of induction by B(*a*)P and TCDD of reporter gene expression in HepG2 cells that were transfected with the NRE-lacking and NRE-containing constructs was assessed. Luciferase activity in cells that were transfected with pMC0LUC⁺ was induced 2- and 13-fold, respectively, after

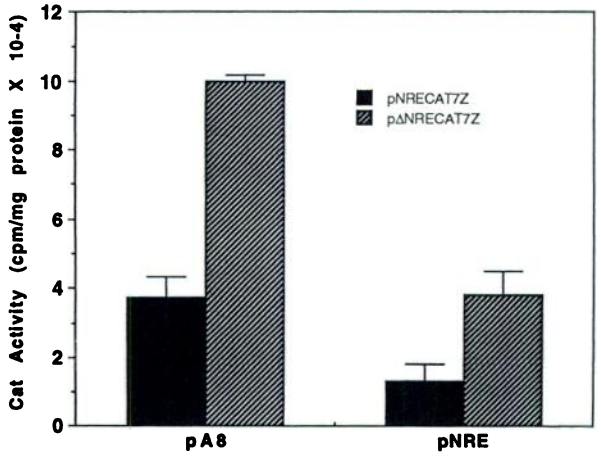

Fig. 8. Effect of co-transfection with pNRE on *cat* activity. HepG2 cells were transfected with either pNRECAT7Z or pΔNRECAT7Z (5 μg) and a control metallothionein promoter, pXGH5 (2 μg), with or without 10 μg of either pA8 (control plasmid) or pNRE. pNRE had no effect on promoter activity of either control construct (i.e., pXGH5 or pRSVCAT). The data represent the mean ± standard error for three experiments.

treatment with B(*a*)P and TCDD (Fig. 7). After transfection of the cells with the NRE-lacking construct (pMC0LUC$^-$), treatment with B(*a*)P and TCDD resulted in 4- and 35-fold increases, respectively, in luciferase expression (Fig. 7). These results indicated that deletion of the NRE from the promoter-*luc* gene constructs was accompanied by a significantly greater level of induction after exposure of the cells to either B(*a*)P or TCDD, suggesting some interference of the NRE-factor complex with additional *cis*-elements that function in the regulation of reporter gene expression (*CYP1A1* expression).

Effect of co-transfected pNRE. The function of the NRE was further studied by co-transfecting the sequence, in the form of pNRE, into HepG2 cells along with a promoter-reporter gene construct. In these studies pA8, which has approximately 80% of the rat *CYP1A1* gene information but does not contain any of the purported regulatory 5′-flanking sequences, was used as a control. The reporter gene constructs included pΔNRECAT7Z (NRE-lacking) and pNRECAT7Z (NRE-containing). pRSVCAT, wherein *cat* expression is driven by the RSV promoter, or pXGH5, wherein *hGH* expression is driven by the mouse metallothionein promoter, were used as negative controls. Co-transfection of pNRE specifically decreased *cat* expression of both pNRECAT7Z and pΔNRECAT7Z, by 2.5- and 3-fold, respectively (Fig. 8). Expression of *cat* in pRSVCAT-transfected cells and *hGH* expression were not affected by co-transfection with pNRE (data not shown). A similar reduction in expression of *hGH* by co-transfection of cells with pMC0GH$^+$ or pMC0GH$^-$ and pNRE was observed (data not shown). These results indicated that pNRE was capable of acting *in trans* to inhibit basal reporter gene activity with either NRE-lacking or NRE-containing promoter-reporter gene constructs.

Constitutive reporter gene activity as affected by various deletion mutations. A series of deletion constructs were created using the *luc* reporter gene in which the rat *CYP1A1* NRE was placed closer to the start of transcription (position +1) by deletions of 242 bp, 390 bp, or 501 bp from bp −658 of the rat promoter (pMC0LUCΔ242$^+$, pMC0LUCΔ390$^+$, and pMC0LUCΔ501$^+$, respectively). A comparable series of deletion constructs lacking the NRE (bp −881 to −658) was also constructed (pMC0LUCΔ242$^-$, pMC0LUCΔ390$^-$, and pMC0LUCΔ501$^-$, respectively) (see Fig. 1). pMC0LUCΔ242$^-$ exhibited 4-fold greater luciferase activity than its comparable NRE-containing construct, pMC0LUCΔ242$^+$ (Fig. 9). Placement of the NRE 390 bp closer to the transcriptional start site, as in pMC0LUCΔ390$^+$, caused a dramatic decrease in constitutive luciferase activity, whereas with the comparable NRE-lacking construct, pMC0LUCΔ390$^-$, a >100-fold increase in luciferase expression was observed. With the deletion of 501 bp, which places the NRE at bp −157 of the rat *CYP1A1* promoter (pMC0LUCΔ501$^+$), no additional effect upon constitutive luciferase activity was observed (Fig. 9). A Hirt supernate/Southern hybridization analysis was conducted with each of the deletion mutant-transfected cell samples (30, 31). This assay revealed that in each case the reporter gene activity was a reflection of the amount of plasmid DNA transfected into the cells.

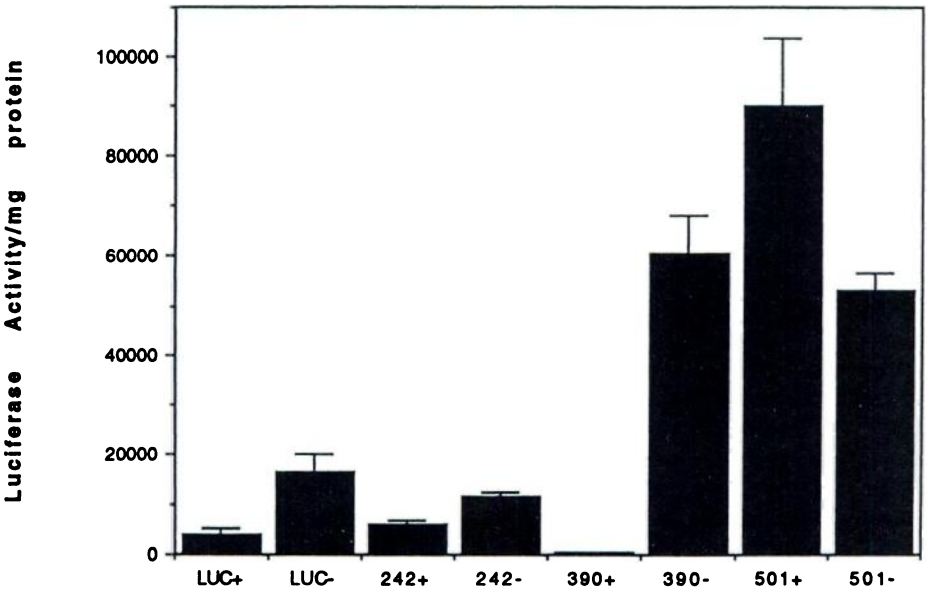

Fig. 9. Luciferase activity after transfection with various NRE-lacking and NRE-containing deletion constructs. HepG2 cells were transfected with 5 μg of one of eight plasmids, i.e., pMC0LUC$^\pm$ (*LUC*±), pMC0LUCΔ242$^\pm$ (*242*±), pMC0LUCΔ390$^\pm$ (*390*±), or pMC0LUCΔ501$^\pm$ (*501*±). All sets of HepG2 cells were co-transfected with pXGH5 as a control for plate to plate transfection efficiency. Promoter activity was expressed as relative luminescence (luciferase activity/mg of protein). Data shown are the mean ± standard error of three experimental values. The relative promoter activities were also corrected for the amount of DNA/dish for each construct, as determined by extraction and hybridization analysis (see text).

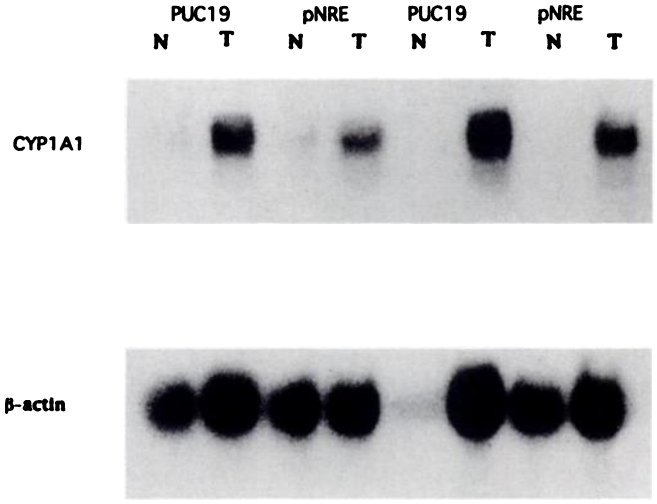

Fig. 10. RNA blot hybridization analysis of HepG2 total RNA. HepG2 cells were transfected with pMC0GH$^+$ (2.5 μg), pMC0CAT (2.5 μg), and either pUC19 (10 μg) or pNRE (10 μg). Total RNA was extracted, separated by gel electrophoresis, and transferred to a nitrocellulose membrane as described in the text. The human *CYP1A1* construct pHMC6B was used as probe. *Lanes 1, 2, 5,* and *6*, RNA from cells co-transfected with pUC19; *lanes 3, 4, 7,* and *8*, RNA from cells co-transfected with pNRE; *lanes 2* and *4*, treated with TCDD (*T*) for 4 hr; *lanes 6* and *8*, treated with TCDD for 24 hr; *lanes 1, 3, 5,* and *7*, treated with vehicle (toluene) alone (*N*).

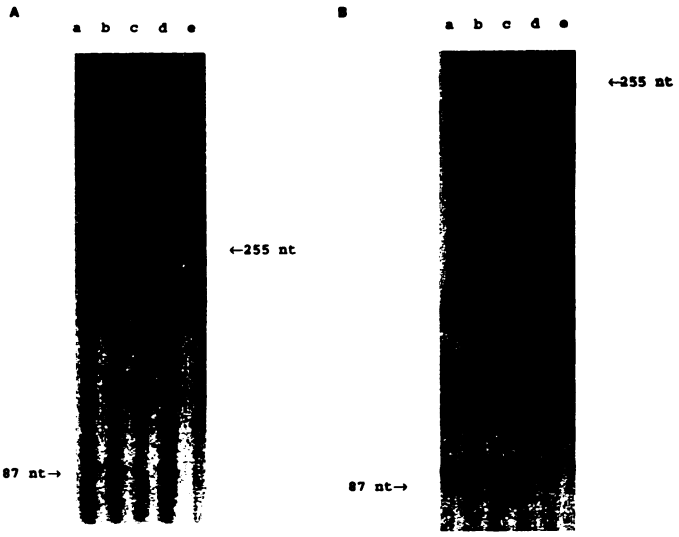

Fig. 11. RNase protection assay of transfected HepG2 cell RNA. A, *Lane a*, RNA (100 μg) from HepG2 cells that had been co-transfected with pMC0CAT, pMC0GH$^+$, and pNRE, hybridized to rat *CYP1A1* antisense RNA (5 × 10^5 cpm); *lane b*, same as *lane a*, except that TCDD was used as inducer; *lane c*, same as *lane a*, except that pUC19 was used instead of pNRE; *lane d*, same as *lane c*, except that cells were induced with TCDD; *lane e*, tRNA (100 μg) only. B, *Lane a*, control RNA (50 μg) from H4IIE cells hybridized to rat *CYP1A1* antisense RNA (5 × 10^5 cpm); *lane b*, same as *lane a*, except that cells had been induced with B(*a*)P; *lane c*, same as *lane a*, except that cells had been induced with TCDD; *lane d*, pRSVCAT-transfected HepG2 cell RNA (50 μg, used as *cat* 255-nucleotide protected fragment control) hybridized to rat *CYP1A1* antisense RNA (5 × 10^5 cpm); *lane e*, tRNA (50 μg) only. Hybridization reactions were digested with RNase A (35 μg/ml) and RNase T1 (2 μg/ml) as described in the text.

TABLE 2

Endogenous *CYP1A1* mRNA levels after transfection with pUC19 or pNRE

HepG2 cells were co-transfected with pMC0CAT, pMC0GH$^+$, and either pUC19 (control) or pNRE and were treated with TCDD for either 4 or 24 hr. Total RNA was isolated and electrophoresed, and RNA blot hybridization analysis was conducted as described in the text (also see related Figs. 10 and 11). After autoradiography, the intensity of the signals was determined by densitometry and expressed in densitometric units.

Plasmid co-transfected	Treatment	*CYP1A1* (×10^2)/β-actin
pUC19	No inducer	1.5
	TCDD, 4 hr	7.3
	No inducer	2.0
	TCDD, 24 hr	11.8
pNRE	No inducer	0.2
	TCDD, 4 hr	3.9
	No inducer	0.1
	TCDD, 24 hr	7.0

The large decrease in luciferase activity observed in cells that were transfected with pMC0LUCΔ390$^+$ but not with pMC0LUCΔ501$^+$ and the abolition of this reduction upon removal of the NRE (pMC0LUCΔ390$^-$) suggested a second site of interaction of the NRE-factor complex occurring between bp −268 and −157 of the rat P450IA1 promoter. This region of the rat *CYP1A1* promoter was reported to contain a 4-S polycyclic hydrocarbon-binding protein element (13) and a DRE (14) as well as a 21-bp repeat of GT.

Effect of co-transfected pNRE on expression of the endogenous *CYP1A1* gene in TCDD-treated HepG2 cells. The *trans* effect of co-transfected pNRE on TCDD-induced *CYP1A1* was determined in HepG2 cells. Total cellular RNA was isolated for RNA blot hybridization analysis or RNase protection analysis at 4 and 24 hr after induction with TCDD. The human *CYP1A1* genomic clone pHMC6B was used to assess the amount of endogenous *CYP1A1* mRNA in the transfected HepG2 cells. The Northern hybridization analysis is shown in Fig. 10 and the results are quantitated in Table 2. TCDD markedly induced the steady state mRNA level for *CYP1A1*, as normalized for β-actin gene expression; 5- and 8-fold increases in mRNA levels were noted at 4 and 24 hr, respectively, after treatment of the HepG2 cells with TCDD. The transfection of pNRE into the HepG2 cells resulted in a marked decrease in the basal expression of *CYP1A1*, in accordance with results described above on the expression of reporter genes. TCDD administration elevated *CYP1A1* mRNA in the pNRE-transfected cells but not to the same extent as in cells transfected with the control plasmid pUC19 (Table 2).

Initiation of transcription at the correct site. The RNase protection assay was carried out to determine whether the correct transcription initiation site was used in cells that were transfected with the *CYP1A1* promoter-*cat* gene fusion constructs with or without pNRE. An antisense RNA probe was designed to give protected fragments of 87 nucleotides (exon 1) and 255 nucleotides (nucleotides 1–255 of *cat*) for a properly initiated gene transcript driven by the rat *CYP1A1* promoter. A portion of the *cat* gene was initially included in the antisense RNA to differentiate between endogenous *CYP1A1* and transfected pMC0CAT fusion gene transcripts. The total RNA from nontransfected H4IIE cells (control RNA) gave the expected protected fragment of 87 nucleotides, i.e., the first exon of the endogenous rat *CYP1A1* (Fig. 11B, *lanes a-c*).

Total RNA from HepG2 cells that had been co-transfected with pMC0CAT, pMC0GH$^+$, and either pUC19 or pNRE also gave the expected protected fragment of 87 nucleotides (Fig. 11A, *lanes a-d*). The signal from TCDD-induced cells co-transfected with pNRE was weaker than that observed for cells co-

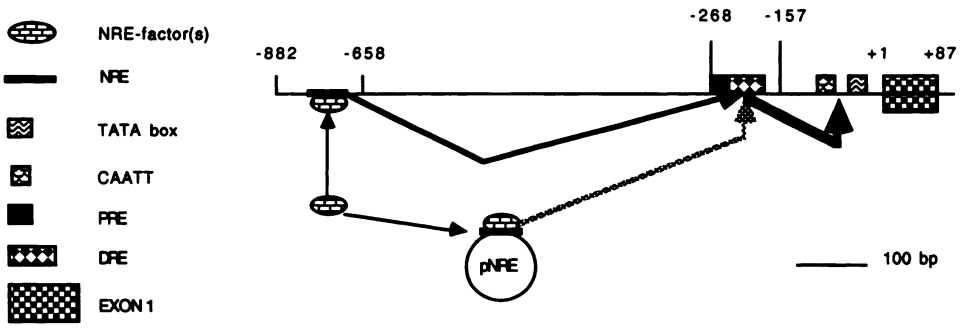

Fig. 12. Schematic diagram of the possible mechanism of action of the NRE-factor complex in regulation of *CYP1A1*. The 5′-flanking portion and first exon of rat *CYP1A1* from bp −882 to +100 are shown. The NRE and regions of *CYP1A1* with which certain *trans* factors interact are shown as *filled blocks*. The positions of the CCAAT and TATA boxes are marked. *Arrow-bearing solid line*, interaction of the NRE-binding factor with the gene; *arrow-bearing hatched line*, interaction of the pNRE-binding factor with the gene.

transfected with pUC19 (Fig. 11A, *lane b* versus *lane d*). This was in agreement with the reduced promoter activity of endogenous human *CYP1A1*. In addition, a less intense signal at 255 nucleotides was observed as a protected fragment in the RNase protection assay (Fig. 11A, *lanes a-d* versus *lane e*). The lower intensity of the signal at 255 nucleotides, relative to that at 87 nucleotides, was expected because both pMC0CAT and pMC0GH$^+$ transcribe the first exon of rat *CYP1A1*, whereas only pMC0CAT transcribes the *cat* gene. Total RNA isolated from HepG2 cells that had been transfected with only pRSVCAT yielded a protected fragment corresponding to the first 255 nucleotides of *cat*, as expected (Fig. 11B, *lane d*). No protected fragment at 87 nucleotides was noted with the endogenously transcribed human *CYP1A1* from HepG2 cells, because the nucleotide sequence of the human gene in this region is divergent from that of the rat (Fig. 11B, *lane d* versus *lanes a-c* and *e*). The results of these experiments indicated that 1) pNRE suppressed *in trans* the induced activity of the transfected *CYP1A1* promoter-reporter gene constructs and the endogenous human *CYP1A1* promoter activity and 2) the transfected rat *CYP1A1* promoter-reporter gene fusion constructs were transcribed at the correct initiation site.

Discussion

The involvement of a NRE in the suppression of *CYP1A1* expression has been reported for the mouse and human genes (8–10). Although the NRE has been postulated to operate in conjunction with a labile protein (8, 9), the existence of a protein that binds to the NRE has not been demonstrated previously. The present study suggests that the rat NRE may specifically bind three or more proteins that are present in H4IIE and HepG2 hepatoma cells and in rat liver. Two of these proteins would interact with the conserved regions of the NRE, i.e., nre_1 and nre_2, whereas additional binding proteins are suggested because of the lack of competition between nre_1 and/or nre_2 and NRE-200 in gel mobility shift assays and DNase I footprinting assays. Restriction fragments of NRE-200 (bp −882 to −842 and bp −755 to −707) that did not contain nre_1 or nre_2 sequences specifically bound a protein from rat liver. It is of interest that in the region between bp −881 and −842 a sequence (CCCCTCC) is found that is very similar to the CCCCATCC motif found in the collagen II gene, i.e., the CIIS2 silencer element (32), and the avian CR1 element (33), which functions as a NRE in the chicken lysozyme gene (34). The CCCCTCC motif of the rat *CYP1A1* NRE is flanked by CT-rich stretches that have similarity to other NRE motifs, i.e., ANCCTCTCTT and ANTCTCCTCC, of the chicken lysozyme gene (34), the human interferon-β gene (35), and the human ϵ-globin gene (36). Similar CT motifs are present as NREs in the angiotensinogen gene (37), the c-*myc* gene (11, 12, 38, 39), and the collagen II gene (32).

In the mobility-shifted NRE-200 restriction fragment (bp −755 to −707), a 16-bp palindromic sequence (CTAAGGTGACCCTTAG) at bp −726 to −711 was juxtaposed to a DNase I-hypersensitive site; this region may be involved in protein binding/transcriptional regulation. In a sequence on the forward strand of the rat NRE, an octamer-binding motif (ATGCAAAACA) (40) is found at bp −797 to −788. The 32-bp sequence of the rat *CYP1A1* NRE (bp −808 to −777) that contains the octamer motif has approximately 88% sequence identity to a 25-bp c-*myc* sequence that binds *fos/jun* and octamer factors; this sequence is localized to a c-*myc* NRE (11, 12). This portion of the rat *CYP1A1* NRE, which is located between the conserved nre_1 and nre_2 sequences, was also protected from DNase I digestion with HepG2, H4IIE, and rat liver nuclear extracts. Whether the sequence between bp −808 and −777 does bind to *fos/jun*-octamer factors has yet to be determined. The conserved nre_2 contains a direct repeat motif, GGGAGCCTACAGGGAGCC, which is located immediately downstream of the 32-bp *fos/jun*-octamer sequence identity. This region may be essential for the binding of additional factors that would act in conjunction with the putative *fos/jun*-octamer factors.

The expression of reporter gene activity after transfection of the non-NRE-containing rat *CYP1A1* constructs was considerably enhanced after treatment of the cells with B(*a*)P and TCDD, compared with the NRE-containing constructs. These results suggested that the putative NRE-factor complex may interact with the *cis* sequences with which 4-S or 8-S binding proteins interact in regulating the level of induction of *CYP1A1* by polycyclic hydrocarbons and dioxins, respectively. Furthermore, basal gene expression may also be regulated by the interaction of the NRE factors with other transcriptional regulatory factors.

Removal of a NRE sequence from the human interferon-β promoter increased basal activity and the level of induction of interferon-β mRNA in a manner that was similar to that seen with the NRE-lacking and NRE-containing rat *CYP1A1* promoter-reporter gene fusion constructs (35). Additional evidence that the NRE-factor complex may interact with another regulatory element or factor derives from the observation that introduction of the cloned NRE into HepG2 cells inhibited basal and induced levels of reporter gene expression in both NRE-containing and non-NRE-containing rat *CYP1A1* constructs. The level of expression of the endogenous human

CYP1A1 in the HepG2 cells was also inhibited by transfection with pNRE.

The findings described above have been incorporated into a model of the regulation of *CYP1A1* (Fig. 12). It is proposed that the NRE complexes with at least three protein factors in hepatoma cells and liver, suppressing transcriptional activity. This suppression could be the result of a conformational folding of the chromatin, placing the complex in association with a downstream regulatory element at bp −268 to −157 and thereby influencing the transcriptional initiation complex of *CYP1A1*.

The introduction of the NRE into cells in the form of pNRE still allowed for the association of this *cis* element with a *trans* factor. The latter complex is capable of interacting *in trans* with the downstream region (bp −268 to −157) in *CYP1A1*, thus down-regulating its expression (Fig. 12). The role of the NRE-protein complex in the negative regulation of basal expression of *CYP1A1* and the alleviation of this suppression by polycyclic hydrocarbons and dioxins suggest multiple interactions within the gene that require additional study.

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