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Partial Characterization of the Human *CYP1A1* Negatively Acting Transcription Factor and Mutational Analysis of Its Cognate DNA Recognition Sequence

PAUL D. BOUCHER, MARIE P. PIECHOCKI, AND RONALD N. HINES*

Department of Pharmacology, Wayne State University School of Medicine, Detroit, Michigan 48201-1998

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Previous studies in our laboratory identified a negative regulatory domain in the 5′-flanking region of the human *CYP1A1* gene containing two negative regulatory elements (NRE). Characterization of one of these elements revealed three nuclear protein binding regions: a 21-bp palindrome with a point of symmetry at −784 and two guanine- and cytosine-rich elements that flank the palindrome. Functional studies suggested the palindrome is critical for transcriptional repression, whereas the guanine- and cytosine-rich sequences play a secondary role. In this study, the interaction between nuclear proteins and the *CYP1A1* NRE was further defined. Electrophoretic mobility shift assays (EMSA) indicated that the NRE −784 palindrome alone, but not the guanine- and cytosine-rich sequences minus the palindrome, was capable of specific nuclear protein binding. Competitive cotransfection experiments confirmed this observation in intact cells. Specific residues important for DNA-protein interactions were identified by site-directed mutagenesis and competitive EMSA. The loss of specific protein binding was also correlated with the loss of negative regulatory activity in a transient-expression assay. Finally, competitive EMSA was performed with consensus oligonucleotides for known transcription factors. An NF-Y consensus sequence efficiently competed with the NRE probe for specific nuclear protein binding. EMSA supershift analyses indicate that a protein immunologically related to NF-Y_B is part of the specific nuclear protein complex binding the human *CYP1A1* NRE. These studies have refined our understanding of the sequences critical for the transcriptional repression of human *CYP1A1*. To our knowledge, this is also the first report implicating a member of the NF-Y transcription factor family in negative gene regulation.

The cytochrome P450-dependent monooxygenases constitute a superfamily of enzymes (30) that play a critical role in the oxidative metabolism of a wide variety of both endogenous (19, 28, 42) and exogenous (11) substances. In mammalian systems, the latter activity is restricted largely to enzymes belonging to families 1 through 4. Although some of these enzymes are constitutively expressed and noninducible, others are constitutively expressed and inducible, while still others are not constitutively expressed but inducible. CYP1A1 belongs to the latter group. Normally not expressed in adult tissues, *CYP1A1* expression is induced severalfold upon exposure to a variety of xenobiotics, including halogenated hydrocarbons (e.g., dioxin) and polyaromatic hydrocarbons (e.g., benzo[*a*]pyrene) (16). Induction is mediated by the Ah receptor, a unique member of the basic helix-loop-helix family of transcription factors (31). The function of CYP1A1 in cellular metabolism is unknown. However, the enzyme is highly active in the metabolism of polyaromatic hydrocarbon procarcinogens (e.g., benzo[*a*]pyrene) into their ultimate carcinogenic forms (3, 20).

In a previous study (15), we identified a 275-bp negative regulatory element (NRE) upstream of the human *CYP1A1* coding region (−833 to −558). The deletion of this fragment resulted in an increase in both constitutive and inducible promoter activity. This observation led to the suggestion that mutations in the NRE or its binding protein may contribute to the large interindividual variation in human *CYP1A1* inducibility (10) and to the aberrant expression noted in several lung carcinoma cell lines (26) and primary pulmonary carcinomas (27). Similar mutations may well contribute to the highly inducible *CYP1A1* phenotype that has been correlated with an increased incidence of lung cancer (21, 22) and to the moderate-to-high constitutive CYP1A1 activity correlated with a poor prognosis in human breast cancer (29, 34).

We have characterized the human *CYP1A1* NRE using both in vitro binding and transient-expression assays (6). Subfragment analysis of the original 275-bp human NRE (15) demonstrated the presence of two functional elements. Both were capable of down-regulating a heterologous enhancer or promoter directing the expression of a reporter gene after transfection into the human hepatoma cell line HepG2. Further analysis of one of these elements revealed a DNase I footprint pattern consisting of three protected regions, a 21-bp palindrome sequence flanked by two distinct guanine- and cytosine-rich elements. Employing transient expression studies, the *CYP1A1* NRE −784 palindrome (−794 to −774) was shown to be capable of significant negative regulatory activity. In contrast, the guanine- and cytosine-rich sequences minus the palindrome had no such activity. However, these sequences may serve a secondary role, since all three elements appeared essential for optimum negative regulatory activity. Further, distinct DNase I hypersensitive sites indicative of potentially critical DNA conformational changes appear only when the guanine- and cytosine-rich sequences are bound. The second NRE element was shown to be dependent on a similar, though less well conserved, palindrome sequence with a point of symmetry at −573.

Given that the NRE −784 palindrome sequence alone is

* Corresponding author. Mailing address: Department of Pharmacology, Wayne State University School of Medicine, 540 East Canfield Ave., Detroit, MI 48201-1998. Phone: (313) 577-8601. Fax: (313) 577-6739. Electronic mail address: rhines@cmb.biosci.wayne.edu.

sufficient for negative regulatory activity, we have extended the previous studies exploring the interaction between specific nuclear proteins and components of the *CYP1A1* NRE. We report that specific residues present in the −784 palindrome are critical for nuclear protein binding and negative regulatory activity. Employing competitive electrophoretic mobility shift assays (EMSA), we also introduce evidence for a protein immunologically related to NF-Y_B interacting with the *CYP1A1* NRE and rule out the involvement of several other known transcription factors.

MATERIALS AND METHODS

Materials. Antipain, aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, tissue culture medium, antibiotics, 4-methylumbelliferyl-β-galactopyranoside, and 4-methylumbelliferone were purchased from Sigma Chemical Co. Fetal bovine serum was obtained from JRH Biosciences. Superscript reverse transcriptase and some restriction endonucleases were acquired from Life Technologies, Inc. Other restriction endonucleases and T4 polynucleotide kinase were obtained from New England Biolabs. T4 DNA polymerase, β-galactosidase and poly(dI-dC) were purchased from Boehringer Mannheim. *Escherichia coli* SURE cells were obtained from Stratagene. Micro bicinchoninic acid protein assay reagents were acquired from Pierce Chemical Co. [α-^{32}P]deoxyribonucleotide triphosphates (3,000 Ci/mmol) and [^{14}C]chloramphenicol (55 Ci/mmol) were purchased from DuPont-New England Nuclear and Amersham Corp., respectively. Fibroblast growth factor 4 (FGF-4) NF-Y consensus oligonucleotides (13) were a gift of A. Rizzino (Eppley Cancer Research Center, University of Nebraska Medical Center). Other consensus sequence oligonucleotides were purchased from either Stratagene or Promega. NF-Y antibodies were a gift of D. Mathis (Laboratoire de Genetique Moleculaire des Eucaryotes du Centre National de la Recherche Scientifique).

Cells and culture conditions. Human HepG2 hepatoblastoma cells were a gift of B. Knowles (Jackson Laboratories) (1). The cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Cell cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

Plasmid constructs. pRNH131 containing a 105-bp fragment (−833 to −728) from human *CYP1A1* cloned into the *Sma*I site of pUC19 was described in a previous study (6). This fragment includes the upstream NRE palindrome exhibiting a point of symmetry at −784. DNA oligonucleotides representing the sense and antisense strands of the three *CYP1A1* NRE components previously identified by DNase I footprinting (6) were custom synthesized by National Biosciences, Inc. Complementary oligonucleotides were annealed by heating to 65°C and then slowly cooled to room temperature, phosphorylated with T4 polynucleotide kinase, and cloned into pUC19. pRNH209 consists of the 21-bp NRE palindrome sequence (−794 to −774) cloned into the *Sma*I site of pUC19. pRNH255 contains both guanine- and cytosine-rich regions (−818 to −798 and −767 to −749) cloned into the pUC19 multiple cloning site such that their relative orientation is the same as that found in *CYP1A1*, but they are separated by 22 bp of vector sequence rather than the *CYP1A1* −784 palindrome.

For transient-expression assays, chloramphenicol acetyltransferase (CAT) expression was directed by the herpes simplex virus thymidine kinase (HSV tk) promoter and simian virus 40 (SV40) enhancer in the reporter plasmid pRNH195c (6). The CAT reporter plasmid, pRNH220c, contains the human *CYP1A1* NRE$_{105}$ fragment cloned immediately upstream of the SV40 enhancer and HSV tk promoter in pRNH195c and has been described previously (6). pRNH483c was constructed in a fashion similar to that of pRNH220c but with the substitution of an NRE$_{105}$ fragment containing a guanine-to-thymine transversion at −787 (see below).

Site-directed mutagenesis of the *CYP1A1* NRE −784 palindrome was performed by an oligonucleotide-directed mutagenesis system and pAlter vector (Promega) with modifications described in detail elsewhere (33). The presence of the desired mutation(s) and the integrity of the remaining DNA fragment were verified by dideoxy sequencing (36).

Plasmid DNA was used to transform *E. coli* SURE cells following the method of Hanahan (12). The presence and orientation of inserts in the various plasmid constructs were verified by restriction endonuclease and DNA sequence analyses (36).

EMSA. The preparation of HepG2 nuclear extract has been described previously (6). Protein concentration was determined by the micro bicinchoninic acid protein assay (39). EMSA probes NRE$_{105}$, NRE21, and NRE57 were isolated from pRNH131, pRNH209, and pRNH255, respectively, by *Sac*I-*Hin*dIII digestion. The NRE$_{105}$ probe consisted of a 150-bp fragment containing *CYP1A1* sequences −833 to −728, the NRE21 probe consisted of a 65-bp fragment containing *CYP1A1* sequences −794 to −774, and the NRE57 probe consisted of a 85-bp fragment containing *CYP1A1* sequences −818 to −767 and −767 to −749 separated by 22 bp of vector sequence. Probes were radiolabeled with Superscript reverse transcriptase, and EMSA were performed as previously described (6). Unlabeled, competing NRE$_{105}$, NRE21, or NRE57 fragments were included in some of the incubations at a 10- to 100-fold molar excess over the labeled probes. In other experiments, a 25- to 1,000-fold molar excess of unlabeled consensus oligonucleotide was used to compete with the NRE$_{105}$ radiolabeled probe. In addition to the oligonucleotides representing transcription factor consensus binding sequences, NRE6 was used in these experiments. This was a 24-bp oligonucleotide representing *CYP1A1* sequences −794 to −774 plus three additional bases to create a unique *Pml*I restriction endonuclease site on the 3′ end.

For competitive EMSA, unlabeled 177-bp wild-type and mutated *Apa*I-*Bpu*1102 NRE fragments (*CYP1A1* sequences −834 to −656) were used to compete for specific protein binding with the radiolabeled 177-bp wild-type probe at a 0- to 1,000-fold molar excess. Following overnight exposure, the autoradiographs were used as templates to excise the specific DNA-protein complexes from the gel. Dried gel slices were solubilized with Solvable (DuPont) and counted in scintillation cocktail as suggested by the manufacturer. Competition was expressed as the percentage of specific bound DNA-protein complex observed in the absence of competitor DNA.

EMSA supershift assays were performed essentially as described above, except nuclear extract was preincubated with antibody overnight at 4°C. One microliter of a monoclonal NF-Y_A antibody raised against recombinant NF-Y_A protein (YA1a), 0.5 μl of a polyclonal antiserum raised against recombinant NF-Y_B protein (pRαYB), 0.5 μl of preimmune rabbit serum, or 0.02 μl of an affinity-purified pRαYB antibody (affinity purified against recombinant NF-Y_B) was added to the nuclear extract. These antibodies have been extensively characterized by Mantovani and colleagues and are described elsewhere (25).

Transient-expression assays. HepG2 cells were plated onto polylysine-treated 100-mm-diameter culture dishes at a density of 10^6 cells per dish and grown overnight. Transfections were carried out as described previously (6). pRNH209 and pRNH255 were included in some transfections at a one- to threefold molar excess relative to pRNH195c or pRNH220c. In the latter experiments, the amount of DNA transfected was kept constant by adjusting the concentration of pUC19 carrier DNA. CAT activity was assayed as described by Seed and Sheen (37). pRSVβgal was cotransfected as an internal control for transfection efficiency. The formation of 4-methylumbelliferone from the substrate, 4-methylumbelliferyl β-galactopyranoside, was used to determine β-galactosidase activity in each of the extracts by fluorometry (2). Protein concentration was measured by the bicinchoninic acid protein assay (39). CAT activity was corrected for transfection efficiency (β-galactosidase activity) and protein content. One-way analysis of variance (Tukey's post test) was performed with the Instat software package (GraphPad). A P value less than 0.05 was accepted as significant.

RESULTS

Previous studies in our laboratory established that a single, specific HepG2 nuclear protein complex forms with the NRE$_{105}$ probe (−833 to −728) and that the palindrome at −784 was critical for activity (6). To determine whether the −784 palindrome was sufficient for specific binding or whether the flanking guanine and cytosine sequences were also necessary, EMSA analysis was performed with NRE21 and NRE57 as probes (see Materials and Methods). When NRE21 was used as the probe, a single specific DNA-protein complex was observed (Fig. 1A). Competition with a 10- to 20-fold molar excess of unlabeled NRE21 or NRE$_{105}$ decreased specific binding to the radiolabeled NRE21 probe. However, unlabeled NRE57 did not compete for this nuclear protein complex, even at a 100-fold molar excess (Fig. 1A). The DNA-protein complexes observed with the radiolabeled NRE57 probe do not represent specific binding, since competition did not occur even with a 100-fold molar excess of unlabeled NRE$_{105}$, NRE21, or NRE57 (Fig. 1B). These results suggest that the NRE palindrome alone is sufficient to form specific complexes with proteins found in HepG2 nuclear extract and that the guanine- and cytosine-rich sequences, in the absence of the palindrome sequence, are incapable of forming specific DNA-protein complexes.

To corroborate the above observation in intact cells, we performed cotransfection competition experiments in HepG2 cells (Fig. 2A and B). Cotransfection of a threefold molar excess of a vector containing either a single copy of the NRE −784 palindrome (pRNH209) or both guanine- and cytosine-rich sequences minus the palindrome (pRNH255) had no significant effect on SV40 enhancer- or HSV tk promoter-di-

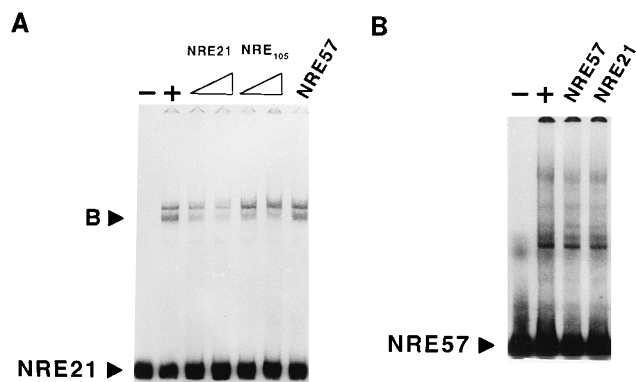

FIG. 1. Specific binding of nuclear proteins on the CYP1A1 NRE palindrome, but not on the conserved guanine- and cytosine-rich elements. The 21-bp NRE −784 palindrome (NRE21) and the guanine- and cytosine-rich sequences minus the palindrome (NRE57) were tested individually for their ability to bind proteins in HepG2 nuclear extract. The reactions in the first lanes (−) contain radiolabeled probe in the absence of nuclear extract. (+) lanes indicate the addition of nuclear extract but no competing DNA. The single specific band is indicated by a B in panel A. This protein complex competed with a 10- or 20-fold molar excess of unlabeled NRE21 or NRE_{105} but not with a 100-fold molar excess of unlabeled NRE57. (B) A 100-fold molar excess of either NRE57 or NRE21 failed to compete with the radiolabeled NRE57 for any of the DNA-protein complexes observed. Incubations were performed with approximately 0.1 ng of radiolabeled probe (50,000 cpm) and 10 μg of nuclear extract.

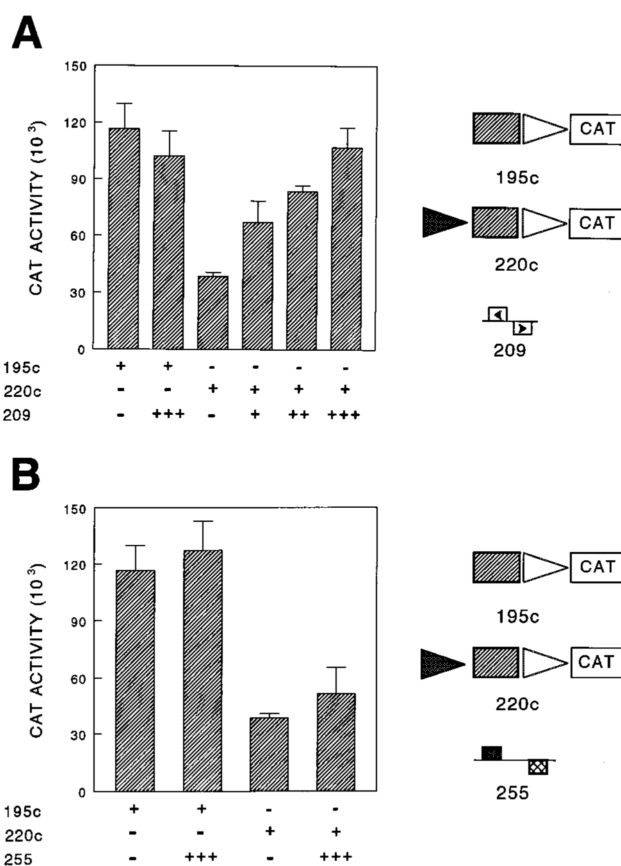

FIG. 2. Competition in intact cells for factors necessary for the negative regulatory activity of NRE_{105} is dependent on the NRE −784 palindrome. The relative positions of the HSV tk promoter (open arrowhead), SV40 enhancer (hatched box), and the NRE_{105} fragment (shaded arrowhead) are shown to the right of the graph. The small boxes representing pRNH209 (209) depict the 21-bp NRE −784 palindrome, and those representing pRNH255 (255) represent the guanine- and cytosine-rich sequences that normally flank the NRE −784 palindrome but here are separated by 22 bp of pUC19 vector sequence instead (see Materials and Methods). The difference in expression of CAT activity observed between pRNH220c (NRE_{105}) and the parent construct pRNH195c (195c) is highly significant ($P = 0.0001$). Relative to pRNH220c (220c), differences in CAT activity were observed with a one- (+), two- (++), or threefold (+++) molar excess of pRNH209 (209), with P values of 0.0013, 0.0028, and 0.31, respectively (A). CAT activity observed after cotransfection of pRNH220c with a threefold molar excess of the guanine- and cytosine-rich elements minus the NRE palindrome (pRNH255) (255) was not significantly different ($P = 0.12$) from that of pRNH220c alone (B). Cotransfection with either pRNH209 (A) or pRNH255 (B) with the control plasmid (pRNH195c) had no significant effect ($P = 0.18$ and 0.38, respectively). CAT activity is expressed as counts per minute per hour per unit of β-galactosidase activity per milligram of protein and represents the mean ± standard deviation ($n = 3$ or 4).

rected CAT expression (pRNH195c). Similar to what has been reported previously (6), the insertion of NRE_{105} immediately upstream of the SV40 enhancer and HSV tk promoter (pRNH220c) down-regulated CAT expression by approximately 70%. Cotransfection of pRNH220c with increasing concentrations of pRNH209 reversed the ability of NRE_{105} to down-regulate CAT expression (Fig. 2A). In contrast, cotransfection with increasing concentrations of pRNH255 had no significant effect (Fig. 2B). These data are consistent with the protein-binding studies described earlier. The NRE palindrome alone is capable of competing for cellular factors necessary for the observed transcriptional repression activity of NRE_{105}, but the guanine- and cytosine-rich elements minus the palindrome are not.

To better understand the observed DNA-protein interactions, point mutations and insertions were introduced into the NRE −784 palindrome, and competitive EMSA were performed (Fig. 3). For these assays, DNA fragments containing specific mutations were used to compete with radiolabeled wild-type probes for specific protein binding. Mutant fragments were used at a 5- to 1,000-fold molar excess over the wild-type fragment. As expected, fragments with substitutions at critical guanine residues identified by methylation interference (6) competed poorly with the wild-type probe for specific nuclear protein binding. At a 500-fold molar excess of PAL1 (−787 guanine to thymidine) and PAL4 (−787 guanine to thymidine and −781 cytosine to adenine), the wild-type DNA-protein complexes still retained 100% of their specific radioactivity. This is in contrast to the 12% of specific radioactivity remaining after competition with a 500-fold molar excess of an unlabeled wild-type fragment. Lengthening the 5-bp spacer by 2 bp (PAL7) also abolished the ability to compete with the wild-type probe for specific protein binding. The point mutation introduced in PAL3 (−781 cytosine to adenine) resulted in a diminished ability to compete. Point mutations in the spacer sequence (PAL5, −786 cytosine to adenine) or at position −788 (PAL16, thymidine to cytosine) also resulted in a more intermediate level of competition, with 53 and 41% of the specific radioactivity, respectively, remaining in the wild-type DNA-protein complex after the addition of a 500-fold molar excess of competing fragment. In contrast, the cytosine to adenine substitution at −789 in PAL13 had little effect; the PAL13 fragment competed as well as the wild-type fragment. To determine whether loss of specific protein binding would correlate with loss of negative regulatory activity, a fragment containing the PAL1 point mutation (−787 guanine to thymidine) was substituted for the wild-type NRE fragment in the heterologous enhancer-promoter reporter gene construct. When examined in a transient-expression assay, this point mutation resulted in a complete loss of negative regulatory activity (Fig. 4).

As a first approach to identifying the *CYP1A1* NRE-binding

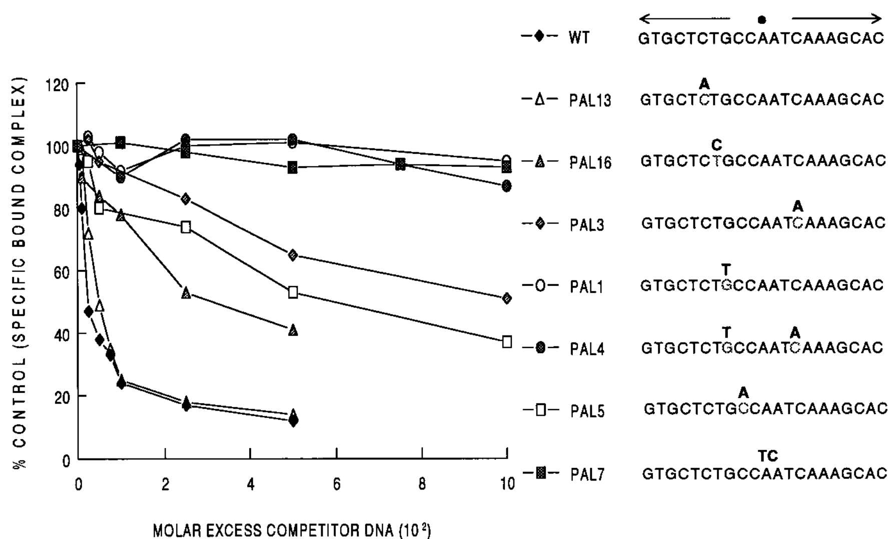

FIG. 3. NRE −784 palindrome point mutations compete poorly for specific nuclear protein binding as measured by competitive EMSA. Both the wild-type (WT) and mutated NRE palindrome sequences are shown to the right of the graph. Substituted bases are indicated by boldface letters above the wild-type bases they replaced. PAL7 has a 2-bp insertion. The inserted bases are shown in boldface above the spaces where they were introduced. EMSA were performed in the presence of a 0- to 1,000-fold molar excess of wild-type or mutated NRE palindrome fragments. Specific protein complexes formed with the wild-type probe were excised from the gels, and the amount of radioactivity in each slice was determined (Materials and Methods). Data are presented as a percentage of radioactive counts per minute contained within the specific complex (B) in the absence of competitor DNA.

proteins, a number of oligonucleotides representing consensus recognition sequences for known transcription factors were used to compete for specific protein binding in EMSA. Previous Southwestern (DNA-protein) blot experiments performed in our laboratory (4) revealed a *CYP1A1* NRE-protein binding pattern similar but not identical to that of another transcription factor, NF-Y (24). Further, the NRE −784 palindrome and NF-Y consensus sequence exhibit homology (Fig. 5) and similar EMSA patterns (5, 8). Competition for specific protein binding between the NRE_{105} probe and an authentic FGF-4 NF-Y oligonucleotide (13) was comparable to the competition observed with the NRE6 oligonucleotide over a 25- to 200-fold molar excess of unlabeled DNA. Specific nuclear protein binding was completely abolished with a 200-fold molar excess of either oligonucleotide (Fig. 6). Not shown, the FGF-4 NF-Y oligonucleotide also was able to compete with the *CYP1A1* NRE −573 palindrome (6) for specific protein binding. A mutated NF-Y oligonucleotide (Fig. 5) failed to compete when used at a 200-fold molar excess over that of the NRE_{105} probe. The NRE −784 palindrome also shows homology with several other transcription factor consensus sequences, particularly NF1-CTF, CREB, and Oct-1 (Fig. 5). We were particularly interested in Oct-1 and AP1, as these transcription factors reportedly bind a rat *CYP1A1* NRE (40, 41). However, oligonucleotides representing these consensus sequences were unable to compete even at concentrations as high as a 1,000-fold molar excess over that of the NRE_{105} probe (Fig. 7).

Given the competition observed with the NF-Y oligonucleotide in the experiments above, we hypothesized that one or more of the NF-Y transcription factor peptides is involved in the *CYP1A1* NRE palindrome-nuclear protein complex. NF-Y consists of three subunits, $NF\text{-}Y_A$, $NF\text{-}Y_B$, and $NF\text{-}Y_C$. All three subunits appear necessary for DNA binding activity, although the $NF\text{-}Y_C$ subunit is normally tightly associated with the $NF\text{-}Y_A$ subunit and copurifies with it (14, 23). Antibodies to the $NF\text{-}Y_A$ and $NF\text{-}Y_B$ subunits (25) were preincubated with nuclear extract prepared from HepG2 cells. The pretreated extract was used in EMSA binding reactions with radiolabeled NRE_{105}. A monoclonal antibody to $NF\text{-}Y_A$ as well as an affinity-purified antibody to $NF\text{-}Y_B$ had no effect on the specific NRE_{105}-nuclear protein complex (Fig. 8). In contrast, pretreatment with the original polyclonal $NF\text{-}Y_B$ antiserum, from which the affinity-purified antibody was prepared, produced both a diminution in the specific NRE_{105}-nuclear protein complex and a second DNA-protein complex with further retarded mobility (Fig. 8). Incubation with preimmune rabbit serum did not alter the NRE_{105}-nuclear protein complexes (Fig. 8, right panel).

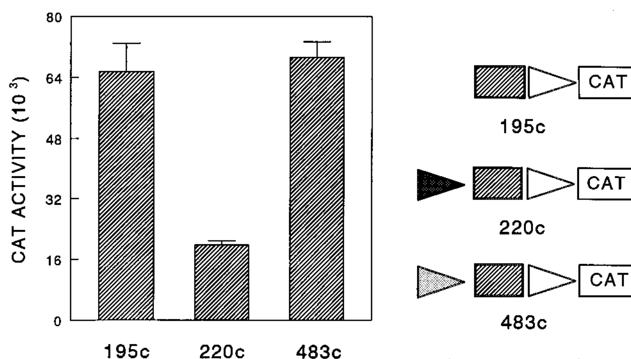

FIG. 4. A point mutation resulting in the loss of specific protein binding also results in the loss of negative regulatory activity. The relative positions of the HSV tk promoter (open arrowhead), SV40 enhancer (hatched rectangle), and NRE fragments (shaded arrowhead, wild type; stippled arrowhead, mutated NRE) are shown to the right with the abbreviated plasmid designation immediately below. A description of the construction and properties of pRNH195c, -220c, and -483c can be found in Materials and Methods. The down-regulation observed with pRNH220c relative to pRNH195c and pRNH483c was highly significant with a P of less than 0.001. No statistical difference was observed between pRNH195c and pRNH438c. CAT activity is expressed as counts per minute per hour per unit of β-galactosidase activity per milligram of protein and represents the mean ± standard deviation (n = 3).

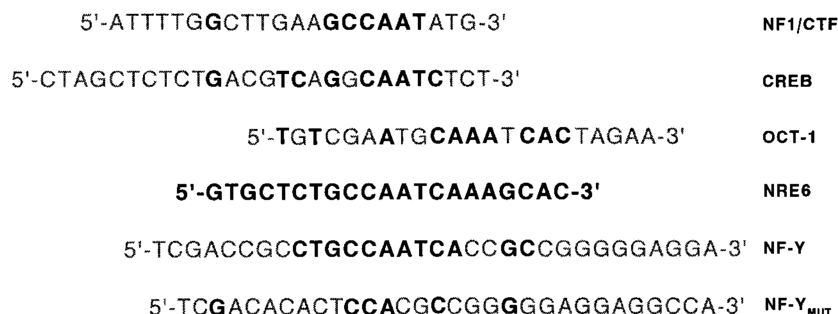

FIG. 5. Sequence comparison of some of the consensus oligonucleotides used in competitive EMSA experiments (Fig. 6 and 7). Sequences correspond to several synthetic consensus oligonucleotides in which sequence similarities with the NRE −784 palindrome are apparent. The oligonucleotides are aligned with the 21-bp *CYP1A1* NRE −784 palindrome (NRE6) which is shown entirely in boldface type. The boldface letters present in the transcription factor consensus oligonucleotides (NF1/CTF, CREB, OCT-1, NF-Y, and an NF-Y mutant [NF-Y_{MUT}]) indicate the position of base matches with the NRE −784 palindrome.

DISCUSSION

The regulation of human *CYP1A1* transcription involves the coordination of several *cis*-DNA sequence elements and their cognate *trans*-acting factors (16). Several early studies implicated an NRE and cognate repressor as one of the factors involved in *CYP1A1* regulation (15, 17, 18). More recently, our group identified two palindromic sequences centered at −784 and −573 that act as highly specific recognition sites for nuclear proteins present in human HepG2 cells and that function as transcriptional silencers. On the basis of preliminary studies with the −784 NRE, the palindrome appeared necessary but not sufficient for optimum activity. Highly conserved guanine and cytosine sequences that flank the palindrome and also exhibit a DNase I protection pattern were hypothesized as ancillary elements. Having no activity on their own, they did enhance the negative regulatory activity of the palindromic sequence, perhaps by stabilizing DNA-protein interactions (6). In this study, this hypothesis was corroborated by both in vitro DNA binding studies and cotransfection competition experiments in intact cells (Fig. 1 and 2). Other observations also are consistent with this hypothesis. First, the appearance of DNase I hypersensitive sites at residues located between the palindrome and guanine- and cytosine-rich elements are dependent upon protein binding to the latter (6), consistent with stabilization through a change in DNA conformation. Second, if protein binding on both elements were relatively stable, one would expect to observe multiple DNA-protein complexes in an EMSA. However, only a single complex is observed with the intact *CYP1A1* NRE.

It is well recognized that interactions between DNA-binding proteins and their recognition sites often depend on specific sequences and sometimes on the spacial orientation of two or more sequences. Site-directed mutagenesis was used in this study to examine the importance of individual bases within and spacing between the −784 palindrome half-sites (Fig. 3). These data demonstrate that binding of HepG2 nuclear protein is dependent upon the integrity of specific DNA residues within both the NRE −784 palindrome half-sites as well as on the spacing separating the half-sites. These observations also are consistent with previously reported methylation-interference experiments (6). Most important, we have demonstrated that a point mutation resulting in the loss of specific DNA-protein interaction also results in a loss of negative regulatory function (Fig. 4). Studies are under way to more thoroughly examine the effect of these mutations within the context of other human *CYP1A1* regulatory sequences.

As a first approach towards identifying potential transcription factors that may bind to the *CYP1A1* NRE, competition with consensus transcription factor binding sequences showing homology with the NRE −784 palindrome was evaluated. We hypothesized that one of these factors might be a component of the human *CYP1A1* repressor, albeit with modifications or heteromeric associations that result in a high degree of specificity for the NRE palindromic sequence, resulting in a negative rather than positive *trans*-regulatory function. Effective competition was observed with an oligonucleotide representing the NF-Y CCAAT-box binding factor recognition sequence present in the FGF-4 (*hst*) promoter (13) (Fig. 6). The decision to use this probe was spurred by the observation of considerable sequence identity between the NRE −784 palindrome and the FGF-4 NF-Y site. This includes the 5-bp spacer separating the NRE palindrome half-sites (a CCAAT-box sequence) as well as critical flanking sequences in each half-site. In addition, EMSA and UV cross-linking data presented for the FGF-4 NF-Y site (13) show some resemblance to our own observations of nuclear protein binding to the *CYP1A1* NRE −784 palindrome (4). We were also able to detect considerable sequence homology between the NRE palindrome and/or one of its half-sites and the consensus recognition sequences for

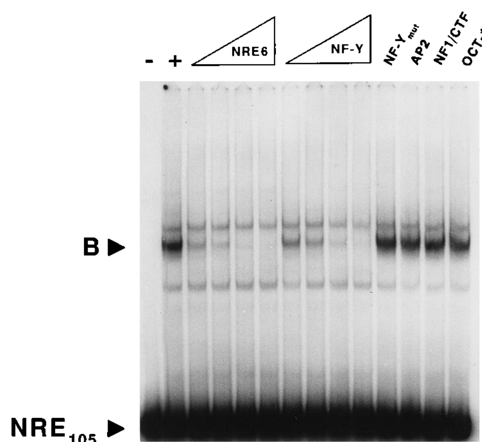

FIG. 6. Competition for NRE-binding proteins with NF-Y consensus oligonucleotides. Reactions were performed essentially as described in Materials and Methods. The single specific complex is indicated with a B. Competition was observed with both unlabeled NRE −784 palindrome (NRE6) and NF-Y oligonucleotides at concentrations of 25-, 50-, 100-, and 200-fold molar excess over the radiolabeled NRE_{105}. Competition was not observed with a 200-fold molar excess of an oligonucleotide with a mutated NF-Y consensus sequence (Fig. 5) or consensus oligonucleotides belonging to AP2, NF1-CTF, or Oct-1.

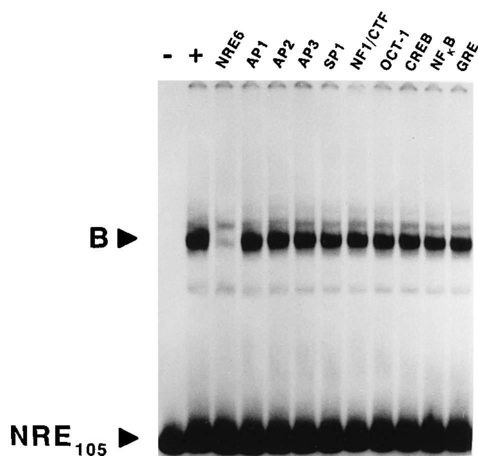

FIG. 7. Competition for NRE-binding proteins with consensus oligonucleotides for known transcription factors. A binding reaction performed with the NRE_{105} probe in the absence of nuclear protein is represented in the first lane (−). A single specific protein band (labeled B) was observed after incubation with the probe and nuclear extract (+). This specific complex was eliminated in the presence of a 100-fold molar excess of an unlabeled oligonucleotide containing the NRE −784 palindrome sequence (NRE6) but not with a 1,000-fold molar excess of any of the nine commercially available consensus oligonucleotides tested. Reactions were performed with approximately 0.03 ng of radiolabeled probe (30,000 cpm) and 10 μg of nuclear extract.

several other transcription factors (Fig. 5). Despite this homology, competition was not observed with the nuclear proteins that bind the *CYP1A1* NRE (Fig. 7). Thus, it is unlikely that any of the cognate DNA binding proteins specific for these other sequences participate in the negative regulation of the human *CYP1A1* gene. This includes Oct-1 and AP1, implicated in the negative regulation of the orthologous rat gene (40, 41), as well as several other CCAAT-box binding factors.

Cross-reactivity with $NF\text{-}Y_A$ and $NF\text{-}Y_B$ antibodies was used to confirm the presence or absence of NF-Y in the *CYP1A1* NRE complex (Fig. 8). Three different antibody preparations previously shown to supershift and/or block NF-Y binding to Y-box sequences in both the major histocompatibility complex class II (25) and FGF-4 (13) genes were tested. However, only the polyclonal rabbit serum to $NF\text{-}Y_B$ reacted with the *CYP1A1* NRE-protein complex, resulting in a diminution of the specific complex and a faint band indicative of a supershift. The latter result is similar to that of the reaction observed with authentic NF-Y sites (13, 25). Combined with the competitive binding observed with the FGF-4 Y-box sequence, these data lead us to conclude that the *CYP1A1* NRE-protein complex involves neither $NF\text{-}Y_A$ nor $NF\text{-}Y_B$ but a protein immunologically related to the latter.

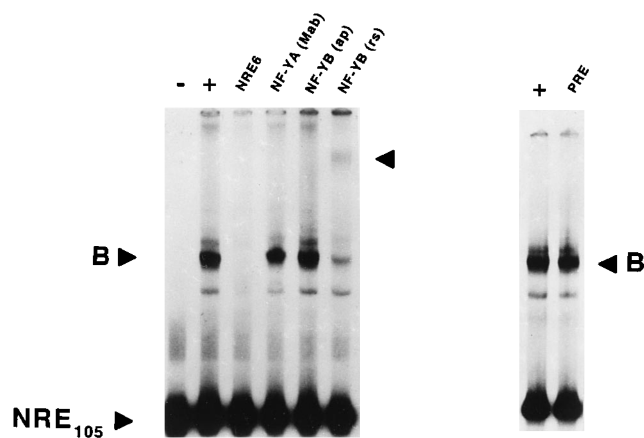

FIG. 8. EMSA performed with nuclear extract pretreated with NF-Y antibodies. HepG2 nuclear extract was preincubated (overnight at 4°C) with preimmune serum (PRE), a monoclonal $NF\text{-}Y_A$ antibody [NF-YA (Mab)], an affinity-purified $NF\text{-}Y_B$ antibody [NF-YB (ap)], and a polyclonal rabbit antiserum raised against $NF\text{-}Y_B$ [NF-YB (rs)]. Control reactions performed with nuclear extract in the absence of antibody (+) and in the presence of a 100-fold-molar-excess competitor (NRE6) were also incubated overnight at 4°C. The unlabeled arrowhead on the right indicates an additional NRE_{105}-protein complex whose migration is further retarded after pretreatment with $NF\text{-}Y_B$ polyclonal antiserum. EMSA incubations were performed with approximately 0.03 ng of radiolabeled probe (30,000 cpm) and 10 μg of nuclear extract.

Several other differences between the binding characteristics of NF-Y to its prototypical recognition sequence and those of the nuclear proteins binding to the *CYP1A1* NRE −784 palindrome support the involvement of an $NF\text{-}Y_B$-related but not identical protein. First, the role of specific DNA residues in protein binding differs. For example, when EMSA are performed with fragments in which the residues flanking the CCAAT-box spacer of the NRE −784 palindrome are mutated (PAL1 and PAL3, Fig. 3), no specific binding is observed (5). In contrast, similar experiments with the NF-Y prototypical recognition sequence, the Eα oligonucleotide, result in specific complex formation at a level at least 10% of that observed with the wild-type sequence (9). Second, the methylation-interference pattern for the FGF-4 promoter Y box is distinct from that observed for the *CYP1A1* NRE (6, 9, 13). Third, three NF-Y subunits (A, B, and C) must be present to generate the DNA-binding form of this transcription factor (23). Thus, the addition of the purified subunits to the binding solution is required to generate any observable binding to an NF-Y DNA fragment in Southwestern blot analyses. In contrast, a binding was observed in a Southwestern blot with HepG2 nuclear extract and a *CYP1A1* NRE fragment without the addition of proteins to the binding solution (4). Finally, we have previously demonstrated the presence of a second functional NRE palindrome at −573 (6). The FGF-4 Y-box oligonucleotide is able to compete with both the NRE −784 and −573 palindromic sequences for specific protein binding (5, 6). However, the −573 palindrome does not contain the CCAAT-box sequence. It does contain a well-conserved upstream half-site and a less-well-conserved downstream half-site relative to the NRE −784 palindrome (see reference 6 for a more complete comparison of these sequences). In contrast to what is observed for authentic NF-Y, this observation suggests the CCAAT box is not critical for specific protein binding to the *CYP1A1* NRE.

Given our evidence for the involvement of an $NF\text{-}Y_B$-related protein in the transcriptional repression of *CYP1A1*, we examined the literature for previous reports on negative-acting transcription factors belonging to this family of proteins. Skalnik et al. (38) have described a CCAAT displacement protein (CDP) that competes with NF-Y for binding to regulatory CCAAT box sequences in the gp91-phox gene, thereby preventing NF-Y-dependent gene activation. However, we do not feel that CDP and the *CYP1A1* NRE-binding factor are related. Several models have been proposed for transcriptional repression, including competition, squelching, blocking of *trans* activation, and silencing (35). We have previously presented evidence that the *CYP1A1* NRE and its cognate repressor act as a silencer, but not through a squelching mechanism or the competitive binding mechanism reported for CDP (6). Consistent with this earlier conclusion, there is no evidence from either our laboratory or others that positive *trans*-acting factors compete for binding at the *CYP1A1* NRE. Further, we consistently observed a single DNA-protein complex by EMSA. Differences

also exist in the number and size of proteins involved. The CDP appears to be a single peptide with a molecular mass of 180 to 200 kDa (38), whereas the *CYP1A1* NRE binding factor appears to be a multisubunit protein, with individual units ranging between 50 and 100 kDa (4).

The suggestion that an NF-Y_B-related protein is critical for *CYP1A1* NRE-dependent transcriptional repression did cause some concern regarding our heterologous expression experiments involving the HSV tk promoter. Chodosh et al. (7) have identified a low-affinity, but functional, NF-Y binding site in this promoter. One could hypothesize that the ability of the *CYP1A1* NRE to down-regulate this promoter (6) might be due to the squelching of the *trans*-acting proteins necessary for HSV tk promoter activity. However, the NF-Y site from the HSV tk promoter was unable to compete with the *CYP1A1* NRE_{105} probe for specific protein binding, even at a 500-fold molar excess. Similarly, when the HSV tk promoter fragment was used as a probe, a 1,000-fold excess of the *CYP1A1* NRE_{105} fragment was unable to compete (5).

Although the Ah receptor is clearly necessary for the normal activation of *CYP1A1* transcription in response to polyaromatic hydrocarbon and halogenated hydrocarbon exposure, our studies have implicated an NRE and cognate *trans*-acting factor as also being important in modulating the expression of this gene. The latter regulatory mechanism may well be involved in the abnormal constitutive expression of this gene observed in several neoplastic human tumors and tumor-derived cell lines (26, 27, 29, 34). It may also play a role in the highly inducible *CYP1A1* phenotype observed in the human population (22, 32). In this study, we have examined the *CYP1A1* NRE function, as well as nuclear protein binding. The role of specific DNA elements and residues within these elements has been resolved. We also present evidence for the involvement of a protein immunologically related to NF-Y_B in the *CYP1A1* NRE-protein complex. Although Skalnik et al. (38) have described a CCAAT-box displacement protein capable of repressing NF-Y-regulated genes, to our knowledge our studies represent the first evidence for an NF-Y-related protein involved in transcriptional repression through a noncompetitive mechanism. Further identification and characterization of this protein and others involved in the *CYP1A1* NRE-protein complex will be critical to our understanding of this regulatory mechanism and, in turn, the role of *CYP1A1* in chemical carcinogenesis and toxicity.

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