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Oct-1 Transcription Factor Is a Negative Regulator of Rat *CYP1A1* Expression via an Octamer Sequence in Its Negative Regulatory Element

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SUMMARY

The rat *CYP1A1* negative regulatory element (NRE) contains AP-1 and Oct-1 motifs at −808 to −788 bp. The *CYP1A1* sequence from −813 to −779 bp and an identical sequence bearing a point mutation in the octamer motif were synthesized. Gel mobility shift assays showed the formation of two complexes with the wild-type *CYP1A1* sequence and nuclear extracts from H4IIE and HepG2 hepatoma cells and from rat liver. The formation of the major complex was significantly reduced with the mutant octamer-containing oligomer and was specifically competed by an Oct-1 oligodeoxyribonucleotide. The addition of Oct-1 antibody caused a supershift of the major complex. The presence of the wild-type sequence, but not the mutant octamer sequence, caused a 3-fold decrease in SV40 enhancerless promoter activity in transfected HepG2 cells. Cotransfection of an Oct-1 expression vector with rat *CYP1A1* NRE octamer-containing, promoter/reporter gene constructs specifically further decreased promoter activity of the wild-type octamer-containing constructs in HepG2 cells. The results indicate that Oct-1 binds to the rat *CYP1A1* promoter NRE and is a negative regulator of rat *CYP1A1* expression.

The *CYP* subgroup 1 gene family is a member of the CYP group 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 *CYP* superfamily consists of more than a dozen gene subgroups (3). The *CYP* subgroup 1 subfamily includes three genes, *CYP1A1*, *CYP1A2*, and *CYP1B1*, all of which are inducible on exposure of the host to polycyclic aromatic hydrocarbons and dioxins (4–6).

Our interest in the regulation of *CYP1A1* has focused on the nature and function of a NRE that is located in the 5′-upstream region of the gene (7–10). In the rat, this region occurs between −843 and −746 bp. Two highly conserved subregions have been identified within the NRE of the rat *CYP1A1*: nre_1 (from −833 to −814 bp) and nre_2 (from −778 to −760 bp) (9). A 32-bp region located between nre_1 and nre_2 in the rat gene has >80% sequence identity to the 26-bp c-*myc* NRE (10–12). The 26-bp c-*myc* NRE was reported to bind both AP-1 and Oct-1 transcription factors (11, 12). Furthermore, a 21-bp region of the rat *CYP1A1* NRE sequence has >80% identity to the SV40 enhancer Sph-octamer motif (13–15). The rat *CYP1A1*-NRE octamer sequence differs from the octamer motif consensus (16) by a single base. The adjacent AP-1 sequence of the rat *CYP1A1*-NRE also differs by a single base from the polyoma AP-1 consensus (17); it also has similarity to the interleukin-2 AP-1 consensus (18).

We (10) have indicated the interaction of the NRE with multiple proteins as judged from the occurrence of several complexes in gel mobility shift assays. In addition, Boucher *et al.* (19) reported a NRE-binding factor(s) that may be involved in the negative regulation of *CYP1A1*. Their report also indicated the presence of a 21-bp palindome (−794 to −774 bp) between the two GC-rich conserved regions; this region predominated in the negative regulation of a heterologous promoter (19).

Based on the similarity of the rat NRE sequence (−813 to −779 bp) to the sequences described above, it was of interest to determine whether the Oct-1 transcription factor would bind to one or more regions of the rat *CYP1A1* and whether the resultant interaction would be associated with some biological activity.

Materials and Methods

The following materials were obtained from the indicated sources: restriction endonucleases, T4 DNA polymerase, the Klenow frag-

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ABBREVIATIONS: CYP, cytochrome P450; NRE, negative regulatory element; MEM, minimal essential medium; *AP-2*, 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′; *AP-3*, 5′-CTACTGGGACTTTCCACACATC-3′; *Oct-1*, 5′-TGTCGAATGCAAATCACTAGAA-3′; bp, base pair(s).

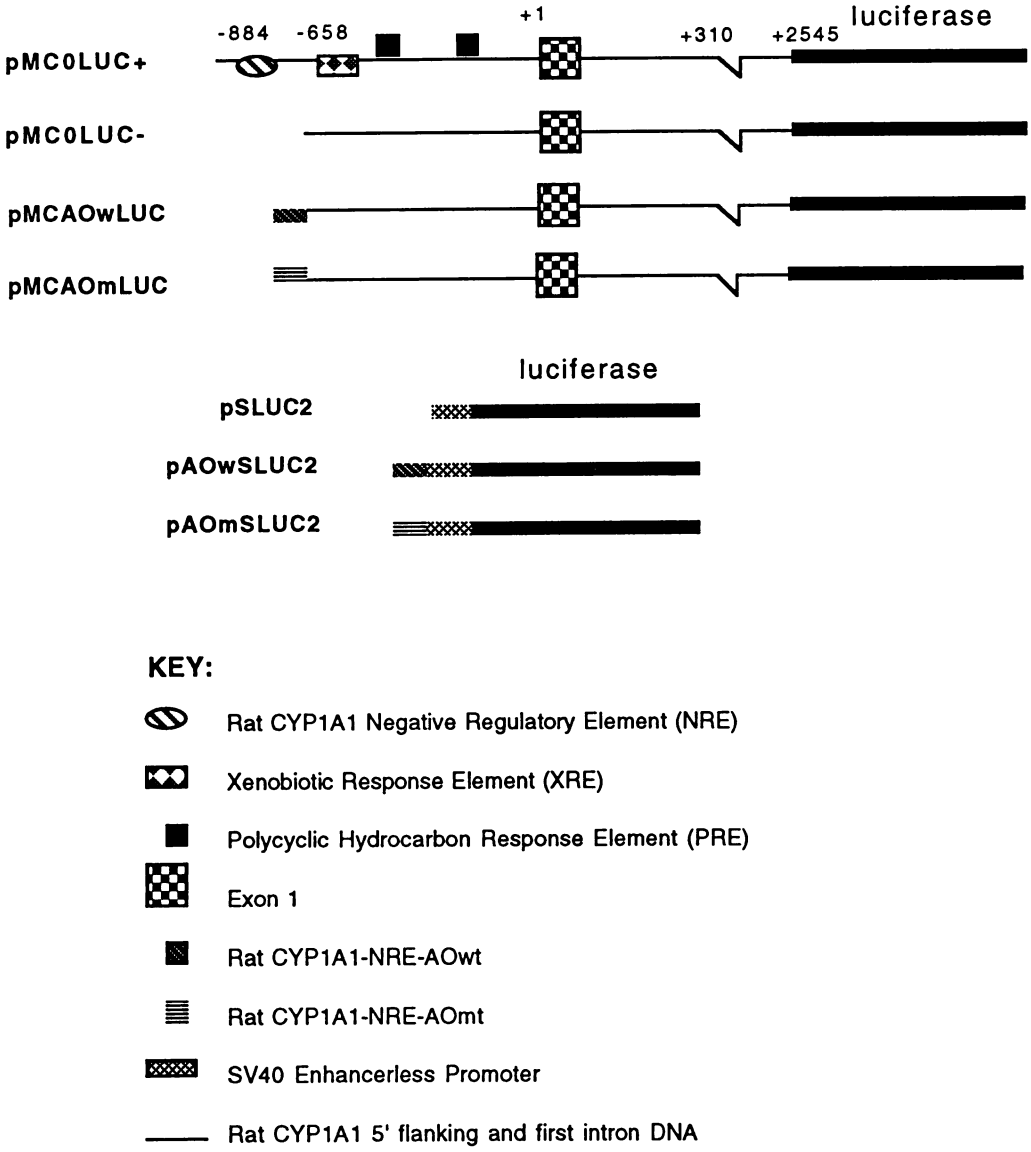

Fig. 1. Schematic representation of the various rat *CYP1A1*-promoter and SV40 enhancerless promoter constructs. Vector sequences are not presented. *Numbers above the DNA fragment*, (from left to right, in bp) the 5′-end of a *cis*-element, the translational start site, a restriction endonuclease site, and the 3′-end of the *CYP1A1* region.

ment, and T4 DNA ligase, New England Biolabs (Beverly, MA) or GIBCO-BRL (Gaithersburg, MD); pTKGH (20), The Nichols Institute (San Juan Capistrano, CA) as a component of their human growth hormone transient gene expression assay system; MEM, methionine-free MEM, gentamycin, penicillin/streptomycin, trypsin, and phosphate-buffered saline, GIBCO-BRL; fetal calf serum, ICN Biomedicals (Costa Mesa, CA); type I collagen (Vitrogen 100), Collagen Corp. (Palo Alto, CA); [γ-^{32}P]ATP and [α-^{32}P]dNTPs, DuPont-NEN (Boston, MA); [^{35}S]methionine/cysteine mixture (Tran^{35}Slabel), ICN (Irvine, CA); benzo[*a*]pyrene, Aldrich Co. (Milwaukee, WI); 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, Chemsyn Laboratories (Lenexa, KS); and luciferin, Sigma Chemical Co. (St. Louis, MO).

DNA sequencing analysis was performed according to the dideoxy method (21) using Sequenase obtained from USB (Cleveland, OH). Oct-1 monoclonal antibodies were a gift from Drs. Neil Segil and Nathaniel Heintz (Rockefeller University, New York, NY) (22, 23).

Synthetic oligodeoxyribonucleotides. The following NRE oligomers and their complements were synthesized by phosphoramidite chemistry using a Cyclone DNA synthesizer (Milligen): (i) wild-type *CYP1A1*-NRE-AOwt: gcg-cgg-atc-cGT-CGC-CTG-CTT-CAG-TAT-GCA-AAA-CAT-CAT-GCA-AGC-gtc-gac-gcg-c (uppercase bases correspond to −813 to −779 bp).[2] A complementary oligomer was synthesized and annealed to the preceding sequence. (ii) Mutant *CYP1A1*-NRE-AOmt: gcg-cgg-atc-cGT-CGC-CTG-CTT-CAG-TAG-GCA-AAA-CAT-CAT-GCA-AGC-gtc-gac-gcg-c. A complementary oligomer was synthesized and annealed to the preceding sequence. The selection of the specific site for the transversion of a T to a G in AOmt (the mutated base is underlined) was based on a previous study in which this mutation resulted in significantly decreased activity of a simple β-globin promoter in transfected MPC 11 cells (24).

AP-2, *AP-3*, and *Oct-1* oligodeoxyribonucleotides were obtained from Promega (Madison, WI).

Plasmid construction. Schematic representations of the rat *CYP1A1* NRE constructs used in this study are shown in Fig. 1. The rat *CYP1A1* promoter/reporter gene plasmids, pMC$_o$LUC$^+$ and pM-

[2] Uppercase letters represent the nucleotide sequence of the rat *CYP1A1* NRE between nre$_1$ and nre$_2$ reported by Hines *et al.* (9), whereas lowercase letters indicate the *Bam*HI or *Sal*I restriction endonuclease sites lengthened by four base extensions. The underlined base represents the specific mutation inserted in the *CYP1A1*-NRE-AP-1/Oct-1 sequence. The synthetic wild-type oligodeoxynucleotide sequence used in our experiments contained a direct repeat, aaCATCATgca, which differs from the rat *CYP1A1* sequence (aaCACTAGca) reported by Sogawa *et al.* (39).

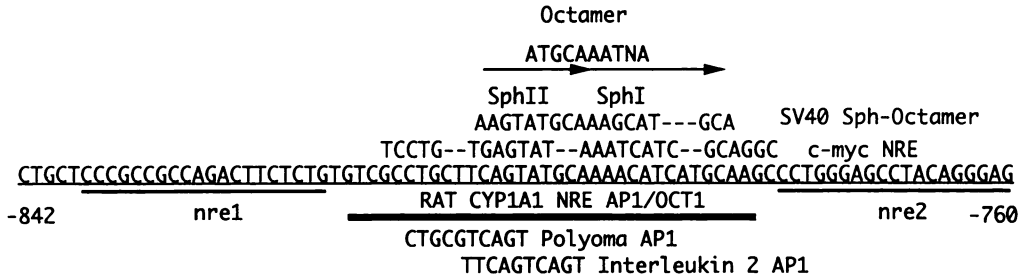

Fig. 2. Sequence of the rat *CYP1A1*-NRE fragment from −842 to −760 bp that contains nre_1 and nre_2 and the intervening AP-1/Oct-1/Sph-like motifs. The intervening AP-1/Oct-1/Sph-like sequences are compared with the octamer consensus sequence (16), SV40 Sph-octamer motif (13–15), c-*myc* NRE (11, 12), polyoma virus AP-1 consensus (17), and interleukin 2 AP-1 consensus (18). *Dashes* indicate gaps in identity between the rat *CYP1A*-NRE AP-1/Oc t-1 sequence and those with which it is compared.

C_oLUC^-, and the plasmids pRSVcat and pSluc2 have been described previously (10, 25, 26). The constructs pMCAOwLUC, pMCAOmLUC, pAOwSluc2, and pAOmSluc2 were formed by ligating the rat NRE wild-type and mutant oligodeoxyribonucleotides into the *Bam*HI/*Sal*I sites of pMC_oLUC^- and pSluc2, respectively. The plasmid constructs were sequenced to verify that a single copy of the wild-type and mutant oligodeoxyribonucleotides was present. The Oct-1 expression vector pCG*Oct-1* (27) was a gift from Dr. Winship Herr (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Cell culture. The rat H4IIE and human HepG2 hepatoma cells were maintained in MEM supplemented with 10% fetal bovine serum and gentamycin or penicillin/streptomycin.

Transfection and reporter gene assays. Transfection was performed using the calcium phosphate/DNA coprecipitation method followed by glycerol shock (28). One million cells were seeded onto Type I collagen-coated (6 mg/cm^2) 60-mm dishes. Twenty hours after seeding, the medium was changed. At 4 hr later, plasmid DNA that had been coprecipitated with carrier DNA and calcium phosphate was added to the cells. pTKGH (2 μg) was cotransfected with each of the test constructs (5 μg), e.g., pAOwSluc2, to account for any differences in plate-to-plate transfection efficiency. In some experiments, cotransfected pCG*Oct-1* was added (0.01 or 0.1 μg) along with the test and control constructs. Sufficient pRSVcat (0.01 or 0.1 μg) was added to maintain an equivalent amount of total transfected plasmid (i.e., ≤0.1 μg) as well as a control for nonspecific sequestration of available basal transcription factors. However, it should be mentioned that pRSVcat includes a Rous sarcoma virus promoter, whereas pCG*Oct-1* contains a cytomegalovirus promoter.

Twenty-four hours after the addition of the DNA, the cells were glycerol-shocked, and fresh medium was added. Forty-eight hours later, the reporter gene activity was assayed. Expression of the pTKGH reporter gene construct was determined as the amount of growth hormone secreted into the culture medium according to the manufacturer's instructions (The Nichols Institute). The luciferase assay was performed as previously described (29). HepG2 cells were used in all transfection studies due to their significantly greater transfection efficiency and the resultant higher basal promoter activities of the transfected rat *CYP1A1*-NRE or AO constructs compared with that seen in transfected H4IIE cells.[3] The use of the rat *CYP1A1* promoter-containing constructs in the human hepatoma cell line was justified by the identical gel mobility shifts and Oct-1 antibody-induced supershifts seen with both H4IIE and HepG2 nuclear extracts and the rat *CYP1A1*-NRE-AO wild-type and mutant octamer oligodeoxynucleotides (see Results).

Protein determination. The protein concentration of the cell lysates was determined according to the method of Groves *et al.* (30).

Preparation of H4IIE and HepG2 nuclear extracts. On reaching 70% confluency, the cells were detached from the plastic flasks with a rubber policeman, collected by centrifugation, and washed in phosphate-buffered saline, and extracts were prepared (31).

Immunoprecipitation. Immunoprecipitation of pCG*Oct-1*-transfected HepG2 cells was carried out as described previously (27). HepG2 cells were transfected with 0.01 or 0.1 μg of pCG*Oct-1*. After incubation at 37° for 44 hr after glycerol shock, the cells were metabolically labeled with 0.4 mCi of [^{35}S]methionine/cysteine mixture in methionine-free medium for 4 hr. The cells were washed in phosphate-buffered saline, collected, and sonicated in RIPA buffer (0.5% NP-40, 0.5% Tween 20, 0.5% deoxycholic acid, 150 mM NaCl, 10 mM KCl, 1 mM EDTA, 20 mM Tris·HCl, pH 7.5) that included 1 mM phenylmethylsulfonyl fluoride and aprotinin (0.2 units/ml). The sonicated cells were boiled for 3 min and diluted 10-fold in the RIPA buffer. The diluted lysates (representing 5 × 10^5 cpm) were precleared with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and immunoprecipitated with Oct-1 antibody. The immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (32) and autofluorography using En-Hance (DuPont-NEN).

Gel mobility shift assays. The specific binding of protein(s) to the oligodeoxyribonucleotide templates was determined by the gel mobility shift assay (33). The hepatoma cell nuclear lysates were preincubated with the labeled oligodeoxyribonucleotide template for 20 min at 25°, after which antibodies to Oct-1 were added. The binding reaction was conducted for an additional 20 min at 25°.

Results

Oct-1 binds to the *CYP1A1*-NRE-AOwt oligodeoxyribonucleotide. The rat *CYP1A1*-NRE-AOwt, conserved GC-rich nre_1 and nre_2, and octamer- and AP-1- consensus sequences are shown in Fig. 2.

Two major protein/DNA complexes, I and II, were observed in gel mobility shift assays (Fig. 3) after incubation of either H4IIE or HepG2 extracts with *CYP1A1*-NRE-AOwt as template. The formation of complex II was effectively competed by the addition of unlabeled oligonucleotide to the binding reaction (Fig. 3, A and B, *lanes 3–5*), whereas complex I was only partially competed by a 200-fold molar excess of the unlabeled Oct-1 oligonucleotide. Complex II, formed with either H4IIE, HepG2, or rat liver nuclear extracts, was significantly competed by a 100-fold molar excess of unlabeled *CYP1A1*-NRE-AOwt oligonucleotide (data not shown). Complex I was not affected by a 100-fold molar excess of unlabeled *CYP1A1*-NRE-AOwt, indicating that this complex was less specific than complex II (data not shown).

As controls, we examined the effects of other oligonucleotides such as AP-2 and AP-3. Complex II, formed with the hepatoma cell extracts, was not competed by a 200-fold molar

[3] K. Sterling and E. Bresnick, unpublished observations.

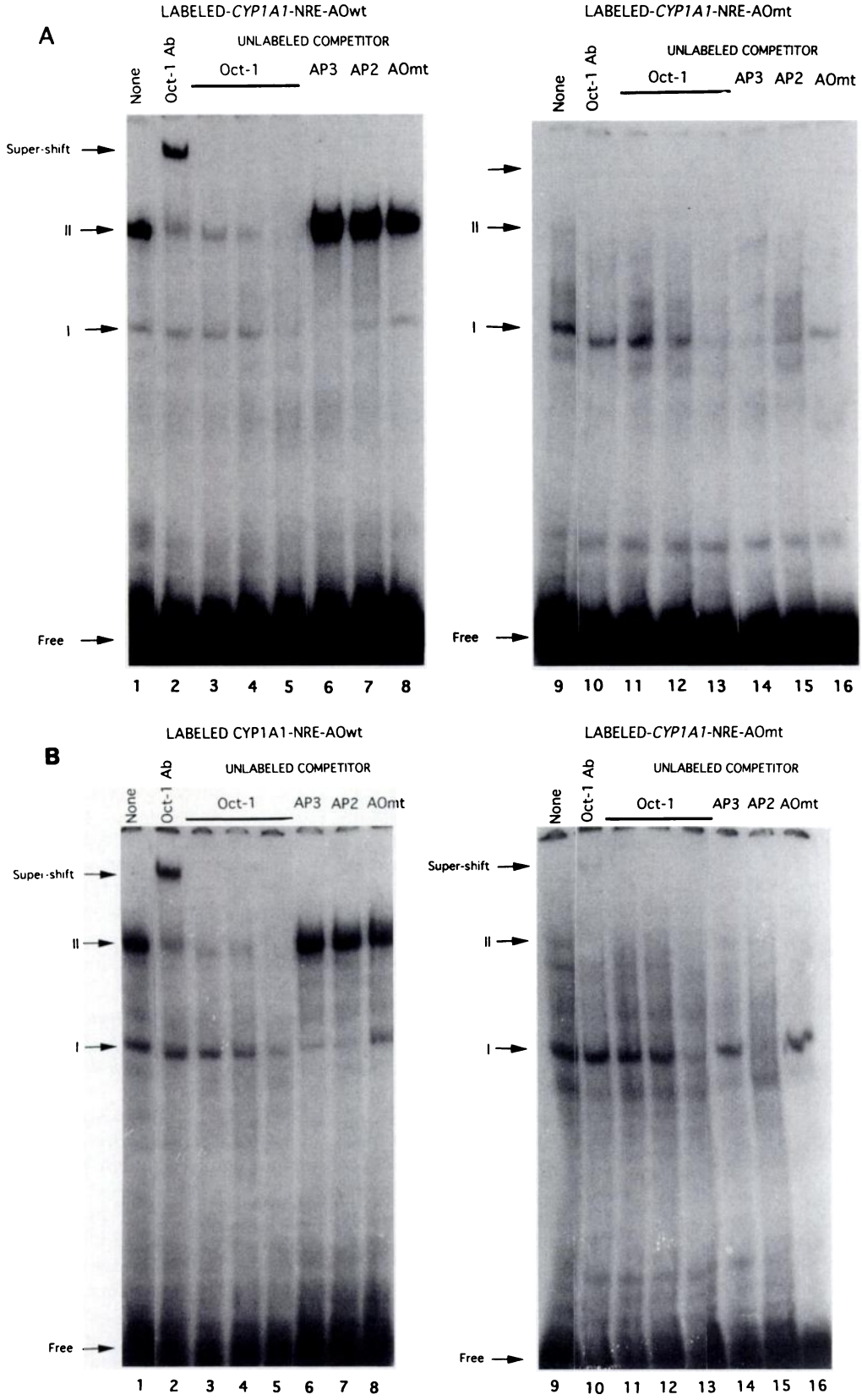

Fig. 3. Gel mobility shift assay of wild-type and mutant *CYP1A1*-NRE-AO oligonucleotides with rat H4IIE and human HepG2 nuclear extracts. ^{32}P-labeled *CYP1A1*-NRE-AOwt (*lanes 1–8*) and *CYP1A1*-NRE-AOmt (*lanes 9–16*) were incubated with 5 μg of an H4IIE (A) or HepG2 (B) extract in the presence of a 10,000-fold molar excess of poly dIdC. The positive controls (*lanes 1 and 9*) did not include an unlabeled competitor DNA or Oct-1 antibody. Oct-1 antibody was added as indicated (*lanes 2 and 10*). A 50-, 100-, and 200-fold molar excess of unlabeled Oct-1 oligonucleotide was included in *lanes 3–5* and *11–13*. A 200-fold molar excess of unlabeled AP-3 (*lanes 6 and 14*) and AP-2 (*lanes 7 and 15*) oligonucleotides was added to these binding reactions. A 100-fold molar excess of unlabeled *CYP1A1*-NRE-AOmt (*AOmt*) was included in the binding reactions of *lanes 8 and 16*. The position of the unbound probe is shown (*Free*). The band migrating just above the free probe in *lanes 9–16* was present in the absence of added nuclear lysate. The shift in the migration of complex I in *lane 9* was due to a "smile" artifact. The faintly shifted complex just below complex I in *lanes 9–16* may indicate an interaction in which one of the factors in complex I or II is missing.

excess of these oligonucleotides; complex I, however, was partially or totally competed by a 200-fold molar excess of these oligomers (Fig. 3, A and B, *lanes 6 and 7*). These data further indicated the specificity of complex II.

The importance of Oct-1 in the interaction with the AOwt sequence was investigated by the use of the supershift assay with antibody to Oct-1. The addition of antibody to Oct-1 to the reaction mixture containing labeled wild-type oligonucle-

otide as a template resulted in a marked supershift of complex II as demonstrated in Fig. 3 (A and B, *lane 2*), implicating Oct-1 as a component of this complex. No alteration in the mobility of complex I was apparent under these conditions.

The formation of complex II with the hepatoma cell extracts was greatly reduced when the mutant construct *CYP1A1*-NRE-AOmt was used as template in the gel mobility shift assay (Fig. 3, A and B, *lane 9*). However, after incubation of the mutant template in a reaction mixture that contained the hepatoma extracts and Oct-1 antibody, a weak supershifted signal was observed (Fig. 3, A and B, *lane 10*).

Immunoprecipitation analysis of pCG*oct*-1-transfected HepG2 cells. Three major bands (I, II, and III; ~42, 64, and 90 kDa, respectively) were observed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography of lysates into which 0.01–1.0 μg of *pCGOct-1* had been transfected and immunoprecipitated with Oct-1 antibody (data not shown). The intensity of the three major bands was greatest with extracts from HepG2 cells that had been transfected with the highest amount of pCG*Oct-1* (Table 1), indicating that the level of the 90-kDa Oct-1 peptide (band III) was proportional to the quantity of cotransfected pCG*Oct-1*. The migration (apparent molecular mass) of band III was in agreement with that previously reported for pCG*Oct-1*-produced peptide in transfected HeLa cells (27). The two faster migrating bands (I and II) probably represented (i) degradation products resulting during the lysis and/or immunoprecipitation procedures, since we have observed similar signals on Western blots of rat liver and hepatoma cell nuclear extracts with the Oct-1 antibody[3] or (ii) peptides that coprecipitate with Oct-1 (34–36). A faint, immunoprecipitated band was noted with HepG2 cells transfected with 1.0, 0.1, or 0.01 μg pCG*Oct-1* corresponding to ~100 kDa apparent molecular mass, the endogenous, metabolically labeled Oct-1 protein (Table 1).

Biological activity of *CYP1A1*-NRE-AOwt and *CYP1A1*-NRE-AOmt. The biological activities of wild-type and mutant NRE sequences were assessed by their incorporation 5′ upstream of the promoters of two different luciferase reporter gene constructs: a SV40 enhancerless promotor/luciferase vector, pSluc2 (resulting in the formation of pAOwSluc2 and pAOmSluc2, respectively), and a *CYP1A1* promoter-driven, NRE-deficient luciferase vector, pMC$_o$LUC$^-$ (resulting in the formation of pMCAOwLUC and pMCAOmLUC, respectively). The results presented in Fig. 4 were obtained after the preparation of all vectors at the same time using the same batch of cells, culture medium, and reagents. These steps were necessary to minimize the inherent variability within the transient transfection experiments.

A 3-fold reduction in the expression of the luciferase gene (promoter activity) was observed in HepG2 cells that had been transiently transfected with pAOwSluc2 (compared with pSluc2), whereas the reporter gene activity in cells into which the mutant pAOmSluc2 had been introduced was not significantly affected (Fig. 4). These results indicated that the Oct-1 transcription factor negatively regulated the SV40 enhancerless promoter via the *CYP1A1*-NRE-AOwt sequence. It is germane to mention that these results have been obtained in at least three separate experiments.

As reported previously (10), the constitutive promoter activity of the NRE-lacking construct pMC$_o$LUC$^-$ was at least 2-fold greater than that of the NRE-containing construct, pMC$_o$LUC$^+$ (see Fig. 4). Inclusion of either wild-type or mutant NRE-AO oligonucleotide, i.e., pMCAOwLUC and pMCAOmLUC, respectively, did not significantly affect constitutive promoter activity. These results suggest that the level of Oct-1 or the extent of phosphorylation of the endogenous Oct-1 in the HepG2 cells or the presence of the AOwt sequence without the additional flanking sequences that occur in pMC$_o$LUC$^+$ was insufficient to bring about negative regulation of the rat *CYP1A1* promoter (−658 bp to +2545 bp).

The effect of overexpression of Oct-1 in HepG2 cells transfected with *CYP1A1*-NRE-AOwt or *CYP1A1*-NRE-AOmt. Cotransfected Oct-1 expression vector (0.01 μg) specifically and significantly reduced the constitutive promoter activity of the NRE-AP-1/Oct-1 wild-type sequence that was present in the SV40 enhancerless promoter-containing construct, pAOwSluc2, without affecting the promoter activity of the parent pSluc2 or the mutant-containing pAOmSluc2 (Fig. 5A). Cotransfected Oct-1 expression vector at 0.1 μg significantly reduced basal promoter activity of the SV40 enhancerless promoter constructs pSluc2, pAOwSluc2, and pAOmSluc2 (Fig. 5A). However, pSluc2 promoter activity was reduced by only 24%, whereas that of pAOwSluc2 and pAOmSluc2 (wild-type and mutant-containing SV40 enhancerless promoter constructs, respectively) was lessened by 70% and 83%, respectively. The results with 0.1 μg of the cotransfected Oct-1 expression vector were not caused by the sequestration of available transcription factors because with the control pTKGH construct, promoter activity was unaffected (see legend to Fig. 5). The effect on promoter activity seen with pSluc2 may be due to binding of the overexpressed Oct-1 to degenerate octamer motifs that are present directly upstream of the SV40 enhancerless promoter (26). The effect on the mutant construct with 0.1 μg of cotransfected Oct-1 expression vector may result from the binding of Oct-1 to the mutant octamer sequence, albeit with much less affinity, as indicated in the gel mobility shift experiments. Consequently, at this level of overexpressed Oct-1 (0.1 μg), the mutant sequence can negatively regulate the SV40 enhancerless promoter.

Cotransfection with 0.01 μg of the Oct-1 expression vector depressed slightly the basal promoter activity of pMC$_o$LUC$^+$, whereas it had no effect on pMC$_o$LUC$^-$, pMCAOwLUC, or pMCAOmLUC. A 10-fold higher amount of cotransfected Oct-1 expression vector selectively and significantly decreased the promoter activity of pMC$_o$LUC$^+$ and pMCAOwLUC while having no effect on pMC$_o$LUC$^-$ or pMCAOm-

TABLE 1

Fluorographic signal intensity of *Oct-1* antibody-immunoprecipitated protein from pCG*oct-1*-transfected HepG2 cells

Relative peak area in arbitrary densitometric units (signal intensity) is presented for the metabolically labeled, immunoprecipitated Oct-1 peptides from pCG*oct*-1-transfected HepG2 cells. The resulting fluorograph from Oct-1 peptides that were precipitated with Oct-1 monoclonal antibody was scanned with an E-C densitometer and Hewlett Packard integrator. The signal intensities are indicated for the major pCG*oct*-1-derived peptides I, II, and III and the putative 100-kDa endogenous Oct-1 peptide from HepG2 cells, transfected with 1.0, 0.1, and 0.01 μg pCG*oct*-1.

	1.0 μg	0.1 μg	0.01 μg
I	21,200	11,805	4,762
II	9,080	3,529	1,081
III	16,969	8,197	7,139
100 kDa	1,017	972	869

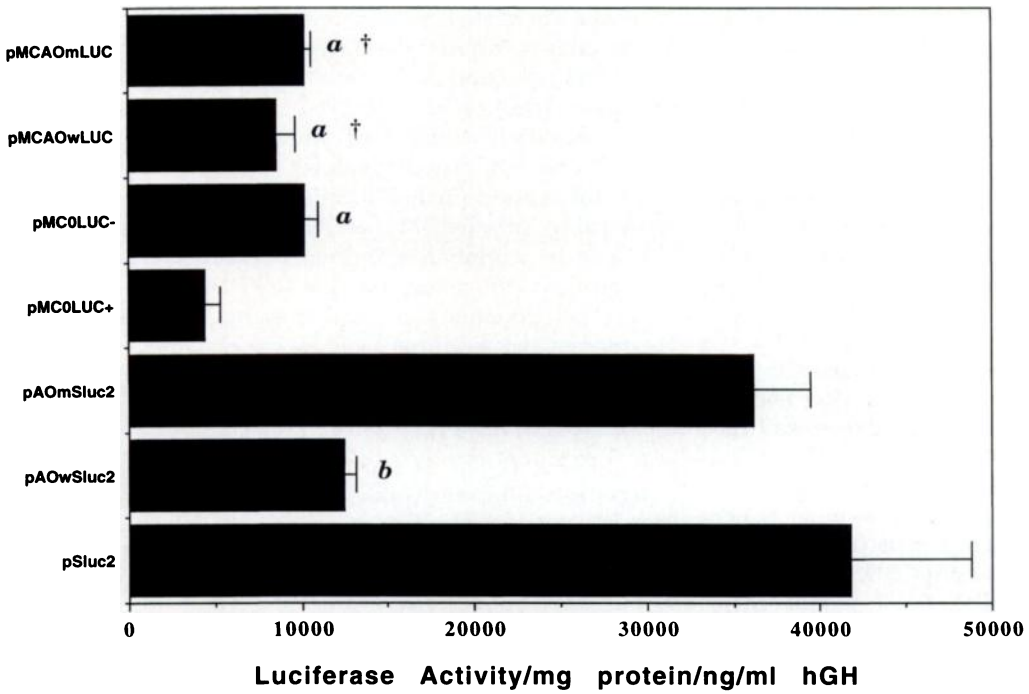

Fig. 4. Luciferase activity after transfection with *CYP1A1* NRE-AP-1/Oct-1 wild-type and mutant promoter/ and SV40 enhancerless promoter/luciferase gene constructs. HepG2 cells were transiently transfected with the constructs indicated, and luciferase reporter gene activity was determined. The HepG2 cells were cotransfected with pTKGH, and the expression of human growth hormone in the cell media was used as a control for plate-to-plate transfection efficiency. The values represent the mean ± standard error of three determinations per set of transfections. *a*, Significant difference from the promoter activity of pMC_oLUC^+ at $p \leq .05$; †, the promoter activities of pMCAOwLUC and pMCAOmLUC were not significantly different from those of the parent pMC_oLUC^-; *b*, significant difference from the promoter activity of pSluc2 at $p \leq .05$, as determined with the Student's nonpaired *t* test.

LUC (Fig. 5B). These data further reinforce the lack of any "squelching" of transfected pCG*Oct-1*.

The promoter activity of the control pTKGH construct was not affected by 0.01 or 0.1 μg of the Oct-1 expression vector (see legend to Fig. 5), even though this plasmid contains octamer and AP-1 motifs (20).

Discussion

Oct-1 binding activity of the *CYP1A1*-NRE-AOwt sequence corresponds to biological activity. Oct-1 in the hepatoma nuclear extracts binds to the *CYP1A1*-NRE-AOwt sequence. When placed immediately 5′ to the SV40 enhancerless promoter, a single copy of *CYP1A1*-NRE-AOwt was sufficient to inhibit basal promoter activity in transiently transfected HepG2 cells. Mutation of a T to a G in the octamer consensus sequence of *CYP1A1*-NRE-AOmt caused a significant reduction in the amount of the major complex (II) that formed with the nuclear lysates. However, the attenuated complex was still supershifted by the addition of Oct-1 antibody, indicating residual binding of this transcription factor to the mutant octamer sequence. In HepG2 cells transiently transfected with pAOmSluc2, the lack of an effect on the activity of the SV40 enhancerless promoter was consistent with the loss of this major complex, as demonstrated in the gel mobility shift assays with the octamer mutant. Further evidence for the negative regulatory effect of Oct-1 mediated by the *CYP1A1*-NRE-AOwt sequence was provided by cotransfection with an Oct-1 expression vector (at 0.01 μg), in which only the promoter activity of pAOwSluc2 was reduced. However, cotransfected Oct-1 expression vector at a 10-fold concentration reduced the basal SV40 enhancerless promoter activity with either the wild-type or mutant oligonucleotide, i.e., with pAOwSluc2 and pAOmSluc2, respectively. Under these conditions, the combined concentration of endogenous Oct-1 and that produced from the cotransfected pCG*Oct-1* might have been sufficient to bind to the mutant sequence, albeit at a lesser affinity, and thus decrease promoter activity. Substantiation of this probability is afforded by the interaction of the mutant oligomer AOmt with Oct-1, as demonstrated in the gel mobility supershift assay; the inability to affect pTKGH promoter activity in cotransfected cells was also in concert with this hypothesis. These observations suggested that the integrity of the octamer motif is critical for the appropriate level of Oct-1 binding and for negative regulation of the SV40 enhancerless promoter [this promoter contained only the proximal AP-1 site and the 21-bp repeats (26)]. The reason for the 24% decrease in promoter activity seen with pSluc2 when cotransfected with 0.1 μg of pCG*Oct-1* is, however, not understood, although the two degenerate octamer consensus sequences (i.e., ATGCAATTGA; Ref. 26) present in the vector immediately upstream of the promoter might be involved in nonspecific inhibition of the simple SV40 enhancerless promoter. Oct-1 does display a certain degree of flexibility in the recognition of a consensus sequence, although the affinity of the interaction can be greatly affected (34).

Effect of a single copy of *CYP1A1*-NRE-AOwt on pMC_oLUC^- promoter activity. Insertion of the wild-type NRE-AP-1/Oct-1 sequence at −658 bp of pMC_oLUC^- did not significantly affect the constitutive promoter activity in transiently transfected HepG2 cells. pMC_oLUC^+ differs from pMC_oLUC^- only by the inclusion of upstream sequences from −659 to −882 bp, i.e., the former contained the complete NRE region. The constitutive promoter activity of the NRE-containing construct ranged from 2- to 5-fold less than that of the NRE-lacking construct (present study and Ref. 10). These observations suggest that additional sequences within the *CYP1A1*-NRE region may contribute to the suppression of transfected *CYP1A1* promoter activity in HepG2 cells, in concert with the occurrence of binding sites for known and unknown transcription factors (10). The presence of the *CYP1A1*-NRE-AOwt sequence, however, caused a significant

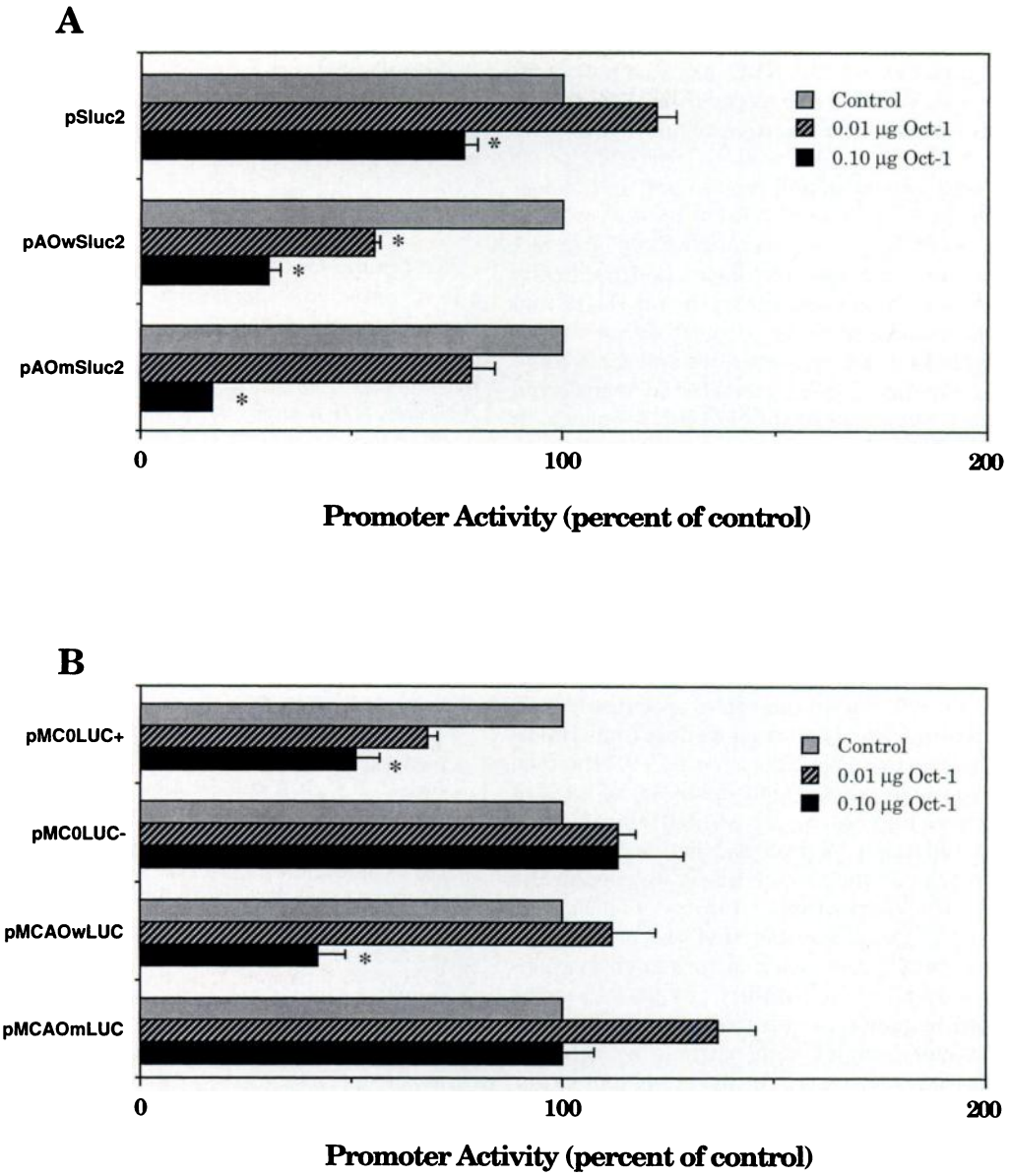

Fig. 5. Luciferase activity in cells transfected with wild-type or mutant-containing constructs as well as an Oct-1 expression vector. A, HepG2 cells were transfected with pSluc2, pAOwSluc2, or pAOmSluc2. Promoter activity was quantified as luciferase activity/mg protein, normalized for human growth hormone gene expression, i.e., luciferase/mg protein/ng human growth hormone/ml. The values are expressed as percentage of control for the purpose of direct comparison of increasing amounts of the cotransfected pCG*oct-1* in the separate set of experiments; the control represented the promoter activity in the absence of Oct-1 overexpression. The representative average values for pTKGH activity in ng human growth hormone/ml medium were 0.25 (average of two values) for control (0.01 μg cotransfected pRSVcat), 0.69 ± 0.27 for control (0.1 μg cotransfected pRSVcat), 0.31 ± 0.06 for cotransfected pCG*oct-1* (0.01 μg), and 0.68 ± 0.15 for cotransfected pCG*oct-1* (0.1 μg). B, HepG2 cells were transfected with rat *CYP1A1* promoter constructs pMC_0LUC^+, pMC_0LUC^-, pMCAOwLUC, and pMCAOmLUC. Luciferase activity/mg protein/ng human growth hormone/ml was determined, and the values are expressed as percentage of control (in the presence of pRSVcat rather than pCG*oct-1*). The representative average values for pTKGH activities, normalized for human growth hormone secretion into the medium, were 0.14 ± 0.01 for the control (0.01 μg cotransfected pRSVcat), 2.6 ± 0.21 for control (0.1 μg pRSVcat), 0.16 ± 0.02 for pCG*oct-1* (O.01 μg), and 2.7 ± 0.22 for pCG*oct-1* (0.1 μg). *, Significant difference at $p \leq .05$ as determined with the Student's nonpaired *t* test.

and specific reduction in the promoter activity of pMC_0LUC^-, i.e., pMCAOwLUC, in the presence of 0.1 μg of a cotransfected Oct-1 expression vector. The promoter activity of pMC_0LUC^+ was also specifically reduced further (beyond that mediated by endogenous Oct-1) by the cotransfection of pCG*Oct-1* at 0.1 μg, thus suggesting a pivotal role of Oct-1 and its potential interplay with other transcription factors in the negative regulation of the rat *CYP1A1* promoter. It is important to note that with the NRE-lacking and AO-mutant constructs, promoter activity was not affected under these conditions. These results indicate that the effect of cotransfected Oct-1 expression vector on promoter activity with wild-type NRE-AO constructs was not artifactual. It is germane to mention that cell cycle-dependent phosphorylation of Oct-1 (22, 23) may also influence its negative regulatory activity on the rat *CYP1A1* expression.

The finding that the Oct-1 transcription factor is involved in the negative regulation of the rat *CYP1A1* expression through the NRE is consistent with previous reports of a putative *trans*-acting factor(s) that interacts at this *cis*-ele-

ment in suppressing expression of this gene (7, 10, 19). In addition, it has been suggested that a negative regulatory factor(s) acting at a site within the NRE might be part of an autoregulatory loop (8, 37). These proposed *trans*-factors may interact with Oct-1 in exerting negative regulation of gene expression.

Boucher and Hines (38) compared the binding and biological activities of the human *CYP1A1* NRE sequence (−831 to −728 bp) with those of the orthologous rat NRE region (−881 to −746 bp) and concluded that the latter nonspecifically bound conserved *trans*-acting factors from the rat H4IIE and human HepG2 cell lysates. They also suggested that the rat sequence was unable to down-regulate a heterologous SV40 enhancer-driven thymidine kinase promoter in transfected HepG2 cells. In their suggestions, the rat NRE sequence, in contrast to the orthologous human region, would not possess biological activity (38). The predominant activity of the human *CYP1A1* NRE would reside in a 21-bp palindromic region centered between the two conserved GC-rich sequences at position −784 bp (38). The rat *CYP1A1* NRE sequence, on the other hand, only contained a palindromic half-site at −794 to −788 bp. These conclusions are in apparent conflict with the present data and a previous report from our laboratory (10) in which the orthologous rat *CYP1A1* NRE sequence from −881 to −707 bp was shown to specifically bind factors from rat liver nuclear lysates as well as from H4IIE and HepG2 nuclear lysates.[3] We also reported (10) that the fusion of this comparable NRE region upstream of each of three different reporter genes in transfected HepG2 cells caused a 2–6-fold reduction in promoter activity. Boucher and Hines (38) did not use the same NRE as we used in the present study, i.e., the region from −745 to −659 bp was omitted. Furthermore, the conclusion that the orthologous rat NRE sequence bound *trans*-acting factors in only a nonspecific manner is based on the inability of a 20-fold molar excess of unlabeled oligomer to effectively compete in the formation of the major complex demonstrable by gel shift assays (38). We have demonstrated in this study and in our previous report (10) that a 100-fold excess of unlabeled oligomer clearly competed in a specific manner in the formation of the major complex between the rat NRE sequence (−881 to −707 bp) and rat liver nuclear proteins but not of a minor complex. Mutation of a single base was sufficient to greatly dampen this competition. The overall results of Boucher and Hines (38) strongly indicate that Oct-1 does not bind to the *human CYP1A* NRE, whereas a nuclear factor-Y-related factor may participate in the "silencing" of this gene. Consequently, Oct-1 may not play any negative regulatory role in the human gene, indicating species specificity of control.

In the present study, the rat *CYP1A1*-NRE-AOwt sequence was shown to specifically bind factors from rat and human hepatoma cells and Oct-1 was identified as interacting with that *cis* region. It is of interest to note that the rat *CYP1A1*-NRE-AOwt sequence contains the palindromic half-site described earlier (19) and that this region contains an octamer-binding motif. We also demonstrated that the rat *CYP1A1*-NRE-AOwt sequence down-regulates an SV40 enhancerless promoter, although it was unable to significantly affect expression from pMC_0LUC^- in the absence of overexpressed Oct-1. This result implies that the *CYP1A1*-NRE-AOwt region exerts negative regulatory action with a simple promoter but that additional flanking sequences (and other putative *trans* factors) may be required for regulation of pMC_0LUC^-.

The negative regulation of the rat *CYP1A1* is clearly a very complex process that involves several *trans*-acting factors, one of which must be Oct-1. The other components involved in this regulatory system await identification.

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