

Evidence That the Coactivator CBP/p300 Is Important for Phenobarbital-Induced but Not Basal Expression of the *CYP2H1* Gene

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ABSTRACT

We have previously identified an upstream 556-bp enhancer domain for the chicken *CYP2H1* gene that responds to phenobarbital and binds several transcription factors, including the orphan chicken xenobiotic receptor (CXR). By contrast, the promoter lacks a CXR site and is not inducible by phenobarbital. Although it has been established that CXR can interact with the coactivator SRC-1, there are no reports as to whether other coactivators may be important for phenobarbital-mediated inducibility. Our studies using the adenovirus E1A wild-type protein, which inhibits the coactivators cAMP response element binding protein (CBP) and CBP associated factor (p/CAF), provide evidence for the involvement of one or both of these coactivators at the enhancer but not at the promoter of the *CYP2H1* gene. The observations that mutant E1A proteins did

not affect the enhancer activity and that inhibition by wild-type E1A was reversed by CBP and p/CAF confirmed the involvement of these coactivators in the induction process. We propose that the intrinsic histone acetyl transferase activity of one or both of these coactivators participates in chromatin remodeling thereby stimulating drug induction of the promoter. This proposal was supported by experiments with the histone deacetylase inhibitor, trichostatin A, which resulted in the superinduction of the drug response but had little effect on basal expression of the *CYP2H1* gene. The work provides evidence for the first time for the involvement of the coactivators CBP and p/CAF in the phenobarbital-mediated induction of the *CYP2H1* gene.

The cytochrome P450 proteins comprise a superfamily of heme-containing enzymes that play an important role in the metabolism of diverse lipophilic compounds, including foreign chemicals, such as pharmaceutical drugs and other xenobiotics (Gonzalez, 1990; Dogra et al., 1998; Waxman, 1999). The synthesis of specific cytochrome P450 enzymes can be selectively induced by their own substrates after the interaction of ligand-receptor complexes with upstream enhancer sequences in these genes (Kliewer et al., 1999; Waxman, 1999; Honkakoski and Negishi, 2000). Phenobarbital is a prototype inducer that markedly increases the expression of *CYP2* genes in mammals (Waxman and Azaroff, 1992; Waxman, 1999; Honkakoski and Negishi, 2000) and chicken (Mattschoss et al., 1986; Hansen et al., 1989; Dogra et al., 1998). In recent years, a great deal of information has been obtained regarding the molecular mechanism of phenobarbital-mediated induction of *cyp2b10*, *CYP2B1*, and *CYP2H1* genes (Dogra et al., 1999; Honkakoski and Negishi, 2000;

Handschin et al., 2001; Kim et al., 2001). These studies have revealed that a nuclear orphan receptor constitutive androstane receptor (CAR) plays a central role in the phenobarbital-mediated induction mechanism. Furthermore, an essential role for CAR in this induction mechanism has been confirmed by the loss of phenobarbital-mediated inducibility of the *cyp2b10* gene in CAR knockout mice (Wei et al., 2000). Interestingly, it has been recently shown that only 50% of phenobarbital responsive genes are affected in CAR-null mice, indicating that CAR has diverse roles (Ueda et al., 2002). Studies in the mouse have shown that in response to phenobarbital, CAR is translocated to the nucleus, where it forms a heterodimer with retinoid X receptor and activates drug response elements in the 5'-flanking region of the *cyp2b10* gene (Honkakoski and Negishi, 2000).

Activation of gene expression involves direct recruitment of coactivator complexes to the enhancer and promoter regions of target genes. Studies have suggested a strong link

ABBREVIATIONS: CAR, constitutive androstane receptor; CBP, cAMP response element binding protein; p/CAF, CBP associated factor; CXR, chicken xenobiotic receptor; TSA, trichostatin A; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGFP, enhanced green fluorescent protein; LMH, leghorn male hepatoma; SV, simian virus 40; FACS, fluorescence-activated cell sorting; HNF-1, hepatocyte nuclear factor-1; SRC-1, steroid receptor coactivator-1; bp, base pair(s); kb, kilobase; PB, phenobarbital; CAT, chloramphenicol acetyltransferase.

between histone acetylation, chromatin remodeling, and gene regulation (Grunstein, 1997; Wade and Wolffe, 1997; Kadonaga, 1998). A number of transcriptional coactivators, including the ubiquitous cAMP response element-binding protein (CBP) and its structural homolog p300 (Bannister and Kouzarides, 1996), CBP-associated factor (p/CAF) (Yang et al., 1996) and steroid receptor coactivator, SRC-1 (Spencer et al., 1997) have been found to possess intrinsic histone acetyltransferase activity, which can modulate chromatin structure and gene transcription (Wang et al., 1998). In addition, CBP/p300 and p/CAF have been postulated to link transcription activators to the basal transcriptional machinery (Eckner, 1996). Human p300 was initially identified by its ability to bind the adenovirus E1A oncoprotein (Moran, 1993). E1A binding to CBP/p300 and p/CAF probably perturbs chromatin structure, which accounts for the transcription modulating effects of these proteins (Goodman and Smolik, 2000). Because E1A interacts with the transcriptional coactivators CBP/p300, its exogenous expression has been used as a tool to study the role of coactivators in specific gene transactivation.

We are studying the molecular mechanism of *CYP2H1* gene induction by phenobarbital in chick embryo livers (Hansen et al., 1989; Dogra et al., 1998, 1999). We previously identified in this gene an upstream enhancer domain (located between -5900 and -1100) that responds to drug and, within this, a 556-bp enhancer region has been analyzed in detail (Dogra et al., 1999). It has been observed that DNA-binding sites for a number of transcription factors including a nuclear receptor are required for the phenobarbital response (Dogra et al., 1999; Handschin and Meyer, 2000). The nuclear receptor binding site in the 556-bp enhancer of *CYP2H1* gene is similar in sequence to that identified as a CAR binding site in *CYP2B* genes and mutagenesis of this motif in the *CYP2H1* enhancer abrogates the phenobarbital response (Handschin et al., 2001). Recently, a nuclear receptor CXR, the chicken homolog of CAR, was cloned and identified as an activator of the chicken *CYP2H1* gene and has activation properties similar to CAR and pregnane X receptor (Handschin et al., 2000). However, there is no information on whether coactivators such as CBP/p300, p/CAF, and SRC-1 are part of the enhancer/promoter complex that drives the phenobarbital-induced activation of the *CYP2H1* gene.

In this study, we show that expression of wild-type E1A in chick embryo primary hepatocytes inhibited only the phenobarbital-induced level of *CYP2H1* mRNA but did not alter the constitutive expression of this mRNA in the absence of drug. Consistent with this, E1A also repressed the phenobarbital-induced activity of the 556-bp *CYP2H1* enhancer but had no effect on the promoter activity of this gene. Trichostatin A (TSA), a selective inhibitor of histone deacetylase activity, further increased the phenobarbital-induced level of *CYP2H1* mRNA. This suggests that drug action involves acetylation, probably mediated through the histone acetyltransferase activity of the coactivator complex recruited to the 556-bp *CYP2H1* enhancer.

Materials and Methods

RT-PCR Analysis of Endogenous *CYP2H1* Gene Expression.

Primary hepatocytes were prepared from 17-day-old chick embryos

by a method described previously (Dogra and May, 1997). Hepatocytes (2×10^7) were cotransfected with 40 μ g of expression clone for E1A (E1A12S) and 5 μ g of pEGFP-C1 (BD Clontech, Palo Alto, CA). After transfection, each sample was split so that approximately 1×10^7 cells were plated onto 60-mm dishes and cultured in Williams' E medium plus 10% Serum Supreme (Edward Keller Ltd., Hallam, Victoria, Australia). Hepatocytes were incubated at 37°C overnight, after which medium was changed and hepatocytes were treated with either phenobarbital (final concentration, 500 μ M in PBS) or solvent. Cells were harvested after 24 h and cells expressing enhanced green fluorescence protein (EGFP) were isolated by FACS ($2-3 \times 10^5$ cells). Total RNA was isolated from these cells by a method described previously (Chomczynski and Sacchi, 1987). cDNA was synthesized from 50 ng of total RNA in 20 μ l using Oligo (dT)₂₃ primer and a two-step enhanced avian RT-PCR kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. An increasing amount of cDNA was used in the semiquantitative PCR reaction containing 400 nM of each primer in a final volume of 50 μ l, as described in the two-step enhanced avian RT-PCR kit (Sigma). The reaction mix was spiked with [³²P]dCTP to quantify *CYP2H1* mRNA using β -actin as an internal control. Primer sequences employed were *CYP2H1*, 5'-CACTGCAGGGAAAGCGGTCAAT-3'; 5'-TGCTGGACTGTACT-TACTGGACC-3', and β -actin, 5'-CCTGAACCCCAAGCCAACAGA-3'; 5'-GGACTCCATACCCAAGAAAGATG-3'.

Northern Blot Analysis. Primary hepatocytes were plated at 6 to 8×10^6 in Williams's E medium supplemented with 10% serum supreme. Medium was changed after overnight culture and cells were treated with TSA at 1 or 2 μ M concentrations for 1 h before phenobarbital was added at a 500 μ M final concentration. Hepatocytes were further incubated for 6 h and used to prepare total RNA (Chomczynski and Sacchi, 1987). Total RNA (15 μ g) was electrophoresed on a 1% agarose gel containing 1.1 M formaldehyde. The fractionated RNA was blotted onto NYTRAN membrane (Schleicher and Schuell, Keene, NH) and UV cross-linked using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The filters were prehybridized for 16 h in 50% formamide, 5 \times standard saline citrate (0.75 M NaCl, 75 mM sodium citrate, pH 7.0), 5 \times Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin), 0.05% sodium pyrophosphate, 0.1% SDS, and 200 μ g/ml salmon sperm DNA, and then hybridized with cDNA probes labeled with [³²P]dATP by random priming using a DNA labeling kit (Amersham Biosciences, Piscataway, NJ). The specific DNA probes were as follows: pCHPB15 for *CYP2H1* mRNA, p105B1 was used to detect ALAS1 (5-aminolevulinic synthase1) mRNA, and a full-length cDNA clone was used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Dogra and May, 1996). Each probe was added at an activity of 0.5 to 1.0×10^6 cpm/ml. Filters were washed and quantified as described previously (Dogra and May, 1996).

Cell Culture and Transfection. For transfection experiments, plasmid DNA was prepared by CsCl/ethidium bromide equilibrium density gradients and quantified by spectrophotometry. The RSV-driven adenovirus E1A12S and mutant clones were a gift from Prof. T. Kouzarides (Cambridge, United Kingdom) and Dr Y. Tsuji (Wake Forest University, NC), respectively. TSA was purchased from Wako Pure Chemical Industries, Japan. For transient transfection assays, primary hepatocytes (2×10^7 cells) in 0.8 ml of electroporation buffer (20 mM HEPES, pH 7.05, containing 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM glucose, and 400 μ g of sonicated Salmon sperm DNA as carrier) were electroporated at 960 μ F and 250 V using a Gene Pulser and Capacitance Extender (Bio-Rad, Hercules, CA). Electroporation efficiency, as observed in cell-sorting experiments using EGFP, was about 8 to 10%. Various CAT reporter constructs used in transient transfection assays to analyze the effect of E1A on the enhancer or promoter regions of the *CYP2H1* gene included p4.8-SVCAT [4.8-kb BamHI enhancer domain (-5900/-1100) SV-CAT], p556-SVCAT [556-bp enhancer region (-1956/-1400) SV-CAT], pCYP-205CAT [205-bp promoter (-205/1)CAT], and enhancerless SVCAT (Dogra et al., 1999). After transfection, each sample

was split so that approximately 1×10^7 cells were plated onto 60-mm dishes and cultured in Williams' E medium plus 10% serum supreme. Hepatocytes were incubated at 37°C overnight, after which the medium was changed and hepatocytes were treated with either phenobarbital (final concentration, 500 μ M in PBS) or solvent. The cells were further cultured for 48 h and then CAT activities were determined. Leghorn male hepatoma (LMH) cells were obtained from the American Type Culture Collection (Manassas, VA). LMH cells were cultured in Williams' E medium supplemented with 10% serum supreme and 50 μ g of gentamicin/ml of medium. Cells were seeded on gelatin-coated 24-well plates at the density of 6×10^4 per well 24 h before transfection. On the next day, the medium was replaced with serum free Williams' E medium and transfections were performed using p556-SV-luciferase, pRL-SV40 (Promega, Madison, WI), CXR expression vector kindly provided by Prof. Urs Meyer (Handschin et al., 2000), E1A12S expression clone (Bannister and Kouzarides, 1996), expression clones for CBP (Chrivia et al., 1993); p/CAF, SRC-1, and hSRC-1A (a kind gift from Dr. B. W. O'Malley, Baylor College of Medicine, Houston, TX) and FuGENE6 transfection agent (Roche Molecular Biochemicals, Mannheim, Germany). The medium was replaced after 6 h with Williams' E containing 10% serum supreme with or without phenobarbital (final concentration, 500 μ M in PBS). After 24 h, cells were lysed and luciferase assays carried out using the Promega dual-luciferase kit.

CAT Assay. Transfected cells were harvested in 40 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 150 mM NaCl, by scraping with a rubber policeman. The cells were pelleted and resuspended in 50 to 100 μ l of 250 mM Tris-HCl, pH 7.6, lysed by three cycles of freezing and thawing, and centrifuged for 5 min to remove cell debris. The protein concentration of each cell was determined by protein microassay (Bio-Rad). For CAT assays, the cell supernatant was heated at 68°C for 6 to 8 min to remove deacetylase activity and CAT activity was then determined (Gorman et al., 1982). The acetylated products of [14 C]chloramphenicol were separated by thin-layer chromatography. After autoradiography, CAT activity was quantified by cutting out the spots from the plate and measuring the radioactivity in a scintillation counter. The results were expressed as percentage of acetylated chloramphenicol or calculated as fold-induction over control values.

Results

E1A Represses Phenobarbital-Induced Expression of Endogenous CYP2H1 mRNA. The orphan receptor CAR underlies phenobarbital-induced activation of the *CYP2B2*, *cyp2b10*, and *CYP2H1* genes (Honkakoski and Negishi, 2000; Handschin et al., 2001; Kim et al., 2001). Activation of genes by receptors generally involves coregulator proteins such as SRC-1, CBP/p300, and p/CAF (Perlmann and Evans, 1997; Torchia et al., 1998). Although there is evidence that SRC-1 is required for CAR activity (Sueyoshi and Negishi, 2001), the participation of other coactivators in the phenobarbital induction process has not been reported. Adenovirus E1A has been shown to abrogate the transcriptional activity of coactivators, including CBP/p300 and p/CAF (Eckner et al., 1994; Reid et al., 1998). Therefore, in this study, we examined the effect of E1A on the phenobarbital-mediated induction of the endogenous *CYP2H1* gene in chick embryo hepatocytes to determine whether CBP or p/CAF participates in the phenobarbital-mediated induction process. Hepatocytes were cotransfected with expression plasmids for E1A (E1A12S) and EGFP (pEGFP-C1), and treated with or without phenobarbital at a final concentration of 500 μ M for 24 h. Transfected cells expressing EGFP were sorted by FACS and the expression of endogenous *CYP2H1* mRNA was determined by semi-

quantitative RT-PCR. RNA from control hepatocytes treated with or without phenobarbital was also analyzed to compare the effect of E1A on the basal and phenobarbital-induced levels of *CYP2H1* mRNA. As shown in Fig. 1A, expression of E1A reduced the steady-state level of drug-induced *CYP2H1* mRNA to that of basal without affecting β -actin mRNA used as a control. The average decrease in phenobarbital-induced levels of *CYP2H1* mRNA by E1A from two independent experiments was from 4.3- to 1.4-fold (Fig. 1B). However, basal expression of the *CYP2H1* mRNA was not altered by E1A (Figs. 1B). These results show that there is an E1A-sensitive

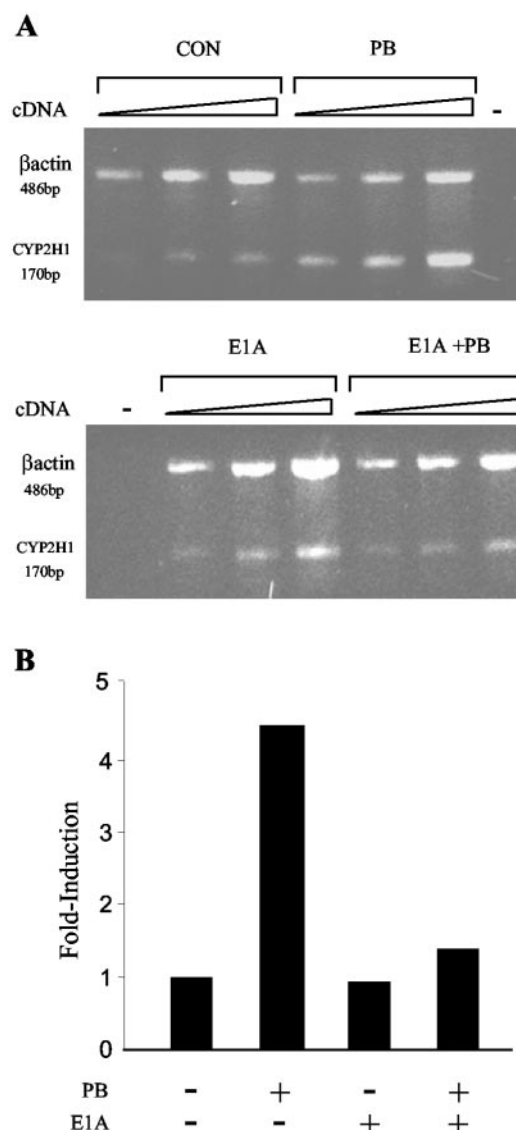


Fig. 1. Effect of E1A expression on the phenobarbital-mediated induction of endogenous *CYP2H1* mRNA. Chick primary hepatocytes were cotransfected with expression clone for E1A and enhanced green fluorescence (pEGFP-C1) by electroporation and treated with and without phenobarbital (PB) for 24h. Transfected hepatocytes expressing EGFP were selected by FACS. Total RNA was isolated from 2×10^5 EGFP expressing cells, reversed transcribed and increasing amounts of cDNA used in the semiquantitative PCR reaction to quantify *CYP2H1* mRNA with β -actin mRNA as an internal control. A reaction was also run with no cDNA to serve as a negative control. A, RT-PCR reactions, spiked with [32 P]dCTP, were resolved on a 1.5% agarose gel and a representative ethidium bromide stained gel is shown. B, PCR reaction products from the agarose gel were cut and quantified in the scintillation counter and the ratio of *CYP2H1* to β -actin as standardized against control is shown.

step in the phenobarbital-mediated induction mechanism that is likely to involve CBP/p300 and/or p/CAF coactivators.

E1A Represses Expression of the Phenobarbital-Responsive Enhancer Region. We have previously identified an upstream 4.8-kb enhancer at $-5900/-1100$ in the chicken *CYP2H1* gene that responds to phenobarbital and have subsequently defined a 556-bp region at $-1956/-1400$ within this domain that retains drug responsiveness when tested in both orientations (Dogra et al., 1999). Maximum induction of this enhancer is dependent on the presence of a number of transcription factor binding sites (Dogra et al., 1999) including a binding site for CXR, the chicken homolog of CAR (Handschin et al., 2000). Basal expression of the *CYP2H1* gene is driven by a number of liver-specific transcription binding sites in the first 205 bp of promoter sequence. This region is not drug-inducible and does not contain a CXR binding site (Dogra and May, 1997).

We investigated the effect of exogenously expressed E1A on the expression of two CAT reporter gene constructs, p4.8-SVCAT and pCYP-205CAT, in chick embryo hepatocytes. pCYP-205CAT is driven by the *CYP2H1* proximal promoter (Dogra and May, 1997) and p4.8-SVCAT by the 4.8-kb upstream drug responsive domain fused to the enhancerless SV40 promoter (Dogra et al., 1999). E1A expressing plasmid at 400 ng markedly repressed phenobarbital-mediated induction of p4.8-SVCAT, but had no effect on the basal expression of this construct (Fig. 2, A and B). When the 4.8-kb enhancer region was replaced with the drug responsive 556-bp enhancer sequence (p556-SVCAT), induction by phenobarbital was again inhibited by E1A (Fig. 2, A and B). A similar inhibition by E1A on the phenobarbital-induced 556-bp enhancer activity was observed when the 556-bp enhancer sequence was tested in the opposite orientation ($5'-3'$) (result not shown). A control vector lacking E1A had no effect on the expression of these constructs (data not shown). Note that E1A did not affect the transcriptional activity of the 205-bp proximal promoter. From these experiments, we conclude that the phenobarbital inducible 556-bp enhancer region is a target for E1A inhibitory action.

Repression by E1A Requires an Intact p300/CBP Binding Domain. The presence of the CR1 domain of E1A (Offringa et al., 1990) is essential for repression of CBP/p300-mediated transcriptional activity. Such repression could involve titration by E1A of CBP/p300 away from the promoter or displacement of p/CAF by E1A (Bannister and Kouzarides, 1996; Reid et al., 1998), because residues in the CR1 domain can directly bind p/CAF independent of CBP (Reid et al., 1998). To test whether E1A repressed the 556-bp *CYP2H1* enhancer by interacting with CBP/p300, mutants of E1A with deletions in the CR1 domain ($\Delta 15-35$ and $\Delta 23-107$) were examined. The inhibitory action of the E1A mutants on the phenobarbital-induced 556-bp enhancer activity in transfected chick embryo hepatocytes, was considerably abrogated compared with that observed by wild-type E1A (Fig. 3). Similarly, an E1A mutant that contained deletion $\Delta 23-150$, thereby abrogating interaction with both CBP/p300 and the retinoblastoma family members, was weakly inhibitory (Fig. 3). These results show that the domain of the E1A protein that interacts with CBP/p300 and p/CAF is required for E1A repression of drug-induced enhancer activity.

If E1A represses drug induction by targeting CBP/p300, excess CBP should reverse the inhibition. To explore this

possibility, E1A expression plasmid (200 ng and 400 ng) was cotransfected with a CBP expression vector (2.5 and 5.0 μg) and p556-SVCAT, a CAT reporter construct containing the 556-bp enhancer. As shown in Fig. 4, CAT activity in re-

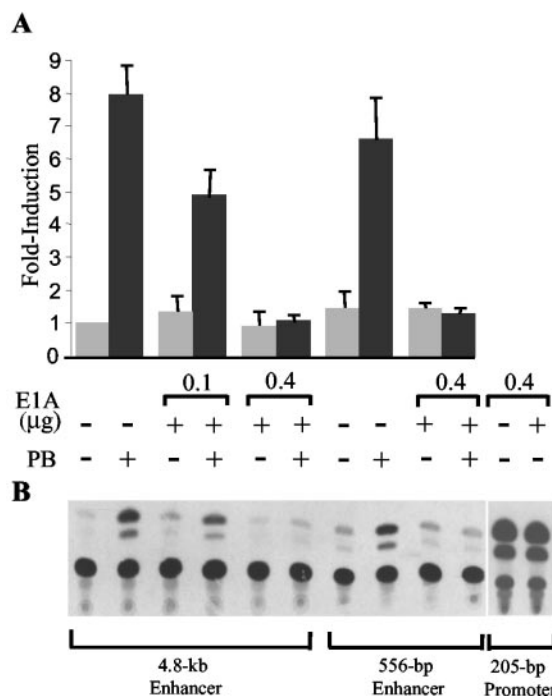


Fig. 2. Effect of E1A on the phenobarbital-mediated induction of p4.8-SVCAT, p556-SVCAT, and pCYP-205CAT. The p4.8-SVCAT, p556-SVCAT, and pCYP-205CAT constructs were cotransfected with 0.4 μg of the expression vector for E1A (p4.8-SVCAT also was transfected with 0.1 μg of E1A) into chick embryo primary hepatocytes. Each sample was halved (but not for the pCYP-205CAT construct), and 500 μM of PB was added to one dish and solvent to the control dish. A, the average of three independent experiments, repeated in duplicate and represented as fold-induction over the activity of control value of the p4.8-SVCAT enhancer construct is shown. The CAT activity of the pCYP-205CAT construct is 12- to 15-fold higher than p4.8-SVCAT and did not change with E1A expression. Therefore, results of the CAT assay only are shown (Fig. 2B). Control vector containing no E1A insert had no effect on phenobarbital induction of either enhancer construct (p4.8-SVCAT and p556-SVCAT). B, a typical CAT assay of one such experiment is shown.

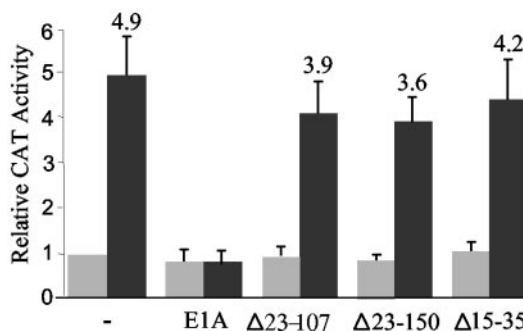


Fig. 3. E1A mutations defective in CBP/p300 binding lose the ability to repress phenobarbital-mediated induction of *CYP2H1* enhancer. Chick embryo primary hepatocytes were transiently cotransfected with the p556-SVCAT enhancer construct and either the control vector, containing no E1A insert; the expression vector for E1A; or mutants of E1A $\Delta 23-107$, $\Delta 23-150$, and $\Delta 15-35$. Each sample was halved and 500 μM of PB was added to one dish and solvent to the control dish. The average of three independent experiments repeated in duplicate and represented as relative CAT activity of the p556-SVCAT enhancer construct is shown. Gray and black bars represent control and phenobarbital-induced activity, respectively, of the p556-SVCAT enhancer construct.

sponse to phenobarbital was repressed by E1A, whereas CBP at 5.0 μg restored this to a maximal level of about 60%. No further reversal was seen with CBP at 10 μg (data not shown). Thus, full restoration of enhancer activity by CBP was not observed. This could reflect an interaction of E1A, not only with CBP but also with other regulatory proteins acting on the 556-bp enhancer region. This possibility was assessed by examining the effect of E1A on enhancer activity when transcription factor binding sites within the 556-bp enhancer were individually mutated. As reported previously (Dogra et al., 1999) mutagenesis of individual binding sites for the factors HNF-1, CCAAT, E-box like protein, and an unknown factor lowered the level of phenobarbital induction. However, E1A repression was still observed in the presence of these mutations (Fig. 5), demonstrating that E1A does not directly inhibit the activity of the transcription factors that interact with the 556-bp enhancer. A functional binding site for CXR is located in the 556-bp enhancer region at -1637/-1622 (Handschin and Meyer, 2000). However, the possibility that E1A inhibits CXR activity could not be tested by this approach because we found that inactivation of the CXR binding site resulted in a very low level of phenobarbital-mediated induction.

E1A Inhibits CXR Activation of the 556-bp Enhancer in LMH Cells. We next examined the effect of exogenous CBP, p/CAF, and E1A on CXR-activation of the *CYP2H1* 556-bp enhancer construct in chicken hepatoma LMH cells. CXR increased expression of the transfected construct by about 5-fold (Fig. 6A). Cotransfection with increasing concentrations of CBP (50–400 ng) further increased CXR-induced activation by about 2.6-fold at 400 ng. At these concentrations, a similar result was seen for p/CAF (Fig. 6A). In the absence of exogenous CXR, neither CBP nor p/CAF altered expression of the 556-bp enhancer construct (data not shown). CXR-driven activation of the 556-bp enhancer construct was inhibited by E1A (Fig. 6B), and this was totally reversed by CBP at 100 ng concentration (Fig. 6B). Interestingly, at this concentration, p/CAF also reversed the E1A inhibition, suggesting that this coactivator can replace CBP

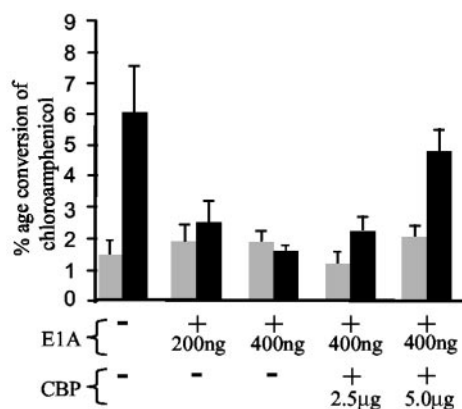


Fig. 4. CBP partially reverses the E1A-mediated repression of phenobarbital-induced *CYP2H1* enhancer. Chick embryo primary hepatocytes were transiently cotransfected with the p556-SVCAT enhancer construct and either the control vector, containing no E1A insert, or expression vector for E1A and the expression vector for CBP. Each sample was halved and 500 μM of PB was added to one dish and solvent to the control dish. The average of three independent experiments repeated in duplicate and represented as percentage conversion of chloroamphenicol to acetylated form is shown. Gray and black bars represent control and phenobarbital-induced activity, respectively, of p556-SVCAT.

in the enhancer complex. Our data provide evidence that CXR activation of the enhancer involves CBP and/or p/CAF. Experiments were also carried out to determine whether phenobarbital-induced enhancer activity in primary chick embryo hepatocytes was altered by exogenous CBP. Only a weak response to CBP expression (about 1.2-fold) was seen in repeated experiments (data not shown), most probably reflecting a high endogenous level of this coactivator in the hepatocytes.

Trichostatin A Stimulates Phenobarbital-induced Expression of *CYP2H1* mRNA but not Basal Expression. It is now well documented that CBP/p300 and p/CAF possess intrinsic histone acetyltransferase activity that can modify chromatin structure through acetylation events (Ogryzko et al., 1996). We therefore examined whether histone acetylation is important in the enhancer-dependent phenobarbital response using TSA, an inhibitor of histone deacetylase activity. Chick embryo primary hepatocytes were pretreated with TSA at a concentration of 1 or 2 μM for 1 h before the addition of phenobarbital and incubation of hepatocytes was continued for a further 6 h. Total RNA was isolated and mRNA amounts determined by Northern blot analysis (Fig. 7). Quantification of the results showed that treatment with phenobarbital induced the levels of mRNA for both *CYP2H1* (15.2-fold) and *ALAS1* (9.5), another phenobarbital-inducible gene (Dogra and May, 1996). TSA alone at 1 μM did not significantly alter the levels of either of the mRNAs, but in the presence of phenobarbital, promoted a super induction of both *CYP2H1* (2.8-fold) and *ALAS1* mRNAs (12.6-fold) (Fig. 7). In this study, phenobarbital, TSA, or a combination of both did not affect mRNA levels for GAPDH. Super induction of *CYP2H1* and *ALAS1* mRNAs with 1 μM TSA treatment was maximum, and no further increase with

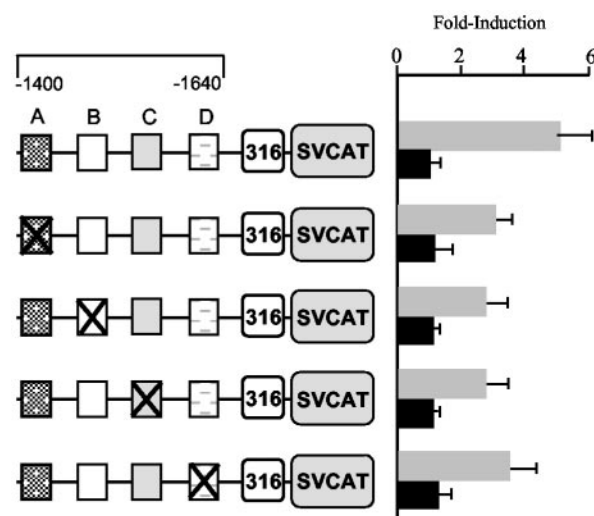


Fig. 5. Identification of *CYP2H1* enhancer element(s) involved in E1A-mediated repression. The p556-SVCAT enhancer construct containing mutations in either of the four functional transcriptional binding sites, denoted as A (E box like), B (HNF-1), C (Unknown), and D (CCAAT), was cotransfected with either 0.4 μg of the expression vector for E1A or control vector containing no insert into chick embryo primary hepatocytes. Each sample was halved and 500 μM of PB was added to one dish and solvent to the control dish. The average of three independent experiments repeated in duplicate and represented as fold-induction over the activity of control value of the p556-SVCAT enhancer construct is shown. Black and gray bars represent phenobarbital-induced activity of various enhancer constructs cotransfected with E1A expression vector or control vector containing no insert, respectively.

2 μ M TSA was observed. The data suggest that one action of phenobarbital induction involves acetylation and chromatin remodeling. The fact that neither basal expression nor the control GAPDH gene was altered by TSA shows that the potentiating response reflects a specificity for the inducing agent and not a global change in chromatin structure.

Discussion

Phenobarbital-induced activation of the mouse *cyp2b10*, rat *CYP2B1*, and chicken *CYP2H1* genes is mediated by

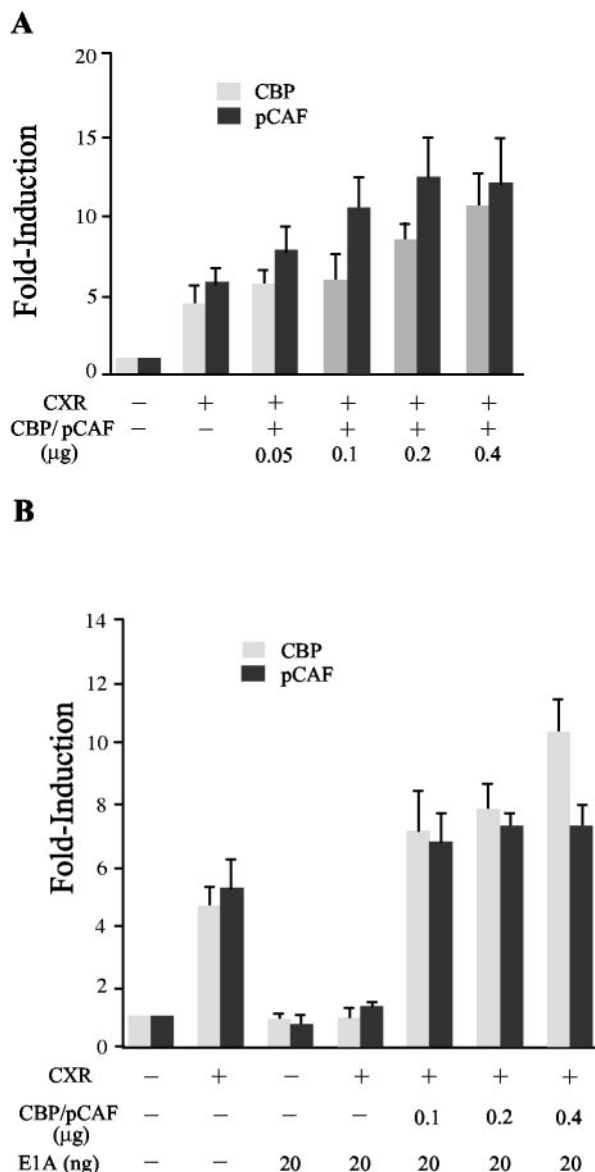


Fig. 6. The E1A inhibition of CXR-induced activity of 556-bp enhancer is reversed by expression of CBP/pCAF. LMH cells were cotransfected with an enhancer-luciferase construct (p556SV-luciferase), CXR, with control vector containing no E1A insert or an E1A expression clone, various concentrations (50–400 ng) of CBP and pCAF expression clones, and pRL-SV40 as an internal transfection control. A, CXR-induced activity of the p556SV-luciferase construct is further induced with the increasing concentrations (50–400 ng) of CBP and pCAF coactivators. B, the CXR-induced activity of the p556SV-luciferase construct is inhibited by E1A, and this inhibition is abrogated by coexpressing CBP and pCAF. Cell extracts were assayed for luciferase activity. The average of three independent experiments repeated in duplicate and represented as fold-induction in activity is expressed as a ratio of test to the control.

complex phenobarbital-responsive enhancer regions, with CAR playing a central role (Dogra et al., 1999; Honkakoski and Negishi, 2000; Handschin et al., 2001; Kim et al., 2001). A 163-bp enhancer region that confers phenobarbital responsiveness to the *CYP2B1* gene contains more than three transcription binding sites that, together with a CAR binding site, are required for maximal phenobarbital responsiveness (Stoltz et al., 1998). A 51-bp enhancer region that independently responds to phenobarbital and induces the *cyp2b10* gene was shown to contain a nuclear factor 1 binding site flanked by CAR sites (Honkakoski et al., 1998). Similarly, the phenobarbital responsive chicken *CYP2H1* gene enhancer region binds at least four transcription factors in addition to CXR (chicken homolog of CAR), with each contributing to enhancer activity (Dogra et al., 1999; Handschin and Meyer, 2000). These studies suggest that phenobarbital-mediated induction requires interactions among multiple regulatory proteins on the enhancer region to constitute a phenobarbital response unit. However, although the coactivator SRC-1 is known to bind CAR (Forman et al., 1998), there is no information as to whether other coactivators are recruited to the phenobarbital-inducible enhancer regions.

In this study, we provide evidence that CBP/p300 and pCAF are required for *CYP2H1* enhancer activity and that E1A as an inhibitor of these components strongly reduces phenobarbital-mediated expression. It was observed that in chick embryo primary hepatocytes, expression of E1A strongly reduced the phenobarbital-induced steady-state level of endogenous *CYP2H1* transcript without influencing basal expression of the mRNA in the absence of phenobarbital. Similarly, in transient expression assays, phenobarbital-induced activity of the 556-bp *CYP2H1* enhancer was inhibited by expression of wild-type E1A but not by E1A mutants defective in CBP/p300 binding. Hence, E1A specifically interferes with phenobarbital-mediated induction of the gene through altering the enhancer activity.

Overexpression of CBP did not completely reverse E1A-

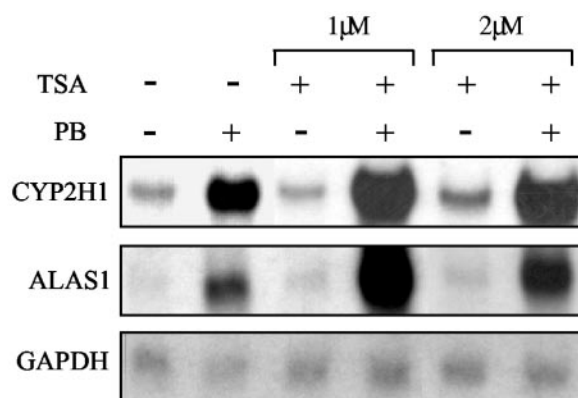


Fig. 7. Effect of TSA on the phenobarbital-induced levels of CYP2H1 mRNA. Representative Northern blot analyses of steady-state levels of mRNAs is shown. Hepatocytes were pretreated with TSA (1 or 2 μ M) for 1 h and then with or without PB at 500 μ M for a further 6 h. Cells without TSA pretreatment were treated with or without PB for 6 h and were included as controls. Total RNA was isolated and 15 μ g analyzed by Northern blotting. The filter was hybridized in turn with 32 P-labeled probes specific for CYP2H1, ALAS1, and GAPDH (as a control). Radiolabeled filters were quantified using a PhosphorImager (model 300A; Amersham Biosciences), the level of CYP2H1 and ALAS1 mRNA was standardized to that of GAPDH mRNA. This experiment was repeated and similar results were obtained.

mediated inhibition of the 556-bp *CYP2H1* enhancer induced by phenobarbital. We speculated that E1A, in addition to interacting with CBP and pCAF, may also directly target other transcription factors bound on the enhancer, but our mutagenesis data did not support this possibility. Perhaps insufficient CBP is produced in transiently transfected primary hepatocytes to completely abrogate the effect of E1A. This may also be the reason that transient overexpression of CBP only marginally (about 1.2-fold) potentiated the effect of phenobarbital exposure in primary hepatocytes (result not shown).

To further examine whether overexpression of CBP and pCAF can activate the 556-bp enhancer, chicken hepatoma LMH cells were employed. We reasoned that LMH cells might have low endogenous levels of these cofactors. The basal level of expression of the 556-bp enhancer construct in LMH cells was substantially induced in the presence of exogenously expressed CXR, whereas E1A repressed this activation. In these cells, coexpression of either CBP or pCAF was able to further activate CXR-mediated transactivation and also completely reverse the inhibitory action of E1A on the 556-bp enhancer. These results strongly indicate that in LMH cells, CBP and pCAF are involved in the CXR-mediated induction process and that the inhibitory action of E1A is dependent on an interaction with these coactivators. Because E1A expression in LMH cells did not affect the basal activity of the 556-bp enhancer, it can be reasoned that in these cells, coactivator assembly and activation of the 556-bp enhancer is dependent on the presence of CXR. Therefore, our studies with LMH cells and primary hepatocytes demonstrate that CXR plays a critical role in the activation of the 556-bp *CYP2H1* enhancer through the recruitment and assembly of the coactivators CBP and pCAF.

The steady-state level of histone acetylation at a promoter is a balance between the action of histone acetyl transferases and histone deacetylases. It is now well-documented that CBP/p300 and pCAF possess intrinsic histone acetyl trans-

ferase activities that can modify chromatin structure at enhancer/promoter sites to facilitate transcriptional activation (Wade and Wolffe, 1997; Grunstein, 1997; Kadonaga, 1998). In keeping with an acetylation role of CBP/p300 and pCAF at the enhancer, we have shown that there is a substantial increase in endogenous *CYP2H1* mRNA in the presence of phenobarbital, but not in its absence, when histone deacetylases are inhibited with TSA. Moreover, in a preliminary study using the chromatin immunoprecipitation assay, we have observed that exposure of chick embryo primary hepatocytes to phenobarbital leads to enhanced acetylation of histone H3 on the 556-bp enhancer region (data not shown).

In summary, our E1A inhibitor and coactivator overexpression studies demonstrate the involvement of CBP/p300 and pCAF in the phenobarbital-mediated induction mechanism, with histone acetylation being a likely step in the phenobarbital response. A speculative model can be proposed for phenobarbital-mediated induction of this gene. As described previously (Dogra et al., 1998), we suggest that the intrinsically strong *CYP2H1* gene promoter in the native chromatin situation is repressed by a nucleosome. After phenobarbital exposure, CBP and pCAF form a higher order complex with transcription factors assembled on the enhancer in response to CXR (see Fig. 8). These coactivators lead to hyperacetylation of the nucleosome at the promoter, with subsequent binding of transcription factors and activation of the promoter. In this model, TSA could superinduce *CYP2H1* promoter activity by inhibiting deacetylases, responsible for removing acetyl groups on the promoter nucleosome, thereby shifting the equilibrium to a higher acetylation state. Further detailed studies using the chromatin immunoprecipitation assay to analyze the acetylation status of the enhancer and promoter regions after phenobarbital exposure in the presence of activators and inhibitors will be of great interest.

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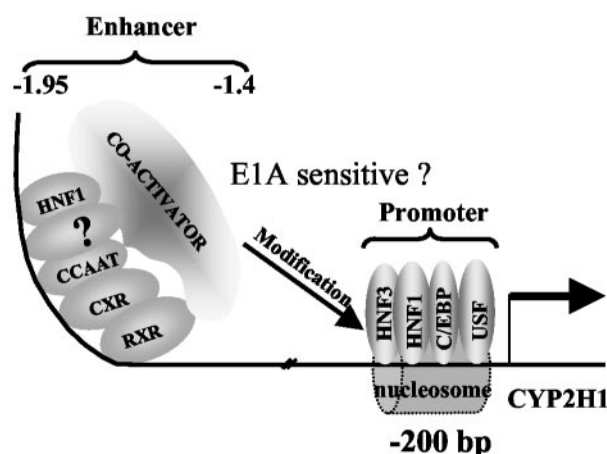


Fig. 8. Speculative model for phenobarbital-induced activation of *CYP2H1* gene. In the absence of phenobarbital, the promoter of the *CYP2H1* gene (–200 bp) is repressed by a nucleosome. After exposure to drug, CXR (chicken homolog of CAR) translocates to the nucleus and, together with retinoid X receptor (RXR) binds to its site on the enhancer. CXR binding nucleates binding of nearby transcription factors, HNF-1, CCAAT, E-box factor, and unknown factor (?), with recruitment of coactivators including CBP/p300 and pCAF. This leads to the acetylation of the nucleosome on the promoter, subsequent binding of liver specific factors, and activation of the promoter.

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