

Drugs Mediate the Transcriptional Activation of the 5-Aminolevulinic Acid Synthase (ALAS1) Gene via the Chicken Xenobiotic-sensing Nuclear Receptor (CXR)*

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Heme is an essential component in oxygen transport and metabolism in living systems. In non-erythropoietic cells, 5-aminolevulinic acid synthase (ALAS1) is the first and rate-limiting enzyme in the heme biosynthesis pathway. ALAS1 expression and heme levels are increased *in vivo* by drugs and other chemical inducers of cytochrome P450 hemoproteins through mechanisms that are poorly understood. In the present studies, a chicken genomic cosmid library was employed to isolate a major portion of the ALAS1 gene. Two drug-responsive enhancer sequences, 176 and 167 base pairs in length, were identified in the 5'-flanking region of the gene in reporter gene assays in the hepatoma cell line LMH. The relative potency of inducers to activate these enhancers corresponds to induction of ALAS1 mRNA levels in LMH cells. Analysis of putative transcription factor binding sites within the enhancers revealed DR5 and DR4 type recognition sequences for nuclear receptors. Drug activation of the enhancer elements was reduced at least 60% after mutagenesis of individual nuclear receptor binding sites and was virtually eliminated following alteration of both recognition sites within the respective elements. Electrophoretic mobility shift assays and transactivation studies demonstrate direct interactions between the nuclear receptor binding sites and the recently described chicken xenobiotic-sensing receptor, (CXR) implicating drug activation mechanisms for ALAS1 similar to those found in inducible cytochrome(s) P450. This is the first report describing direct transcriptional activation of ALAS1 by drugs via drug-responsive enhancer sequences.

5-Aminolevulinic acid synthase (ALAS)¹ is the first and rate-

limiting enzyme in the heme biosynthesis pathway (1). In eukaryotes, there exist two isoforms of ALAS that are encoded by distinct genes located on different chromosomes. The erythroid form ALAS2 is expressed in hematopoietic tissue and is essential for the generation of functional hemoglobin in erythrocytes, whereas ALAS1 is the drug-responsive, housekeeping form that is expressed ubiquitously, providing heme for CYPs and other hemoproteins. Defects in genes encoding enzymes in the heme biosynthesis pathway are associated with a family of serious disorders known as porphyrias, in which neuropsychiatric symptoms are precipitated by drugs and are associated with increased ALAS1 (2). Because ALAS is the rate-limiting enzyme in the heme pathway, it has been the focus of numerous studies examining the mechanisms of coordinated heme and apocytochrome synthesis during drug induction of cytochromes P450 (1, 3–5). The mechanism of ALAS transcriptional regulation by xenochemicals has remained enigmatic.

Under normal physiological conditions, free heme levels are low and tightly regulated, as toxicity can occur with increased cellular concentrations of unincorporated heme. Following administration of drugs such as phenobarbital (PB) or other prototypical CYP inducers, heme concentrations are elevated in the liver to accommodate the increased levels of heme-dependent enzymes (2, 6). This is achieved by induction of ALAS1 and assures an adequate and apparently coordinated supply of heme for the generation of functional cytochrome holoproteins. After accumulation of ALAS1 mRNA and protein, free heme represses hepatic ALAS1 by a number of negative feedback mechanisms that can inhibit the transport of ALAS1 into the mitochondria, increase heme degradation by inducing heme oxygenase, and decrease ALAS1 mRNA stability directly (5). In this way, the cell can provide an adequate supply of heme when required while preventing the potentially dangerous accumulation of heme and heme precursors.

Recent studies have demonstrated that the induction of CYPs by drugs is mediated by several orphan nuclear receptors (NRs), members of a superfamily of DNA-binding proteins that act as transcription factors. NRs regulate genes as homodimers, heterodimers, or monomers by binding to specific DNA response elements (7–9). A number of NRs heterodimerize with retinoid X receptor (RXR), and these dimers then bind to cognate DNA recognition elements, which normally consist of two hexamer half-sites spaced by a variable number of nucleotides, and subsequently modify transcription rates of the targeted genes. In particular, NRs such as chicken xenobiotic receptor (CXR), pregnane X receptor (PXR), and constitutive androstane receptor have been shown to play crucial roles in the induction of members of the drug metabolizing cytochromes from the subfamilies 2H and 2C in chicken (10, 11) 3A and 2C in humans (12–16), and 2B in rodents (17, 18).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF536192.

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¹ The abbreviations used are: ALAS, 5-aminolevulinic acid synthase; ADRES, aminolevulinic acid drug-responsive enhancer sequence; PB, phenobarbital; DR, hexamer half-site direct repeat; LMH, leghorn male hepatoma; NF1, nuclear factor 1; CYP, cytochrome(s) P450; CXR, chicken xenobiotic receptor; PXR, pregnane X receptor; RXR, 9-*cis*-retinoic acid receptor; PIA, propylisopropylacetamide; PCN, 5-pregnen-3 β -ol-20-one-16 α -carbonitrile; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; LUC, luciferase; mifepristone, RU-486; clotrimazole, 1-[*o*-chlorotriptyl]-imidazole; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase; NR, nuclear receptor.

In the present work, we describe the characterization of two drug-responsive elements isolated from the 5'-flanking region of the gene encoding ALAS1. These regions respond to a wide range of drugs and are referred to as aminolevulinic acid synthase drug-responsive enhancer sequence (ADRES) elements. Site-directed mutagenesis data demonstrate ADRES-mediated drug response to be conferred by DR4 and DR5 NR recognition sequences. Our data also suggest an important role for additional transcription factors including potential co-activators and/or co-repressors in conferring full drug response. Gel-shift assays and transactivations support the hypothesis that CXR is responsible for the transcriptional activation of the ALAS1 gene by drugs. The observed effects of drugs on ALAS1 mRNA transcription in LMH cells closely mirror the pattern of induction exhibited by the ADRES elements in response to diverse chemical inducers. These studies are the first to demonstrate the direct transcriptional activation of the ALAS1 gene by drugs via well defined drug-responsive enhancer units.

EXPERIMENTAL PROCEDURES

Reagents—Dexamethasone, 2-methyl-1,2-di-3-pyridyl propadone (metyrapone), 5-pregnen-3 β -ol-20-one-16 α -carbonitrile (PCN), and rifampicin were purchased from Sigma. Propylisopropylacetamide (PIA) was a gift from Dr. Peter Sinclair (Veterans Affairs Hospital, White River Junction, VT). Glutethimide was purchased from Aldrich. Mifepristone (RU-486) was obtained from Roussel-UCLAF. 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) was generously provided by U. Schmidt (Institute of Toxicology, Bayer, Wuppertal, Germany). Phenobarbital sodium salt (5-ethyl-5-phenylbarbituric acid sodium salt) was purchased from Fluka. Tissue culture reagents, media, and sera were purchased from Invitrogen. All other reagents and supplies were obtained from standard sources.

Plasmids—The pGL3LUC luciferase reporter containing an SV40 promoter was purchased from Promega. The reporter plasmid was modified by the addition of the fragment spanning the *SacI* to the *XhoI* restriction endonuclease sites of the multiple cloning site of the pBlue-script SK vector (Stratagene) to the pGL3LUC vector, thus greatly enhancing the cloning versatility of the new pLucMCS reporter. The pBLCAT5 chloramphenicol acetyltransferase reporter vector was described previously (19). Chicken CXR and RXR were cloned into the pSG5 expression vector (Stratagene) as previously reported (10). The pRSV β -galactosidase vector used for normalization of transfection experiments was kindly provided by Anastasia Kralli (Biozentrum, University of Basel, Basel, Switzerland).

Cosmid Isolation—A specific probe for the ALAS1 gene was generated via PCR using chicken embryo liver genomic DNA as template and forward primer 5'-cgg gca gca ggt cga gga ga-3' and reverse primer 5'-cag gaa cgg gca tt ttt agc a-3'. The probe was ³²P-radiolabeled using the random primer labeling kit (Roche Molecular Biochemicals) according to instructions from the manufacturer. A genomic cosmid library generated from adult male Leghorn chicken liver was purchased from CLONTECH. The ALAS1 probe was used to identify an individual cosmid clone containing the ALAS1 gene, and at least 15 kb of 5'-flanking region was isolated and confirmed by sequencing.

Construction of Vectors—The cosmid containing the ALAS1 gene and flanking region was digested with *EcoRI* restriction endonuclease, and subfragments of the ~35 kb of new sequence were cloned into the *EcoRI* site of the pLucMCS vector. Eight fragments ranging in size from 10 kb to 900 bp in length were cloned. In addition, a 3282-bp *SmaI* fragment encoding the ALAS1 promoter region and proximal 5'-flanking region was cloned into pLucMCS. The drug-responsive 8-kb *EcoRI* region was then further subdivided using standard subcloning procedures and restriction endonucleases to isolate the *Sau3AI-SmaI* 176-bp element and the *PvuII-HaeIII* 167-bp element. Single copies of the 176- and 167-bp wild type and mutated elements were cloned into pBLCAT5 by excising a 222-bp fragment containing the desired sequences with *BamHI* and *BglII* restriction endonucleases and ligating them into *BamHI*-linearized pBLCAT5 vector. Multiple repeats of the 176-bp wild type and mutant elements were subcloned by inserting the 222-bp fragment four times in succession into the *BamHI*-linearized pBLCAT5 vector.

Cell Culture—Leghorn male hepatoma (LMH) cells were obtained from the American Type Culture Collection and cultivated in 10-cm dishes in Williams E medium supplemented with 10% fetal calf serum, 1% glutamine (2 mM), and 1% penicillin/streptomycin (50 IU/ml).

Dishes coated with 0.1% gelatin were used for routine culture of LMH cells to facilitate proper seating of the cells onto the plastic plate surface. For transfections, cells were seeded onto 12-well Falcon 3043 dishes and expanded to 70–80% surface density. Cells were then maintained in serum-free Williams E media for 24 h and transfected using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the protocol from the manufacturer.

Analysis of Reporter Gene Expression—Cells were treated with drugs or vehicle for 16 h and harvested. For luciferase assays, lysis was performed with 200 μ l/well Passive Lysis Buffer (Promega) and extracts were centrifuged for 1 min to pellet cellular debris. Luciferase assays were performed on supernatants using the luciferase assay kit (Promega) and a Microlite TLX1 luminometer (Dynatech). Relative β -galactosidase activities were determined as described (20). For CAT assays, cells were lysed with 600 μ l/well CAT lysis buffer and extracts were centrifuged for 1 min to pellet cellular debris. Assays were performed using a CAT enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals) according to the protocol from the manufacturer.

Site-directed Mutagenesis—Mutations in the putative NR binding sites were introduced into the ADRES elements by PCR using standard overlap techniques. Briefly, subfragments were amplified with overlapping primers carrying the desired mutations and vector primers. These subfragments were then combined and used as template in a second PCR using vector primers to amplify the full-length mutated fragment, which was subsequently digested with appropriate enzymes and cloned into pLucMCS. The forward vector primer was the RV primer 3, and the reverse vector primer was the GL primer 2 within the pGL3 luciferase vector (Promega). All mutations are shown in *bold*. DR4-1 double mutation constructs were generated with 5'-gga gga **act cga cac gat acc aac ata gca at-3'** forward and 5'-cta tgt **tgg tat cgt gtc gag ttc ctc cct g-3'** reverse primers. DR5 double mutants were amplified with 5'-**gaa ttc gcc aac tgc agc cag gct gtc c-3'** forward and 5'-cag cct **ggc tgc agt tgg cga att ctc ctc-3'** reverse primers. DR4-2 double mutants were generated with 5'-**ccc cac gca gcc cca cgc ctc gta act cgt g-3'** forward and 5'-**gtg ggg ctg cgt ggg gca gca gaa gtt cag g-3'** reverse primers. DR4-3 double mutants were amplified using a 5'-**gaa ttc aca gcc atg gtc aag atc agc-3'** forward primer and a 5'-**cca tgg ctg tga att gca tca cga g-3'** reverse primer. Avian NF1 consensus sequence was generated using 5'-ggt taa agc **tgg cac tgt ccc aag-3'** and 5'-ctt **tgg cac agt gcc agc ttt aaa c-3'** forward and reverse primers (21). Following PCR overlap, the products were digested with *BglII* and either *EcoRI* or *NotI* restriction endonucleases and cloned into pLucMCS. All constructs were verified by sequencing.

Quantitative PCR—LMH cells were plated onto 12-well plates, expanded to 70–80% surface density, and incubated in serum-free media for 24 h. Cells were then exposed to either drug or vehicle, and RNA was isolated with TRIzol reagent (Invitrogen) according to the protocol from the manufacturer. One μ g of total RNA was reverse transcribed with the Moloney murine leukemia virus reverse transcriptase kit (Roche Molecular Biochemicals). PCR was performed using the Taqman PCR core reagent kit (PerkinElmer Applied Biosystems) and transcript levels quantitated with an ABI Prism 7700 sequence detection system (PerkinElmer Applied Biosystems). Relative transcript levels were determined using the relative quantitation method measuring the $\Delta\Delta Ct$. The following primers and probes were used in these reactions: ALAS1 (probe, 5'-ttc cgc cat aac gac gtc aac cat ctt-3'; forward primer, 5'-gca ggg tgc caa aac aca t-3'; reverse primer, 5'-tcg atg gat cag act tct tca aca-3') and glyceraldehyde-3-phosphate dehydrogenase (probe, 5'-tgg cgt gcc cat tga tca caa ttt-3'; forward primer, 5'-ggt cac gct cct gga aga tag t-3'; reverse primer, 5'-ggg cac tgt caa ggc tga ga-3'). Transcript levels were measured in separate tubes, and glyceraldehyde-3-phosphate dehydrogenase values were used for normalization of ALAS1 values.

Gel Mobility-shift Assays—Chicken CXR and RXR proteins were expressed using the TNT T7 quick coupled translation system (Promega) according to the protocol from the manufacturer. Ends were filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of radiolabeled [α -³²P]ATP and purified over a Biospin 6 chromatography column. A volume of labeled oligonucleotide corresponding to 100,000 cpm was used for each reaction in 10 mM Tris-HCl, pH 8.0, 40 mM KCl, 0.05% Nonidet P40, 6% glycerol (v/v), 1 mM DTT containing 0.2 μ g of poly(dI-dC) and 2.5 μ l *in vitro* synthesized proteins as described previously (10, 12). To test for supershifts, 0.5 μ l of monoclonal anti-mouse-RXR rabbit antibody (kindly provided by P. Chambon, Universit  Louis Pasteur, Illkirch, France) were added to the reaction mix. This antibody has been previously tested for interactions with chicken RXR in Western blots (data not shown). The reaction mix was incubated for 20 min at room temperature and electrophoresed on

a 6% polyacrylamide gel in 0.5× Tris borate/EDTA buffer followed by autoradiography.

Transactivations—Experiments to determine the ability of the nuclear receptors CXR, human PXR, and mouse PXR to mediate induction of ALAS-1 were done in COS-1 monkey kidney cells according to methods previously described (10). PXR clones were the generous gift of S. Kliewer (University of Texas Southwestern Medical Center, Dallas, TX). Briefly, cells were expanded for 3 days on 10-cm Falcon 3003 dishes in Dulbecco's modified Eagle's medium/F-12 medium (Invitrogen) without phenol red supplemented with 10% charcoal-stripped fetal bovine serum. Cells were then plated onto six-well dishes and expanded overnight to ~30% density. Cells were then rinsed with PBS and maintained for transfection in OptiMEM (Invitrogen) without further additions. Transfection of 1 µg of reporter plus 800 ng of pSV β-galactosidase construct and 50 ng of CXR expression vector was performed using 3 ml/well LipofectAMINE, according to the protocol from the manufacturer. After a 24-h incubation, cells were rinsed with PBS and Dulbecco's modified Eagle's medium/F-12 containing 10% delipidated/charcoal-stripped fetal bovine serum containing either drugs or vehicle control was added. After a 16-h induction, cells were rinsed with PBS, lysed in 600 µl of CAT lysis buffer, and assayed for CAT enzyme using the CAT enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals). CAT levels were then normalized against β-galactosidase levels to compensate for variations in transfection efficiency.

RESULTS

A cosmid clone containing an insert ~35 kb in length, spanning the chicken ALAS1 gene and 15 kb of the 5'-flanking region, was isolated and its sequence analyzed. Three major subclones were generated from the region upstream of the transcriptional start site, including a 3282-bp *Sma*I fragment and 5056- and 7973-bp *Eco*RI segments (Fig. 1A). The *Sma*I clone extends from bp -167 to bp -3449, whereas the *Eco*RI subfragments span the regions from bp -2347 to bp -7402 and bp -7403 to bp -15376, respectively. These subfragments were cloned into the pLucMCS modified luciferase vector containing an SV40 promoter as described under "Experimental Procedures." Drug inducibility was measured in transiently transfected LMH cells treated with 600 µM PB and compared with control values. The results revealed the 7973-bp subfragment to be highly inducible with PB, displaying a 32-fold increase in transcriptional activation relative to control values. In comparison, the 5056- and 3282-bp subfragments exhibited virtually no transcriptional activation in response to drug treatment (Fig. 1C). The 7973-bp subfragment (-15376/-7403) was chosen for further analysis and was divided into numerous subclones in the pLucMCS reporter vector, resulting in the isolation of 176-bp *Sau*3AI-*Sma*I and 167-bp *Pvu*II-*Hae*III elements (Fig. 1, A and B). These sequences routinely exhibit 25–60-fold induction over control values in reporter gene assays when exposed to PB in LMH cells (Fig. 1C). All other portions of the 7973-bp fragment were also subcloned but displayed no drug response when tested in LMH cells (data not shown). Because the 176- and 167-bp fragments retain high drug response regardless of orientation or distance from the promoter (data not shown), they are referred to as ADRES enhancers.

Recent discoveries have implicated NRs in drug-mediated enzyme induction (10, 13, 18). For this reason, we scanned the responsive elements for potential nuclear receptor response sites using a computer algorithm based on a weighted nucleotide distribution matrix compiled from published functional hexamer half-sites (22). Two potential binding sites for orphan NRs were identified in each ADRES element, having two direct repeats with 4 nucleotide (DR4) and 5 nucleotide (DR5) separations between half-sites in the 176-bp sequence and two direct repeats with 4 nucleotide (DR4) separations between half-sites in the 167-bp sequence (Fig. 1B). For clarity, the three DR4 binding sites are labeled according to their occurrence in the gene, with the furthest upstream from the tran-

scription start site called DR4-1 and the closest to the start site DR4-3. The putative DR4-1 is defined by one perfect half-site (AGGTCA) and one imperfect half-site (AGTTGA) at -14186/-14181 and -14176/-14171 respectively, whereas the DR5 site is characterized by an imperfect upstream half-site (AGCTGA) and a perfect downstream half-site (AGGTCA) at -14251/-14246 and -14240/-14235. In the 167-bp sequence, DR4-2 consists of one imperfect upstream half-site (GGATGA) and one perfect downstream half-site (AGTTCA) at -13563/-13558 and -13553/-13548 and DR4-3 has two imperfect half-sites (GTGTCA and GGGGCA) at -13526/-13521 and -13516/-13511. It is interesting to note that the 176-bp ADRES also contains a putative binding site for nuclear factor 1 which overlaps the DR5, spanning bp -14255 to bp -14242, whereas the 167-bp ADRES does not.

We next wanted to compare ADRES-mediated ALAS1 induction levels from reporter gene assays with stimulation of transcription in a physiological system. Therefore, ALAS1 mRNA levels were quantified in LMH cells cultured in serum-free medium and 16 h of exposure to a variety of chemical inducers and compared with the induction pattern observed with the same compounds in transient transfections of the ADRES (Fig. 2). The compounds examined include PB (600 µM) and the PB-like inducers PIA (250 µM), glutethimide (500 µM), and the potent mouse CYP 2B inducer TCPOBOP (10 µM). In addition, the common CYP3A inducers dexamethasone (50 µM), metyrapone (400 µM), and 10 µM mifepristone (RU-486) were employed for comparison. We were also interested in the effects of 10 µM PCN and rifampicin (100 µM) because of their species-specific effects on PXR activation and CYP3A induction. Messenger RNA was reverse transcribed, and levels of ALAS1 cDNA were quantified using the Taqman real-time PCR quantification system as described under "Experimental Procedures." PB was a strong inducer of ALAS1 in LMH cells, increasing RNA levels an average of 16-fold relative to basal transcript levels (Fig. 2). This value was chosen to represent 100% induction, against which all other values are compared. The general inducers PIA and glutethimide, as well as the 3A-specific inducer metyrapone, exhibited the strongest effects upon the ADRES elements, stimulating transcription in excess of levels obtained from PB treatment. In comparison, dexamethasone, PCN, RU-486, and rifampicin had minor or no effects on either mRNA levels or ADRES activation. Moreover, the mouse-specific compound TCPOBOP elicited no response in either mRNA transcription or stimulation of the ADRES in reporter assays. When comparing the induction profiles of the two ADRES elements to each other, very few differences are in evidence. The 167-bp element responds to PB with twice the activation when compared with the 176-bp element. Additionally, the 176-bp element has slightly more affinity for glutethimide than metyrapone, whereas the 167-bp element exhibits a stronger response to metyrapone than glutethimide. These experiments indicate a high degree of similarity in the relative activation of the ADRES elements in reporter gene assays to each other and to mRNA transcript levels from chemically induced LMH cells.

Site-specific mutagenesis was used to examine the roles of specific nucleotides within the putative DR5 and DR4 recognition sequences in conferring drug response to the ADRES elements (Fig. 3). Mutant constructs of the DR4 and DR5 core recognition sites destroying the putative NR binding sites were generated as described under "Experimental Procedures." Briefly, primers were used in conjunction with PCR to convert the 5' and 3' half-sites of the DR5 to *Eco*RI and *Pst*I restriction endonuclease sites, respectively. Similarly, the DR4-3 half-sites were converted to *Eco*RI and *Nco*I restriction endonucle-

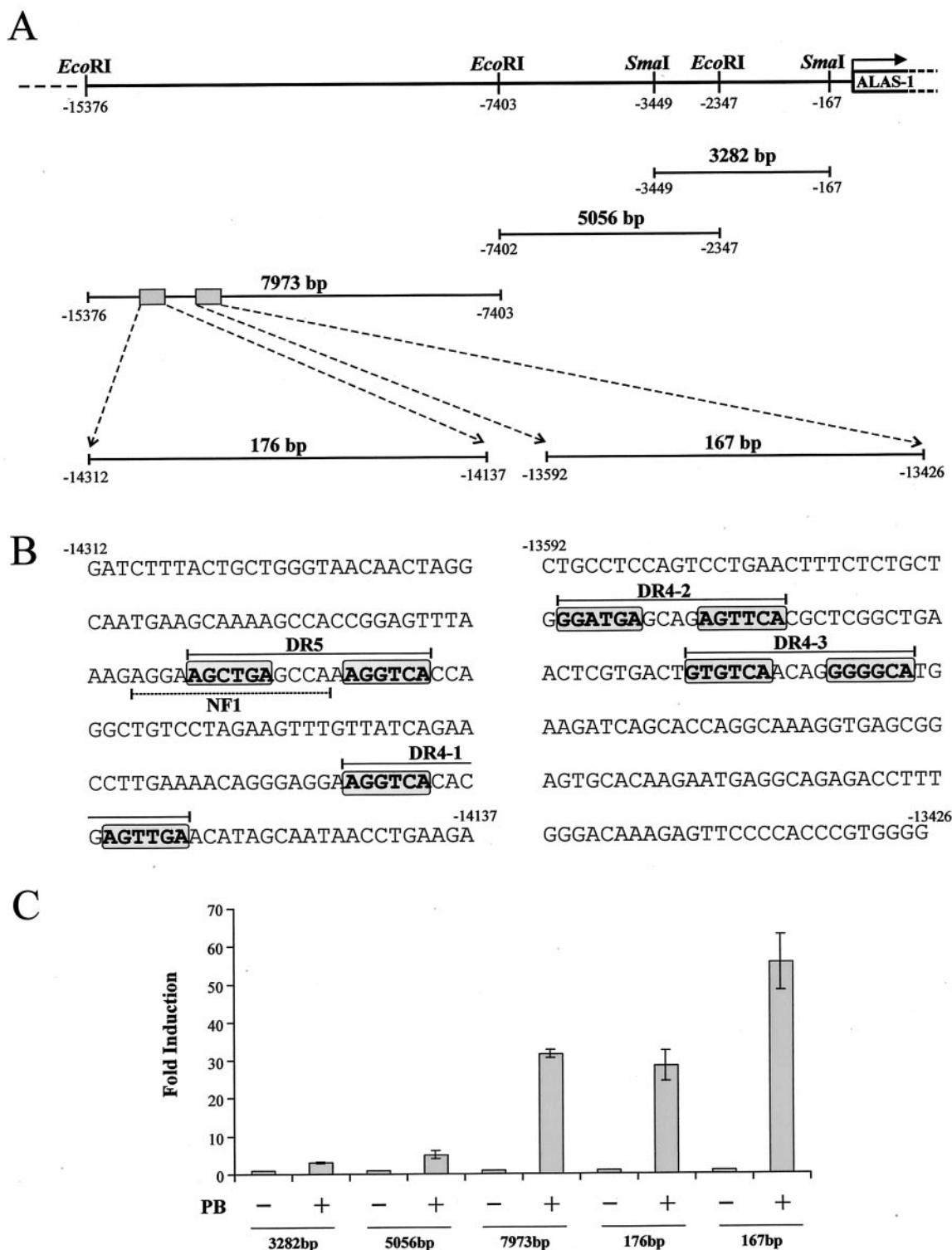


FIG. 1. Analysis of the 5'-flanking region of chicken ALAS1. *A*, isolation of 176- and 167-bp drug-responsive enhancer sequences within the first 15 kb upstream of the ALAS1 transcription start site by restriction endonuclease digestion and subcloning as described under "Experimental Procedures." Fragments were cloned into the pLucMCS luciferase reporter vector containing an SV-40 promoter. *B*, DNA sequences of the 176- and 167-bp enhancers. *Numbering* refers to sequence positions relative to the transcriptional start site of the chicken ALAS1 gene. *Solid lines* identify DR4 and DR5 NR binding sites. *Shaded boxes* contain individual half-sites. A *hatched line* marks the NF1 binding site. *C*, reporter gene assays of the fragments. The constructs were transfected together with a transfection-control construct expressing β -galactosidase into LMH cells. Cells were then treated with 600 μ M PB for 16 h, and luciferase assays were performed on the cell extracts. Relative luciferase levels are standardized against cells transfected with vector containing no insert and expressed as -fold induction. Experiments were repeated at least three times, and data from a representative experiment tested in triplicate are shown here. *Error bars* represent standard deviations.

ase sites. Data from a nucleotide distribution matrix for half-sites developed by M. Podvinec in this laboratory was applied to ascertain that the mutated half-sites least resemble func-

tional half-sites. DR4-1 half-sites were obliterated by converting AGGTCA and AGTTGA half-sites to unconserved ACTCGA and ATACCA bases, respectively. Similarly, DR4-2 half-sites

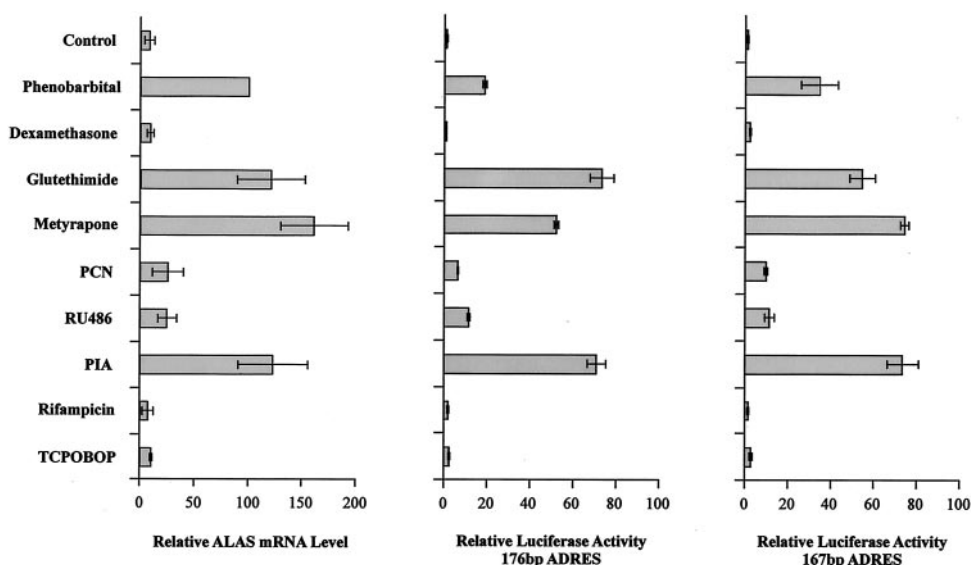


FIG. 2. Comparison of ADRES and mRNA activation by different drugs. LMH cells were treated for 16 h with inducers, and cells were harvested. RNA was recovered and reverse transcribed as described under "Experimental Procedures." Relative ALAS1 mRNA levels standardized against glyceraldehyde-3-phosphate dehydrogenase levels were obtained by real time PCR using the Taqman system. Values are the average of three individual experiments and phenobarbital induction of ALAS1 was chosen as 100% activation. The ALAS1 RNA induction levels obtained after PB treatment in the three individual experiments were 8-, 16-, and 24-fold, respectively, and *error bars* represent standard deviations (*left panel*). The 176- and 167-bp ADRES were tested with a battery of known ALAS1 and CYP inducers. Reporter constructs were transfected into LMH, and cells were treated for 16 h with the specified compounds as described under "Experimental Procedures." β -Galactosidase was used as an internal transfection control, and cell lysates were tested for luciferase activity. Relative luciferase levels are standardized against cells transfected with vector containing no insert and expressed in -fold induction. Experiments were repeated at least three times, and data from a representative experiment tested in triplicate are shown here. *Error bars* represent standard deviations.

were both converted from GGATGA and AGTTCA nucleotides to CCCAC bases. Primers were used to generate constructs mutated at each individual and both NR binding sites within both of the ADRES elements as shown in Fig. 3.

The modified enhancers were examined for response to 600 μ M PB in luciferase reporter gene assays, and the results are presented in Fig. 3. These findings indicate that both the DR5 and DR4 recognition sites in the 176-bp ADRES and both DR4 recognition sites in the 167-bp ADRES element are essential to elicit full drug response. Mutation of the DR5 reduced activity of the 176-bp ADRES element by over 85% from 44- to 6.4-fold activation by PB, whereas changes in the DR4-1 limited activation by over 60% from 44- to 16-fold stimulation (Fig. 3A). As depicted in Fig. 3B, both DR4-2 and DR4-3 sites in the 167-bp ADRES element were found to be required for full activation by PB. Alteration of the DR4-2 site resulted in the reduction of PB response by over 90% from 60- to 5.4-fold. Mutations in the DR4-3 site caused PB response to be diminished 75% from 60- to 15-fold induction. These studies demonstrate an essential contribution of the sequences within the putative DR4 and DR5 NR binding sites to PB activation of the ADRES elements.

Because the DR5 overlaps a putative binding site of NF1, a transcription factor that has been implicated in modifying drug induction, we tested the possibility that NF1 confers activation to the 176-bp ADRES element rather than NRs binding to the DR5 (23). A mutant construct converting the putative NF1 site to a consensus avian NF1 binding site, thus destroying the first half-site of the DR5, was generated and tested in luciferase assays. As seen in Fig. 3A, the NF1 consensus sequence does not increase the response of the 176-bp ADRES element to drugs. Rather, the induction is decreased by 66% from 44- to 15-fold induction, presumably because of the destruction of the DR5 NR binding site. To confirm these findings, chicken NF1-A was amplified from a cDNA library generated from LMH cells and cloned into pSG5 expression vector. Coding sequence fidelity was confirmed by sequencing, and the construct was co-

transfected both in induction experiments in LMH cells and transactivations in COS-1 cells, resulting in no changes in induction or transactivation (data not shown).

Recent findings have implicated a number of orphan NRs in drug induction of cytochromes P450 (for reviews, see Refs. 24–26). Our group has successfully cloned and expressed chicken CXR and has demonstrated CXR-RXR interactions with CYP enhancers in EMSAs (10). As the DR4 and DR5 sites clearly contribute to the transcriptional activation exhibited by the ADRES elements, gel-mobility shift assays were used to determine whether CXR might bind the responsive enhancers (Fig. 4). Neither *in vitro* transcribed/translated chicken CXR nor chicken RXR alone bound to the 32 P-radiolabeled 176-bp ADRES (Fig. 4A, lanes 2 and 3) or to the 167-bp ADRES (Fig. 4B, lanes 2 and 3). In contrast, CXR/RXR heterodimers bind the drug-responsive enhancers, and these complexes could be supershifted with anti-RXR antibodies (Fig. 4, A and B, lanes 4 and 5). Nuclear receptor binding to the 167-bp ADRES element was reduced when the double mutant DNA sequences were used, as demonstrated by the reduced band intensities of the shifted and supershifted components (Fig. 4B, lanes 6 and 7). Moreover, the binding of CXR/RXR heterodimers was virtually eliminated when both binding sites in the 176-bp ADRES elements were mutated (Fig. 4A, lanes 6 and 7). Additional gel-shift assays using increasing amounts of CXR were done to determine whether the binding of heterodimers to the NR half-sites occurs in an independent or a competitive fashion. There is no evidence for an additional complex band of increased molecular mass at higher CXR concentrations, suggesting that binding occurs in a mutually exclusive fashion (data not shown). In summary, these findings demonstrate interactions of CXR/RXR heterodimers with the ADRES elements through the DR4 and DR5 NR binding sites.

To confirm the role of CXR in the activation of the ADRES elements, transactivation experiments were done in COS-1 monkey kidney cells. These cells express RXR but exhibit no

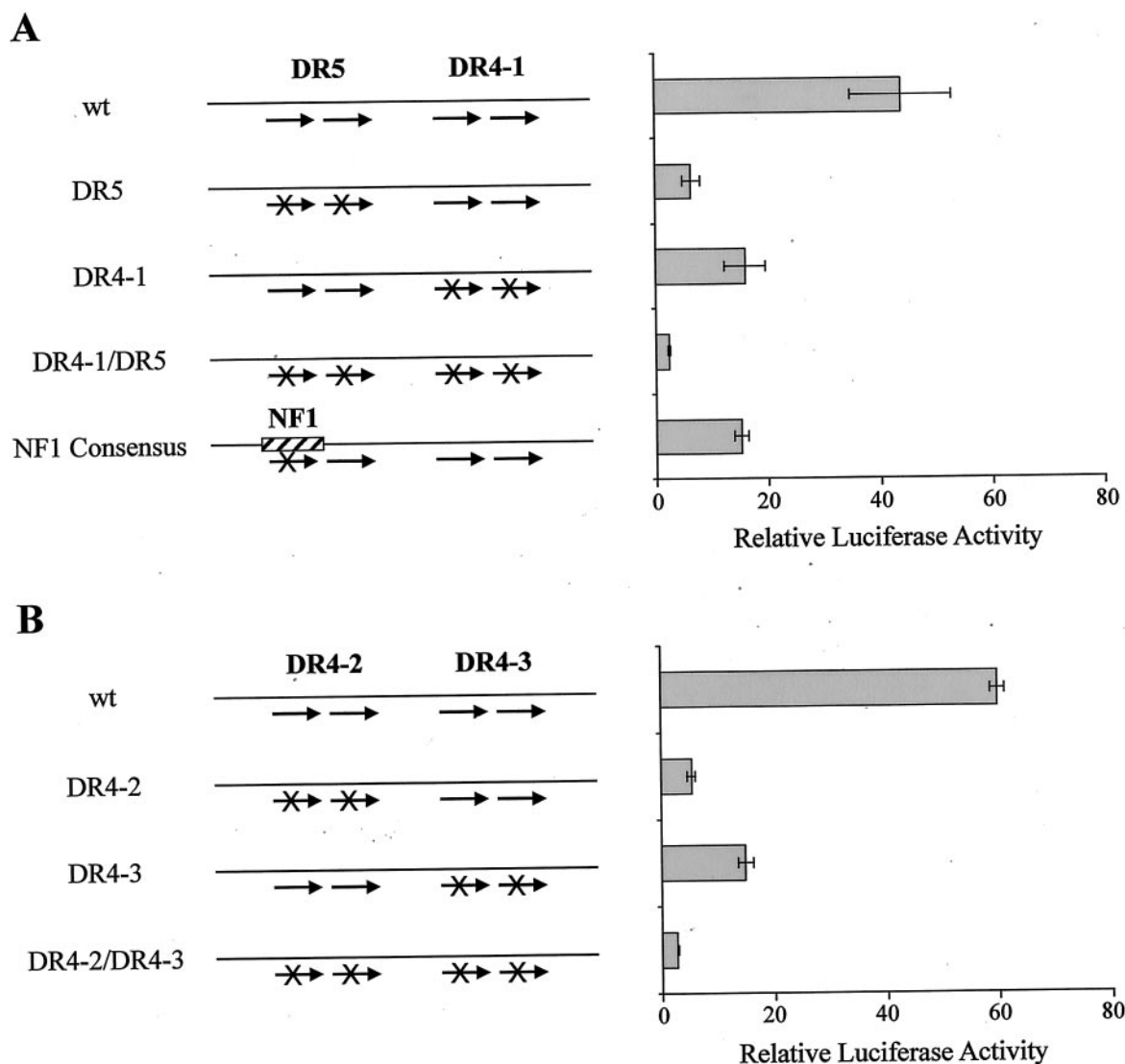


FIG. 3. Site-directed mutagenesis of the DR4 and DR5 sites within the 176- and 167-bp ADRES elements. Mutations in the DR4 and DR5 half-sites of the 176-bp (A) and 167-bp (B) sequences were introduced via PCR-based site-directed mutagenesis. Mutations are labeled in the *left column* and depicted with crosses in the scheme. Specific base pair alterations are described under "Experimental Procedures." LMH cells were transfected with the mutant constructs along with a β -galactosidase expression construct as an internal control. The cells were exposed to 600 μ M PB for 16 h, and cell lysates were tested for luciferase activity. Relative luciferase levels are standardized against cells transfected with vector containing no insert (control set to 1.0) and expressed as percentages of the 176-bp ADRES. Experiments were repeated at least three times, and data from representative experiments tested in triplicate are shown here. Error bars represent standard deviations.

induction response in the absence of CXR transfection (data not shown). Four copies of the wild type and mutated 176-bp element or a single copy of the mutated and wild type 167-bp element were cloned into the pBLCAT5 plasmid containing a thymidine kinase minimal promoter as described under "Experimental Procedures." CAT vectors were used for transactivations rather than luciferase because CAT provided more stable expression and showed higher drug response. These constructs were cotransfected along with a pSG5 expression vector containing the coding sequence for CXR and a β -galactosidase expression construct to correct for variations in transfection efficiency. After a 24-h incubation to allow for the expression of CXR, induction of the wild type and mutant sequences was tested with glutethimide, metyrapone, and PIA, the three best inducers identified in Fig. 2. As shown in Fig. 5, both the 176- and 167-bp ADRES elements are transactivated by CXR. In the 176-bp element, the induction ranged from 3.7- to 6.9-fold and was reduced to a range of 2–3.5-fold in the constructs carrying the mutant DR5 NR binding site. The

mutations in the DR4-1 binding site reduced the induction by all drugs to less than 1.6-fold. Moreover, the alteration of both NR binding sites in the 176-bp element resulted in the complete elimination of drug response. Because the 167-bp element was found to respond better to drugs in transactivations than the 176-bp element, a single copy was sufficient for these experiments. The induction of the wild type sequence was strong for all three drugs, ranging from 4.0- to 8.3-fold over uninduced levels (Fig. 5B). The DR4-2 mutants exhibited lower induction after drug exposure, reduced by 58–66% when compared with wild type values. Similarly, the DR4-3 mutant sequences responded to drugs with diminished capacity, exhibiting 55–66% of the 167-bp activity. The double mutant 167-bp element did not respond to drugs, confirming the role of CXR in activating the ADRES elements via the DR4 and DR5 NR binding sites.

In addition to CXR, we examined whether mammalian nuclear receptors involved in drug-mediated increases in transcription of cytochromes P450 can transactivate the ADRES elements. Human and mouse PXR exhibit distinctive activa-

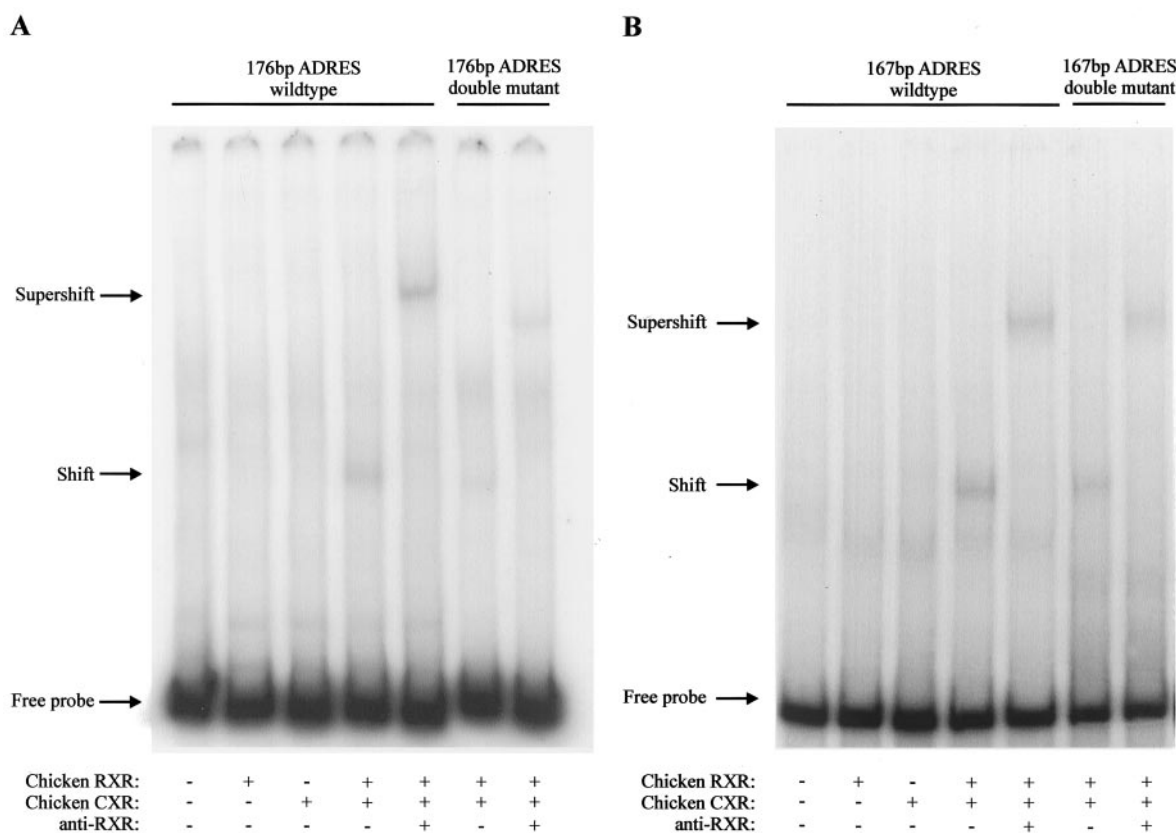


FIG. 4. Gel-mobility shift assays demonstrating that CXR binds the ADRES elements. Gel setup was the same for the 176-bp (A) and the 167-bp (B) elements. Radiolabeled ADRES wild type (lanes 1–5) and mutant (lanes 6 and 7) sequences were incubated with *in vitro* transcribed/translated CXR (lanes 3–7), chicken RXR (lanes 2 and 4–7), and anti-RXR antibody (lanes 5 and 7), as indicated. Arrows depict the unbound probe, the shifted CXR-RXR-probe complex, and the supershifted CXR-RXR-probe-anti-RXR antibody complex.

tion patterns of cytochromes P450, with the human isoform activated by rifampicin but not PCN, and the mouse isoform activated by PCN but not rifampicin (13). We therefore undertook studies to examine whether these differences are reflected in induction patterns for ADRES elements. The 176- and 167-bp CAT constructs were cotransfected along with expression constructs of CXR, human PXR, or mouse PXR and a β -galactosidase expression construct to correct for variations in transfection efficiency. After a 24-h incubation to allow for the expression of CXR, induction by metyrapone, PCN, and rifampicin was measured. As shown in Fig. 6, the 176- and 167-bp ADRES elements are activated by metyrapone 3.4- and 5.0-fold, respectively, but are not stimulated by PCN or rifampicin, findings that are in agreement with the RNA and reporter gene assay data in Fig. 2. In comparison, mouse PXR activates transcription of the 176- and 167-bp ADRES elements in the presence of PCN 2.7- and 2.5-fold, respectively, whereas metyrapone and rifampicin do not stimulate activity above 1.4-fold for the 176-bp element and have no statistically significant effect on the 167-bp element. The human isoform of PXR stimulates transcription of the 176- and 167-bp sequences 1.7- and 2.9-fold with rifampicin, respectively, whereas PCN has no effects on either ADRES element. Interestingly, metyrapone also stimulates transcription of the 176- and 167-bp enhancers via hPXR 1.4- and 2.2-fold, respectively. These experiments provide evidence that the species-specific patterns of transcriptional activation of ALAS1 enhancers by NRs are similar to those observed for induction of cytochromes P450 and are determined by the ligand-binding domains of the nuclear receptors.

DISCUSSION

The direct up-regulation of genes in the heme biosynthesis pathway by PB and PB-like inducers has been the subject of numerous studies, but has not led to the identification of discrete drug-responsive enhancers (1, 27). Indirect evidence, however, suggests that chemical inducers mediate direct transcriptional activation (28, 29). Because the majority of the drug-responsive elements in the CYP genes and other genes for drug-metabolizing enzymes or drug transporters reside within the first few kilobases of the transcriptional start site, similar findings were expected for ALAS1. Previous studies of ALAS1 regulation, however, did not result in the identification of a drug-responsive enhancer in these regions (1, 27). In light of reports in different systems identifying enhancers far upstream of the coding region or, alternatively, within introns of the gene itself, we decided to examine a larger portion of the ALAS1 gene for enhancer elements that confer direct drug response. The data presented here demonstrate that the ADRES elements lie farther upstream from the ALAS1 transcription start site when compared with its CYP counterparts, explaining previous failures in their identification. Moreover, these findings have stimulated successful ongoing studies examining the flanking regions of mouse and human ALAS1 for related sequences.

Because of their considerable activation by drugs and xenobiotics, it is suspected that the ADRES elements described here account for a majority of the transcriptional activation of the ALAS1 gene *in vivo*. A modest response is consistently observed from the 5056-bp subfragment that remains to be investigated, but its muted response relative to that exhibited by the ADRES elements suggests a limited role for this region in

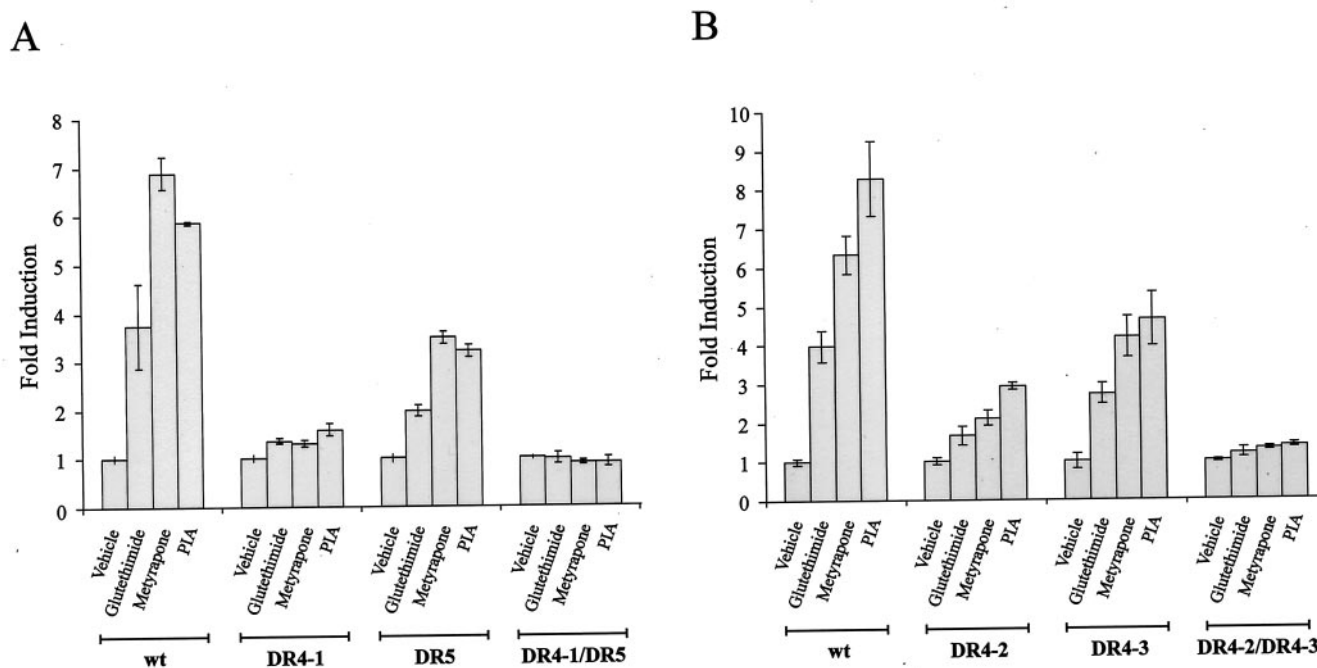


FIG. 5. Transactivation of the ADRES elements by CXR. COS-1 cells were transfected with constructs containing either four repeats of the 176-bp wild type, DR4-1, DR5, and DR4-1/DR5 mutants (A) or a single copy of the 167-bp wild type, DR4-2, DR4-3, and DR4-2/DR4-3 mutants (B) cloned into the pBLCAT5 vector containing a thymidine kinase minimal promoter. The chicken CXR coding region cloned into the pSG5 expression vector was cotransfected along with a vector expressing pSV β -galactosidase as control. Cells were then treated for 16 h with either drugs or vehicle control, and extracts were analyzed for CAT expression normalized against β -galactosidase levels as described under "Experimental Procedures." Experiments were repeated at least three times, and data from representative experiments tested in triplicate are shown here. All constructs were verified by sequencing, and *error bars* represent standard deviations.

drug induction. Additional experiments examining subclones of the entire 35-kilobase cosmid encoding the ALAS gene in reporter gene assays also indicated the only sequences to exhibit drug induction were the ADRES elements presented here (data not shown). Moreover, previous studies examining similar regions upstream of the ALAS coding region but not encompassing the ADRES sequences did not result in the identification of specific drug-responsive DNA sequences (1). The data presented here describing multiple drug response elements are in line with evidence from a number of inducible CYP genes from the 3A, 2B, and 2H subfamilies, where multiple distinct drug response elements have been identified as reviewed in Ref. 30.

The ADRES was then analyzed using the MatInspector program to detect putative regulatory element and transcription factor binding sites. This program scans DNA sequences for matches to nucleotide distribution matrices that define putative transcription factor binding sites registered in the TRANSFAC data base (31). Using a core similarity of 0.75 and an optimized matrix similarity of 0.85, a total of 77 independent matches were identified within the two sequences, including a nuclear factor 1 site and C/EBP binding sites within the 176-bp ADRES and COUP sites within both elements. A number of these sites linked to transcriptional modulation have been identified in other drug-responsive enhancers. The recognition sequence for COUP found at $-14248/-14235$ in the 176-bp element and $-13561/-13549$ in the 167-bp sequence could provide a means of transcriptional modulation through competition with other nuclear receptors such as RXR (32). However, given the high level of similarity between RXR and COUP recognition sequences, it is not surprising to find that sequences which bind CXR-RXR heterodimers would also contain potential COUP binding sites. Our analysis also revealed a CCAAT/enhancer-binding protein (C/EBP) site at $-14284/-14273$ in the 176-bp sequence. Members of the C/EBP family are involved in regulating mammalian cell differentiation and

can interact with a number of other transcription factors including AP-1 (33). These factors are prime targets for future studies to determine the role of sequences flanking the nuclear receptor binding sites within the ADRES elements, which have been shown in deletion experiments aimed at reducing the size of the response elements to be essential for full induction response (data not shown). However, the potential roles of COUP and C/EBP sites in drug-mediated transcriptional activation of ALAS are beyond the scope of the work presented here and remain to be investigated. The role of NF1 in drug induction has been somewhat controversial, but a number of different studies have found that site-specific mutagenesis of NF1 recognition sequences reduces the drug-mediated transcriptional activation of drug-responsive enhancers (18, 23). Our studies do not support a role for NF1 in the induction process, but that may reflect limitations of the experimental system.

The mutagenesis studies demonstrate the role of the DR4 and DR5 recognition sites in conferring drug-response to the ADRES elements. The mutation of either the DR4 or the DR5 sites results in substantial reductions in transcriptional activation, and mutation of both sites resulted in an almost total loss of induction (Fig. 3). The remaining activity may be the result of residual binding of transcription factors to these sites or, alternatively, could indicate the presence of other unidentified elements able to bind trans-activating factors and activate the transcriptional machinery. Efforts to reduce the size of the ADRES elements while keeping the DR4 and DR5 binding sites intact resulted in the loss of drug response, indicating an important role of the flanking regions in induction. Moreover, splitting either element in half to separate the NR binding sites resulted in the drastic reduction of drug response. Taken together, these data support the general concept of complex cooperative behavior between proteins at different sites within the ADRES acting in concert to generate maximal transcriptional activation.

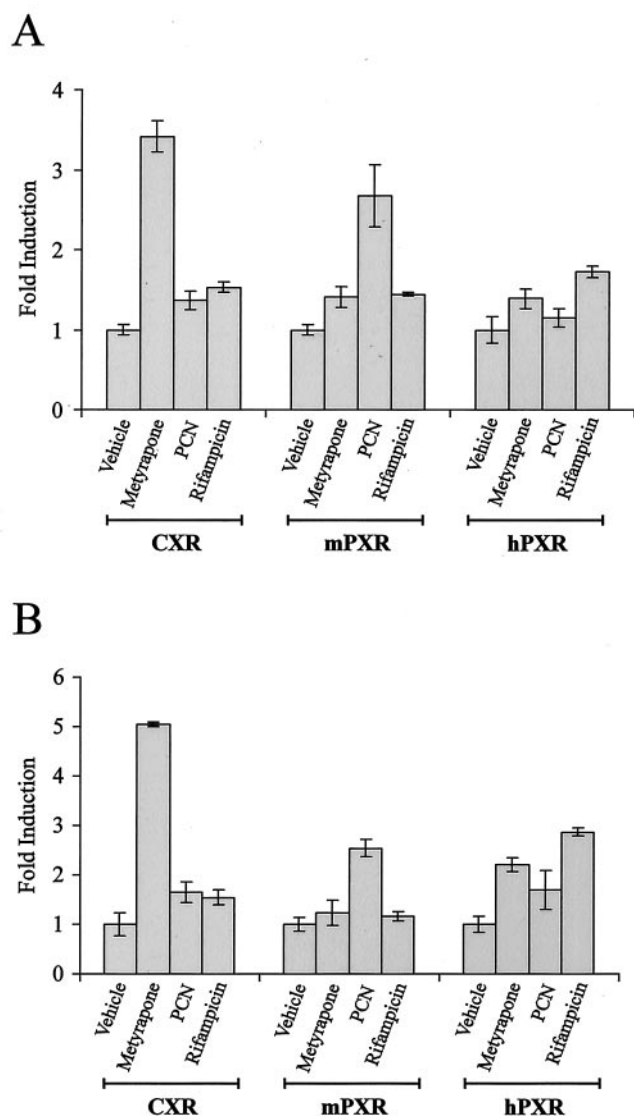


FIG. 6. Transactivation of the ADRES elements by human and mouse PXR. COS-1 cells were transfected with constructs containing either four repeats of the 176-bp element (A) or a single copy of the 167-bp element (B) cloned into the pBLCAT5 vector containing a thymidine kinase minimal promoter. DNAs encoding chicken CXR, human PXR, or mouse PXR cloned into the pSG5 expression vector were co-transfected along with a vector expressing pSV β -galactosidase as control. Cells were then treated for 16 h with either drugs or vehicle control, and extracts were analyzed for CAT expression normalized against β -galactosidase levels as described under "Experimental Procedures." Experiments were repeated at least three times, and data from representative experiments tested in triplicate are shown here. Error bars represent standard deviations.

To determine whether the effects elicited by the ADRES elements are a true reflection of the *in vivo* situation, we compared the pattern of induction in reporter gene assays with the mRNA induction in LMH cells treated with a variety of known ALAS1 inducers. The data indicate that the transcriptional activation mediated by the ADRES in reporter gene assays is comparable with that observed at the mRNA level (Fig 2). In comparison, our previous studies of the chicken CYP2H1 induction also revealed strikingly similar induction patterns in LMH cells when compared with those of ALAS1 both at the mRNA level and in ADRES reporter gene constructs (10). High induction with PB, glutethimide, PIA, and metyrapone was observed, with the other inducers listed exhibiting only modest or no effects (Fig 2). These findings were

reflected in subsequent transactivation assays where positive induction responses were obtained only for compounds that increased transcription in reporter gene assays in LMH cells.

The binding of CXR as a heterodimer with RXR to the ADRES elements in EMSAs supports a role of CXR in ALAS1 regulation. Double mutant constructs still bind NR heterodimers in the 167-bp ADRES, but at reduced levels when compared with the wild type sequences. In comparison, the binding of CXR to the 176-bp ADRES is virtually eliminated when the DR4 and DR5 sites within this sequence are mutated. It is unclear why the 167-bp mutant retains residual binding of CXR/RXR complexes in gel-shift assays. This might indicate that the NR binds at other position(s) on the element that is not conducive for transcriptional activation. The gel shift results with the 167-bp fragment with varying CXR concentrations suggest that the binding of CXR/RXR heterodimers to each of the DR4 elements is mutually exclusive. The question of whether the 167- and 176-bp elements act in a cooperative fashion also remains unresolved. We conclude that CXR can indeed bind to both the 167- and 176-bp elements and that these interactions can be reduced when the DR4 and DR5 binding motifs are eliminated. These findings are consistent with the concept that CXR activates transcription of ALAS1.

Transactivation studies demonstrate a role for CXR in the activation of the ADRES elements in COS-1 cells. Mutation of the DR4 and DR5 transcription factor binding sites eliminated transactivation, providing compelling evidence of the interactive role of CXR with these sequences. The requirement of multiple copies of the 176-bp element for strong drug activation remains unexplained, but may reflect the involvement of different coactivators *in vivo*. Notably, transactivations of the ADRES elements performed in related CV-1 cells resulted in no drug activation of the 176-bp element but strong activation of the 167-bp sequence. Because the pSG5 vector contains an SV-40 origin of replication, these vectors will be amplified in COS-1 cells, which express the T-antigen, but not in CV-1 cells. Thus, CXR may be expressed in higher concentrations in COS-1 than in CV-1 cells. These findings imply that different amounts of nuclear receptor(s) may be required for the different enhancers. Studies of drug induction of cytochromes P450 using these systems have resulted in similar levels of activation, providing another compelling argument that CXR is responsible at least in part for the drug-mediated activation of ALAS transcription *in vivo* (13, 34, 35).

The observations that human PXR and mouse PXR can transactivate the ADRES elements are compelling evidence that the regulation of ALAS1 is similar to that found for the cytochromes P450 (36). Our data indicate that ALAS1 induction profiles are most likely determined by the specific ability of drugs to bind and activate individual nuclear receptors via their ligand binding domains. We conclude from these studies that the induction profiles for the ADRES elements are determined more by the nuclear receptors used and that the DNA response elements are interchangeable. Moreover, the high correlation between the transactivation of ADRES elements with PXR reported here and previous studies with enhancers of the cytochromes P450 validate our system as a reliable tool for the evaluation of transactivation potential by CXR and other nuclear receptors involved in the regulation of the ALAS1 gene.

We conclude that the regulation of chicken ALAS1 expression, and therefore heme synthesis, is mediated through the direct transcriptional activation of a drug-responsive enhancer unit. This ADRES responds to a wide range of inducer compounds and closely reflects the *in vivo* induction of ALAS1 mRNA. The stimulation of heme synthesis provides an efficient

mechanism of drug induction similar to that found in inducible CYP systems. The coordination of heme to the needs of apoprotein synthesis is then achieved by feedback regulation of ALAS1 mRNA and ALAS1 transport into mitochondria (1). This relationship may explain the orchestrated up-regulation of heme and CYPs observed in mammalian systems exposed to xenobiotics. Moreover, because the up-regulation of heme synthesis always accompanies CYP induction, ADRES-mediated regulation of ALAS1 may serve as an excellent model system for the analysis of new chemicals and their effects on enzyme induction, including those that initiate acute attacks of porphyria.

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