Functional Analysis of a Metal Response Element in the Regulatory Region of Flounder Cytochrome P450 1A and Implications for Environmental Monitoring of Pollutants

Nick A. Lewis, Tim D. Williams, and James K. Chipman

School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom

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Cytochrome P450 1A (CYP 1A) is a member of a multigene family of xenobiotic metabolizing enzymes. CYP 1A is highly inducible by numerous environmental contaminants including polycyclic aromatic hydrocarbons (PAHs) and is widely used in biomonitoring studies. Therefore, understanding the regulation of this gene is important for accurate interpretation of biomarker data. We describe here the functional role of a metal response element (MRE) in the European flounder CYP 1A promoter region. To help elucidate the potential role of this MRE, reporter gene constructs, with or without site-directed mutagenesis, were used in conjunction with a dual-luciferase assay. The electrophoretic mobility shift assay (EMSA) was also used to investigate potential protein binding at this MRE site. Treatment with the prototypical PAH 3-methylcholanthrene (3MC) (1.0 μM) produced a dose-dependent response at the CYP 1A promoter, whereas treatment with cadmium (0–1.0 μM) produced little transcriptional activity at either the wild-type or mutated promoter. Cotreatment with cadmium (1.0 μM) and 3MC (1.0 μM) reduced induction at this promoter to 1.83-fold compared to 3MC treatment alone (4.0-fold induction). Mutation of the MRE site resulted in abolishment of this cadmium-related loss of 3MC-dependent activity. Furthermore, a retarded band was observed in the EMSA when the MRE was used as a probe and incubated with liver nuclear protein from flounder treated with cadmium. The results not only add to knowledge of the diversity of vertebrate CYP 1A regulation but also raise the complexity of interpretation of CYP 1A induction in monitoring studies that involve mixtures of PAHs and metals.

Key Words: flounder; CYP 1A; cadmium; metal response element; gene expression.

Cytochrome P450s (CYPs) constitute a superfamily of microsomal monooxygenases capable of catalyzing a variety of reactions generally leading to the oxidation of endogenous and exogenous substrates (Morel and Barouki, 1998). Several genes of this superfamily are inducible by xenobiotics that bind to and activate specific intracellular receptors, consequently modulating gene expression (Berndtson and Chen, 1994). The CYP 1 family is inducible by, and involved in, oxidative metabolism of exogenous chemicals including polycyclic aromatic hydrocarbons (PAHs) (Hahn and Stegeman, 1994; Hahn et al., 1996; Minsavage et al., 2003).

The induction of the Cytochrome P450 1A (CYP 1A) gene by PAHs is a paradigm of xenobiotic gene regulation and is known to be controlled at the level of transcription. PAHs bind to and activate the cytoplasmic aryl hydrocarbon receptor (AhR) including that of fish (Leaver et al., 1993). Following activation, the AhR translocates to the nucleus where it forms a heterodimer with the aryl hydrocarbon nuclear translocator protein. This dimer binds to specific DNA sequences, known as xenobiotic response elements (XREs), upstream of the CYP 1A promoter (Huang et al., 2000; McFadyen et al., 2003). This interaction with consensus XREs in the CYP 1A promoter and other AhR-responsive genes and subsequent recruitment of coactivators and general transcription factors results in transcription (Kumar et al., 1999; Mimura and Fujii-Kuriyama, 2003; Swanson, 2002). XREs are conserved among many species including mouse, rat, human, trout, and European flounder CYP 1A genes and have been shown to be required for inducer-dependent transcription. Eight XRE consensus sequences have been identified in the European flounder promoter, of which four appear to be functional (Lewis et al., 2004). In addition, a number of consensus sequences corresponding to other response elements have been identified in the European flounder CYP 1A promoter. Due to the habitat of the European flounder and its use as a sentinel species, of particular interest is the identification of two metal response elements (MREs), which indicates potential regulation of this CYP 1A gene by heavy metals (Williams et al., 2000). As one of these MREs is in close proximity to the TATA box, it is hypothesized that binding of a metal-induced transcription factor at this site could block binding of the TATA-binding protein. For the purposes of this study, attention was focused on the MRE situated close to the TATA box.
Numerous classes of pollutants, including PAHs and heavy metals, are present in aquatic environments, and exposure of aquatic organisms is not on an individual basis but rather to a multitude of xenobiotics simultaneously (George and Young, 1986). One such class of pollutants may modulate xenobiotic-metabolizing enzymes and, therefore, exhibit stimulatory or inhibitory effects on the metabolism and detoxification of another class. This can have adverse effects on an organism’s health (George and Young, 1986). As CYP 1A levels are considered to be a useful biomarker of exposure to environmental PAHs within numerous national monitoring programs (Kirby et al., 2004), an understanding of the regulation of this gene and its responsiveness to various classes of pollutant is an important factor in the interpretation of biomonitoring information.

The aim of the present study was to elucidate the functional roles for the putative MRE that has previously been shown to exist within this promoter. Site-directed mutagenesis, dual-luciferase reporter gene assays, and the electrophoretic mobility shift assay (EMSA) were employed to examine the role of these DNA sequences and to assess if they have the potential to bind and regulate transcription at the European flounder CYP 1A promoter. 3-Methylcholanthrene (3MC) and cadmium were selected as stressors because they are effective model inducers of CYP 1A and metal transcription factors, respectively, in aquatic organisms (Billiard et al., 2002; Schlenk and Rice, 1998). Both 3MC and cadmium are associated with contaminated aquatic sediment and are, therefore, of environmental relevance. Furthermore, the flounder is a bottom-dwelling fish and is readily exposed to these xenobiotics in its natural habitat.

**MATERIALS AND METHODS**

**Chemicals.** Minimum Eagle’s medium, penicillin-streptomycin, L-glutamine, fetal bovine serum (FBS), cadmium, and 3MC were purchased from Sigma (Gillingham, UK). FuGene6 was from Roche (Lewes, UK). Poly(dI-dC)-(dI-dC) synthetic competitor DNA homopolymer was purchased from Amersham Pharmacia Biotech (Amersham, UK). γ-32P-Adenosine-5'-triphosphate (ATP) specific activity, 7000 Ci/mmol, was obtained from ICN Pharmaceuticals (Basingstoke, UK).

**Cell culture.** PLHC-1 cells (*Poeciliopsis lucida*), a Top minnow hepatoma cell line, were cultured in minimum Eagle’s medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and L-glutamine (2 mM) and maintained as previously described (Ackerman et al., 2004). Cell lines were routinely maintained at 30°C (0–1.0 μM) and cadmium (0–1.0 μM) alone or in combination was added; cells were incubated for 16 h in a 30°C humidified chamber (5% CO2; 95% air). In addition, cells were treated with DMSO vehicle alone.

**Reporter gene construction and site-directed mutagenesis.** The European flounder CYP 1A promoter (EMBL Acc. No. AJ132353) was ligated into the pGL3-basic plasmid (Promega, Southampton, UK) and designated P1. To analyze the functional properties of the MRE (centered at −41), site-directed mutagenesis using mismatched primers (Table 1) was employed. PCRs performed (temperature profile, 1 min at 95°C, 1 min at 58°C, and 2 min at 72°C) in a DNA thermal cycler (Techne, Stone, UK). PCR products were purified, restriction digested (Table 1) according to the manufacturers’ instructions, and triple ligated into the linearized pGL3-basic plasmid. This construct was designated P10 and sequenced using the GLPrimer2 (Promega), which is complementary to the N-terminal of the luciferase gene.

**Transfection and cell treatments.** For transient transfection, PLHC-1 cells were seeded at a density of 5.0 x 104 cells per well (2 ml media per well). Cells were transiently cotransfected using FuGene6 with one of the experimental (P1 and P10) plasmids (1 μg) and the pRL-CMV control vector (1 μg). Following transfection, cells were washed in phosphate-buffered saline (PBS, 200 μl), and fresh vehicle containing 3MC (0–1.0 μM) and cadmium (0–1.0 μM) alone or in combination was added; cells were incubated for 16 h in a 30°C humidified chamber (5% CO2; 95% air). In addition, cells were treated with DMSO vehicle alone.

**Reporter gene assay.** Cells were harvested using Passive lysis buffer (Promega) and centrifuged and treated with DMSO vehicle alone. The cells were treated with DMSO vehicle alone.

**Preparation of nuclear extracts from European flounder liver tissue.** The livers of European flounder treated with corn oil or cadmium were kindly donated by Sheader et al. (2004). Following thawing on ice, livers were macerated and washed in ice-cold PBS. All solutions, tubes, and centrifuges were maintained at 4°C. PBS was then removed by centrifugation at 250 x g for 10 min. The cells were resuspended in five volumes of buffer A (10M HEPES [pH 7.9], 10mM KCl, 1.5mM MgCl2, 0.5mM dithiothreitol [DTT]), 0.5mM phenylmethylsulfonyl fluoride [PMSF], 0.5 μg/ml leupeptin, and 0.5 μg/ml pepstatin A) and left on ice for 10 min. The cells were pelleted by centrifugation at 250 x g for 20 min. The supernatant was removed, and cells were resuspended in three volumes of buffer B; Nonidet P-40 was added to 0.05%. The nuclei were then homogenized with 20 strokes of a tight-fitting Ultra-Turrax T8 homogenizer (IKA Laboratories, Staufen, Germany) and the dual-luciferase assay kit (Promega) according to the manufacturer’s instructions. Firefly luciferase expression was normalized to that of Renilla, and data were expressed as mean fold induction over DMSO controls.

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**Electrophoretic mobility shift assay.** DNA probes were prepared by PCR across the MRE, and thus, the resulting fragment possessed a single response element. Probes were prepared by PCR from P1 and P10 constructs. The forward primer 5'-CACAGACAGATATCCACATAAC-3' (containing a EcoRV site) and the reverse primer 5'-GGTGGAGGACGCATGACGTGAT-3'.
were used to generate DNA probes containing the MRE (P1*) and a mutated version of the MRE (P10*). The PCR products were digested with EcoRV and dephosphorylated with Calf intestinal alkaline phosphatase (New England Biolabs, Hitchin, UK), according to the manufacturer’s instructions. The resulting dephosphorylated DNA probes were 5′ end labeled with \( ^{32} \text{P}-\text{ATP} \) by incubation with T4 polynucleotide kinase (New England Biolabs) according to the manufacturer’s instructions. DNA-protein binding reactions containing 2 \( \mu \)g nuclear protein extract, 50,000 cpm of \(^{32}\text{P}\)-labeled DNA probe, 1 \( \times \) gel-binding buffer (20% glycerol, 5mM MgCl\(_2\), 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, 50mM Tris-HCl [pH 7.5]), and 2 \( \mu \)g of poly (dl-dC) were incubated for 15 min on ice to allow complex formation. Where indicated, the unlabeled fragment was added at 100-fold molar excess to act as a specific competitor. Unlabeled DNA fragments were incubated for 10 min before the addition of the radiolabeled probe. Resultant protein-DNA complexes were resolved by 4% nondenaturing electrophoresis at 200 V for 1–2 h. The gel was transferred to Whatman 3M paper and dried for 1–2 h under vacuum at 80°C.

\[ \text{Statistical analysis.} \] Data were analyzed using parametric tests (ANOVA, two-sample t-test, or linear regression) using the PRISM statistical analysis package. A value of \( p = 0.05 \) was taken as the level of significance.

RESULTS

Expression of European Flounder (Platichthys flesus) CYP 1A Promoter in PLHC-1 Cells

In preliminary experiments, PLHC-1 cells were transiently cotransfected with the P1 and pRL-CMV reporter plasmids and treated with 3MC (0–1.0 \( \mu \)l) in DMSO or DMSO alone. CYP 1A induction was measured as the normalized firefly to Renilla luciferase ratio. DMSO and untreated negative controls showed that there was constitutive expression of the firefly luciferase from the CYP 1A promoter and that DMSO had no statistically significant effect on induction and was, therefore, a suitable vehicle for 3MC (data not shown). To allow for the constitutive expression from the CYP 1A promoter, it was necessary to classify CYP 1A induction as fold induction over the DMSO control.

Treatment of PLHC-1 cells with 3MC produced a dose-dependent response, with measurable inductions at exposure levels as low as 0.05\( \mu \)M (Fig. 1). Treatment with 3MC (0.5–1.0\( \mu \)M) resulted in a statistically significant increase in CYP 1A induction (2.9- and 4.0-fold, respectively) compared to control incubations (\( p < 0.05 \), paired t-test).

Effect of 3MC and Cadmium on Expression of European Flounder CYP 1A Promoter in PLHC-1 Cells

PLHC-1 cells were transiently cotransfected with the P1 and pRL-CMV reporter plasmids and treated with cadmium (0–1.0\( \mu \)M) in DMSO, cadmium and 3MC (1.0\( \mu \)M) in DMSO, or DMSO in FBS-free medium. Although treatment with cadmium (0.05–1.0\( \mu \)M) resulted in a measurable decrease in reporter gene expression, this reduction was not found to be statistically significant compared to control incubations (Fig. 2) (\( p > 0.05 \), paired t-test).

Co-treatment with cadmium (0.5–1.0\( \mu \)M) and 3MC (1.0\( \mu \)M) produced a statistically significant inhibition of reporter gene activity compared to the control (3MC, 1.0\( \mu \)M) (\( p < 0.01 \), Dunnett’s ANOVA) (Fig. 3).

Mutagenesis of MRE

The putative MRE (centered at \( -41 \)) is believed to provide a binding site for a transcription factor induced in response to a metal insult. Mutations within this MRE were created using site-directed mutagenesis. Either the full-length promoter (P1) or the full-length mutated promoter (P10) was then transiently transfected in PLHC-1 cells.

Effect of cadmium treatment on Luc induction in PLHC-1 cells transfected with the P1 reporter vector.

\[ \text{FIG. 1. Dual-luciferase assay of the effect of 3MC on transcriptional activity at the flounder CYP 1A promoter. Results are expressed as a fold induction over a DMSO control for Luc activity normalized to Ren activity. Results are means of six independent experiments \pm SDs. \text{*} p < 0.05 \text{ paired t-test.} \]

\[ \text{FIG. 2. Dual-luciferase assay of the effect of cadmium on transcriptional activity at the flounder CYP 1A promoter. Results are expressed as a fold induction over a DMSO control for Luc activity normalized to Ren activity. Results are means of six independent experiments \pm SDs.} \]

\[ \text{Dunnett’s ANOVA} \] (Fig. 3).
As expected, mutation of this MRE did not affect transcription activity following treatment with 3MC (0–1.0 μM) compared to construct P1, with both constructs producing a similar reporter gene activity (Fig. 4A), thus, confirming that the site was not involved in inducing 3MC-dependent transcriptional activity at the CYP 1A promoter. The effect of cell treatment with cadmium on transcription activity at the CYP 1A P10 construct can be seen in Figure 4B. At non-cytotoxic concentrations no effect on constitutive transcription was observed in this study.

To determine if the reduction of 3MC-dependent CYP 1A activity by cadmium, described earlier, was due to a metal-induced transcription factor binding at the MRE, PLHC-1 cells transfected with the CYP 1A P10 construct were treated with both cadmium (0–1.0 μM) and 3MC (1.0 μM) (Fig. 5). The mutation of the MRE resulted in the abolishment of the statistically significant effect that cadmium had on 3MC-dependent transcriptional activity at the CYP 1A promoter. Mutation of the MRE led to reporter gene activity returning to a level similar to that with 3MC (1.0 μM) treatment alone.

Electrophoretic Mobility Shift Assay

The DNA-binding abilities of the wild-type and mutated MRE fragments were compared to determine whether the cadmium-related reduction of 3MC-dependent reporter gene activity was due to a metal-induced transcription factor binding at the MRE. Sequence-specific, DNA-binding proteins have been identified as binding to the CYP 1A promoter by using 32P-end-labeled promoter fragments and nuclear protein extracts from liver of normal flounders and of those treated with cadmium. By using the gel shift assay, a specific DNA-binding protein was identified which recognizes a sequence in the promoter region equivalent to position – 41 upstream of the transcription start site (Fig. 6). As expected, liver nuclear protein from untreated flounder did not bind to the MRE, whereas protein from cadmium-treated flounder produced a shift in the banding pattern that was abolished when in the presence of a molar excess of unlabeled probe. Furthermore, the banding pattern was also abolished when protein from cadmium-treated flounder was incubated with the mutated MRE.

**DISCUSSION**

In aquatic organisms that inhabit environments often contaminated with high levels of organic pollutants, induction of CYP 1A levels is considered a useful biomarker (Goksøyr, 1995). Therefore, a great deal of data on the enzymatic activity including that of the European flounder has been reported. As the enzymatic activity can often be influenced by many factors, measurement of transcribed RNA is considered to be a reliable alternative when measuring induction. To interpret...
data obtained in monitoring programs, an understanding of the regulation of this gene and its responsiveness to various classes of pollutants is a necessity.

The European flounder CYP 1A promoter contains numerous consensus sequences corresponding to response elements; eight XREs of which four appear to be functional, and two putative MREs (Lewis et al., 2004). The purpose of this study was to elucidate the functional role of the putative MRE that exists in close proximity to the TATA box within this promoter.

The 3MC dose-dependent inducibility of the CYP 1A reporter gene construct concurs with the general observation that CYP 1A expression is regulated at the transcriptional level via the AhR (Todd et al., 1995; Whitlock et al., 1996; Williams et al., 2000). Induction characteristics of the CYP 1A gene may be species dependent as treatment of both Atlantic tomcod (Microgadus tomcod) and smooth flounder (Pleuronectes putna) with β-naphthoflavone resulted in a 97- and 14-fold induction of the CYP 1A gene, respectively. These differences are substantiated by the observation that liver tumors are more frequent in these two species when habituating in the same polluted environment (Wirgin et al., 1996).

Both simultaneous and sequential exposures to heavy metals such as cadmium and AhR ligands are common environmental problems with biological consequences. Yet, there have been relatively few studies of combined effects of heavy metals and PAHs on CYP 1A regulation. As far as we are aware, the flounder is one of only three species found to contain MREs in the CYP 1A promoter. Although the numbers of these MREs vary, the common theme among the flounder, Japanese medaka (Oryzias latipes), and Rivulus (Rivulus marmoratus) is that the CYP 1A promoter contains both proximal and distal MREs (Kim et al., 2003). Coexposure to high doses of both cadmium and 3MC produced a statistically significant reduction of 3MC-induced CYP 1A reporter gene activity. The presence of a putative MRE site in the CYP 1A promoter does not appear to confer any activation effect on transcriptional induction. Mutation of this site does not alter transcriptional activity at this promoter in response to 3MC or cadmium alone. However, this site (in close proximity to the TATA box) does appear to confer a repression effect in response to a mixture of 3MC and cadmium. Cadmium reduced the transcriptional activity induced by 3MC, and mutation of this MRE resulted in abolishment of this cadmium-related effect. Since the MRE and TATA box consensus sequences are only separated by two bases, binding of a heavy metal–induced transcription factor at the MRE may inhibit binding of the TATA-binding protein. Indeed, we found in EMSA experiments using the MRE as a probe that inclusion of nuclear protein from livers of cadmium-treated fish resulted in the appearance of a retarded complex. This suggests that a metal-induced transcription factor is capable of binding to this MRE site. To the best of our knowledge, results presented here are unique in showing inhibition of CYP 1A expression by a heavy metal and identifying the CYP 1A promoter sequence to which a transcription factor can bind in response to a heavy metal insult. The findings may provide a mechanism for the recently reported observation that coexposure to metals modulates CYP 1A mRNA inducibility in Atlantic tomcod (Sorrentino et al., 2005). Because differential effects on MREs have been reported for different metals, future studies on alternative metals such as zinc would be valuable as would studies on insertion of the MRE into the promoters of non–MRE-containing CYP 1A genes using different host cells. To date, MREs have not been identified in the promoter regions of mammalian CYPs. Other inhibitory effects of metals on CYP expression have been previously attributed to metal-induced oxidative stress such as those seen in black sea bream (Spondylus canthus) hepatocyte cultures with 3MC (1.0 µM) and cadmium (1.3 µM) (Risso-de Faverney et al.,...
The mechanism of heavy metal–mediated CYP expression in fish appears to function at both the transcriptional and posttranscriptional levels. Organotins such as tributyltin and triphenyltin have been shown to inhibit CYP1A activity in rainbow trout (*Oncorhynchus mykiss*), European eel (*Anguilla anguilla*), bullhead (*Ictalurus nebulosus*), and scup (*Stenotomus chrysops*) (Fent and Hunn, 1996; Fent and Stegeman, 1991, 1993; Fent *et al.*, 1998). Likewise, the heavy metal cadmium has been shown to inhibit CYP1A activity in flounder and plaice (George, 1989; George and Young, 1986; Sandvik *et al.*, 1997), but this was thought to be posttranslational. Since AhR-dependent gene expression may be inhibited by cellular oxidative stress (Maier *et al.*, 2000), the observed inhibitory effects of heavy metals on CYP1A 1A activity and activity could be related to cellular thiol status (Ghosh *et al.*, 2001; Maier *et al.*, 2000). Although the reason for the functional MRE in flounder CYP1A promoter is unknown, it is possible that its purpose is to limit oxidative stress that might occur from combined high CYP expression and prooxidant metal exposure. However, in contrast to findings in our system, CYP1A induction in European eel due to cadmium treatment has been observed, and this illustrates the complexity of interspecies differences in regulation of gene expression (Lemaire-Gony *et al.*, 1993). Studies of the CYP1A gene in its native chromosomal setting suggest that the CYP1A transcriptional control region assumes a nucleosomal configuration, a structure which restricts access to the TATA box (Durrin and Whitlock, 1989; Ko *et al.*, 1997; Wu and Whitlock, 1992). Should this be the case in the European flounder, then as the TATA box and MRE are in close proximity, access to this MRE may be limited. Further in vivo studies would be required to elucidate this issue of promoter accessibility and occupancy.

Results presented here, in combination with the posttranslational effects discussed, not only add to the considerable knowledge and diversity of CYP1A regulation but also raise questions over the use of CYP1A induction of flounder and possibly other fish in biomonitoring studies. If the presence of heavy metals cannot be discounted and complex mixtures of pollutants are present, then the use of CYP1A activity in flounder would not be appropriate as data may not be reflective of true PAH exposure and effect. Field organisms are exposed to pollutant mixtures including heavy metals, and therefore, for an accurate evaluation, a battery of biomarkers consisting of both generic and specific stress indices should be utilized.

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