RALDH-independent generation of retinoic acid during vertebrate embryogenesis by CYP1B1

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Several independent lines of evidence have revealed an instructive role for retinoic acid (RA) signalling in the establishment of normal pattern and cellular specification of the vertebrate embryo. Molecular analyses have previously identified the major RA-synthesising (RALDH1-3) and RA-degrading (CYP26A-C1) enzymes as well as other components involved in RA processing (e.g. CRABP). Although the majority of the early effects of RA can be attributed to the activity of RALDH2, many other effects are suggestive of the presence of an as yet unidentified RA source. Here we describe the identification, expression, biochemistry and functional analysis of CYP1B1, a member of the cytochrome p450 family of mono-oxygenases, and provide evidence that it contributes to RA synthesis during embryonic patterning. We present in vitro biochemical data demonstrating that this enzyme can generate both all-trans-retinal (t-RAL) and all-trans-retinoic acid (t-RA) from the precursor all-trans-retinol (t-ROH), but unlike the CYP26s, CYP1B1 cannot degrade t-RA. In particular, we focussed on the capacity of CYP1B1 to regulate the molecular mechanisms associated with dorsoventral patterning of the neural tube and acquisition of motor neuron progenitor domain identity. Concordant with its sites of expression and biochemistry, data are presented demonstrating that CYP1B1 is capable of eliciting responses that are consistent with the production of RA. Taken together, we propose that these data provide strong support for CYP1B1 being one of the RALDH-independent components by which embryos direct RA-mediated patterning.

KEY WORDS: Cyp1B1, Retinoic acid, Hindbrain, Motor neurons, Dorsoventral, Shh, Hoxb1, Chick

INTRODUCTION

Retinoic acid (RA), the biologically active derivative of vitamin A (retinol), is well established as a potent regulator of many aspects of early embryo development. Both gain- and loss-of-function studies have provided direct evidence that endogenous retinoids are required for appropriate specification and patterning of several tissues including the hindbrain (Maden et al., 1996; Gale et al., 1999; Dupé and Lumsden, 2001; Niederreither et al., 1997; Niederreither et al., 2000; Sirbu et al., 2005) (reviewed by Gavalas and Krumlauf, 2000; Maden, 2002), spinal cord (Sockanathan and Jessell, 1998; Pierani et al., 1999; Novitch et al., 2003; Diez del Corral et al., 2003; Wilson et al., 2004) (reviewed by Wilson and Maden, 2005), eye (Hyatt et al., 1996; Dickman et al., 1997; Wagner et al., 2000) (reviewed by Drager et al., 2001) and branchial arches (Maden et al., 1996; Niederreither et al., 1999; Niederreither et al., 2003; Quinlan et al., 2002) (reviewed by Mark et al., 2004). The ability of RA to exert its full range of effects relies upon its capacity to signal in both an autocrine and paracrine fashion and alter gene expression in the recipient cell.

In the embryo, RA is initially produced intracellularly by the stepwise oxidation of maternally derived retinol to retinal and subsequently of retinal to RA. The first reaction is catalysed by the retinol or alcohol dehydrogenases (RoDHs or ADHs), and the second by retinal dehydrogenases (RALDH1-4) (reviewed by Duester, 2000; Maden, 2002). The newly synthesised RA is bound in the cytoplasm by cellular RA-binding protein (CRABP), whereupon it enters the nucleus and binds to the nuclear RA receptors (RARs) and retinoid X receptors (RXRs), which are ligand-dependent transcriptional regulators. Following heterodimerisation, the RA-RAR-RXR complex modulates the expression of target genes by binding to RA response elements (RAREs). RA can be further metabolised by oxidative inactivation via three members of the cytochrome p450 enzyme family (CYP26A-C1) to products such as 4-αx-RA, 4-OH-RA and 5,8-epoxy-RA (Fujii et al., 1997; White et al., 1996; Tahayato et al., 2003). The RA metabolites are generally considered to be biologically inactive (e.g. Niederreither et al., 2002a), but other studies have reported that they might themselves be capable of evoking a signalling response (Pijnappel et al., 1993; Nikawa et al., 1995; Reijntjes et al., 2005). ADH3, a member of the alcohol dehydrogenase family, is expressed ubiquitously, presumably producing retinal to be supplied as a substrate to the RALDHs throughout the embryo (Molotkov et al., 2002). By contrast, accumulated data show that the genes encoding the RALDHs, CYP26s, RARs and RXRs, all have discrete sites of expression and activity during early development and that the availability of each of these factors contributes to the observed specificity of retinoids (e.g. Blentic et al., 2003).

Genetic inactivation, biochemical and expression approaches have revealed that the majority of early RA-signalling activity in the embryo can be attributed to RALDH2 (Niederreither et al., 1997; McCaffery and Dräger, 1995; Blentic et al., 2003). However, compounding all of the sites of the RALDH2 activity with Raldh1 and Raldh3 expression and their inferred activity is insufficient to account for all of the early patterning defects seen following full vitamin A deprivation in either the quail (Maden et al., 1996; Gale et al., 1999) or rat (White et al., 1998; White et al., 2000). Thus, it is unlikely that these three enzymes alone can account for all of the observed RA-patterning processes. Using conditional rescue of Raldh2-null embryos carrying a RA-responsive lacZ transgene, recent work has identified several sites of tissue-specific non-RALDH-dependent synthesis in early mouse embryos (in mouse, ¹Wellcome Trust Functional Genomics Development Initiative, MRC Centre for Developmental Neurobiology and ²MRC Centre for Developmental Neurobiology, 4th Floor New Hunt's House, King's College London, Guy's Campus, London SE1 1UL, UK.  
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RALDHs are also known as ALDH1a/8a) and this has raised the possibility that new classes of RA-synthesising enzyme exist (Niederrreither et al., 2002b; Mic et al., 2002; Rossant et al., 1991). The main sites of RALDH-independent RA-synthesis were seen throughout the lumbar and cervical levels of the ventral spinal cord, extending into ventral regions of the posterior hindbrain. Such sites indicate a potential function for RALDH-independent synthesis in both dorsoventral (DV) and anteroposterior (AP) patterning. Other studies in chick have reported that the distribution of endogenous retinoids is wider than would be predicted (e.g. in the mesoderm surrounding the hindbrain) from the expression of Raldh1-3 alone (Maden et al., 1998). To date, the identity of the enzyme(s) responsible for these additional RA activities remains elusive.

We have previously observed the spatiotemporal distribution of Cyp1B1, a member of the cytochrome p450 family, expression during mouse embryonic development (D.C. and A.L., unpublished). Cytochrome p450s constitute a large gene family of monomeric multifunctional mono-oxygenases that function by coordinating one oxygen atom in a substrate to generate a new functional group. In this study, we describe the identification of the chick Cyp1B1 orthologue and describe its expression during early development as being associated with many known sites of RA activity. We provide biochemical evidence that Cyp1B1 alone is sufficient to efficiently oxidise retinol to retinal and subsequently to RA but that, unlike the CYP26s, CYP1B1 cannot participate in the breakdown of RA. Furthermore, we show that Cyp1B1 can regulate DV patterning of the hindbrain and spinal cord in a manner that is consistent with both its endogenous expression and its RA-synthetic properties. We also provide evidence that Cyp1B1 activity can contribute to AP patterning as well as being involved in the regulation of epibranchial placode neurogenesis. Consequently, these data describe for the first time the presence of an RALDH-independent RA-signalling mechanism operating during early embryogenesis that can account for some of the non-RALDH RA activity that has previously been observed.

MATERIALS AND METHODS

Embryo staging

Chicken embryos were collected from fertilised brown chicken eggs (Needles Egg Farm, Hertfordshire, UK) and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951).

Molecular analyses

Whole mount in situ hybridisation with digoxigenin-labelled riboprobes was performed as described by Wilkinson (Wilkinson, 1992) with the exception that the embryos were fixed in MEMFA [100 mM MOPS, 2 mM EGTA, 1 mM MgSO₄, 3.7% (v/v) formaldehyde]. Embryos analysed by sectioning were embedded in 20% gelatin and fixed in 4% PFA in PBS for at least 3 days. Sections were cut at 40 µm on a vibratome and mounted in 80% glycerol in PBS before being photographed using a digital camera.

Vitamin A-deficient (VAD) embryos and rescue experiments

VAD embryos were obtained from Japanese quail (Coturnix coturnix japonica) hens fed on a semi-purified diet containing 10 mg/kg t-RA as the only source of retinoids. Normal quail eggs were obtained from a local supplier. Vitamin A rescue experiments were carried out by windowing an incubated egg and injecting 10 µl of a retinol solution as described by Maden et al. (Maden et al., 1996).

Oxidation of retinoids catalysed by CYP1B1

The ability of CYP1B1 to oxidise RA precursors was measured using microsomes prepared from a baculovirus expression system containing the in vitro-translated hCyp1B1 cDNA as well as a human p450 reductase (BD Biosciences). Microsomes that did not contain recombinant CYP1B1 were used as a control for the activity of the human p450 reductase alone. Reactions were set up as described by Chen et al. (Chen et al., 2000) using 35 µM t-ROH as a substrate, and retinoids were subsequently extracted with n-butanol and methanol. t-RA, t-RAL and t-RA generated from t-ROL, and t-RAL or 4-oxo-RA generated from t-RA, were confirmed by HPLC analysis using a Beckman System Gold Hardware system with a UV detector (351 nm) in series with a solid scintillant radioisotope detector as described by Maden et al. (Maden et al., 1998).

In ovo electroperoration

In ovo electroporation was typically performed on stage HH10-11 embryos as described previously (Gilthorpe et al., 2002). The Cyp1B1 expression construct was generated by inserting the full-length mouse cDNA (Accession number: gi6753567 and NM_009994.1) with an enhanced Kozak sequence (J. Gilthorpe, personal communication) into the pCAB expression vector that contains an internal ribosome entry site linked to a GFP gene. Briefly, plasmids were electroperorated at a minimum concentration of 2 µg/µl with a single pulse of 15V into the left-hand side of the neural tube, followed by overnight incubation at 38°C.

Implants of FGFR, t-RA, t-RA and TMS beads

Implantation of beads coated in FGF protein was performed as described by Chambers et al. (Chambers et al., 2000). For control experiments, beads were incubated in L-15 for 1 hour prior to implantation. Beads soaked in either t-RA, t-RA, or 2,3,4,5'-Tetramethoxystilbene (TMS; Cayman Chemical Company), a highly-specific chemical inhibitor of CYP1B1, were implanted in the embryo as described by Reijntjes et al. (Reijntjes et al., 2005).

RESULTS

Identification of Cyp1B1 as a developmentally expressed gene

We have previously identified cytochrome p450 1B1 (Cyp1B1), a monomeric multifunction mono-oxygenase, as a gene expressed at discrete sites during early development of the mouse CNS (E8-12.5; D.C. and A.L., unpublished). Correlation of the Cyp1B1 expression pattern with the sites of known signalling processes suggested that this gene might be associated with retinoid-mediated patterning. Given the pivotal role of retinoids during early development and the involvement of CYP26A-C1 in this process, Cyp1B1 was selected for further investigation. To facilitate functional analysis of this gene, the chick Cyp1B1 orthologue was isolated using a database screening strategy. This approach yielded a partial cDNA (ChEST107a; 603130733F1; www.chick.umist.ac.uk) with 84% similarity at the protein level (across ~250 amino acids) to its mouse counterpart (see Fig. S1 in the supplementary material).

Cyp1B1 has a temporally and spatially dynamic expression pattern

As a prelude to functional studies, we studied the spatiotemporal expression of Cyp1B1 (Fig. 1A-V). Expression of Cyp1B1 was first detectable in the ectoderm and mesoderm of the posterior primitive streak (Fig. 1A,B, black arrowheads). By the four-somite (4s) stage, transcripts were localised to all of the newly formed somites, as well as to the mediolateral ectoderm of the sinus rhomboidalis (Fig. 1C-F). Expression throughout the somites persisted at HH9–10, but was lost from the posterior ectoderm. By HH10, transcripts were abundant in the neuroepithelium of the mid-hindbrain boundary (MHB) region (Fig. 1H,I, white arrows), in addition to weak expression in rhombomere (r) 2 (Fig. 2, II, blue arrow) and the paraxial mesoderm adjacent to the developing hindbrain (Fig. 1H, black arrow). Flat-mount preparation of a 10s embryo revealed that the expression extends across the entire DV axis of the MHB and r2 (Fig. 1I, white and blue arrows, respectively). Transverse sections through the MHB confirmed the DV extent in the neural tube and revealed strong expression in both ectoderm and mesoderm, including possibly...
neural crest-derived cells immediately adjacent to the MHB (Fig. 1J,K, blue arrow). At HH10, the somite expression was enriched in the dermamyotome, and this became more pronounced by HH12+ (Fig. 1M, white arrow). At HH12+, the MHB expression narrowed to a thin band in the posterior midbrain and an additional band of neuroepithelial expression observed in the anterior midbrain (Fig. 1L, blue arrowhead). Strong expression of Cyp1B1 in the ectoderm abutting the entire mesencephalic vesicle was seen by this stage (Fig. 1L-O). The presence of Cyp1B1 message in the mesoderm adjacent to the hindbrain persisted at HH12+ (Fig. 1P, blue arrow), as did

Fig. 1. Expression of Cyp1B1 in the chick embryo. (A,B) Expression in the ectoderm and mesoderm (black arrowheads) of the posterior primitive streak of a HH4 embryo. Black lines denote plane of sections. (C-F) 4s: transcripts are localised to all of the newly formed somites (D, black arrow), as well as to the mediolateral ectoderm of the sinus rhomboidalis (E, white arrow). Black lines denote planes of section shown in D-F. (G) 6s: at HH9+, expression persists in the somites but is lost from the posterior ectoderm. (H) HH10: transcripts are abundant in the neuroepithelium of the mid-hindbrain boundary (MHB) region (white arrowhead), in addition to weak expression in r2 (blue arrow) and the paraxial mesoderm adjacent to the developing hindbrain (black arrow). (I) HH10: flat-mount preparation showing that the expression extends across the DV axis of the MHB and r2. (J,K) HH10: transverse sections though the hindbrain showing strong expression in both ectoderm and mesoderm, possibly including neural crest-derived cells immediately adjacent to the MHB (blue arrow). (L) HH12+: MHB expression has narrowed to a thin band in the posterior midbrain, and an additional band of neuroepithelial expression is seen at the anterior midbrain (blue arrowhead). Expression is evident in the ectoderm immediately anterior to the otic vesicle (white arrowhead). Black lines denote planes of section shown in N-P. (M) Expression is enriched in the dermomyotome (white arrow). (N,O) Transverse sections showing strong expression of Cyp1B1 in the ectoderm abutting the entire mesencephalic vesicle (white arrows). (P,Q) Cyp1B1 message in the mesoderm adjacent to the hindbrain persists at HH12+ (blue arrow), as does expression in the somites. Cyp1B1 expression is also strong in the notochord (black arrows). The section in Q is more posterior than can be shown in L. (R) 30s: new sites of Cyp1B1 expression emerge, including a highly localised expression pattern in the developing eye (white arrowhead) and the epiphysis (blue arrow). (S) Longitudinal sections show expression in lens vesicle (black arrow) and neural retina (blue arrows). There is continued expression in all of the somites. (T) Transverse section at the level indicated in R shows expression throughout the notochord (black arrow) and somites. (U-W) 41s: other sites of expression include a pocket of mesodermal cells lateral to r2 (blue arrow), the sinus venosus of the heart and asymmetrically in the endoderm of the pharyngeal arches (V, blue arrows). Cyp1B1 is also expressed in the mesoderm of the forelimb bud (W, white arrow). (X) Dorsal view of Raldh2 expression in a 12s embryo. (Y) Cyp26C1 is expressed in r2 (black arrow) in a 12s embryo.
expression in the somites (Fig. 1Q, white arrowhead). Expression in
the ectoderm immediately anterior to the otic vesicle could now be
detected (Fig. 1L, white arrowhead). At this stage, Cyp1B1
expression was also strong in the notochord (Fig. 1Q, black arrow).
By 30s, several new sites of Cyp1B1 expression had emerged; most
notably, a highly localised expression pattern in the developing eye
(Fig. 1R, white arrowhead). Expression in the neural retina (Fig. 1S, black arrows). There was continued
expression in all of the somites, enriched in the dermamyotome, but
also weakly in the sclerotome (Fig. 1T). Interestingly, pronounced
expression was evident throughout the AP extent of the notochord
(Fig. 1T, black arrow). Other sites of expression included that in a
pocket of mesodermal cells lateral to r2 (Fig. 1R, blue arrowhead),
the sinus venosus of the heart and asymmetrically in the endoderm of
the pharyngeal arches (Fig. 1R, blue arrowheads). At later stages,
Cyp1B1 was expressed in the mesoderm of the forelimb bud (Fig.
1W, white arrow).

**Cyp1B1 expression is strongly correlated with known sites of RA production**

To determine likely sites of CYP1B1 activity, its expression was
correlated with known areas of RA accumulation in the developing
chick embryo (Maden et al., 1998). These findings, presented in Table
1, were also compared with the described sites of Raldh1-3 expression
with the aim of identifying exclusive regions patterned by CYP1B1. To summarise, there is strong concordance between the temporally
and spatially recorded presence of RA and the expression of Cyp1B1. Although some of these finding can be attributed to the overlapping
expression of Raldh1-3, there are some regions where there are high
RA levels associated with Cyp1B1 expression but not Raldh gene
expression. For example, Cyp1B1 is abundantly expressed in the
hindbrain paraxial mesoderm and this region contains high RA levels
(~50% of somite level), but is negative for the expression of Raldh
genes. Other sites included the presence of RA in the branchial arches at HH15 (~15% of somite level), again a region where Cyp1B1 is
found at distinct sites (i.e. endoderm of pharyngeal arches; Fig. 1V,
blue arrowheads), but which is devoid of Raldh expression at this time.
Furthermore, the posterior limb bud of an HH24 embryo, although
deficient in Raldh gene expression, was found to contain significant
RA levels (~65% of somite level). This finding is concordant with the
presence of Cyp1B1 in this territory but its absence from the anterior
limb bud (Fig. 1U, white arrow). Together, these data provide
correlative evidence that Cyp1B1 is expressed at sites of RA
production throughout the embryo, and may also be exclusively
responsible for its synthesis in some tissues. Consistent with the
Cyp1B1 biochemistry (see below), these regions have a lower level of
RA compared with those that express a member of the Raldh family.

**Table 1. Correlation between regions of RA production in the developing chick embryo and known sites of Cyp1B1 and Raldh1-3 expression**

<table>
<thead>
<tr>
<th>Retinoid-producing tissue*</th>
<th>Raldh1-3 expression in tissue†</th>
<th>Cyp1B1 expression in tissue‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH4/5 chick embryos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posterior end of embryo</td>
<td>✓ (2 and 3)</td>
<td>✓</td>
</tr>
<tr>
<td>HH10-12 chick embryos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Somites 1-3</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>Somites 3-8</td>
<td>✓ (2)</td>
<td>✓</td>
</tr>
<tr>
<td>Heart (low)</td>
<td>✓ (2 and 3)</td>
<td>✓</td>
</tr>
<tr>
<td>Forebrain mesenchyme</td>
<td>X (3: in epithelium)</td>
<td>✓</td>
</tr>
<tr>
<td>Midbrain mesenchyme</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>Hindbrain mesenchyme</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>Spinal neural tube</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>HH15 chick embryos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Branchial arches (low)</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>Heart (low)</td>
<td>✓ (2)</td>
<td>✓</td>
</tr>
<tr>
<td>Somites</td>
<td>✓ (2)</td>
<td>✓</td>
</tr>
<tr>
<td>Lateral plate</td>
<td>✓ (2)</td>
<td>X</td>
</tr>
<tr>
<td>HH20 chick embryos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hindbrain mesenchyme</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>Somites</td>
<td>✓ (2)</td>
<td>✓</td>
</tr>
<tr>
<td>Spinal neural tube</td>
<td>✓ (2: motor neurons and roof plate)</td>
<td>X</td>
</tr>
<tr>
<td>HH24 chick embryos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Branchial arches (low)</td>
<td>✓ (2: first branchial groove)</td>
<td>✓</td>
</tr>
<tr>
<td>Heart (low)</td>
<td>✓ (2 and 3)</td>
<td>✓</td>
</tr>
<tr>
<td>Posterior limb bud</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>Eye</td>
<td>✓ (1, 2 and 3)</td>
<td>✓</td>
</tr>
<tr>
<td>Somites</td>
<td>✓ (2)</td>
<td>✓</td>
</tr>
<tr>
<td>Midbrain mesenchyme</td>
<td>X</td>
<td>✓</td>
</tr>
<tr>
<td>Forebrain mesenchyme</td>
<td>✓ (2: mesenchyme next to eye)</td>
<td>✓</td>
</tr>
</tbody>
</table>

X, sites where RA has been described but expression of either Cyp1B1 or Raldh genes is missing.
✓, sites where there is concordance between expression and RA accumulation.
The bracketed number indicates which member(s) of the Raldh family is represented in that region.
*Data on RA distribution are taken from Maden et al. (Maden et al., 1998).
†e.g. Blentic et al. (Blentic et al., 2003).
‡Figs 1, 3, 6 and data not shown.
CYP1B1 is capable of converting retinol to retinal and to all-trans-RA, but not to 4-oxo-RA or other breakdown products

In view of the association of Cyp1B1 with sites of RA abundance (Table 1), we tested whether CYP1B1 was capable of participating in the major steps of RA synthesis and/or breakdown by measuring its ability to metabolise retinol (vitamin A; ROL) to retinal (t-RAL), retinal to the biologically active all-trans-RA (t-RA), and RA to its degradative products 4-oxo-RA, 4-OH-RA and 5,8-epoxy-RA (Fig. 2).

Fig. 2A shows an HPLC trace of the metabolites generated from 35 μM ROL in the in vitro system containing NADPH, a necessary co-factor for mono-oxigenase activity. All incubations were repeated in the absence of NADPH or CYP1B1, and no metabolic conversion of the input substrate was recorded in either case (data not shown). The data support a model in which CYP1B1 can actively convert retinol to retinal and then to RA, but cannot participate in the breakdown of either the newly formed RA or RA from other anabolic sources.

Fig. 2. Determining the role of CYP1B1 in the biochemistry of t-RA synthesis and breakdown. (A) Reverse phase HPLC profile of the metabolism of t-retinol by CYP1B1 (0.025 nM CYP1B1 in 0.1 M potassium phosphate buffer pH 7.4, plus NADPH-cytochrome p450 reductase; 20 minutes at 37°C). Black arrow, input retinol; blue arrow, output retinal. (B) Reverse phase HPLC profile of the metabolism of t-retinal by CYP1B1 under the same conditions for differing lengths of time (20, 60 and 240 minutes). Black arrow, input retinal; blue arrow, output RA. The rates of synthesis of RA are given in Table 2. (C) Reverse phase HPLC profile of the metabolism of t-RA by CYP1B1 under the same conditions. All incubations were repeated in the absence of NADPH or CYP1B1, and no metabolic conversion of the input substrate was recorded in either case (data not shown).
repeated in the absence of NADPH or CYP1B1, under which circumstances no metabolic conversion of the input substrate was seen (data not shown). The data presented were performed in duplicate and found to be concordant between each study. After 20 minutes, CYP1B1 was capable of the efficient conversion of ROL to t-RAL (Fig. 2A, black and blue arrows, respectively). Similarly, HPLC analysis of the metabolites using 35 μM RAL as a substrate in the presence of CYP1B1 generated a reproducible peak consistent with the generation of t-RAL, albeit at a reduced rate than its capacity to make RAL from ROL (Fig. 2B, 20 minutes, black and blue arrows, respectively). We additionally tested for the production of RA after 60, 120 and 240 minutes, to fully reflect the length of time that embryonic tissues may be exposed to signalling from CYP1B1, as well as calculating the rate of RA production at each time point (Fig. 2B, 60 and 240 minutes, arrows labelled RA; Table 2). At each interval, a peak characteristic of RA accumulation was recorded and rates were found to vary between 55.2 and 147 ng t-RA/mg CYP1B1/hour (Table 2).

To assess whether CYP1B1 could convert t-RA to its less active metabolites, 35 μM t-RA was incubated under the same conditions described above. In duplicate experiments, CYP1B1 was found to be incapable of metabolising t-RA to any altered state after 20 (Fig. 2C, showing location of 4-oxo-RA peak if present), 60, 120 or 240 (Table 2 and data not shown) minutes. Taken together, these findings conclusively demonstrate that CYP1B1 is capable of directing the synthesis of t-RA, but, unlike the CYP26 p450s, it does not degrade t-RA.

**Cyp1B1 expression is dependent on RA at some but not all sites**

To test the dependence of Cyp1B1 expression on retinoid signalling, expression was examined in vitamin A-deprived (VAD) quail embryos and compared with the normal chick pattern (Fig. 3). Early sites of Cyp1B1 expression in the VAD quail appeared largely normal (data not shown). However, by HH10, expression at the MHB was weaker and totally absent from r2 (Fig. 3Ab,Ac, white arrows). Expression throughout the somites, hindbrain paraxial mesoderm and ectoderm surrounding the midbrain seemed to be unaffected (e.g. Fig. 3Ac, blue arrowhead). At HH12, the pattern remained similar, but the strong enrichment encapsulating the midbrain failed to manifest and expression in the ectoderm anterior of the presumptive otic placode was also lacking (data not shown). In comparison to the normal expression pattern at 23s, the most striking differences are seen in the developing eye (Fig. 3Bc, white arrow), pharyngeal arches (Fig. 3Bc, blue arrow) and sinus venosus (Fig. 3Bc, red arrow). A more detailed analysis at this stage revealed that expression is maintained in the paraxial mesoderm beside the hindbrain territory (Fig. 3Be, green arrow) and in the somites (Fig. 3Bf, yellow arrow), but that it is absent from the notochord (Fig. 3Be, orange arrow). In contrast to the 30s normal embryo where marked expression in the anterior retina and lens vesicle was observed, in the VAD embryo no Cyp1B1 transcripts could be seen (Fig. 3Cc, white arrows). Similarly, expression in the pharyngeal arches (Fig. 3Cc, blue arrow) and sinus venosus (Fig. 3Cc, red arrow) was absent, as was the pocket of expression in the mesoderm adjacent to r2 (data not shown). Thus, Cyp1B1 expression displays a differential requirement for retinoids depending on location. These findings were validated using VAD quails in which the phenotype was rescued by the exogenous addition of RA and the normal sites of Cyp1B expression were restored (Fig. 3Db and data not shown).

Although some of these changes in expression can be attributed to an initial lack of specification of the expressing tissue in the VAD quail (e.g. pharyngeal arches and sinus venosus), other changes, such as that observed in the notochord, reflect a true dependence on retinoids for the initiation of Cyp1B1 expression.

**CYP1B1 contributes to RA signalling and can pattern motor neuron domains**

To test for a functional role, we chose to study the effects of Cyp1B1 overexpression in a region where no Raldh gene expression has been recorded, yet RA has been found (Table 1). As such, the observed effects could be attributed to CYP1B1 and not to the enhanced activity of RALDHs by supplementation of retinal produced from CYP1B1. Thus, ectopic expression of Cyp1B1 was induced in a region adjacent to where it was normally expressed (i.e. hindbrain neuroepithelium versus hindbrain paraxial mesoderm, and pharyngeal ectoderm versus pharyngeal endoderm), to determine the intrinsic role rather than what CYP1B1 might be capable of doing in a region where it was not normally present. Recent data on the role of retinoids in the DV patterning of the neural tube provide a sound framework with which the results of this study can be compared (Pierani et al., 1999; Novitch et al., 2003; Wilson et al., 2004).

Following focal electroporation of the Cyp1B1-IRES-GFP construct (Fig. 4Aa), GFP expression was readily detected in the hindbrain and neural tube (Fig. 4Ab, white arrowhead, and see Fig. S3Ab,Bb,Cb in the supplementary material). Also, transcription of the Cyp1B1 cassette from the vector was confirmed by in situ hybridisation using the mouse-specific cDNA (Fig. 4Ac, blue arrowhead). Thus, Cyp1B1 is expressed in a spatially restricted and detectable manner. In all cases (Fig. 4B-Eb), control embryos were electroporated with the pCAB construct lacking the Cyp1B1 cDNA and were found to have no changes in target gene expression. Even after a more detailed examination of the neural tubes, no defects were observed (Fig. 4B-Eb).

Following the introduction of the Cyp1B1 expression construct unilaterally into the hindbrain and spinal cord of an HH10-11 embryo, the effects on Shh expression were determined. In all cases (n>20) where Cyp1B1 was expressed in the neuroepithelium, as determined by GFP expression (see Fig. S3 in the supplementary material), we noted a pronounced downregulation or complete ablation of Shh expression. Fig. 4Bd shows the isolated neural tube of a Cyp1B1-electroporated embryo where Shh expression is lost from the floorplate, as compared with normal or control electroporated embryos (Fig. 4Bb,Bc versus Bd, black arrows). Transverse sections revealed that Shh expression is depleted in the floorplate, but retained in the underlying notochord (Fig. 5Ac versus Cd). Comparison of the Cyp1B1-expressing cells with the registered effect on Shh showed this to be a non-cell-autonomous phenomenon (see Fig. S3 in the supplementary material). In addition, a small number of embryos (n=3/20) lost Shh expression from the notochord as well as from the floorplate (data not shown). These data are consistent with CYP1B1 metabolising sufficient levels of RA that can signal at a distance to negatively regulate the transcription of

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**Table 2. Rate of RA synthesis by CYP1B1 during a 4 hour period**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>RA synthesis rate*</th>
<th>RA degradation rate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>147</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>65.8</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>68.4</td>
<td>0</td>
</tr>
<tr>
<td>180</td>
<td>55.2</td>
<td>0</td>
</tr>
</tbody>
</table>

*ng t-RA/mg CYP1B1/hour. †ng 4-oxo-RA/mg CYP1B1/hour.
**Shh.** The antagonistic relationship between RA and Shh has been established previously, both directly and indirectly. In the VAD quail, a dorsal expansion of the Shh expression domain has been reported (Wilson et al., 2004), whereas in Xenopus embryos, RA strongly downregulates Shh expression, with the reciprocal response upon treatment with an RA-antagonist (Franco et al., 1999).

To further assess the influence of RA, we asked whether downstream determinants of DV identity were altered by ectopic Cyp1B1. The homeodomain transcription factor Nkx6.1 is a crucial regulator in the establishment of the appropriate motor neuron (MN) progenitor domains. Focal electroporation of Cyp1B1 caused a marked downregulation of Nkx6.1 in its normal expression domain (Fig. 4Cd, blue arrow). Interestingly, in some places (Fig. 4Cf, blue arrow) the boundaries of ventral expression had extended towards the midline, possibly reflecting a reduced level of SHH signalling from floorplate and notochord. The pronounced reduction in Nkx6.1 expression was seen towards the caudal end of the spinal cord (Fig. 4Cd, blue arrow), an area that was more recently induced by SHH, and hence less mature at the time of electroporation. Thus, CYP1B1 signalling exerts a repressive effect on Nkx6.1, although whether this is via a lack of SHH inducing activity, or via a direct downregulation or repression by other upregulated Class I transcription factors, is not clear (see Briscoe et al., 2000) (reviewed by Jessell, 2000).

SHH and NKX6.1 function are required for the establishment of the correct MN progenitor domains, in part by controlling the expression of the basic helix-loop-helix (e.g. OLIG2) and HD transcription factors (e.g. MNR2, ISL1) that direct successive steps in MN differentiation. Therefore, we next examined whether the disruption of Shh and Nkx6.1 affected the specification of MNs as determined by these markers. We found that the misexpression of Cyp1B1 exerted a suppressive effect on the expression of Isl1 (Fig. 4Dd,Df, green arrows), and thus the appearance of post-mitotic MNs. A similar effect was also noted on the expression of Gata2, a marker of the motor neurons of r4 (Fig. 4Db, green arrow). Isl1 was also found to be expressed in the placodal cells of the trigeminal and vestibular ganglia at these stages (Fig. 4Dg, white arrow). Overexpression of Cyp1B1 in the neural tube adjacent to these placodes (r2-4) also caused a suppression of Isl1 expression (Fig. 4Dh, white arrow) that was not observed in any of the controls (Fig. 4Dg). These findings can be interpreted in two ways: either CYP1B1 signals extracellularly, and represses target gene expression in the

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**Fig. 3. Expression of Cyp1B1 in normal and vitamin A-deficient (VAD) quail embryos.** Arrows represent regions where expression of Cyp1B1 is seen in normal embryos but not in the VAD embryos. (Aa) Dorsal view of a normal 10s (HH10) embryo. (Ab,Ac) Equivalent view of a HH10 VAD embryo showing Cyp1B1 expression in the MHB (white arrow), paraxial mesoderm of the hindbrain (blue arrowhead) and all of the somites. Expression is absent from the r2 region as compared with normal embryos (white arrow). (Ba) Cyp1B1 expression in a HH16 embryo and (Bb) in a transverse section in the plane indicated by the white line. (Cc) Sites of altered expression are also observed in a 30s (HH17) VAD embryo compared with its normal counterpart. These include the ephysis, the eye and heart (Ca compared with Cc, orange, white and pink arrows, respectively). The green arrows in Be indicates mesoderm beside the hindbrain. (Ca-Cc) Sites of altered expression are also observed in a 30s (HH17) VAD embryo compared with its normal counterpart. These include the ephysis, the eye and heart (Ca compared with Cc, orange, white and pink arrows, respectively). The blue arrows in Ca and Cc indicate loss of expression in the branchial arches. (Da,Db) Rescue of Cyp1B1 expression in the anterior of the embryo and particularly the eye (white arrow) by the exogenous addition of RA.
Fig. 4. Overexpression of Cyp1B1 alters DV patterning and motor neuron specification. (Aa) Full-length Cyp1B1 (1629 nt) with a modified Kozak sequence was subcloned into the pCAB IRES-GFP vector (Gilthorpe et al., 2002) to generate Cyp1B1-IRES-GFP. (Ab, Ac) Following electroporation of Cyp1B1-IRES-GFP into HH11 embryos, both a high level of GFP (Ab, white arrowhead) and Cyp1B1 (Ac, blue arrowhead) transcripts are detected in the left-hand side of neuroepithelium. (Ad) Endogenous expression of cCyp1B1 at HH12 in the hindbrain. (B-E) Embryos were electroporated with Cyp1B1-IRES-GFP at HH10-12, cultured for 16-20 hours and analysed by in situ hybridisation for (Ba-Bf) Shh, (Ca-Cf) Nkx6.1, (Da-Dh) Isl1 and (Ea-Ed) Gata2. Wild-type expression of each gene is shown in the ‘normal’ column and embryos electroporated with the pCAB IRES-GFP vector alone in the ‘control’ column. Isolated neural tubes from either the control or Cyp1B1-IRES-GFP-electroporated embryos are shown in Bc,Bd, Cc,Cd and Dc,Dd, respectively. Arrows in Bd and Cd indicate domains of loss of expression. (Be,Bf) Comparison of hindbrain flat-mounts of a normal and a Cyp1B1-IRES-GFP-electroporated embryo analysed for Shh showing that expression is reduced in the floorplate cells (Bf, green arrow). (Ce,Cf) Comparison of flat-mount hindbrain preparation of a normal and a Cyp1B1-IRES-GFP-electroporated embryo analysed for Nkx6.1 shows alteration of expression domains in the electroporated embryo (Cf, green arrow). (Dc,Dd) Comparison of hindbrain flat-mount preparation of a normal and a Cyp1B1-IRES-GFP-electroporated embryo analysed for Isl1 expression shows downregulation (Df, green arrow). (Dg) Lateral view of the non-electroporated control side of an embryo processed for Isl1 expression (white arrow, placodal expression). (Dh) Expression of Isl1 in the placodes is reduced on the Cyp1B1-IRES-GFP-electroporated side (white arrows). (Ec,Ed) Gata2 expression is reduced in hindbrain of Cyp1B1-IRES-GFP-electroporated embryo. The location of the facial motor nuclei is indicated by VII (green arrow).
neighbouring ectoderm in a manner consistent with RA as the effector; or, the identity of the neural tube is transformed by Cyp1B1 activity such that an endogenous placode patterning signal is disrupted.

**All-trans-RA but not all-trans-retinal phenocopies Cyp1B1 overexpression**

To gain further insight into the mechanism of Cyp1B1-induced effects on neural tube development, we applied either RAL or RA in ovo to normal embryos to see if the effects of Cyp1B1 overexpression could be phenocopied. Under these conditions, beads soaked in either DMSO or RAL did not elicit any detectable change in the normal expression domains of the DV-patterning genes Shh and Nkx6.1, or of the post-mitotic motor neuron marker Isl1 (Fig. 5Aa-Ac, white or black arrow marks location of inserted bead). By contrast, beads soaked in RA caused a severe downregulation of Shh (Fig. 5Ad, blue arrows), Nkx6.1 and Isl1 (data not shown). Therefore, these responses contribute support for the antagonist role of RA in the regulation of Shh, and similarly support the proposal that Cyp1B1 exerts its effects by the production of RA and not RAL. These findings are also consistent with the observation that retinal is ubiquitously available in the embryo (Molotkov et al., 2002).

To compare the effects of RA production by Cyp1B1 with that by RALDH2, we performed similar experiments with a Raldh2-expressing construct (A. Blentic and M.M., unpublished). Using the same conditions as those described for Cyp1B1 and in a vector carrying the same β-actin promoter, we determined the extent of Shh repression as a result of Raldh2 overexpression in the hindbrain and spinal cord region (Fig. 5Ba-Bd). Consistent with our ectopic bead experiments, electroporated Raldh2 repressed Shh expression, presumably as a result of RA production. Importantly, in all the experiments performed [(n=6)×2], the AP extent of repression was comparable with that seen for Cyp1B1 overexpression (Fig. 5Bb-Bd, black arrows), although a stronger degree of repression was seen in the underlying notochord compared with Cyp1B1 overexpression (data not shown).

We next assessed the consequences of a lack of retinoid signalling on DV patterning of the hindbrain. Compared with its normal expression domain (Fig. 5Ca), the dorsal limit of Shh expression was expanded throughout the DV axis of the VAD hindbrain (n=6; Fig. 5Cb,Cc, black arrows indicating direction of expansion). Conversely, in regions where Cyp1B1 was overexpressed, the domain of Shh expression was shifted ventrally (Fig. 5Cd, black arrows indicating direction of reduction). These results are consistent with a repressive role for RA in the maintenance of Shh expression in the floorplate.

**Cyp1B1 antagonises the formation of epibranchial placodes**

Next, we examined the role of Cyp1B1 activity in the branchial arches, another region that has been shown to contain RA but is negative for Raldh expression at an equivalent time (HH15 (Maden et al., 1998; Blentic et al., 2003); Table 1). Furthermore, both gain- and loss-of-function studies have previously demonstrated an instructive role for RA signalling in many aspects of branchial arch morphogenesis, such as AP specification and endodermal directed patterning (reviewed by Mark et al., 2004). We carried out a detailed examination of Cyp1B1 expression throughout the period of arch morphogenesis, such as AP specification and endodermal directed patterning (reviewed by Mark et al., 2004). We carried out a detailed examination of Cyp1B1 expression throughout the period of arch morphogenesis, such as AP specification and endodermal directed patterning (reviewed by Mark et al., 2004).
emergence and found that *Cyp1B1* was specifically expressed in the endoderm of the first and second pharyngeal pouches and grooves at HH115 (Fig. 6Aa-Ac, white arrows). Moreover, whilst *Raldh2* was shown to be absent from these territories (Fig. 6Ba, black arrow), the overlapping expression of *Cyp26C1* in the endoderm of the pharyngeal pouches (Reijntjes et al., 2004) (Fig. 6Bc, white arrows) was suggestive of a regulated RA signal occurring here. Given the established role of the pharyngeal endoderm in directing the induction of the overlying epibranchial placodes (Begbie et al., 1999), we investigated the contribution of CYP1B1 activity to this process. *Cyp1B1* was overexpressed in the ectodermal region of the epibranchial placodes by electroporation of the presumptive placodal field at HH10/11 (Fig. 6C), and embryos were then allowed to develop for a further 16 hours. Following electroporation, analysis of *Phox2a* expression, a neuronal cell marker of the geniculate, petrosal and nodose placodes, showed marked downregulation in regions of *Cyp1B1*-expressing cells, as compared with the non-electroporated side [Fig. 6D compared with 6Db; 6Dc, white arrows (n=7)]. An equivalent response was not seen with a construct containing only the GFP gene. Consistent with CYP1B1 producing RA, a similar disruption of epibranchial placode neurogenesis was observed when embryos were treated in ovo with t-RA (data not shown).

This suggests a role for RA in the regulation of neurogenesis of the epibranchial placodes. We propose that the activity of RA in this region is itself antagonised by CYP26C1.

**CYP1B1 contributes to AP patterning**

Previous studies have shown that the initial expression of Hox genes and the demarcation of their spatial domains in the hindbrain are dependent upon a variety of transient inputs such as RA [e.g. Dupé and Lumsden (Dupé and Lumsden, 2001) and Bel-Vialar et al. (Bel-Vialar et al., 2002) and references therein]. To try to understand the contribution of CYP1B1 activity to RA-mediated AP patterning of the hindbrain and spinal cord, we exposed chick embryos to a highly specific chemical inhibitor of CYP1B1 [2,3‘,4,5‘-Tetramethoxystilbene (TMS)], and assayed for its effects on *Hoxb1* expression. In our hands, either the direct in ovo application of TMS or chick embryo culture in TMS-supplemented medium produced inconsistent effects. Therefore, to endogenously disrupt CYP1B1 activity, we implanted TMS-soaked beads in ovo at stage HH10/11 across a range of concentrations (10⁻⁴ to 10⁻⁶ nM) and assessed the effects on *Hoxb1* expression 16 hours later.

Following implantation of beads carrying 10⁻⁶ nM TMS into the dorsal margins of r4, a minor but consistent downregulation of *Hoxb1* was observed as compared with either the stage-matched control or DMSO-only-treated embryos (n=11/14). In all experiments, both TMS-treated and untreated embryos were processed simultaneously through an in situ protocol in which the *Hoxb1* probe was derived from a common mix, colour reactions were performed for an identical time, and images were obtained under identical settings. When the concentration of TMS on the implanted bead was increased to either 10⁻³ or 10⁻⁵ nM, the expression of *Hoxb1* was significantly reduced as compared with the untreated and control counterparts (Fig. 7Aa-Ac versus Ba-Bc and Ca-Cc; n=5/7 and 5/6, respectively; blue arrows) or the 10⁻⁶ nM-treated embryos (data not shown). For example, the endogenous weak expression seen in r6 of the normal embryos was extinguished in both the 10⁻³ and 10⁻⁵ nM TMS-treated embryos (Fig. 7Ac versus Bc and Cc, red arrows). Thus, the reduction in *Hoxb1* expression levels positively correlates with increasing CYP1B1 inhibitor concentration. In some cases, there was a reduction in the size of the r4 expression domain (Fig. 7Cc, black arrows). Furthermore, even when the TMS-treated embryos were developed for several hours longer, the intensity of *Hoxb1* staining did not reach that of the controls (data not shown).

Conversely, we determined the effects of overexpression of *Cyp1B1* on *Hoxb1* by electroporation into HH10/11 hindbrains and anterior spinal cords. Here, no additional sites of *Hoxb1* expression were observed outside the normal domains of r4 and the spinal cord. However, the intensity of staining, particularly in the region of the anterior spinal cord, seemed to be enhanced in the electroporated
implantation of a bead soaked in 10–4 nM TMS, a specific inhibitor of colour reactions were performed for the same length of time.

Ba-Bc

Hoxb1

the hindbrain (Ac). (Fig. 7Aa-Ac) shows distinct expression in the eye with Raldh1 and Raldh3 having defined dorsal and ventral boundaries in the neural retina, respectively, whereas Cyp1B1 shows an even DV distribution in the anterior retina as well as exclusive expression in the anterior segment. Interestingly, by HH15, Cyp1B1 is extended to include not only the neural retina, but also the neuroepithelium of MHB (Fig. 1R) (Blecinti et al., 2003).

We have provided a detailed description of Cyp1B1 expression and shown that many sites overlap with those of the Raldh genes, most notably with Raldh2 in the somites and with Raldh1 and Raldh3 in the eye. However, Cyp1B1 is also expressed in several unique sites not shared with the Raldhs (e.g. the hindbrain paraxial mesoderm, Fig. 1L). Furthermore, studies on the endogenous distribution show these regions to contain significant levels of RA (RALDH1-3 and CYP1B1) have a tissue-specific profile. RA (RALDH1-3 and CYP1B1) have a tissue-specific profile.

Cytochrome p450s in normal development

Cytochrome p450s are a large family of evolutionarily conserved proteins (57 functional genes in human; see http://drnelson.utmem.edu/CytochromeP450.html) that are used to make steroids and lipid-derivatives as well as having a prominent role in xenobiotic metabolism. In eukaryotes, they are heme-containing membrane-bound proteins capable of catalysing multiple reactions including mono-oxygenation (reviewed by Denisov et al., 2005). Prominent cytochromes during normal development are the CYP26s, which attenuate the spatiotemporal extent of RA signalling (e.g. Sirbu et al., 2005; Reijntjes et al., 2005). Although the majority of RA production is via the oxidative activity of the RALDHs, there is also good evidence that other RA-generating enzymes must exist (Maden et al., 1998; Mic et al., 2002; Niederreither et al., 2002b). Here, we report the characterisation of an RA-synthesising enzyme, CYP1B1, and show for the first time that a cytochrome p450 can positively direct RA-mediated patterning processes.

Cyp1B1 is expressed at some but not all sites of Raldh activity

Identification of the sites of expression of Cyp1B1 was a prerequisite to understanding its involvement in normal development. To this end, we have described expression from HH4 to HH22 (Fig. 1A-V). Although there have been previous reports of Cyp1B1 expression in mouse and human at various developmental stages (Bejjani et al., 2002; Stoilov et al., 2004; Doshi et al., 2006), there has been no comprehensive description in chick across the stages examined here.

Cyp1B1 is expressed in endodermal, mesodermal and ectodermal derivatives during development, including the somites (and subsequently dermamyotome), hindbrain paraxial mesoderm, notochord, sinus venous, forelimb bud mesenchyme, MHB, anterior retina and anterior segment of the eye and the endoderm of the posterior pharyngeal arches. This profile bears strong similarity to that of the Raldh genes and to other known sites of RA-signalling activity (Table 1). At the earliest stages (HH4), the Cyp1B1 expression domain in the ectoderm and mesoderm defines a similar domain to the characteristic pattern of Raldh2 transcription seen in the newly innervated mesoderm. Later, expression is coincident in the newly-forming and existing somites. However, from early stages (HH9–11), Raldh2 expression is progressively downregulated in the first somites (1–4), whereas Cyp1B1 persists (Fig. 1A–V compared with W). At stages HH4–11, the Cyp1B1 pattern is distinct from that of Raldh3 (Fig. 1A–V) (cf. Blecinti et al., 2003). Later, the similarity in expression of Cyp1B1 and Raldh2 in the somites persists, such that they are both enriched in the dermamyotome (Fig. 1U) (Blecinti et al., 2003), although Raldh2 is also present at several other locations (e.g. the rostral end of the anterior intestinal portal). By HH18, Cyp1B1, Raldh1 and Raldh3 show distinct expression in the eye with Raldh1 and Raldh3 having defined dorsal and ventral boundaries in the neural retina, respectively, whereas Cyp1B1 shows an even DV distribution in the anterior retina as well as exclusive expression in the anterior segment. Interestingly, by HH15, the similarity between Cyp1B1 and Raldh3 is extended to include not only the neural retina, but also the neuroepithelium of MHB (Fig. 1R) (Blecinti et al., 2003).

Fig. 7. CYP1B1 contributes to maintaining the appropriate expression of Hoxb1. (Aa-Ac) Hoxb1 expression is reduced (n=5/7) by implantation of a bead soaked in 10–4 nM TMS, a specific inhibitor of CYP1B1 (green arrow represents bead position). Expression is less intense in r4, r6 (red arrow) and the anterior spinal cord (blue arrow). (Ca-Cc) A similar reduction was noted with 10–5 nM TMS (n=5/6). In some cases (Cc), the borders of the Hoxb1 r4 expression domain appeared to be smaller in the treated embryos (black arrows). These effects were not seen in either untreated embryos (Aa-Ac) or embryos where a DMSO-soaked bead was implanted into r4 (data not shown). TMS-treated and untreated embryos were processed simultaneously using an in situ protocol in which the Hoxb1 probe was derived from a common mix and colour reactions were performed for the same length of time.
*Cyp1B1* is present in regions where Raldhs are not expressed (Table 1). However, despite the lack of RALDH activity in some tissues, evidence from RA ablation (e.g. using pan-receptor antagonists), deprivation (e.g. VAD quail and rat) studies or direct measurement of endogenous RA, indicate that retinoid signalling acts in the patterning of these tissues. For example, studies using HPLC and F9-RARE-lacZ reporter cells in chick have shown high levels of RA synthesis (~50% of the levels found in somites) in the mesenchyme surrounding the hindbrain (Maden et al., 1998), although no Raldhs are concordantly expressed. By contrast, chick *Cyp1B1* is highly expressed in the mesoderm adjacent to the hindbrain (Fig. 1L,P) and is thus well positioned as a candidate for synthesising the RA activity. Similarly, enriched RA synthesis was recorded in the posterior versus the anterior half of chick limb buds, also a site of exclusive *Cyp1B1* expression (Fig. 1W, white arrow).

A potential inconsistency is that *Cyp1B1* is expressed, albeit weakly, in r2 at HH10 and no significant RA has been reported in this region at this time. One explanation is that here the low levels of RA produced by *Cyp1B1* are further nullified by the activities of CYP26C1 and CYP26A1, which are also highly expressed in r2 at the equivalent time (Fig. 1Y, black arrow and data not shown, respectively). Alternatively, *Cyp1B1* in this region may be metabolising a different substrate for which it has a greater catalytic activity.

**CYP1B1 can synthesise but not degrade RA**

*Cyp1B1* is capable of mediating the stepwise oxidative metabolism of retinol to retinal and subsequently from retinal to RA (Fig. 2A,B, Table 2). Although, the overall amount of RA produced is low compared with the equivalent conversion of retinol to retinal, it is nonetheless synthesised at a relatively constant rate across the time period assayed (between 55.2 and 147 ng r-RA/ng CYP1B1/hour; Table 2). This raises the intriguing question of how much RA is physiologically required to elicit a biological response. Consistent with ectopic bead experiments, electroporated *Raldh2* represses *Shh* expression, presumably as a result of RA production. Importantly, in all the experiments performed ([n=6]×2), the extent of repression was comparable to that seen for *Cyp1B1* overexpression (Fig. 5Ba-Bd for control and electroporated data). Thus, although *Cyp1B1* only produces relatively small amounts of RA, when expressed in the embryo it is sufficient to elicit a transcriptional response of equivalent biological potential to RALDH2. However, it is a possibility that the amount of RA produced by overexpression of *Cyp1B1* in the embryo was unusually high owing to the electroporation strategy employed.

Unlike CYP26A-C1, *Cyp1B1* is unable to further metabolise RA to any of its less active products (Fig. 2C). The data shown in Fig. 2 are supported by other in vitro studies that have shown CYP1B1 to be capable of synthesising RA from retinol (Chen et al., 2000; Zhang et al., 2000; Choudhary et al., 2004), but incapable of degrading RA (Choudhary et al., 2004).

Together, these data are consistent with the identification of a new RA-synthesising pathway operating during normal development that complements the RALDH2 pathway. The biochemical data point to a mechanism that is not reliant on ADHs, unlike the RALDHs, to generate a locally concentrated source of retinal for further conversion to RA. However, in addition to the role reported here, *CYP1B1* has also been shown to be involved in the detoxification of xenobiotics, tryptophan metabolism and to play an important role in the modulation of estrogenic activity. *CYP1B1*, itself induced by estradiol, catalyses the 4-hydroxylation of estrogen that leads to a decrease of the estrogen activity but an increase in the production of toxicologically active metabolites (reviewed by Tsuchiya et al., 2005). Thus, *CYP1B1* is potentially capable of generating other signals that can influence cell behaviour, and this is a consideration when interpreting the observations described here.

Interestingly, it has previously been suggested that RALDH1, itself relatively inefficient at RA synthesis, may also have initially functioned as a detoxifying enzyme and only subsequently been co-opted into the RA-synthetic pathway within specific tissues (Niedereither et al., 2002b).

**Cyp1B1 expression is differentially regulated by maternal RA**

Further support for the interplay with retinoid signalling comes from the reliance of *Cyp1B1* on maternal retinoids for some sites of endogenous expression (Fig. 3). The absence of *Cyp1B1* expression at several sites (eye, pharyngeal arches, sinus venosus, mesoderm adjacent to r2) in the VAD quail embryo may be attributed to either a requirement for retinoids in initial gene induction, or a failure to correctly specify the whole *Cyp1B1*-expressing tissue (e.g. third and fourth PA). In either case, these data are consistent with *Cyp1B1* being involved in regionalisation directed by retinoids.

**A role for Cyp1B1 in the RA patterning of the neural tube**

During specification of neuronal identity in the ventral CNS, progenitors express a distinct combinatorial pattern of homeodomain and basic-helix-loop-helix transcription factors. SHH, expressed in the notochord and floorplate, positively regulates the expression of a subset of these genes (Class II; e.g. *Nkx6.1*) and represses others (Class I; e.g. *Pax6*). Subsequently, the Class I and II genes mutually repress each other, establishing discrete domains that go on to generate specific neuronal classes that are themselves characterised by a unique transcription factor profile [e.g. for MNs: *Mnr2* and *Isl1*, reviewed by Jessell (Jessell, 2000) and Shirasaki and Pfaff (Shirasaki and Pfaff, 2002)]. In addition to the role of SHH, recent studies have elucidated a pivotal role for RA in the regulation of ventral pattern in the spinal cord as well as control of neuronal differentiation (Pierani et al., 1999; Sockanathan and Jessell, 1998; Sockanathan et al., 2003; Diez del Corral et al., 2003; Novitch et al., 2003; Wilson et al., 2004) (reviewed by Wilson and Maden, 2005). Based on the evidence presented here, we proposed that CYP1B1-mediated RA production in the paraxial mesoderm is ideally positioned to contribute to DV patterning of the hindbrain and neural tube. *Cyp1B1* overexpression in both the hindbrain and spinal cord was sufficient to repress *Shh* expression non-cell-autonomously in both the floorplate (Fig. 4Bd) and notochord. This is consistent with indirect evidence that RA plays an inhibitory role in Shh expression (Wilson et al., 2004), as has also been observed in *Xenopus* embryos, where RA strongly downregulates Shh expression, whereas an RA antagonist upregulates expression in the midline tissues (Franco et al., 1999). Consistent with these findings, ectopic *Cyp1B1* expression also caused a marked reduction or deletion of the expression domains of *Nkx6.1* (Fig. 4Cd, blue arrow), *Mnr2* (data not shown), *Isl1* (Fig. 4Dd, green arrow) and *Gata2* (Fig. 4Ed, green arrow). These findings represent a significant alteration of ventral progenitor domain identity and subsequent failure to progress through to a differentiated motor neuron phenotype, as assessed by *Isl1* expression. It is possible that the observed effects on *Nkx6.1* are...
the result of reduced SHH activity, or of an expansion of the ventral limit of the Class I gene Pax6 because RA has been shown to be capable of promoting expression of this class of genes in vitro (Novitch et al., 2003). Furthermore, in embryos deficient for RA signalling, expression of the Class I genes Pax6 and Irx3 is severely reduced (Diez del Corral et al., 2003; Wilson et al., 2004) and electroporation of a dominant-negative RA receptor reduces the expression of Class I genes in vitro (Novitch et al., 2003). Furthermore, in embryos deficient for RA signalling, expression of the Class I genes Pax6 and Irx3 is severely reduced (Diez del Corral et al., 2003; Wilson et al., 2004) and electroporation of a dominant-negative RA receptor reduces the expression of Class I genes in vitro (Novitch et al., 2003).

However, the complete ablation of Nkx6.1 expression was rarely seen (n=2; Fig. 4Cd, blue arrow), presumably owing the fact that it only requires a short pulse of SHH signal to roughly establish its domain of expression (Ericson et al., 1996; Ericson et al., 1997; Briscoe et al., 2000). The alteration of neuronal progenitor identity by Cyp1B1 following overexpression at HH10-11 is also consistent with previous studies showing that cell fate in the ventral neural tube can be influenced by RA signalling at this stage (Novitch et al., 2003).

Thus, we propose that specification of appropriate initial ventral progenitor domains in the spinal cord is a multifaceted process that involves CYP1B1- and RALDH2-derived RA to establish normal pattern. The presence of Cyp1B1 in the mesoderm adjacent to the hindbrain offers a unique source of RA to direct DV pattern here (where Raldh2 is absent) and to explain the observed distribution of retinoids in the chick embryo (Maden et al., 1998). A contribution of CYP1B1 to later RA-dependent neuronal subtype specification (Novitch et al., 2003) (reviewed by Appel and Eisen, 2003; Wilson and Maden, 2005) (see Fig. 8) remains to be determined.

**Fig. 8.** The role of CYP1B1 in RA biochemistry and its potential role in patterning the ventral neural tube and motor neuron differentiation. (A) Oxidative cascade leading to the synthesis and breakdown of RA. CYP1B1 can generate retinal and RA but does not subsequently catabolise RA. The other major components of the pathway are shown. In this model, only the ADHs are thought to be ubiquitously expressed, with the others showing developmentally restricted patterns of expression. (B) Expression domains of Shh, homeodomain and bHLH transcription factors in the ventral spinal cord. Mutual cross-repressive interactions between class I and II proteins establish a motor neuron progenitor domain exclusively in the region of PAX6 and NKX6 that is defined by the expression of OLIG2. Later steps in acquisition of a committed motor neuron phenotype are marked by MN2 and ISL1. RA is known to play a role in the regulation of this process (reviewed by Jessell, 2000). (C) Following overexpression of Cyp1B1 in the ventral neural tube, the phenotypic effects can be explained by a disruption of the pMN domain via the excess production of RA. (D) RA is known to be involved in many other steps (green arrows) of MN specification/maturatio.
CYP1B1 and AP patterning

The data presented here demonstrate that loss of CYP1B1 activity via the application of a chemical inhibitor (TMS) caused a significant reduction in the intensity of Hoxb1 expression (Fig. 7Ba-Bc, Ca-Cc). Several previous studies have shown a requirement for RA in the induction and spatial regulation of Hoxb1. Thus, the data are consistent with a reduction in the available levels of RA, which resulted in the reduced capacity to express/maintain Hoxb1 in its normal domains. It is noteworthy that here, where the inhibitor is applied at a relatively late stage (HH10-11), it would not be expected to dramatically change the Hoxb1 domains owing it being in a maintenance phase involving auto- and cross-regulatory loops.

Interestingly, Cyp1B1 overexpression was not able to induce ectopic Hoxb1 sites, but it might be able to enhance transcription of Hoxb1 at its normal sites. This observation lends further support to CYP1B1 being able to produce RA at meaningful levels. The inability to induce ectopic Hoxb1 might be due to tightly regulated activating and/or repressing transcription factor crosstalk and feedback mechanisms operating during at this time (e.g. Ferretti et al., 2005). Therefore, the RA provided by CYP1B1 might be insufficient to overcome these maintenance mechanisms outside of the normal sites of expression. In other experiments where Hoxb1 is induced anterior to r4, the embryos are treated with RA earlier during the induction phase of expression (e.g. HH7) (Bel-Vialar et al., 2002).

Together, these findings suggest a mechanism in which under normal circumstances, CYP1B1 activity complements that of RALDH2 by the supply of retinal to RALDH, or by supplying RA directly, or both, so as to set up and/or maintain appropriate Hox domains. The precise timing and extent of this cooperative interaction in the chick embryo is currently under investigation. It may be significant that Cyp1B1 expression is maintained in the first few somites, whereas Raldh2 expression is downregulated. Thus, CYP1B1 is well positioned to supply retinoids to the hindbrain and anterior spinal cord.

CYP1B1 exerts its effects through RA not retinal

In the light of the capacity of CYP1B1 to efficiently synthesise retinal from retinol (Fig. 2A, blue arrow), we sought to establish whether the observed effects on DV patterning could be explained by an increased supply of retinal for some other, as yet unidentified, RA-synthesising enzyme. The data shown in Fig. 5Aa-Ad demonstrate that the localised enrichment of retinal was insufficient to elicit any of the responses seen by Cyp1B1 electroporation. By contrast, the ectopic supply of t-RA into the hindbrain or spinal cord consistently produced an identical response, albeit of a greater magnitude, to Cyp1B1 overexpression. This observation supports the notion that overexpression of Cyp1B1 can coordinate the production of a RA signal sufficiently strong to elicit a biological response. However, the levels of repression seen here may in part be due to high levels of RA produced by Cyp1B1 associated with the electroporation strategy. Secondly, the effects we have observed could also be explained by the ectopically expressed Cyp1B1 supplying continuously elevated levels of retinal to an as yet unidentified RA-synthesising enzyme, as opposed to retinal supplied on a bead that may have a short half life. Alternatively, CYP1B1 may elicit a biological response that is similar in effect to the production of RA, but is due to the production of a different unidentified signalling molecule or diffusible metabolic by-products (see above).

Other potential roles of CYP1B1

It has been established that CYP1B1 can catalyse the conversion of retinol to retinal and subsequently to RA. Where the expression of Cyp1B1 and Raldhs overlap (e.g. in the somites (Raldh2) and the mid-hindbrain boundary (Raldh3)), we propose that it is possible that CYP1B1 supplies the supplement of retinal to the RALDHs as well as making RA. However, where Cyp1B1 is expressed in exclusion to any other known Raldhs (e.g. hindbrain paraxial mesoderm, limb buds and pharyngeal pouches), then here it is propose that CYP1B1 is the sole supplier of RA. However, it is possible that CYP1B1 may mediate the functions described above via the synthesis of an alternative regulatory factor, which is not RA, but has similar profound effects on development. For example, CYP1B1 has a well documented role in the 4-hydroxylation of estradiol and this may in some way be linked to altered cell-signalling events (reviewed by Tsuchiya et al., 2005).

Future directions

The identification of a major new RA-signalling pathway in development raises many intriguing questions. What is the exact extent and use of the CYP1B1 pathway and does it produce any signals other than RA in the developing embryo? What would be the loss of signal or the phenotype associated with Cyp1B1-null versus RARE-lacZ, or Cyp1B1-null versus Raldh1-, 2- or 3-null mutants? Similarly, it would be informative to know if a Cyp1B1 knock-in strategy could rescue the embryonic lethality of the Raldh2-null.

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Supplementary material

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References


