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Expression pattern of *BXR* suggests a role for benzoate ligand-mediated signalling in hatching gland function

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ABSTRACT The *Xenopus laevis* nuclear receptor BXR has recently been shown to be activated by a class of endogenous benzoate metabolites, indicating the presence of a novel and unsuspected benzoate ligand-dependent signalling pathway. The receptor is expressed ubiquitously in blastula and gastrula stage embryos, and its expression declines during neurula stages. In order to examine further this novel vertebrate signalling system, we have examined the expression of the *BXR* gene in tailbud stage embryos and adults. We show here that in *Xenopus* tailbud stage embryos expression is restricted to the hatching gland, suggesting a role in hatching gland function. Neither BXR nor a BXR-VP16 fusion is sufficient to specify hatching gland in neurally-induced tissue. In adults, *BXR* expression is abundant in the brain and gonads. This expression pattern in adults is distinct from any of the putative mammalian homologues. A nuclear receptor that mediates benzoate signalling has yet to be found in mammals.

KEY WORDS: *Xenopus, nuclear receptor, BXR, benzoate, hatching gland*

Nuclear receptors comprise a superfamily of ligand-modulated transcription factors. They transduce the signals provided by a range of lipophilic ligands, including such signalling molecules as steroids, retinoids, thyroid hormones and vitamin D, all of which co-ordinate complex gene expression programs in higher eukaryotes.

In addition to the nuclear receptor superfamily members for which ligands are known, there is an even larger class (amounting to about 30 examples in vertebrates) of superfamily members, which have been discovered by homology-led cloning, but for which no ligand is known. These are the orphan nuclear receptors. The search for hormonal activators of the orphan nuclear receptors has been productive in a number of cases. For several receptors, their orphan status has been changed to mediators of previously unknown signalling pathways. Thus, the nuclear receptor superfamily now includes more recently discovered members which participate in signalling by farnesoids (Forman *et al.,* 1995a), fatty acyl-CoA thioesters (Hertz *et al.,* 1998), prostaglandins (Forman *et al.,* 1995b; Kleiwer *et al.,* 1995), oxysterols (Janowski *et al.,* 1996) and pregnanes (Kleiwer *et al.,* 1998).

We first isolated the *Xenopus laevis* nuclear receptor BXR by homology to the human vitamin D receptor, but as it showed no ability to bind vitamin D_3 in assays, it was designated an orphan nuclear receptor, and was originally named xONR1 (Smith *et al.,* 1994). Subsequently, Blumberg and co-workers showed that BXR is activated by a number of benzoate metabolites including alkyl esters of amino and hydroxy benzoic acid. Such benzoates are found endogenously in *Xenopus* embryos (Blumberg *et al.,* 1998). This suggests that the benzoate-activated receptor, BXR, mediates a novel and a previously unsuspected signalling pathway.

Since the isolation of BXR in *Xenopus*, other nuclear receptors have been discovered, in mammals, which are more similar to BXR than are the vitamin D receptors (Fig. 1). These related receptors include CARβ, which is a constitutively active transcription factor (Baes *et al.,* 1994; Choi *et al.,* 1997) that is a ligand-inactivated by androstane metabolites (Forman *et al.,* 1998), and PXR, which defines a novel steroid signalling pathway activated by progesterone and other pregnanes (Kleiwer *et al.,* 1998). Most recently SXR has been identified, which activates transcription in response to a diversity of structurally unrelated natural and xenobiotic compounds, including numerous steroids (Blumberg *et al.,* 1999). Like BXR, all these receptors bind DNA as heterodimers with RXRs. Despite intensive efforts, we have not yet been able to isolate undoubted orthologues of the *Xenopus BXR* gene from mammals.

It has been shown previously that the *BXR* gene is expressed in the oocyte, giving rise to maternally-inherited transcripts in the egg. Zygotic expression leads to a peak of transcript abundance during blastula and gastrula stages, followed by a decline in the neurula (Smith *et al.,* 1994). In view of the concept that BXR mediates novel benzoate ligand-dependent signalling pathways

Abbreviations used in this paper: RT-PCR, reverse transcriptase-polymerase chain reaction.

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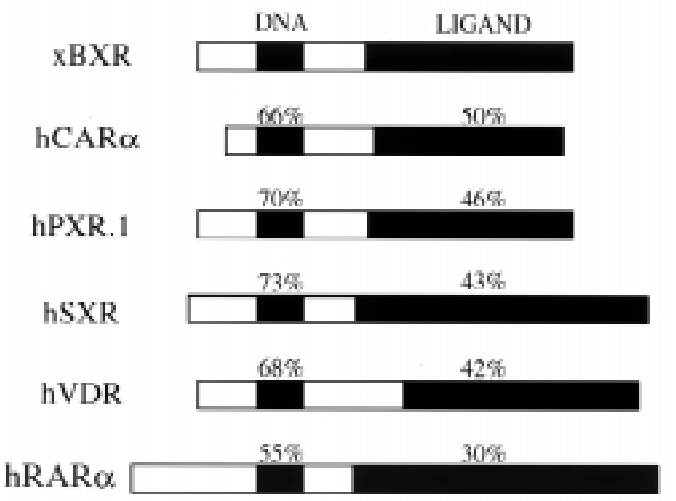

Fig. 1. Homology of BXR to other nuclear receptors. *Schema of Xenopus BXR showing the DNA-binding zinc finger domain and the ligand binding domain, and homology within these domains to other members of the nuclear receptor superfamily. The closely-related human CARα, PXR.1, SXR and VDR receptors are shown. The human RARα is included as a representative of more distantly-related members of the superfamily. Percentages refer to amino acid identity in the DNA and ligand binding domains.*

in development, we have examined the expression pattern of *BXR* further, in developing embryos and in adult *Xenopus* tissues.

Expression pattern of the BXR gene in tailbud stage embryos

In situ hybridisation was carried out to localise *BXR* transcripts in developing *Xenopus laevis* embryos. The *in situ* hybridisation results with embryos up to neurula stages did not give a clearly localised signal, presumably because the transcripts are generally distributed, but by the early tailbud stage embryos showed strong, localised expression on the front of the head (Fig. 2). The expression pattern starts at the top of the head, level with the pharyngeal arches, forming a thick band down the centre of the head until it reaches eye level, where it forks. It continues down the front of the head, close to the eyes, until it stops, apparently abutting the cement gland. The overall appearance is of an inverted Y from the top of the head to the cement gland. This pattern corresponds to the hatching gland (Carroll and Hedrick, 1974; Sato and Sargent, 1990; Drysdale and Elinson, 1991).

BXR is not sufficient for specification of the hatching gland

Given the localisation of BXR transcripts in the *Xenopus* hatching gland, we were curious about a possible role for BXR in the specification of the gland. Hatching gland cells are specified at around stage 12 (gastrulation). They arise at the surface of the neural plate boundary in the same area where deep cells give rise to neural crest (Drysdale and Elinson, 1991). We therefore investigated the ability of BXR, in the presence of neural inducers, to induce hatching gland in explanted animal caps. We used expression of the hatching enzyme gene *Xhe* as a specific marker for hatching gland tissue (Katagiri *et al.,* 1997). The neural inducers in this experiment were provided either by injection of *noggin* mRNA, which results in neural induction of an anterior character (Lamb and Harland, 1995), or by exposure of *noggin*-injected caps to bFGF, which transforms neural tissue to a more posterior character (Lamb and Harland, 1995). Neither alone, nor in combination with bFGF, was noggin able to induce expression of the hatching gland marker in animal caps (Fig. 3). The role of nuclear receptors as transcriptional regulators makes them amenable to investigation by the generation of chimeric constructs that contain constitutive activation or repression domains. We therefore made constructs encoding BXR linked to the Herpes virus VP16 activation domain (Xu *et al.,* 1993; Blumberg *et al.,* 1997), and encoding BXR linked to the *Drosophila* Engrailed repressor (Han and Manley, 1993; Conlon *et al.,* 1996; Kessler, 1997). There was no induction of *Xhe* by overexpression of BXR, BXR-VP16 or BXR-EnR in such caps (Fig. 3).

The hatching gland contains epidermal cells which transiently secrete a zinc metalloprotease (hatching enzyme, the *Xhe* gene product) which partially digests the fertilisation envelope and jelly coat. Regulation of the *Xenopus* hatching enzyme synthesis is not well characterised, but it is tempting to speculate that although BXR is not sufficient to specify hatching gland development in neuralised ectoderm, it may have a role in regulating transcription of the *Xhe* gene. In the quail, the expression of a homologous metalloendopeptidase with a role in hatching, is under the control of 1,25-dihydroxyvitamin D-3 (Elaroussi and Deluca, 1994).

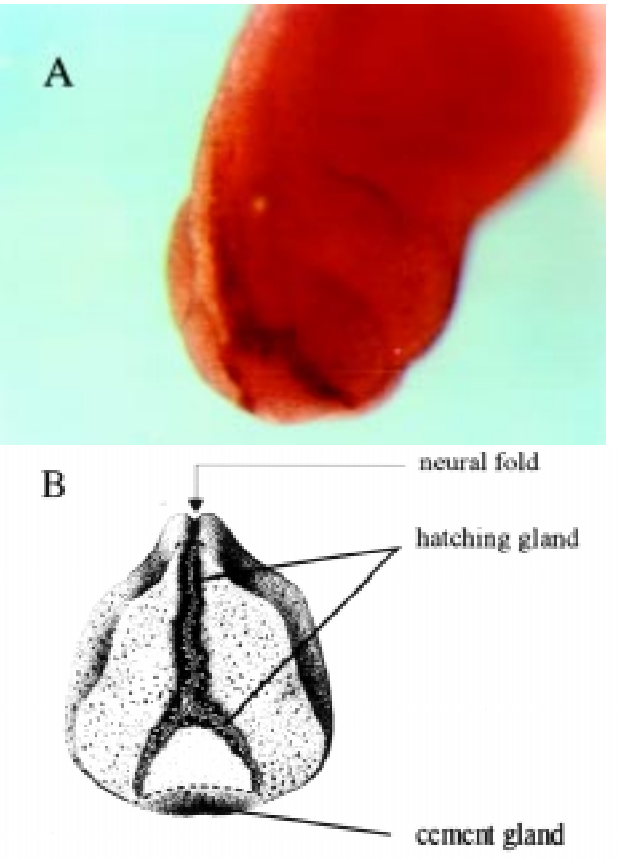

Fig. 2. Whole-mount *in situ* hybridisation with a BXR probe. *X. laevis embryos were hybridised with a BXR antisense mRNA probe, and the embryos stained according to Harland (1991).* **(A)** *Head of a tailbud stage albino embryo (stage 25) showing inverted Y staining of the hatching gland.* **(B)** *Schema of the embryo head region showing the positions of the hatching gland and cement gland (adapted from Drysdale and Ellinson 1991).*

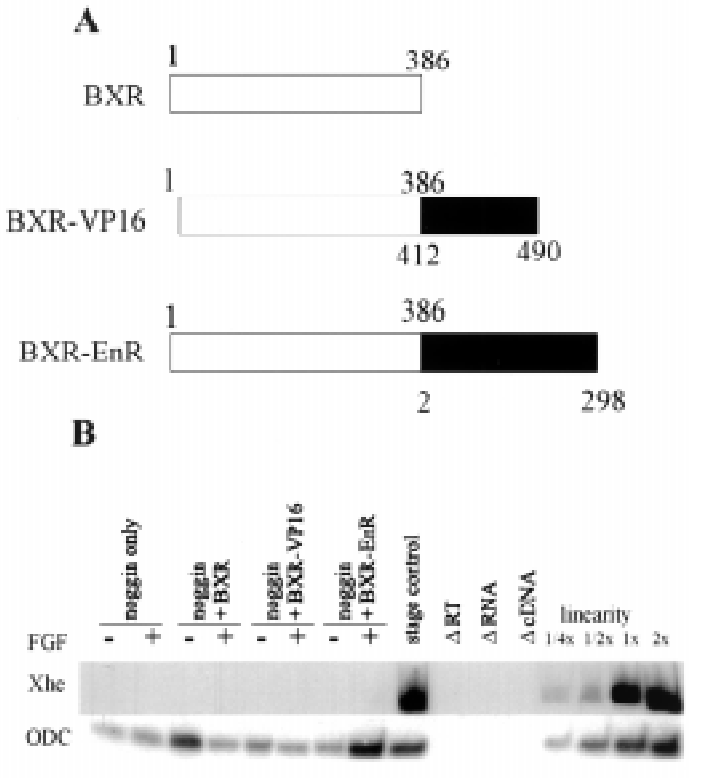

Fig. 3. BXR expression in neurally-induced ectoderm does not specify hatching gland tissue. (A) *Schema of the wild -type BXR and chimeric derivatives. DNA constructs were made which encoded the activator and repressor domains of VP16 and Drosophila Engrailed fused to the carboxy terminus of BXR. Amino acid positions are numbered.* **(B)** *Synthetic noggin mRNA was injected into embryos at the two-cell stage, either alone or together with wild-type BXR, BXR-VP16, or BXR-EnR mRNA. Animal cap explants were taken at stage 9, and half were treated with bFGF. Caps were incubated to stage 20 for RT-PCR analysis of Xhe gene expression. ODC was assayed to show that comparable amounts of RNA were isolated for each sample.*

Expression of the BXR gene in adult tissues

The expression in adult tissues was examined by RT-PCR with primers based on the hinge region of the receptor. The hinge region of nuclear receptors generally shows the lowest homology with other receptors, enabling primers to be designed that are specific for BXR. Figure 4 shows that there was a higher level of expression in the brain, and in the gonads of both male and female *Xenopus*. There was a lower level of expression in other tissues examined, including the heart, skin, and kidney, with low but detectable expression in the liver and muscle. The receptor is not restricted to the oocyte, or early embryo. A role in gametogenesis in both testis and ovary is suggested by the results of the survey presented here. This pattern of expression is distinct from that of the related mammalian nuclear receptor genes: *CAR* is expressed predominantly in the liver (Baes *et al.,* 1994; Choi *et al.,* 1997), whereas *PXR* and *SXR* are both restricted to the liver and intestine (Kleiwer *et al.,* 1998; Blumberg *et al.,* 1999).

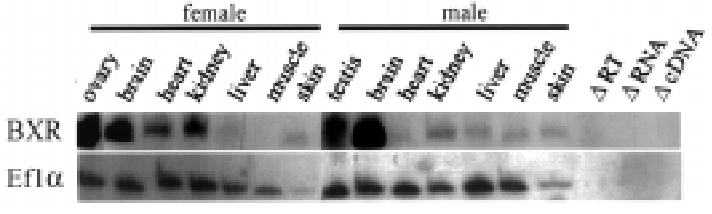

Fig. 4. Distribution of BXR transcripts in adult *Xenopus laevis* tissues. *A range of tissue RNAs was used as templates for reverse transcriptase. The resultant cDNAs, along with no RNA, no reverse transcriptase and no cDNA negative controls, were used as a template for radioactive PCR, with a primer pair designed against the hinge region of BXR. The resulting fragments were visualised by PAGE and autoradiography. EF1α was assayed to show that comparable amounts of RNA were isolated for each sample.*

Experimental Procedures

Whole mount in situ hybridisation

Antisense RNA was synthesised from linearised plasmid containing *BXR* cDNA (Smith *et al.,* 1994). Embryos were probed with the DIG probes according to a standard procedure (Harland, 1991).

Plasmid construction

For both the *BXR-En* and *BXR-Vp16* constructs, a 1262 nucleotide fragment containing the coding sequence and partial 3' UTR of *BXR* was amplified by the polymerase chain reaction from the cDNA clone isolated previously (Smith *et al.,* 1994). A *Hind*III site was introduced at the beginning of the fragment, and the TGA stop site was destroyed with the introduction of a *Cla*I site. (Primers, gene sequence underlined, were: 5'-GGCCAAGCTTGAGGAGCTGCTCAGTGAA-3' and 5'-CCATCGATTT-CAGGGGATCCAAAGAC-3'.) The fragment was ligated into pGEM-T (Promega) and an *Hind*III/*Nde*I fragment containing the cDNA was ligated into *Hind*III/*Nde*I digested expression vector, pSPJC2L, which contains the 5' and 3' UTR from the *Xenopus* β-globin cDNA (Cook *et al.,* 1993), to create pSPJC2L-BXR. The initial 896 nucleotides of the *Drosophila engrailed* coding region, containing the repressor domain, were amplified by PCR from EnR-pBS, (Conlon *et al.,* 1996) (a gift of F. Conlon) in which the initial methionine has been replaced by a *Cla*I site. An *Nde*I site was introduced at the 3' end of the molecule. (Primers, T7 primer and 5'-CGGCCATATGC-TAGGGCTGCATAGATCCCAG-3'.) This fragment was also cloned into pGEM T and excised with *Cla*I and *Nde*I, this was inserted into *Cla*I/*Nde*I digested pSPJC2L-BXR to give pSPJC2L-BXR-EnR (encoding BXR-EnR). A fragment corresponding to amino acids 412 to 490 (the final 79 amino acids) of the *Herpes simplex* virus VP16 transactivator (Triezenberg *et al.,* 1988) was amplified by PCR. A *Cla*I site was introduced at the start of the sequence and an *Nde*I site at the 3' end. (Primers 5'-GCGCATCGA-TACGGCCCCCCCGACCGAT-3' and 5'-GCCGCATATGCTACCCACCG-TACTCGTC-3'.) The fragment was cloned into pGEM T and inserted into *Cla*I/*Nde*I digested pSPJC2L-BXR to give pSPJC2L-BXR-VP16 (encoding BXR-VP16). The individual pGEM T clones were sequenced, and TNT (Promega) translation products were visualised by SDS-polyacrylamide gel electrophoresis, to check the protein size (not shown). The mRNAs from the chimeric constructs were shown to be biologically active in embryos, where they specifically disrupted normal development (not shown).

Messenger RNA generation for microinjection

The plasmids encoding BXR-En and BXR-VP16 were linearised with *Xho*I, and an expression construct of *BXR* in pSP64T (Smith *et al.,* 1994) was linearised with *Sst*I. These linearised plasmids were used as templates for the synthesis of capped mRNA, using a commercial kit (Amicon).

Embryo culture and RNA microinjection

All embryos used in this study were obtained by *in vitro* fertilisation of hormonally stimulated *Xenopus laevis.* Standard embryological procedures were as described (Jones and Woodland, 1986). Microinjections were carried out with mRNA transcribed *in vitro* as described previously and diluted to approximately 50 μg/ml. Approximately 20 nl was injected into both blastomeres of dejellied 2-cell embryos under 3% Ficoll in Barth X.

Total RNA isolation

Embryos were staged according to Nieuwkoop and Faber (1967). Total RNA was prepared from animal caps and dissected adult tissues as described (Smith *et al.,* 1994).

RT-PCR analysis

Approximately 500 ng of total RNA were used as the template for cDNA synthesis. Random hexamers were used to prime the reaction, catalysed by 'Superscript' reverse transcriptase (Gibco). The *BXR* primers were designed based on the hinge region of the receptor. *BXR* cDNAs were normalised to *Ef1*α transcript levels. Amplification took place in the presence of [α^{32}P]dGTP and 3.0 mM $MgCl_2$. *BXR* and *Xhe* cycle times were: 1 min at 94°C; 30 sec at 94°C, 30 sec at 60°C, 30 sec at 72°C repeated 25 *(BXR)* or 24 *(Xhe)* times; 72°C for 5 min. *ODC* and *EF1*α cycle times were as above, but with 18 cycles and an annealing step at 55°C. Radiolabelled products were visualised by polyacrylamide gel electrophoresis and autoradiography. The *BXR* primers were: hinge, upstream, GATCATGTCCGATGCAGCG and downstream, 5'-CCGAAAGTTCTTGGAGAAGG-3'. *EF1*α primers were: upstream, 5'-CAGATTGGTGCTGGATATGC-3' and downstream, 5' CACTGCC-TTGATGACTCCTA-3' (Mohun *et al.,* 1989). Primers for *Xhe* were: upstream 5'-TGTGCCTTACAACCTCTCCT-3', downstream 5'-TCCAACTCCAATCTCTGAGC-3'. Primers for *ODC* were: upstream 5' GGAGCTGCAAGTTGGAGA-3', and downstream 5'-TCAGTTGCCA-GTGTGGTC-3'.

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