A member of the RXR nuclear receptor family is expressed in neural-crest-derived cells of the developing chick peripheral nervous system

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Summary

Retinoic acid (RA) affects differentiation and morphogenesis in various developmental systems and is believed to act through nuclear RA receptors that belong to the steroid/thyroid hormone family of ligand-binding transcription factors. Three closely related receptors, RAR-α, -β and -γ, with distinct expression patterns, have been identified and a fourth receptor, hRXR-α, which responds to RA but which has low homology to RAR-α, -β and -γ, was recently discovered. Here we report the isolation of a cDNA clone encoding a chicken homologue of hRXR-α (cRXR) and show that a cRXR transcript of 2.5 kb is expressed in a range of embryonic chick tissues. By in situ hybridization to sections from stage 24 and stage 27 chick embryos, we show that cRXR transcripts are expressed at high levels in the liver and in elements of the developing peripheral nervous system derived from the neural crest, including dorsal root ganglia, cranial ganglia, enteric ganglia and peripheral nerve tracts. At stage 16, in the posterior trunk region, cRXR transcripts are expressed by cells in the neural crest and in neural crest cells migrating into the sclerotome, indicating that neural crest cells express cRXR transcripts before overt differentiation into peripheral nervous tissue. This distribution suggests a novel role for RA in the developing peripheral nervous system, mediated by cRXR. In addition, it identifies cRXR as a marker for a specific population of neural-crest-derived cells.

Key words: RXR, retinoic acid receptor, peripheral nervous system, neural crest.

Introduction

The retinoids are a group of related compounds, including vitamin A (retinol) and its metabolite retinoic acid (RA), that profoundly affect differentiation and morphogenesis in various developmental systems. Local application of RA to developing chick limb buds induces duplication of the digits (Tickle et al. 1982) and there is evidence to suggest that RA may be a natural signalling substance in the chick limb bud (Thaller and Eichele, 1987; Brickell and Tickle, 1989; Brockes, 1989). Local application of RA also induces specific malformations of the chick face (Wedden, 1987). In Xenopus laevis embryos, RA acts on the developing central nervous system to transform anterior neural tissue to a posterior neural specification (Durston et al. 1989). In mammals, retinoids are potent teratogens, producing malformations of craniofacial structures, the limb, the heart, the thymus and the central nervous system (Morriss and Thorogood, 1978; Lammer et al. 1985; Satre and Kochhar, 1989). In addition to these effects, retinoids markedly affect the differentiation and maintenance of epithelial cells both in vivo and in vitro (Asselineau et al. 1989; Kopan and Fuchs, 1989) and are used in the treatment of a number of chronic dermatoses (Lammer et al. 1985).

RA acts by binding to nuclear RA receptors, which are members of the steroid/thyroid hormone receptor superfamily. The resultant ligand–receptor complexes are capable of regulating the transcription of target genes (Green and Chambon, 1988; La Rosa and Gudas, 1988; de Thé et al. 1990). Three closely related RA receptors, encoded by distinct genes, have been identified in humans and mice. These are RAR-α (Petkovich et al. 1987; Giguère et al. 1987), RAR-β (Brand et al. 1988; Benbrook et al. 1988) and RAR-γ (Zelent et al. 1989; Krust et al. 1989). A number of isoforms of RAR-γ, generated by alternative splicing of the primary transcript, have also been identified (Kastner et al. 1990; Giguère et al. 1990). The expression patterns of the three RAR genes are quite distinct (Dolle et al. 1989; Ruberte et al. 1990; Zelent et al. 1989). Recently, a fourth species of nuclear receptor that responds to RA (hRXR-α) was identified in humans (Mangelsdorf et al. 1990). Whilst hRXR-α is still a member of the steroid/thyroid hormone receptor superfamily, it has no significant homology to RAR-α, RAR-β and RAR-γ in the RA-binding domain and
therefore appears to represent an evolutionarily distinct RA response pathway. Transcriptional transactivation by hRXR-α is considerably less sensitive to RA than is transactivation by hRAR-α, an approximately five-fold higher concentration of RA being required for a 50% maximal response with hRXR-α (Mangelsdorf et al. 1990). This suggests that a closely related metabolite or structural analogue of RA, rather than RA itself, may be the natural ligand of hRXR-α. High level expression of hRXR-α was detected in rat liver, by northern blotting analysis (Mangelsdorf et al. 1990).

Here we report the isolation of a cDNA clone encoding a chicken homologue of hRXR-α (cRXR) and show by in situ hybridisation that, in addition to high level expression in liver, cRXR transcripts are unexpectedly expressed in elements of the developing peripheral nervous system derived from the neural crest.

Materials and methods

Isolation of cDNA clones

Approximately $2 \times 10^5$ recombinant bacteriophage from a 10 day (stage 36) chick embryo cDNA library constructed in λgt11 (Clontech) were screened with a 380 bp KpnI–PstI fragment of the human RAR-α cDNA clone p63 (Petrovich et al. 1987), which had been labelled with [α-32P]dCTP (New England Nuclear) by random priming (Feinberg and Vogelstein, 1984). This probe contained sequences encoding the A, B and most of the C (DNA-binding) domain of human RAR-α. Plaque lifts on to Hybond-N (Amersham International) were performed according to the manufacturer’s instructions. Hybridisation to duplicate filters was performed for 16h at 65°C in a solution containing 6xSSC, 1% (w/v) SDS, 5x Denhardt’s solution, 0.1 mg ml$^{-1}$ yeast total RNA and 10$^6$ cts min$^{-1}$ ml$^{-1}$ of radiolabelled probe. The most stringent post-hybridisation wash was for 30 min at 55°C in 1xSSC, 0.1% (w/v) SDS. The filters were autoradiographed and positive clones were purified by rescreening under the same conditions. The EcoRI insert of clone λR2 was subcloned into the plasmid vector pBluescript SK$^+$ (Strategene) for subsequent analysis, yielding plasmid pR2.

Nucleotide sequencing

Dideoxy sequencing was performed using Sequenase (US Biochemicals) according to the manufacturer’s instructions, either from double-stranded pR2 DNA or following transfer of selected fragments into M13 mp18 or mp19. Oligonucleotide primers were synthesised in our laboratory. Nucleotide sequences were analysed using the Microgenie programme (Beckmann).

RNA isolation and northern blotting

Fertilised chicken eggs were obtained from Poyndon Farm, Waltham Cross, Herts, and were incubated at 38±1°C. The embryos were staged according to Hamburger and Hamilton (1951), dissected into fresh PBS and snap-frozen in liquid nitrogen. Total RNA was isolated from snap-frozen tissue by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) and was then fractionated on a 1% agarose MOPS-formaldehyde gel (10 µg per track) and blotted on to Genescreen Plus membrane (Dupont). Sample quality and quantity were checked by ethidium bromide staining of non-denaturing agarose gels and by hybridisation of filters with probes for type I or type II collagen transcripts, as described previously (Devlin et al. 1988). Filters were hybridised with a 32P-labelled RNA probe synthesised with T7 RNA polymerase (BCL), as previously described (Devlin et al. 1988), using the BamHI–EcoRI fragment of λR2 (Fig. 1; nucleotides 786–1713) as a template, after subcloning into pBluescript SK$^+$ (Strategene) and linearisation with BamHI. Hybridisation was performed for 16h at 65°C in a solution containing 60% (v/v) formamide, 6xSSC, 20 mm sodium phosphate, 1% (w/v) SDS, 5x Denhardt’s solution, 7% (w/v) dextran sulphate, 0.1 mg ml$^{-1}$ denatured herring testis DNA, 0.1 mg ml$^{-1}$ yeast total RNA, 0.01 mg ml$^{-1}$ poly(A) and 10$^6$ cts min$^{-1}$ ml$^{-1}$ of radiolabelled probe. The most stringent post-hybridisation wash was for 30 min at 80°C in 0.1xSSC, 0.1% (w/v) SDS.

In situ hybridisation

Chick embryos, staged according to Hamburger and Hamilton (1951) were embedded in wax as described by Davidson et al. (1988). 7µm sections were then cut, collected on slides coated with 3-aminopropyltriethoxysilane (TESPA, Sigma) and baked for 6–16h at 60°C. In situ hybridisation was performed essentially as described by Hogan et al. (1986), using a 35S-labelled antisense RNA probe synthesised from the same cRXR template as for the northern hybridisation probe. Hybridisation was performed at 55°C. As a negative control, adjacent sections were hybridised with a 32P-labelled sense strand RNA probe synthesised from the same template. After post-hybridisation washes and treatment with 40 µg ml$^{-1}$ RNAase A (Wilkinson et al. 1987), the slides were autoradiographed (Hogan et al. 1986) and stained with 0.05% (w/v) malachite green.

Results

Isolation of a cDNA clone for a chicken homologue of hRXR-α

In order to isolate chicken homologues of the human and murine RARs, a stage 36 (10 day) chick embryo cDNA library was screened at low stringency with a radiolabelled probe derived from the human RAR-α cDNA clone p63 (Petrovich et al. 1987). Amongst the clones isolated was λR2. The restriction map and nucleotide sequence of the insert of AR2 and the clones isolated was AR2. The restriction map and nucleotide sequence of the insert of AR2 and the predicted amino acid sequence of the protein that it would encode are shown in Fig. 1. The predicted amino acid sequence is strongly homologous to that of the human nuclear retinoic acid receptor hRXR-α (Mangelsdorf et al. 1990), not only in the putative DNA-binding domain, but also in the putative ligand-binding domain (Fig. 2). It is also strongly homologous in these domains to the predicted protein product of the cDNA clone H-2RIIBP (Hamada et al. 1989), which Mangelsdorf et al. (1990) have suggested might represent a murine RXR-β species (Fig. 2). In contrast, the predicted amino acid sequence of λR2 is less homologous to those of human RAR-α, RAR-β and RAR-γ in the DNA-binding domain, and shows no significant homology to the ligand-binding domains of these proteins (Fig. 2) or to those of any other known members of the steroid/thyroid hormone receptor superfamily (data not shown). The cDNA clone λR2
A) The amino acid sequence derived from cDNA clone AR2. The putative DNA-binding domain is underlined. Sequences that are conserved in evolution are shown in upper case. Sequences that are conserved only within the RXR family are shown in lower case.

B) A restriction map of the insert of cDNA clone AR2. 5' and 3' untranslated regions are shown as solid lines. The coding region is boxed. The shaded region represents the putative DNA-binding domain. Sequences present in this probe used in northern blotting and in situ hybridisation analyses are indicated. B, BamHI; Ba, Balti; E, EcoRI; H, HindII; N, NcoI; P, PstI; Pv, PvuII; S, Smal; Sa, SaeI; X, XhoI. (B) Nucleotide sequence and predicted amino acid sequence derived from cDNA clone AR2. EcoRI linker sequences are shown in lower case. The putative DNA-binding domain is underlined.

Fig. 1. (A) Restriction map of the insert of cDNA clone AR2. 5' and 3' untranslated regions are shown as solid lines. The coding region is boxed. The shaded region represents the putative DNA-binding domain. Sequences present in this probe used in northern blotting and in situ hybridisation analyses are indicated. B, BamHI; Ba, Balti; E, EcoRI; H, HindII; N, NcoI; P, PstI; Pv, PvuII; S, Smal; Sa, SaeI; X, XhoI. (B) Nucleotide sequence and predicted amino acid sequence derived from cDNA clone AR2. EcoRI linker sequences are shown in lower case. The putative DNA-binding domain is underlined.

therefore appears to derive from a mRNA encoding the chick homologue of hRXR-α, or a very closely related protein. The protein product of AR2 will be referred to as cRXR. The homology between cRXR and hRXR-α is significant, but less marked, in the amino-terminal region (Fig. 2). This might simply indicate that the amino-terminal region has been relatively poorly conserved in evolution. Alternatively, cRXR might be derived from a second member of the RXR family, or could represent an alternatively spliced transcript of the chicken RXR-α gene, as has been observed for RAR-γ (Kastner et al. 1990; Giguere et al. 1990). The resolution of these issues must await the cloning of other members of the RXR gene family from human and chicken.

Northern blotting analysis of cRXR gene expression

A BamHI–EcoRI fragment of AR2 (Fig. 1A,B, nucleotides 786–1713) was used as a template for the synthesis of a radio-labelled antisense RNA probe for cRXR transcripts. This probe was complementary to sequences encoding the putative ligand-binding domain of cRXR, which are not highly conserved amongst members of the steroid/thyroid hormone receptor family. The probe did not contain sequences complementary to those encoding the putative DNA-binding domain, which are conserved amongst members of this family. This minimised the risk of cross-hybridisation of the probe to transcripts of other genes within the family. Northern blotting analysis (Fig. 3) identified a single cRXR transcript of approximately 2.5 kb in chick limb buds, heads and bodies at stages 22 and 25. This transcript was also found at low levels in a range of adult chick tissues, and at high levels in adult liver (data not shown). The distribution in the adult chicken is similar to that found by northern blotting in the adult rat, using the human RXR-α probe (Mangelsdorf et al. 1990).
Fig. 3. Northern blotting analysis of cRXR transcript expression in chick embryos. Tracks contain 10 μg of total RNA isolated from stage 22 embryo wing bud (1), stage 22 embryo leg bud (2), stage 22 embryo head (3), stage 25 embryo head (4), stage 22 embryo body (5) and whole stage 36 embryo (6). The size of the transcript is indicated in kilobases.

Distribution of cRXR transcripts in stage 24 and stage 27 chick embryos

In order to determine in more detail the cellular pattern of cRXR expression, in situ hybridisation to tissue sections from stage 27 chick embryos was performed using a 35S-labelled RNA probe synthesised from the same template as the northern blotting probe. A negative control RNA probe synthesised in the opposite orientation, from the same template, did not hybridise specifically to any cells within the tissue sections (Fig. 4E,F). The cRXR probe hybridised strongly to liver, as expected from the northern blotting data (Fig. 4A,C). However, there was also strong hybridisation to elements of the peripheral nervous system (Fig. 4A,B,C,D), including the dorsal root ganglia, the dorsal root, the ventral root, the spinal nerve and its dorsal and ventral rami and the sympathetic chain. In contrast, there was no hybridisation to the neural tube or to the developing ventral horn. Punctate regions of hybridisation were visible in the gut wall (Fig. 4A,C), consistent with hybridisation to cells of the developing enteric ganglia, and in the wing buds (Fig. 4A,C), consistent with hybridisation to the developing innervation of this tissue. In transverse sections of stage 24 embryos, a similar pattern of hybridisation was seen (data not shown). In frontal and sagittal sections of stage 24 chick embryos, such as those shown in Fig. 5 (C,D), cRXR transcripts were found in

Fig. 4. Distribution of cRXR transcripts in stage 27 chick embryo. (A–D), Transverse section of a stage 27 embryo hybridised with the probe for cRXR transcripts; (E,F), adjacent section hybridised with the negative control probe. Exposure times were 18 days. Sections are shown under light-field (A,B) or dark-field (C–F) illumination at low (A,C,E) or high (B,D,F) magnification (all scale bars: 200 μm). c, sympathetic chain; d, dorsal root; dg, dorsal root ganglion; dr, dorsal ramus of spinal nerve; g, gut; l, liver; n, neural tube; s, spinal nerve; v, ventral root; w, wing bud.
**Fig. 5.** Distribution of cRXR transcripts in stage 24 chick embryo. Sagittal sections (A,C) of stage 24 chick embryos were hybridised with the probe for cRXR transcripts and adjacent sections (B,D) were hybridised with the negative control probe. Exposure times were 18 days. Sections are shown under light-field illumination. dg, dorsal root ganglion; g, geniculate ganglion; o, otic ganglion; ot, otocyst; s, spinal nerves; t, trigeminal ganglion; X, tenth cranial nerve. The disposition of the cranial ganglia in the stage 24 embryo has been described by Kuratani (1990). The apparent high background in section B is due to the density of malachite green staining and not to the presence of silver grains. Scale bars: 200 μm.

All of the dorsal root ganglia along the body axis and in the spinal nerves associated with them. As shown in Fig. 5 (A,B), cRXR transcripts were also present within sensory ganglia located on cranial nerves, including V (trigeminal ganglion), VII (geniculate ganglion), VIII (otic ganglion) and X. Punctate hybridisation to the gut wall and high level expression in the liver was also apparent at stage 24, as at stage 27 (data not shown). Since there was hybridisation to nerve roots at stage 24 and stage 27, cRXR transcripts must be expressed by peripheral glial cells. However, we cannot exclude the possibility that neurons also express cRXR transcripts. In either case, it is apparent that expression of cRXR transcripts within nervous tissue is limited to those cells that are derived from the neural crest (Le Douarin and Smith, 1988).

**Distribution of cRXR transcripts in migrating neural crest cells**

At stage 16, in the posterior trunk of the chick embryo,
neural crest cells continue to migrate into the sclerotome, where some will differentiate into cells of the dorsal root ganglia and other elements of peripheral nervous tissue (Le Douarin and Smith, 1988). As shown in Fig. 6, cells in the neural crest and cells migrating from the neural crest into the sclerotome at stage 16 expressed cRXR transcripts. In more anterior regions of the stage 16 embryo, the development of the peripheral nervous system is more advanced and cRXR transcripts were found, for example, in the trigeminal ganglion (data not shown).

Discussion

We have described the isolation and characterisation of a cDNA clone encoding a chicken homologue (cRXR) of the recently discovered human nuclear receptor hRXR-α, and have shown that cRXR transcripts are expressed in the liver and in elements of the peripheral nervous system that are derived from the neural crest. We have also shown that cRXR transcripts are present in migrating neural crest cells, prior to the overt development of peripheral nervous tissue.

It is probable that there will prove to be a family of closely related RXR-like receptors, analogous to the RAR-like family (Mangelsdorf et al. 1990). Whilst the other members of this putative family have not yet been characterised, Mangelsdorf et al. (1990) have suggested that the cDNA clone H-2RIIBP, which was isolated by Hamada et al. (1989), might represent a murine RXR-β species. Whilst cRXR and the H-2RIIBP product are clearly related, the homology between them is less strong than that between cRXR and hRXR-α (Fig. 2).

Mangelsdorf et al. (1990) showed that hRXR-α can respond to RA by activating transcription from a promoter containing a palindromic thyroid hormone response element, although transactivation by hRXR-α is less sensitive to RA than is transactivation by human RAR-α. Our preliminary data show that cRXR can also activate transcription from this promoter in response to RA.

The distribution of cRXR transcripts, which we have described, is quite different to that of RAR-α, RAR-β and RAR-γ transcripts. This indicates that the biological roles of the RXR-like and RAR-like receptors are different. In the mouse, RAR-γ transcripts are present in various tissues whose development is sensitive to retinoid treatment, including primary mesenchyme, cartilage and differentiating squamous keratinizing epithelia (Zelent et al. 1990; Ruberte et al. 1990). Cells expressing RAR-γ do not have a common embryological origin and include derivatives of the endoderm, mesoderm, ectoderm and cranial neural crest. The distribution of RAR-α and RAR-β transcripts has not been described in such detail, but RAR-α transcripts appear to have a rather general distribution, whilst RAR-β transcripts are restricted to particular regions of mesenchyme that tend not to express RAR-γ transcripts (Dolle et al. 1989; Ruberte et al. 1990). RAR-α, RAR-β and RAR-γ transcripts have not been found in the peripheral nervous system at any stage of development (Dolle et al. 1989; Ruberte et al. 1990). The distribution of cRXR transcripts does, however, show some similarity to that of cellular retinoic acid-binding protein (CRABP) in stage 24 chick embryos (Maden et al. 1989) and in mouse embryos at similar stages (Maden et al. 1990). For example, CRABP is expressed in dorsal root ganglia, sensory nerves, sympathetic ganglia and enteric ganglia. It is not clear whether the roles of cRXR and CRABP in these tissues are integrated in any way. However, CRABP is not expressed in the ventral root, which does express cRXR transcripts and, conversely, high levels of CRABP are present in the neural tube, which does not express cRXR transcripts. The distribution of cRXR transcripts therefore correlates more closely with neural crest origin than does CRABP expression, and cRXR clearly...
RXR in the peripheral nervous system

provides a valuable molecular marker for a specific population of neural-crest-derived cells.

Mangelsdorf et al. (1990) speculated that the high level expression of hRXR-α in the liver may indicate a role for the hRXR-α family of receptors as regulators of vitamin A storage, metabolism or mobilisation by this organ. Our data support this idea, but also indicate an important role in the neural-crest-derived cells of the peripheral nervous system. The nature of this role is unclear. RA can induce nerve growth factor receptor expression in immature chick sympathetic neurons (H. Rohrer, personal communication), but it is not known whether this is a transcriptional effect or whether these cells express cRXR. Retinoids are known to have teratogenic effects on the central nervous system (Durston et al. 1989; Lammer et al. 1985) and on tissues derived from cranial neural crest cells, including craniofacial structures, heart and thymus (Lammer et al. 1985). However, retinoid-induced malformations of the trunk neural crest-derived structures of the peripheral nervous system have not been described (Lammer et al. 1985; Smith-Thomas et al. 1987). Retinoids do induce decreased cell-substratum adhesion in both cranial and trunk neural crest cells in vitro (Smith-Thomas et al. 1987) and it has been suggested this inhibits neural crest cell migration in vivo (Pratt et al. 1987). We have shown that cells migrating from the neural crest of the posterior trunk at stage 16 express cRXR transcripts, raising the possibility that cRXR mediates these effects of retinoids. However, it is likely that the effects of retinoids on neural crest cells in vitro are mediated by the disruption of cell membrane structure rather than by the induction of changes in gene expression (Smith-Thomas et al. 1987). Similarly, experimental vitamin A deficiency in animals results in peripheral nerve degeneration, but this is secondary to a selective cessation of bone growth, rather than representing a direct effect on peripheral nervous tissue (Duchen and Jacobs, 1984). It may be that the effects of RA upon the development and function of the peripheral nervous system are subtle, and a search for such effects might be informative. Alternatively, it is possible that a closely related metabolite or structural analogue of RA, rather than RA itself, is the natural ligand of cRXR (Mangelsdorf et al. 1990). However, as with the RAR family of RA receptors, a fuller understanding of function will require the identification of target genes regulated in vivo.

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References


Kastner, P., Kastner, P., Petkovich, M., Zelent, A. and...


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