Retinoic acid receptors and nuclear orphan receptors in the development of *Xenopus laevis*

CHRISTINE DREYER* and HEIDRUN ELLINGER-ZIEGELBAUER

Max Planck-Institut für Entwicklungsbiologie, Tübingen, Germany

ABSTRACT  Nuclear hormone receptors are ligand-activated transcription factors that regulate the expression of target genes by binding to hormone responsive elements (HRE) in their 5’ upstream region. Retinoids which are known for their teratogenicity and which have a potential role in the specification of the anteroposterior axis of vertebrate embryos regulate transcription via a hormone-like mechanism by activating nuclear retinoic acid receptors, designated RAR and RXR. Of the several isoforms of RAR found in embryos of *Xenopus laevis*, xRAR2 appears to be the most abundant. During the early retinoic acid-sensitive period of development, the total amount of xRAR2 transcript and protein is increased and a highly specific pattern of expression emerges. During neurulation, the receptor is predominantly found in the dorsal posterior region, in the head endomesoderm, and in the rostral hindbrain. The dependence of this pattern on mesoderm induction and on neural induction is discussed. Contrasting with the elaborate pattern of xRAR2, the FTZ-F1-related nuclear orphan receptors (xFF1rA/B) are ubiquitous nuclear proteins in *Xenopus* embryos, as are the peroxisome proliferator-activated receptors xPPARα and B. PPARs are activated by polyunsaturated fatty acids and regulate the synthesis of enzymes involved in lipid metabolism. Later in development, the isoforms xPPARα, B, and γ attain different tissue specificities.

KEY WORDS: retinoic acid receptor, nuclear orphan receptor, PPAR, FTZ-F1, GCNF, *Xenopus laevis*

Introduction

Nuclear hormone receptors are ligand-dependent transcription factors that regulate the expression of a limited number of target genes by binding to specific hormone responsive elements (HRE) in their upstream regulatory region (for reviews see Wahli and Martinez, 1991; Glass, 1994). As was first shown for steroid hormone receptors, and later for a family of related proteins, these transcription factors consist of the same array of domains, A to F, whose function was tested in isolation or after recombination of functional domains originating from different receptors. The most highly conserved domain between receptors of different specificities is the DNA-binding domain (DBD) C with its characteristic Zn-finger structure. Within the Zn-finger structure, the P-box, a motif comprising the second pair of cysteine residues, is essential for sequence-specific binding of the DNA target. The amino acid sequence of the P-box allows for a prediction of the sequence of the 6 bp half-site element that is characteristic of a given HRE. Hormone responsive elements consist of imperfect direct repeats or of palindromes or inverted palindromes of a given half-site element, separated by a characteristic number of nucleotides (Martínez et al., 1991; Stunnenberg, 1993). In some orphan receptors (see below), monomeric extended half site elements (Wilson et al., 1993) are bound by a DBD consisting of domain C and an adjacent part of domain D (Ueda et al., 1992). Domain D often contains a nuclear localization signal. Several functions can be ascribed to the ligand-binding domain E, which is followed by a shorter and less conserved C-terminal region called F. Besides ligand binding, dimerization, transcriptional activation or silencing and, in the subgroup of the GR-like receptors, a ligand-dependent NLS function (Picard and Yamamoto 1987) have been ascribed to the E domain. The N-terminal A/B domain is highly variable, especially between differentially spliced variants of receptors derived from the same gene (see e.g. Leid et al., 1992), and may contribute to transcriptional enhancement.

Corresponding to the bipartite structure of the hormone responsive elements, the receptor proteins form homo- or heterodimers, and their response is modulated by a variety of other proteins. As examples, the GR (classical glucocorticoid receptor) is stabilized and becomes transcriptionally active upon ligand binding and association with the coactivator CBP (C/EBP binding protein) (Fournier et al., 1994), and the AP-1 complex is activated by the receptor RAR-α upon ligand binding (Koopman et al., 1992).

Abbreviations used in this paper: CRABP, cellular retinoic acid binding protein; DBD, DNA-binding domain; ftz, fushi tarazu; GCNF, germ cell nuclear factor; GR, glucocorticoid receptor; HRE, hormone responsive element; mELP, marine embryonic long terminal repeat binding protein; mSF-1, marine steroidogenic factor 1; PPAR, peroxisome proliferator activated receptor; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoic X receptor; xFF1rA and B, *Xenopus* FTZ-F1-related receptor A and B.

*Address for reprints: Max Planck-Institut für Entwicklungsbiologie, Postfach 2109, D-72011 Tübingen, Germany. FAX: 7071-601449. e-mail: dreyer@gen.mpib-tuebingen.mpg.de.*

*Present address: Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA.*
erodimers thereby enhancing the combinatorial possibilities of gene regulation (Forman and Samuels, 1990; Stunnenberg, 1993; Glass, 1994). Moreover, a potential antagonism between nuclear receptors competing for the same response element (Katz and Lazar 1993), and activation of receptors by protein phosphorylation (Legoff et al., 1994), or inhibition by binding of calreticulin (Dedhar et al., 1994) may contribute to the fine tuning of transcriptional regulation. Not only thyroid and steroid hormones, or the steroid vitamin D3, but also retinoic acids (see Stunnenberg, 1993, and Giguere, 1994 for recent reviews) and fatty acids (Keller et al., 1993a,b; Dreyer et al., 1993, and references therein) are able to mediate transcriptional regulation by activation of transcription factors that by their structure and function belong to the nuclear hormone receptor superfamily (Laudet et al., 1992). Moreover, homologs of the nuclear hormone receptors have been described for which no ligand has yet been identified. Such orphan receptors may either function constitutively or they could be activated by metabolites present in most cells that are used for the functional studies on novel receptors.

The RA-sensitive phase during embryogenesis of X. laevis

The effects of retinoic acid (RA) on the development of different vertebrate species has been studied systematically. Low doses of RA lead to compression of the rostral hindbrain of the zebrafish (Holder and Hill, 1991) and of X. laevis (Papalopulu et al., 1991). In addition, in the frog higher doses result in severe malformation of facial structures, including the eyes, which are reduced in size, fused, or even missing (Fig. 1). In embryos of X. laevis, a RA-sensitive phase which lasts up to midneurula stages has been defined with respect to these anterior defects (Durston et al., 1989). Later pulses of RA mainly affect tail differentiation (Ruiz i Altaba and Jessell, 1991; Pfeffer and De Robertis, 1994), whereas the trunk region appears to be more resistant to RA (Fig. 1a). Beside all-trans-RA, 9-cis-RA (Creech-Kraft et al., 1994), 4-oxo-RA (Pijnappel et al., 1993), didehydro-RA and the synthetic retinoid Am80 (Dreyer, unpublished observations) cause similar malformations in X. laevis.

Analysis of the developmental defects caused by exogenous RA on vertebrates has led to the hypothesis that endogenous RA is potentially involved in the specification of the anteroposterior axis (Durston et al., 1989; Ruiz i Altaba and Jessell, 1991; Simeone et al., 1995). Endogenous all-trans-RA and 9-cis-RA can be detected in whole embryos of X. laevis at the neurula stage (Durston et al., 1989; Creech-Kraft et al., 1994). X. laevis embryos also contain 4-oxo RA (Pijnappel et al., 1993) and a source of endogenous active retinoids is established in the dorso-posterior region of the embryo during neurulation (Y.P. Chen et al., 1994).

Retinoic acid receptors in embryos of X. laevis

Enzymes involved in vitamin A metabolism, cellular retinoic acid binding proteins (CRABP), and nuclear retinoid receptors are potentially involved in the transmission of retinoid signals. Whereas the function of the CRABP remains a matter of debate, two subfamilies of nuclear receptors have been functionally characterized, the RA-receptors RARα, β and γ, and the retinoid X receptors RXRA, RXRB and RXRC, which are specific for 9-cis-RA and act as heterodimers in conjunction with receptors of RA, thyroid hormone, VitD3, peroxisome proliferators, and some of the orphan receptors (Stunnenberg, 1993; Giguere, 1994; Leblanc and Stunnenberg, 1995 and references therein). In embryos of X. laevis, CRABP, xRARα and γ and xRXRa and γ have been described (see Table 1 for references). Transcripts of xRARα1 and γ2, and of xRXRa and γ are found in the egg. Paternal xRARα1 appears to be replaced by zygotically transcribed xRARα2. Zygotic transcripts of xRXRa and xRARγ2 are also found in the embryo after midblastula transition. The amount of xRARγ2-specific transcripts greatly increases between early gastrula and midneurula (Fig. 3a). Although a β isoform of RAR has not yet been identified in X. laevis, minor amounts of specific transcripts were detectable with a heterologous mouse RARβ probe at early tailbud stages (Fig. 3b).

By means of in situ hybridization, we detected transcripts of xRARα throughout the ectoderm and mesoderm at the midgastrula stage. Starting at the end of gastrulation, an anterior and a posterior region of xRARα transcription emerge, which become prominent in the course of neurulation (Fig. 2). Posteriorly, transcripts are found in the entire circumblastoporal area but predominantly in the dorsal posterior region. Anteriorly, xRARα is expressed in the head endomesoderm and in the prechordal mesoderm, but not in the differentiating notochord and somites.
TABLE 1
Retinoic acid receptors and CRABP mRNA expressed in embryos of Xenopus laevis

<table>
<thead>
<tr>
<th>Receptor</th>
<th>egg cleavage</th>
<th>blastula</th>
<th>gastrula</th>
<th>neurula</th>
<th>tailbud</th>
</tr>
</thead>
<tbody>
<tr>
<td>xRARα1</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
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<td>xRARα2</td>
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<td>+++</td>
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<td>+</td>
<td>++</td>
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<tr>
<td>xRXRα1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
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<td>+/+</td>
<td>+/+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

1) Blumberg et al., (1992); 2) Sharpe, (1992); 3) Ellinger-Ziegelbauer and Dreyer, (1991); for differences between γ1 and γ2 isoforms see Pfeffer and De Robertis, (1994); 4) Dekker et al. (1994). *) Detected with heterologous mouse probe (Fig. 3a).

xRARγ transcripts are also not detected in the anlagen of the eyes and prosencephalon, whereas label is found in the neuroectoderm of the hindbrain area (Ellinger-Ziegelbauer and Dreyer 1991). With isoform-specific probes xRARγ1 was localized in the anterior region of embryos exclusively, whereas xRARγ2 was found anteriorly and posteriorly (Pfeffer and De Robertis, 1994).

To demonstrate the expression of xRARγ at the protein level, we have raised a specific polyclonal serum against the D/E/F domains of xRARγ2 which detects a single protein band of the expected MW on developmental Western blots during the phase of increasing xRARγ mRNA expression (Fig. 3b). A second antigen band of higher MW emerges at later stages. This could represent another isoform of xRARγ, or a putative xRARβ which is also detected by the antisemum. Between gastrula and late neurula stages, we consider this polyclonal serum to be a selective reagent for xRARγ in the embryo.

The expression pattern of the corresponding nuclear antigen closely follows the pattern observed by in situ hybridization, except that nuclear staining in the head endomesoderm (Fig. 4c,d) and the hindbrain area (Fig. 4b) precedes nuclear staining in the posterior region (Fig. 4a; Ellinger-Ziegelbauer and Dreyer, 1993), despite the abundance of the RNA signal in the dorsal-posterior region from stage 13 onwards (Fig. 2; Ellinger-Ziegelbauer and Dreyer, 1991). With the superior resolution of immunohistological detection, the xRARγ protein is seen in the sensorial layer of the rostral hindbrain, posterior to the engrailed stripe (Ellinger-Ziegelbauer and Dreyer 1993), and in a more anterior region of the epithelial layer of the hindbrain between midneurula and late neurula stages (Fig. 4b). Remarkably, the anlagen of the forebrain and the eyes do not express xRARγ to a measurable extent (Fig. 4d), although these structures are affected by excess RA. Similarly, the region subjacent to the cement gland, but not the cement gland itself contains nuclear xRARγ (Fig. 4c,d).

At later stages of development, transcripts of xRARγ2 are found in the head mesenchyme, which is a derivative of the head endomesoderm and the neural crest, and in the tailbud (Ellinger-Ziegelbauer and Dreyer 1991; Pfeffer and De Robertis, 1994). The marked increase of xRARγ transcripts during the RA sensitive phase up to midneurula and the specific localization of the receptor mRNA and protein in the regions which are most sensitive to excess of RA, including the hindbrain, as well as the anterior and posterior region of the neurulating embryo, make xRARγ a likely mediator of exogenous and endogenous retinoid signals.
Effects of exogenous RA on the hindbrain are probably direct because the xRAR\(_y\) protein is expressed in the rostral hindbrain (Fig. 4b; Ellinger-Ziegelbauer and Dreyer 1993). Defects observed on the forebrain, eyes and cement gland could either be mediated by other receptors or be a consequence of RA action on the subjacent head endomesoderm (Fig. 4c,d). Alternatively, RA might ectopically induce RAR\(_\gamma\) or \(\alpha\), as has been observed in the zebrafish (Joore et al., 1994). The later defects in the development of the tail are most probably associated with the persistence of xRAR\(_y\) in the tip of the tail (Ellinger-Ziegelbauer and Dreyer 1993; Pfeffer and De Robertis 1994). Null mutant mice for RAR subtypes revealed a functional redundancy among RARs. Despite this fact, null mutant mice for RAR\(_y\) showed that the sensitivity of tail development to RA is associated with the RAR\(_y\) gene (Lohnes et al., 1993, and references therein).

The higher sensitivity of the anterior region of Xenopus embryos to exogenous RA could be due to the fact that the endogenous source of active retinoids is posterior (Y.P. Chen et al., 1994), and therefore the presence of RA anteriorly represents an unphysiological situation. Moreover, the xRAR\(_y\) protein is detected in the anterior region first (Ellinger-Ziegelbauer and Dreyer 1993). Interestingly, the region-specific localization of CRABP in the embryo resembles that of xRAR\(_y\) in the hindbrain and in the tip of the tail. Overexpression of CRABP leads to defects resembling those caused by an excess of RA (Dekker et al., 1994), suggesting that CRABP may sequester RA in some areas that are highly responsive to retinoids due to their content of nuclear receptors. Among the potential target genes of RAR are RAR\(_\beta\), CRABP, and a number of HOX-genes (Dekker et al., 1993; Stunnenberg, 1993). The orphan receptor COUP-TFI may counteract RA-signaling (Schuh and Kimelman, 1995).

**Establishment of region-specific expression of xRAR\(_y\)**

We have investigated how manipulation of axis formation might influence the elaborate pattern of xRAR\(_y\) expression in the embryo (Ellinger-Ziegelbauer and Dreyer, 1993). After UV-irradiation, xRAR\(_y\) protein was immunolocalized in the symmetrical blastoporal lips. In dorsalized embryos, we detected xRAR\(_y\) predominantly in the undifferentiated endomesoderm (Ellinger-Ziegelbauer and Dreyer, unpublished). In explanted animal caps, we could demonstrate that transcription of the xRAR\(_y\)-specific mRNA occurred independent of mesoderm induction. Yet treatment of the explants with activin increased the amount of xRAR\(_y\) mRNA and triggered the specific pattern of protein expression, mainly in the undifferentiated endomesoderm at both ends of the elongating explants, but not in the differentiated notochord or somites (Ellinger-Ziegelbauer and Dreyer 1993).

To investigate whether the regional expression of xRAR\(_y\) depends on vertical interactions between the endomesoderm and the neuroectoderm, which are established during the course of gastrulation, we have examined exogastrulae and Keller sandwich explants of dorsal tissue. In both experimental systems, the axes of the neuroectoderm and of the endomesoderm point in opposite directions, with merely the posterior ends of both parts apposing one another. In exogastrulae, as well as in Keller sandwiches, expression of xRAR\(_y\) in the head endomesoderm was easily detectable. Expression of xRAR\(_y\) protein in the neuroectodermal region of the Keller sandwich corresponding to the hindbrain was weak, yet significant (Ellinger-Ziegelbauer and Dreyer, 1993). In conclusion, expression of xRAR\(_y\) in undifferentiated endomesoderm does not depend on contact with the ectoderm. For region-specific expression in the hindbrain area of the neuroectoderm, a planar signal emanating from the dorsal lip appears to be sufficient. Pattern formation is, however, likely to be enhanced or stabilized by vertical signals in the whole embryo.

**Peroxisome proliferator-activated receptors (PPAR)**

We have identified three different PPARs of *X. laevis* by cDNA cloning, and named them xPPAR\(_{\alpha}\), \(\beta\) and \(\gamma\) (Dreyer et al., 1992), according to their similarity with the previously identified murine PPAR\(_{\alpha}\) (Iseman and Green, 1990). Activators of PPARs include xenobiogenic hypolipidemic drugs, e.g. clofibrate, and plasticizers, e.g. Wy 14.643, that induce proliferation of peroxisomes in rodent liver. In tadpoles of *X. laevis*, peroxisomal enzymes are induced by clofibrate without a detectable augmentation in size or number of the organelles (Ciolek and Dauc, 1991). A peroxisome proliferator responsive element (PPRE) was first detected in a rat gene encoding peroxisomal acyl-CoA oxidase, the key enzyme of the fatty acid \(\beta\)-oxidation pathway (Osumi et al., 1991). Consistently, long chain polyunsaturated fatty acids including arachidonic acid, have been identified as potent natural activators of PPARs (Keller et al., 1993a, b). Thus fatty acids provide a first example of nutrients which may induce the synthesis of enzymes required for their catabolism by a hormone-like mechanism. A number of genes encoding enzymes for lipid metabolism contain a PPRE consisting of a direct repeat motif (DR1) in their regulatory region (Krey et al., 1993). Reporter genes containing this PPRE are bound and activated most efficiently by heterodimers of PPAR and RXR in the presence of their respective activators. Thus 9-cis-RA and polyunsaturated fatty acids cooperate in the activation of target genes (Keller et al., 1993a).

The cDNAs specific for xPPAR\(_\alpha\) and \(\beta\) mRNAs are found in oocytes and embryos, while only traces of xPPAR\(_y\) mRNA were detected. During oogenesis and embryogenesis, transcripts of xPPAR\(_{\beta}\) prevail (Dreyer et al., 1992), and the xPPAR\(_{\beta}\) protein can be localized by immunofluorescence in nuclei of immature...
oocytes (Fig. 4e) and in all nuclei of the neurula stage embryo (Fig. 4f). Whereas xPPARα and β mRNAs are rather ubiquitously expressed in organs of the adult frog, xPPARγ mRNA is found mainly in adipose tissue, kidney and liver (Dreyer et al., 1993). A second transcript of xPPARγ we have exclusively found in the fat body, and this longer transcript likely corresponds to the fat-specific murine isof orm mPPARγ2. Expression of this mPPARγ2 isof orm in fibroblasts in presence of specific activators is sufficient to induce adipocyte differentiation (Tontonoz et al., 1994).

Beside their different tissue specificities, some differences in responsiveness to different activators have been detected in cotransfection studies. The most potent activator of xPPARα known so far is eicosatetraynoic acid (ETYA), a stable homolog of arachidonic acid (Keller et al., 1993a). Activation of xPPARγ or β, however, requires 100 times the amount of ETYA as compared to xPPARα (Krey et al., 1993). Similar observations were reported on the murine PPARα, γ, and δ (Kiewer et al., 1994). Recently, antidiabetic thiazolidinediones were identified as specific ligands of PPARγ (Lehmann et al., 1995; P. Devchand and W. Wahl, personal communication).

The ubiquitous occurrence of xPPARs in embryos and in organs of the adult corroborates their postulated role in metabolic homeostasis. xPPARα and β, which are abundant through- out oogenesis, are potential regulators of a maternal pool of peroxisomes. This would be an essential function because peroxisomes multiply by growth and fission (Lazarow and Fujiki, 1985). Of special interest is the question whether growth factors are targets of PPAR, since peroxisome proliferators have been described as nongenotoxic hepatocarcinogens in rodents (Lock et al., 1989).

FTZ-F1-related organ receptors

The transcription factor FTZ-F1 was first identified in the fruit fly D. melanogaster as a regulator involved in the expression of the ftz gene product in seven stripes early in embryogenesis (Lavorgna et al., 1991). At least two homologs of FTZ-F1 exist in vertebrates, one is represented by the steroidogenic factor-I (mSF-I; Ikeda et al., 1993) and its splice variant mELP (Tsukiyama et al., 1991). By means of null mutants, SF-I has been proven to be essential for the development of gonads and adrenal glands (Luo et al., 1994). The other vertebrate counterpart of DmFTZ-F1 is the mouse liver receptor homolog (mLRH1) and its X. laevis homologs xFF1rA and B, which are likely to be pseudallelic forms of the same receptor (see Ellinger-Ziegelbauer et al., 1994 and references therein). The function of these orphan receptors is presently unknown. On developmental Northern blots, at least four different xFF1-related transcripts were detected and the amounts of mRNA increased between gastrulation and tailbud stages. The complex pattern of transcripts may be explained by the expression of both the A and the B gene, and the existence of differentially spliced isoforms (Ellinger-Ziegelbauer et al., 1994). During early tailbud stages, when the xFF1rA/B transcripts are most abundant, xFF1-related receptors are ubiquitous nuclear antigens (Fig. 4h), and protein binding to FTZ-F1 response elements (FRE) can be detected in nuclear extracts of tailbud embryos (Ellinger-Ziegelbauer et al., 1994). In adult X. laevis, we find xFF1r protein mainly in the liver (data not shown).

Whereas steroid hormone receptors bind to palindromic response elements as homodimers, and RAR and PPAR bind as heterodimers with RXR to direct repeats of 6bp half site elements (see Wahl and Martinez, 1991; Stunnenberg, 1993 and references therein), xFF1rA, like other FTZ-F1-related orphan receptors, binds as a monomer to monomeric FRE in vitro (Ellinger-Ziegelbauer et al., 1994). Interestingly, the C-terminal E-domain influences the efficiency of FRE-binding by the DNA binding domain (DBD), which consists of the Zn-finger domain C and the adjacent FTZ-F1 box.

Minor amounts of a transcript encoding a C-terminally truncated receptor, xFF1rA short have been detected in embryos by RT-PCR. The truncated receptor xFF1rA short diverges from the full-length form in the conserved region II of the E-domain, and it neither activates nor silences FRE-containing reporter genes. An excess of xFF1rA short interferes with transcriptional activation of the full-length form, probably by competitive DNA binding (Ellinger-Ziegelbauer et al., 1995).

GNCF: a novel orphan receptor subfamily

By means of PCR-aided cDNA cloning, we have identified a novel orphan receptor of X. laevis, whose closest relative is the germ cell nuclear factor of the mouse (mGCNF; F. Chen et al., 1994), also described as mRTR (Hirose et al., 1995). XGCNF and mGCNF share 84% amino acid sequence identity and both are only distantly related to other members of the receptor superfamily and can therefore be said to form a novel subgroup. Transcripts of mGCNF are predominantly localized in round spermatids and in diplotene oocytes (F. Chen et al., 1994, Hirose et al., 1995). In addition to oocytes and testes, we find xGCNF expression in embryos of X. laevis. During embryogenesis, transcription of xGCNF reaches a maximum at midneurula stages. In neurula stage embryos xGCNF transcripts are found mainly in the ectoderm, with a gradient that decreases from anterior to posterior. Highest levels of expression are obvious in the neural plate and in neural crest cells (T.O. Joos, R. David and C. Dreyer, in preparation).

Concluding remarks

Analysis of the molecular structure and function of nuclear hormone receptors has enhanced our understanding of hormone action and has revealed that other low MW lipophilic compounds, especially retinoids and fatty acids, may regulate target genes in a manner very similar to steroid and thyroid hormones. Activation of PPARα by fatty acids may involve as yet unidentified mediators, since direct binding of these activators to the receptor could not be demonstrated. In addition to endocrine signals like hormones acting on distant target organs, low MW lipophilic signals may also act as local mediators, or even as intracellular regulators of metabolic homeostasis. During early embryogenesis, prior to the establishment of blood circulation, paracrine signalling might be of special importance, e.g. for the establishment of embryonic fields.

Further investigations into numerous orphan receptors may reveal novel signalling molecules, although we cannot exclude the possibility that orphan receptors represent constitutive activators or silencers. Even without binding of ligands, the conserved structural features of the nuclear hormone receptors...
might be exploited by the orphans in order to function as versatile regulators of transcription. Furthermore, the identification of a broader spectrum of target genes, and of potential binding partners by means of two-hybrid screening may reveal further interconnections between different signalling pathways in embryogenesis and cell differentiation.

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References


