Dimerization is required for transactivation by estrogen-receptor-related (ERR) orphan receptors: evidence from amphioxus ERR

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Abstract

The estrogen-receptor-related (ERR) receptors are orphan members of the nuclear receptor superfamily that bind to their specific DNA target sites as homodimers. However, it has not been shown whether this mode of binding is required for the transcriptional activation they drive. We here show that heterodimerization can also occur between these receptors. Furthermore, we demonstrate that the unique amphioxus ortholog of ERR genes (AmphiERR) is expressed as two isoforms differing by an in-frame insertion. While the short isoform behaves like its mammalian counterparts, the long isoform (AmphiERR(L)) displays divergent transcriptional properties according to the target site to which it binds. Indeed, AmphiERR(L) binds as a monomer but does not activate transcription through the SF1 response element (SFRE). On the contrary, this isoform binds as a homodimer and activates transcription through the classical estrogen-response element. Our results strongly suggest that dimerization is required for transactivation exerted by the ERR receptors.

Introduction

In the human genome, 48 nuclear receptors (NR) have been identified that are generally defined as ligand-dependent transcription factors (Robinson-Rechavi et al. 2001, Laudet & Gronemeyer 2002). However, besides receptors for such diverse ligands as steroid hormones, retinoic acids, thyroid hormone or vitamin D, the NR family comprises a number of ‘orphan’ receptors, for which no ligand has been identified to date (Giguère 1999). NR are organized in several functional domains, two of which are highly conserved between the different members of the family and also between orthologs found in different animal species. The DNA-binding domain (DBD), located in the central part of the primary structure, comprises two zinc-finger modules that specifically recognize the target DNA sites, whereas the C-terminus of the protein encompasses the ligand-binding domain (LBD), responsible for ligand-dependent transactivation. Ligand binding induces conformational changes in the LBD that allow the recruitment of coactivators, such as members of the p160 class, bridging the NR to the basal transcriptional machinery. For many orphan receptors, the question is still open as to whether or not they require an up-to-now unknown ligand to activate transcription.

Depending on the subtype, NR can bind to DNA as monomers, homodimers or heterodimers with common partners, the RXRs, that are also members of the family (Laudet & Gronemeyer 2002). The classical NR DNA target sites are composed of derivatives of a core AGGTCA element. Binding sites for dimers generally consist of two copies of the core element organized as direct or inverted repeats, with a defined spacing between them. For example, the estrogen-response element (ERE) contains two core elements in a head-to-head orientation spaced by three nucleotides, and it binds the estrogen receptors (ER) α and β as homodimers (Kumar & Chambon 1988,
Tsai et al. (1988) or as α/β heterodimers (Cowley et al. 1997, Pettersson et al. 1997). It is commonly assumed that dimerization of ERs is required for stable DNA recognition (Lees et al. 1990) and therefore for efficient transcriptional activity (reviewed in Sanchez et al. 2002). Response elements have also been identified that bear a single core sequence and for which the 5’ extension is important in defining specific recognition by a given NR. One example is the SF1-response element (SFRE), in which a TCA trinucleotide preceding the core element is required to mediate the monomeric binding of the SF1 orphan receptor (Wilson et al. 1995).

Estrogen-receptor-related (ERR) α and β were the first orphan NRs to be identified on the basis of their sequence similarity to the ER (Giguère et al. 1988). A third member of the subfamily has been more recently cloned (ERRγ) (Hong et al. 1999). At the amino-acid level, these receptors are over 90% identical in the DBD and over 60% in the LBD. Little is known about their physiological functions. Both ERRα and ERRγ are highly expressed in muscle, heart and adipose tissues as well as in the central nervous system (Vega & Kelly 1997, Bonnelye et al. 1997b, Lorke et al. 2000, Ichida et al. 2002). ERRα is also highly expressed in bone (Bonnelye et al. 1997a). Knockout animal analysis has shown that ERRβ is required for placentation (Luo et al. 1997). In addition, recent data have pointed to a role for ERRα in energy storage, mitochondrial biogenesis and oxidative phosphorylation (Luo et al. 2003, Mootha et al. 2004, Schreiber et al. 2004). Results from various laboratories have also suggested that ERR receptors interfere or collaborate with the estrogen signaling (reviewed in Giguère 2002). Indeed, though ERR receptors are still orphan in the sense that no endogenous ligand has been identified that regulates their activities, synthetic molecules such as tamoxifen and diethylstilbestrol might inhibit their transcriptional activities in a subtype-specific manner (Coward et al. 2001, Tremblay et al. 2001, reviewed in Horard & Vanacker 2003). Furthermore, ER and ERR share common DNA target sites, such as the classical ERE and the SFRE, and common target genes such as osteopontin (Vanacker et al. 1999b), lactoferrin (Yang et al. 1996) and the breast cancer marker pS2 (Lu et al. 2001). Conflicting results have been published concerning the mode (monomer or homodimer) of binding of ERR proteins to their target sites. However, cotranslation of wild-type and N-terminal truncation mutants of ERR proteins have shown that these receptors bind as homodimers to an ERE and an SFRE sequence (Vanacker et al. 1999a, Hentschke et al. 2002), although the latter site contains only a single core element. Nevertheless, two questions remain unanswered. First, it is not known whether ERRs can also form heterodimers, as do the ERs. We here address this issue by using mouse ERRα and ERRβ. Second, it has not been determined whether homodimer binding is actually required for ERR-driven transactivation, again as it is for ERs. In this report, we present evidence on this question, using, as a molecular tool, the unique ERR gene cloned from amphioxus.

Amphioxus (Branchiostoma floridae) is a member of the phylum Cephalochordata; it is the invertebrate closest in relation to the vertebrates. Furthermore, this animal has not undergone the waves of gene duplication that led to the vertebrates. Thus, instead of the two to four paralog genes found in vertebrates, amphioxus frequently contains a single corresponding gene. These two properties are appealing when one wants to study the evolution of gene function and expression. Genetic simplicity in amphioxus has been demonstrated for Hox genes (Holland et al. 1994), but also for nuclear receptors, since a single COUP-TF gene (instead of the two in mammals) and a single RAR gene (ortholog of the three mammalian RARs) have been reported in this species (Langlois et al. 2000, Escriva et al. 2002).

Accordingly, we have identified a unique ERR receptor in the amphioxus (AmphiERR). This receptor is expressed as two mRNA isoforms that diverge by only an in-frame insertion 5′ to the LBD-encoding region. The resulting proteins (AmphiERR(S) for the short isoform and AmphiERR(L) for the long isoform) have divergent transcriptional behaviors. Whereas AmphiERR(S) binds as a homodimer to and activates transcription through the SFRE and the ERE, we found that AmphiERR(L) binds only as a monomer to and is incapable of transactuation through the SFRE. However, the transactivation capacities of AmphiERR(L) are not impaired since transcription stimulation can be observed via the ERE, to which AmphiERR(L) binds as a homodimer. Moreover, pull-down assays demonstrate that both AmphiERR isoforms interact with mammalian coactivators. Altogether, this suggests that dimerization is
required for transactivation by ERR orphan receptors.

Materials and methods

Molecular cloning

Cloning of AmphieERR cDNA by degenerate PCR will be described elsewhere (P-L Bardet and J-M Vanacker, unpublished observation). Two isoforms were identified during the process of AmphieERR cDNA cloning. 5'-RACE experiments were performed with the 5'3'-RACE kit (Roche, Meylan, France), according to the manufacturer’s instructions. Complete cDNAs were subcloned in the BamHI and BglII sites of plasmid pSG5. Mutant receptors deleted of the A/B domain were created by PCR using specific oligonucleotides, one of which localized upstream of the DBD, thereby deleting 68 amino acids from the original constructs, and the other one overlapped the end of the LBD. PCR products were sequenced and cloned in BamHI–BglII sites of pSG5 vector.

The oligonucleotides used for AmphieERR A/B domain deletion were as follows:

5': GGATCCTCCAGTGAGATACTTACA
3': AGATCTCTAGTACATCTTTTTACA

Plasmids encoding GST-hTIF1α, GST-SRC1 and GST-RIP140 have been described elsewhere (L’Horset et al. 1996, Thénot et al. 1999). The vector allowing expression of GST-GRIP, which contains residues 415–812 of GRIP-1 (Hong et al. 1996), was a gift from M. Stallcup (University of Southern California, Los Angeles, CA, USA). Human TRα promoter derivatives have been described elsewhere (Vanacker et al. 1998). EREtkLuc contains two copies of the consensus ERE in front of the thymidine kinase minimal promoter; it was a gift from P. Balaguer (Montpellier, France).

Protein sequences were aligned automatically by ClustalW (Thomson et al. 1994) with manual correction in Seaview (Galtier et al. 1996).

Semiquantitative RT-PCR

An amount of 1 µg total RNA extracted from different embryonic stages of amphioxus was retrotranscribed using MMLV reverse transcriptase (Gibco/Invitrogen, Cergy) and submitted to non-saturating (27 cycles) PCR using specific primers either for the common parts of the cDNA or for the insertion (strategy shown in Fig. 3A). Cytoplasmic actin was used as control (data not shown). For genomic PCR, 500 ng amphioxus DNA was used. Fragments were loaded on an 1% agarose gel and revealed by ethidium bromide staining.

The oligonucleotides shown in Fig. 3 were as follows

A: GTCAAGCTTGGCCGCTTCACC
B: TTGGACAAGTGACGACCTGT
C: GTCTTCAGCTCAGTGCCTGG
D: TTGGACAAGTGACGACCTGT

Electrophoretic mobility shift assays (EMSA)

ERR proteins were translated in vitro by the transcription/translation-coupled reticulocyte lysate system (TNT T7 kit; Promega). Crude reactions (3 µl) were incubated with [γ32P]-ATP-labeled oligonucleotide probes in a buffer containing 10% glycerol, 10 mM HEPES, 30 mM KCl, 4 mM spermidine, 0·1 mM EDTA, 0·25 mM dithiothreitol and 1 mM Na2HPO4 (pH 7·9). Single-stranded salmon sperm DNA (1 µg) and poly(dI-dC) (0·2 µg) were added. Unlabeled competitor oligonucleotides were included at the indicated molar excesses. Samples were loaded and run on a 5% native polyacrylamide gel. For dimerization experiments, wild-type and ΔA/B ERR were cotranslated at a 0:100, 25:75, 50:50, 75:25 or 100:0 ratio.

Oligonucleotides used as probes (only one strand is indicated; consensus elements in boldface) were as follows:

ERE: ACGGGCAAGGTCACTGTGACCTCTGCCCG
SFRE: AGTGGCGATTTGTCAAGGTCA

The last-named oligonucleotide originates from the human TRα promoter (Vanacker et al. 1998).

The unrelated oligonucleotide used as a control was as follows:

CGGAGAAGGGACCCGAACTCCCAACAAAC

Cell transfections

Rat osteosarcoma ROS 17/2·8 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS). For transient transfections, 105 cells were seeded into 24-well plate and
transfected with 3 µl of ExGen 500 (Euromedex, Souffelweyersheim, France), 100 ng reporter plasmid and various amounts of ERR-encoding plasmid. CMV-βGal plasmid (50 ng) was added to normalize transfection efficiency, and pSG5 plasmid was added as a carrier up to 550 ng. Cells were lysed 48 h after transfection, and reporter activities were determined by standard methods. All transfections were performed in triplicate.

Pull-down assays

In vitro binding assays were performed essentially as previously described (Thénot et al. 1999). Briefly, 35S-methionine-labeled ERR proteins were cell-free-synthesized using the TNT lysate system (Promega) and incubated overnight at 4 °C with bacterially expressed transcription cofactors purified as GST-fusion proteins. Binding reactions were performed in NETN buffer (NaCl 300 mM, EDTA 1 mM, Tris 20 mM (pH 8) and NP40 0·5%) supplemented with 1 mM DTT and protease inhibitors (complete cocktail, Roche). Protein interactions were analyzed by SDS–PAGE. The gel was stained with Coomassie brilliant blue (BioRad) to visualize the GST fusion proteins present in each track prior to radioactivity quantification with a Phosphorimager (Fujix BAS1000).

Figure 1 (A).
Results

Heterodimerization of ERR receptors

ER (α and β) bind to DNA as homodimers and heterodimers (reviewed in Sanchez et al. 2002). Since ERR also form homodimers on DNA (Petterson et al. 1996, Hong et al. 1999, Vanacker et al. 1999a, Hentschke et al. 2002), we investigated the possibility of heterodimer formation within members of this subgroup. To this end EMSA were performed with in vitro synthesized derivatives of ERR proteins. Full-length ERRβ was cotranslated with ERRα deleted from its A/B domain, which is not necessary to DNA binding (construct ΔA/BERRα) in various ratios. The reaction mixtures were then allowed to bind to ERE or SFRE (Fig. 1A) radioactive probes. When used alone, ERRβ and ΔA/BERRα each yielded a single specific band on each probe. When both constructs were cotranslated, an intermediate migrating complex appeared that revealed heterodimerization between ERRα and ERRβ. We thus conclude that ERR receptors can heterodimerize at least on ERE and SFRE probes.

We next investigated whether heterodimerization modifies the transcriptional properties of the ERR receptors as compared with their effect as homodimers. To this end, transient cotransfections were performed on ROS17/2·8 cells. Plasmids comprising two copies of the ERE in front of a minimal promoter driving the expression of the luciferase reporter gene were transfected together with varying amounts of ERR-encoding plasmids (Fig. 1B). In this assay, each ERR receptor was able to activate transcription on its own. However, no correlation could be observed between the level of fold activation and the combination of ERR plasmids transfected. Instead, fold activation in the presence of both receptors reflects a combination of the activations driven by the individual ERR proteins. Identical results were obtained with a plasmid containing three copies of the SFRE in front of the minimal promoter (data not shown). This indicates that ERR heterodimerization, as likely to occur in cells as it does in vitro, does not result in transcriptional synergy or interferences.

Two isoforms of AmphiERR

We have isolated a single ERR receptor in the amphioxus B. floridae (P-L Bardet and J-M Vanacker, unpublished observation), that is a homologue of all three vertebrate ERRs. During the cloning of AmphiERR cDNA, we isolated two cDNA species differing by a 123 bp insertion. 5’ RACE experiments were then performed to recover full length cDNAs that were subsequently sequenced. ORFs in the cDNAs were predicted to produce putative proteins of 438 and 479 amino acids. These sequences were aligned with the published human ERRα, β and γ sequences (Fig. 2). The unique 123 bp of AmphiERR(L) are inserted at a region equivalent to the junction between exon 4 and exon 5 in human ERRα (Shi et al. 1997). Since AmphiERR(S) is the equivalent in length of mammalian ERR, the AmphiERR(L)
isoform could therefore arise by addition of an alternative exon. At the protein level, the insertion is located at the end of the hinge domain, upstream from the first helix of the putative LBD.

**Figure 2** Sequence alignment of the two isoforms of AmphiERR. Human and amphioxus ERR sequences were aligned with ClustalW with Seaview manual corrections. A dot represents identical amino acids; a dash symbolizes a gap, using AmphiERR sequence as a reference. The DNA-binding domain is in large brackets, with its conserved cysteines appearing in boxes. The putative helices of the ligand-binding domain are labeled and boxed. The unique 123 bp insertion present in AmphiERR(L) is underlined and in boldface characters.
Figure 3  Two AmphiERR isoforms are expressed during amphioxus development. (A) PCR strategy. Oligonucleotides were designed to bind specifically in putative exons (E) 4a, 4b and 5. Note that this numbering refers to the published human ERRα genomic sequence. Expected sizes of the amplicons on amphioxus cDNAs are indicated. The size of the intron between exon 4 and exon 5 was deduced from genomic PCR. (B) RT-PCR. RNA extracted at the indicated time of development was retrotranscribed and submitted to PCR with oligonucleotides A and C. Estimated sizes of the amplicons are indicated. Ad: RNA extracted from adult; V: no substrate reaction added. (C) Control PCR. Genomic (gen) or retrotranscribed RNA extracted from 36 h embryos (RT) were submitted to PCR with oligonucleotides A and B. Estimated sizes of the amplicons are indicated. V: no substrate added.
not shown). The existence of internal insertion in ERR proteins could thus be restricted to cephalochordates.

We next verified that both isoforms were expressed in amphioxus. To this end, total RNA was extracted from whole embryos at different stages of development or from adult animals. RT-PCR experiments were performed with primers designed so as to frame the insertion (oligonucleotides A and C; see strategy in Fig. 3A) and to amplify both long and short species. For each stage of development, two PCR fragments of the predicted size were obtained, indicating that both isoforms were expressed (Fig. 3B). Amphi-ERR(S) might be slightly predominant over AmphiERR(L) in early phases, a trend which reversed as development proceeds. Only a fainter signal corresponding to AmphiERR(S) could be detected with adult RNA, whereas AmphiERR(L) is still present. To rule out that the detected bands, and especially the larger one, originated from a genomic DNA contamination, PCR experiments were performed on amphioxus DNA with a primer annealing in the insertion (oligonucleotide B; see Fig. 3A). The obtained fragment has an estimated size (1·2 kbp) much larger than the one generated with retrotranscribed RNA (142 bp; Fig. 3C). Taken together, these observations indicate

(A)  

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<th>Isoform</th>
<th>AmphiERR(S)</th>
<th>AmphiERR(L)</th>
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<td>Competitor</td>
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<td>Molar excess</td>
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Figure 4 (A)
that AmphiERR is expressed as two mRNAs produced by alternative splicing. Since AmphiERR(S) corresponds in structure to the bona fide mouse ERR in organization and size, AmphiERR(L) is therefore a naturally occurring insertion variant.

**DNA-binding properties of AmphiERR**

To determine the binding capacities of both AmphiERR isoforms, we performed EMSA, using the ERE or the SFRE as probes. *In vitro* translated AmphiERR(S) and AmphiERR(L) efficiently bound to the ERE sequence (Fig. 4A). The bindings were specific since the generated complexes could be displaced by an excess of unlabeled ERE or SFRE oligonucleotides, but not by an unrelated one (that is, not containing any core element). We also observed specific binding of both isoforms on the SFRE probe (Fig. 4B). However, AmphiERR(L)-SFRE complexes had a higher electrophoretic mobility as compared to the AmphiERR(S)-ERE complexes.

### Table 1: Binding capacities of AmphiERR isoforms

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<th>Competitor</th>
<th>ERE</th>
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<td>molar excess</td>
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**Figure 4** AmphiERR isoforms bind to the ERE and the SFRE probes. A volume of 3 µl *in vitro* synthesized AmphiERR(S) (left panels) or AmphiERR(L) (right panels) proteins was allowed to bind on radiolabeled consensus ERE (A) or SFRE (B) oligonucleotides. The molar excess of unlabeled oligonucleotide is indicated. unr.: unrelated oligonucleotide used as a control; C: control reticulocyte lysate, that is, without DNA construct added to reaction. Position of the putative homodimeric AmphiERR(S) (left panel) and putative monomeric AmphiERR(L) (right panel) on SFRE probe is indicated by arrowheads.
mobility than AmphiERR(S)-SFRE ones. This suggests that, while AmphiERR(S) bound as a homodimer as do its mammalian counterparts, AmphiERR(L) bound as a monomer to the SFRE.

To test this hypothesis, we constructed a deletion mutant of each isoform, removing the A/B domain. These mutants were then in vitro cotranslated with their corresponding full-length construct and subjected to EMSA. Under these conditions, we observed intermediately migrating complexes (wt-Δ) representing heterodimers of full-length and mutant proteins for both AmphiERR isoforms on the ERE probe (Fig. 5A). This demonstrates that both AmphiERR bind as homodimers on the ERE site. When we used SFRE as a probe (Fig. 5B), an intermediate complex was observed only with AmphiERR(S), but not AmphiERR(L). We thus concluded that AmphiERR(L) binds as a homodimer on the ERE, but as a monomer on the SFRE.

We next tested whether the isoforms were capable of heterodimerization. To this end, full-length AmphiERR(L) was cotranslated together with deleted AmphiERR(S), and the mixture was subjected to EMSA (Fig. 5C). An intermediate complex was
observed only with the ERE probe, but not with the SFRE one. The same result was obtained with the converse combination of full-length and mutant species (that is, full-length ERR(S) cotranslated with deleted ERR(L); data not shown). Heterodimerization between the AmphERR isoforms is therefore restricted to the ERE site.

Transcriptional activities of AmphERR
As a first attempt to explore the consequences of homodimeric versus monomeric binding on transcriptional regulation exerted by ERR proteins, we investigated whether the two AmphERR isoforms can recruit the coregulators essential to achieve transactivation. To this end, a series of glutathione S-transferase (GST)-based pull-down assays was performed (Fig. 6A). The ERR isoforms exhibited similar direct interactions in solution with various coregulators (SRC1, GRIP1, TIF1α and RIP140) known to contact NRs. As a control, GST alone was unable to interact with either AmphERR isoform. This demonstrated that the 41 aa insertion in AmphERR(L) did not alter the cofactor-binding properties of AmphERR.

We next performed transient cotransfection experiments in rat osteosarcoma ROS17/2·8 cells.
As a reporter, we first used a plasmid containing two ERE sequences cloned in front of the minimal tk promoter driving the luciferase reporter gene (Fig. 6B). Under these conditions, both AmphiERR isoforms were able to activate transcription, indicating that AmphiERR is a bona fide transcriptional activator to the same extent as mouse ERR used as a control. To determine the consequences of monomeric binding, we chose to use the human TRα gene promoter. This promoter is a target gene of mouse ERRα and comprises a single SFRE on which mouse ERRα bind as a homodimer (Vanacker et al. 1998). With this reporter system, transactivation was obtained only with AmphiERR(S) and mouse ERRα, but not with AmphiERR(L) (Fig. 6C), indicating a correlation between monomer binding and transactivation capacity. As a control, we also used a version

**Figure 5 (C)**

**Figure 5** Divergent binding properties within the isoforms of AmphiERR. (A and B) Constructs encoding a wild-type (wtERR) and deletion mutant (ΔERR) lacking the A/B domain. AmphiERR proteins were in vitro cotranslated. Reaction products were tested in EMSA on ERE (A) and SFRE (B) probes. Positions and nature of the dimeric complexes are indicated (wt-Δ: heterodimer between wild-type and deletion mutant). No intermediately migrating complexes could be observed between wild-type and ΔA/B AmphiERR(L) on SFRE probe (B). (C) Same type of experiment, using combinations of wtAmphiERR(L) and ΔA/BAmphiERR(S), allowed to bind on ERE (left panel) or SFRE probe (right panel). Heterodimerization is observed on the ERE probe, but not on the SFRE one. C: control reticulocyte lysate, that is, without DNA construct added to reaction.
of the TRα promoter harboring a mutation that disrupts the SFRE (pTRmut-Luc) and does not respond to mouse ERRα. No transactivation could be observed by any AmphiERR isoform (Fig. 6D), indicating a dependence toward the integrity of the SFRE site.
Both AmphiERR isoforms bind to the SFRE sequence present in the human TRα promoter, but transactivation is achieved only with AmphiERR(S), and not AmphiERR(L). It could therefore be anticipated that the latter will repress activation driven by the former. To test this hypothesis, we performed cotransfections, using pTRLuc and varying amounts of AmphiERR isoforms (Fig. 7A). As predicted, AmphiERR(L), inactive by itself, reduced the level of AmphiERR(S)-driven transcriptional activation. In contrast, using the EREtkLuc as a reporter plasmid did not result in any interference between AmphiERR isoforms (Fig. 7B).

Altogether, our results show a correlation between homodimerization and transactivation capability, suggesting that the dimerization is indeed required for activation of transcription by ERR receptors.

Discussion

The unique amphioxus ERR receptor is expressed as two isoforms

Expression of NRs as various isoforms is a common phenomenon that can result in the production of proteins displaying differential transcriptional activities. This is assumed to contribute to the fine tuning of the expression of their target genes. For example, alternative promoter and exon usage has been described in the 5′ part of human ERα gene (Flouriot et al. 2000, Denger et al. 2001). The resulting protein isoforms compete for binding to the same DNA element and activate transcription at a different level, leading to a modulation of downstream promoter activity. In the mouse TRα gene, an internal promoter localized in intron 7 drives the expression of truncated isoforms that inhibit the activity of RAR and TRα itself (Chassande et al. 1997).

In the case of ERR receptors, isoforms have been described in the N-term part of human ERRα and γ proteins (Johnston et al. 1997, Heard et al. 2000). In contrast, the two isoforms of AmphiERR differ by the presence or absence of an internal 123 bp insertion in the hinge domain (that is, between the DBD and LBD). Computer-assisted secondary structure prediction suggests that the 41 additional amino acids could adopt a beta sheet conformation (data not shown). Insertion of such a structure could enhance the distance between the DBD and the LBD, thereby suggesting a mechanistic explanation of the altered transcriptional capacities observed in AmphiERR(L) versus AmphiERR(S).

It is likely that the two AmphiERR isoforms arise by alternative splicing. Indeed, PCR reaction performed on amphioxus genomic DNA indicates a distance of at least 1200 bp between the insertion and the previous 5′ exon (Fig. 3). The genomic distance between the insertion and the next downstream exon could not be measured by PCR.
(data not shown), suggesting a size exceeding the capacity of the reaction. It is noteworthy that the insertion is localized at the junction between human and mouse ERRα exon 4 and exon 5 (Shi et al. 1997 and data not shown). Computer-assisted scanning of mouse and human ERR genes has not raised the possibility of alternate splice variants such as the ones expressed in the amphioxus (data not shown). It is therefore probable that central insertion in ERR is restricted to the cephalochordate amphioxus.

**Dimerization is a feature of ERR transactivation**

Depending on the subtype, NRs can bind to DNA as monomers, homodimers or heterodimers with the promiscuous partner RXR, with some receptors exhibiting several of these behaviors (reviewed in Laudet & Gronemeyer 2002). In addition, ER not only bind as homodimers on their cognate target sequences but can also heterodimerize with each other (Pettersson et al. 1996, Cowley et al. 1997). ERR orphan receptors have been shown to form homodimers on ERE and SFRE sites (Pettersson et al. 1996, Vanacker et al. 1999a, Hentschke et al. 2002). In the present report, we document the possibility of heterodimerization among ERR receptors. This phenomenon occurs in vitro between ERRα and ERRβ. Given the sequence conservation among ERR receptors (90% identity in the DBD, over 60% in the LBD), it is likely that this is also true for ERRγ. However, in the transient transfection assays reported here, ERR heterodimerization did not result in any synergy or transcriptional interference. It could be that such occurrences would be revealed only in certain cellular systems where the different ERR proteins display divergent activities. Indeed, it has been shown that ERRα transcriptional activity depends on the cellular context (Bonnelye et al. 1997b). Moreover, synthetic ER ligands such as tamoxifen and diethylstilbestrol have been suggested to exert different effects according to the ERR receptor subset considered (Coward et al. 2001, Tremblay et al. 2001, reviewed in Horard & Vanacker 2003). During the writing of this paper, Huppunen and Aarnisalo (2004) demonstrated that ERRγ heterodimerizes with ERRα. However, the physiological relevance of ERR heterodimerization is unknown to date.

The main DNA-protein interaction generated with AmphiERR(L) on the SFRE probe represented a monomeric complex. Additional bands were also detected that might reflect homodimeric binding. However, the low intensity of the corresponding signal does not support the hypothesis that these complexes play a decisive role in transcriptional regulation. Two regions necessary for mouse ERRγ dimerization have recently been mapped in the hinge and LBD domains (Hentschke et al. 2002). Interestingly, the unique 41 amino acid stretch of AmphiERR(L) disrupts the equivalent of the hinge domain. This could account for lack of dimerization of AmphiERR(L). However, this is restricted to the SFRE sequence, since this receptor binds as a homodimer on ERE. The intrinsic capacity of dimerization is thus not impaired in AmphiERR(L). Instead, since ERR generate very stable homodimers that can be formed in solution, we think that the DNA site on which the receptor binds influences the stability of the complex, with disrupting activities exerted by the SFRE sequence.

Interestingly, AmphiERR(L) is unable to activate transcription via a single consensus SFRE site, as observed with the human TRα promoter. However, the intrinsic transactivation capacities of AmphiERR are not modified by the insertion. Indeed, no differences were found in the physical interactions with coactivators in the absence of DNA between AmphiERR isoforms. Furthermore, AmphiERR(L) stimulated transcription via multimerized ERE or SFRE sequences cloned in front of a minimal promoter (Fig. 6 and data not shown). In the latter context, multimerized SFRE provide multiple core AGGTCA elements on the promoter that could be bound as homodimers. In agreement with this, an oligonucleotide that comprises two tandem SFRE sequences was found to bind AmphiERR(L) as a homodimer (data not shown).

We cannot formally exclude that AmphiERR isoforms might differ in their transcriptional capacities in vivo in the amphioxus context. However, using AmphiERR as a tool in mammalian cells, we show a correlation between dimeric binding and transactivation capacities. Since the intrinsic transactivation capacities of the AmphiERR isoforms are not different, it is tempting to hypothesize a causal link between these occurrences, and to propose that dimerization is necessary to transcriptional activation exerted by ERR receptors. Amphioxus and mouse ERR
receptors are highly conserved in sequence. Furthermore, in terms of domain organization, AmphierR/S is the exact equivalent of its mouse counterparts. We therefore propose that homodimerization is required for transactivation not only in the case of AmphierR but also for all ERR proteins.

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ERR-driven transactivation requires dimerization

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