Characterization of oestrogen receptors in zebrafish (*Danio rerio*)

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Abstract

We cloned the cDNAs corresponding to three oestrogen receptors (ERs) in zebrafish (*Danio rerio*). Sequence analysis and phylogenetic studies demonstrated that two of these genes, ERβ.1 and ERβ.2, arose from duplication of the original ERβ in many species of the fish phylum, whereas ERα is unique. Zebrafish ERs behaved as oestrogen-dependent transcription factors in transactivation assays. However, their reactivity to various oestrogen modulators was different compared with that of mouse ERs. ER mRNA expression during zebrafish development is restricted to distinct time periods, as observed by RNase protection assays. ERβ.2 is initially expressed as maternally transmitted RNA, until 6 h after fertilization, when expression disappears. Between 6 and 48 h after fertilization, no ER expression could be observed. After 48 h after fertilization, all ERs, but predominantly ERα, began to be expressed. We conclude that oestrogen signal transduction can operate during zebrafish development only within discrete time windows.

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Introduction

Over the past 10 years, zebrafish (*Danio rerio*) has received growing attention as an alternative animal model that is particularly suitable for the study of embryonic development, mostly in its early phases. In addition, zebrafish is also being used as an *in vivo* model for the identification of small molecules that can interfere with normal developmental (Peterson *et al.* 2000) or physiological processes, including the detection of water pollutants and endocrine disruptors. To the latter class of compounds belong molecules that interfere with reproductive functions and mimick or antagonize the effects of endogenous hormones such as oestrogens. A growing number of so-called xeno-oestrogens, present in food or the environment, have been identified that jeopardize the reproductive capacities of various animals, including humans. Adequate transmission of the signal conveyed by oestrogen is indeed necessary for reproduction. This is illustrated by the fact that mice lacking the aromatase gene (the key enzyme for oestrogen production *in vivo*) or oestrogen receptors (ERs) are sterile or exhibit reduced fertility (Fisher *et al.* 1998, Couse & Korach 1999, Couse *et al.* 1999, Dupont *et al.* 2000).

Oestrogens are small lipophilic molecules that cross the cell membrane and are bound by specific nuclear receptors. ERs act as ligand-dependent transcription factors that regulate the expression of their target genes either by binding to specific sequences (the oestrogen response elements, ERE) or by interfering with other transcription factors such as Sp1 or the AP1 complex (Paech *et al.* 1997, Saville *et al.* 2000, reviewed in Hall *et al.* 2001). In mammals, two oestrogen receptors have been identified: ERα (Green *et al.* 1986) and ERβ (Kuiper *et al.* 1996) (official names NR3A1 and NR3A2 respectively). The ERs display both overlapping and distinct ligand-binding capacities (Kuiper *et al.* 1997), patterns of expression during development (Lemmen *et al.* 1999) and in the adult (Couse & Korach 1999), transcriptional properties (Paech *et al.* 1997, Vanacker *et al.* 1999, Saville *et al.* 2000), and knockout phenotypes (Lubahn *et al.*

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1993, Krege *et al.* 1998, reviewed in Couse & Korach 1999). In addition, mouse (m) ERs have been suspected to have opposite physiological roles in some tissues such as the uterus and the prostate (Weihua *et al.* 2000, 2001).

Oestrogen receptors are organized in modular domains, two of which have been particularly conserved during evolution: the DNA-binding domain (DBD), and the ligand-binding domain (LBD), which also mediates coactivator binding, dimerization and ligand-dependent transactivation through the activation function 2 (AF2) region (for reviews see Gronemeyer & Laudet 1995, Mangelsdorf *et al.* 1995). Human and trout ERα, for instance, share 90% sequence identity in their DBD (Pakdel *et al.* 1989). Conversely, ERα and ERβ are also highly related, as sequence comparison between orthologues indicates more than 90% sequence identity in the DBD and 65% in the LBD (Kuiper *et al.* 1996, Laudet 1997). Another activation function (AF1) that mediates ligand-independent transactivation resides in the N-terminal part of the protein and is poorly conserved between receptors and between species.

In an effort to determine the conditions under which zebrafish can be used as a model for the detection of molecules interfering with oestrogen signalling, we have characterized the oestrogen receptors in this species. We herein report the isolation of three ERs in zebrafish, two of which (ERβ.1 and ERβ.2) have arisen after specific duplication of the *ERβ* gene in part or all of the fish phylum. In common with their mammalian counterparts, all three zebrafish receptors can be transcriptionally activated by 17β-oestradiol (E_2). However, other molecules activating all or some of the mERs, such as 4OH-E_2 and 4OH-tamoxifen, are inactive toward the zebrafish (zf) ERs. This suggests that the spectrum of molecules activating the ERs is different between mouse and zebrafish. During development of the zebrafish, ERβ2 is expressed in a maternally inherited manner. After the onset of zygotic transcription, no ER expression could be detected until 48 h post fertilization, when all ERs, but predominantly ERα, start to be detected in a diffuse manner. Our results suggest that oestrogenic signalling could only be efficiently transduced during very early development and in later embryonic phases.

Material and methods

Molecular cloning

One microgram of total RNA pooled from male and female adult zebrafish was retrotranscribed using Moloney murine leukaemia virus reverse transcriptase (Gibco Brl) and submitted to nested PCR using degenerated primers located in the conserved DBD and LBD. PCR products were cloned into pCR2.1 vector (Invitrogen) and individual clones were sequenced. An expressed sequence tag (EST) was also found in Genbank (access number AW134052) corresponding to a zfER (ERβ2). The corresponding clone (2601181; Incyte Genomics Inc., Palo Alto, CA, USA) was ordered and sequenced. Before transfection experiments, cDNAs were cloned in the blunted EcoRI site of pCMX plasmid.

Rapid amplification of cDNA ends (RACE) experiments were performed using the 5′/3′ RACE kit (Roche) and following the manufacturer's instructions.

Degenerate oligonucleotides used were:
ER 5′4: (A/C)GIAA(A/G)AG(C/T)TG(C/T)CA(G/A)GCITG
ER 5′5: A(C/T)GAAGTIGGIATG(A/G)TGAAAG
ER 5′6: T(C/T)GAAGT(A/G)GG(A/C)ATG(A/G)T(G/C)AAGT
ER 3′1: GIIGTIG(T/C)CA(C/G)IA(G/A)CATGTC
ER 3′2: CAITTICC(T/C)T(G/C)(A/G)(T/C)(T/C)CCTGTCCA

Phylogenetic analysis

Protein sequences were aligned automatically by Clustal W (Thomson *et al.* 1994) with manual correction in Seaview (Galtier *et al.* 1996). Trees were constructed by neighbour-joining (Saitou & Nei 1987), with Poisson-corrected distance in amino acid sequences of only the well-conserved DBD and LBD domains, implemented in Phylo_win (Galtier *et al.* 1996). Support for branches in the tree was investigated by bootstrap (Felsenstein 1985), with 1000 replicates.

Cells and transfections

Rat osteosarcoma ROS 17/2.8 cells were maintained in DMEM supplemented with 10% fetal calf

serum (FCS). Before transfections, cells were cultivated for 2 weeks in phenol-red-free DMEM supplemented with 10% charcoal-treated FCS, to ensure steroid clearance. For transient transfections, 10^5 cells were seeded in 24-well plates and transfected using 3 μl ExGen 500 (Euromedex, Souffelweyersheim, France), 50 ng reporter plasmid and 50 ng ER-encoding plasmid. pCMX plasmid was added as a carrier up to 500 ng. After 5 h, cells were supplemented with fresh medium to which 10^{-8} M hormone was added as needed. Cells were lysed 48 h after transfection and reporter activity was determined, using standard methods. All transfections were performed in triplicate.

RNase protection assays

For total RNA extraction, 200–400 embryos were scraped in 3 ml 4 M guanidinium thiocyanate solution. RNA was extracted by phenol/chloroform, precipitated by isopropanol and resuspended in RNase-free water. Antisense RNA probes were prepared by *in vitro* transcription of linearized templates with T7 (MAXIscript, *in vitro* transcription kit, Ambion) or T3 (Promega) RNA polymerase using phosphorus-32-labelled UTP. After phenol/chloroform extraction and precipitation, probes were hybridized with 15 μg of each stage RNA and digested with RNase A/T1 (RPAIII kit, Ambion). Samples were separated on a 5% denaturing polyacrylamide gel, dried, analysed by PhosphorImager (Amersham Pharmacia) and quantified using ImageQuant software (Amersham Pharmacia).

Results

Identification of ERs in zebrafish

To isolate ER cDNAs, we first performed RT-PCR using total RNA extracted from pooled male and female adult zebrafish. Degenerated primers located in conserved regions (DBD and LBD) were designed to amplify both ERα and ERβ. These experiments gave rise to three distinct species of the expected size. 5′ and 3′ RACE were then performed to produce complete cDNAs that were subsequently sequenced. One of the PCR fragments was also present in a zebrafish EST (accession number AW134052). Open reading frames in the cDNAs were predicted to produce putative proteins of 570, 558 and 554 amino acids (ERα, ERβ.1 and ERβ.2 respectively). The predicted sequences were then aligned with mERα and mERβ (Fig. 1). The zfER cDNAs bore the typical DBD of nuclear receptors organized in two zinc-finger modules and LBD located in the C-terminal part of the protein. As expected, sequence identity within these domains is increased between species (over 95% for the DBD, close to 70% for the LBD when comparing mouse and zfER).

Using the neighbour-joining method, we then analysed the phylogenetic relationships of zfERs to oestrogen receptors previously cloned in other species, in particular in various fish (Fig. 2). All ERα were homogenously grouped within a single group. In contrast, fish ERβ proteins appeared to be clustered in two distinct groups, equally related to mammalian ERβ. Furthermore, in some species such as zebrafish, goldfish (*Carassius auratus*) or atlantic croaker (*Micropogonias undulatus*), two different ERβ proteins have been identified, one from each group. This indicates that the *ERβ* gene has undergone duplication in an ancestor common to various teleosts, after the emergence of the fish phylum from the remainder of the vertebrates. In agreement with current nomenclature procedure, we hereafter refer to zebrafish ERβs as ERβ.1 and ERβ.2 (official nomenclature: NR3A2-A and NR3A2-B, respectively).

Transcriptional activities of zfERs

ERs activate transcription in an oestrogen-dependent manner through a variety of response sites, the most typical of which is composed of two head-to-head AGGTCA half sites separated by three nucleotides (hereafter referred to as the ERE). To analyse the transcriptional properties of the zfERs, we cotransfected a reporter plasmid containing two EREs in front of a minimal *tk* promoter driving the luciferase reporter gene (EREtkLuc), together with a zfER encoding plasmid (Fig. 3). mERs were used as controls. In the absence of exogenously added oestrogen, no significant ER-driven transactivation could be detected. In contrast, all ERs activated the expression of the luciferase reporter gene in the presence of E_2 (10^{-8} M), the natural ligand of mammalian ERs. We also analysed the effects of other natural or synthetic oestrogenic agents

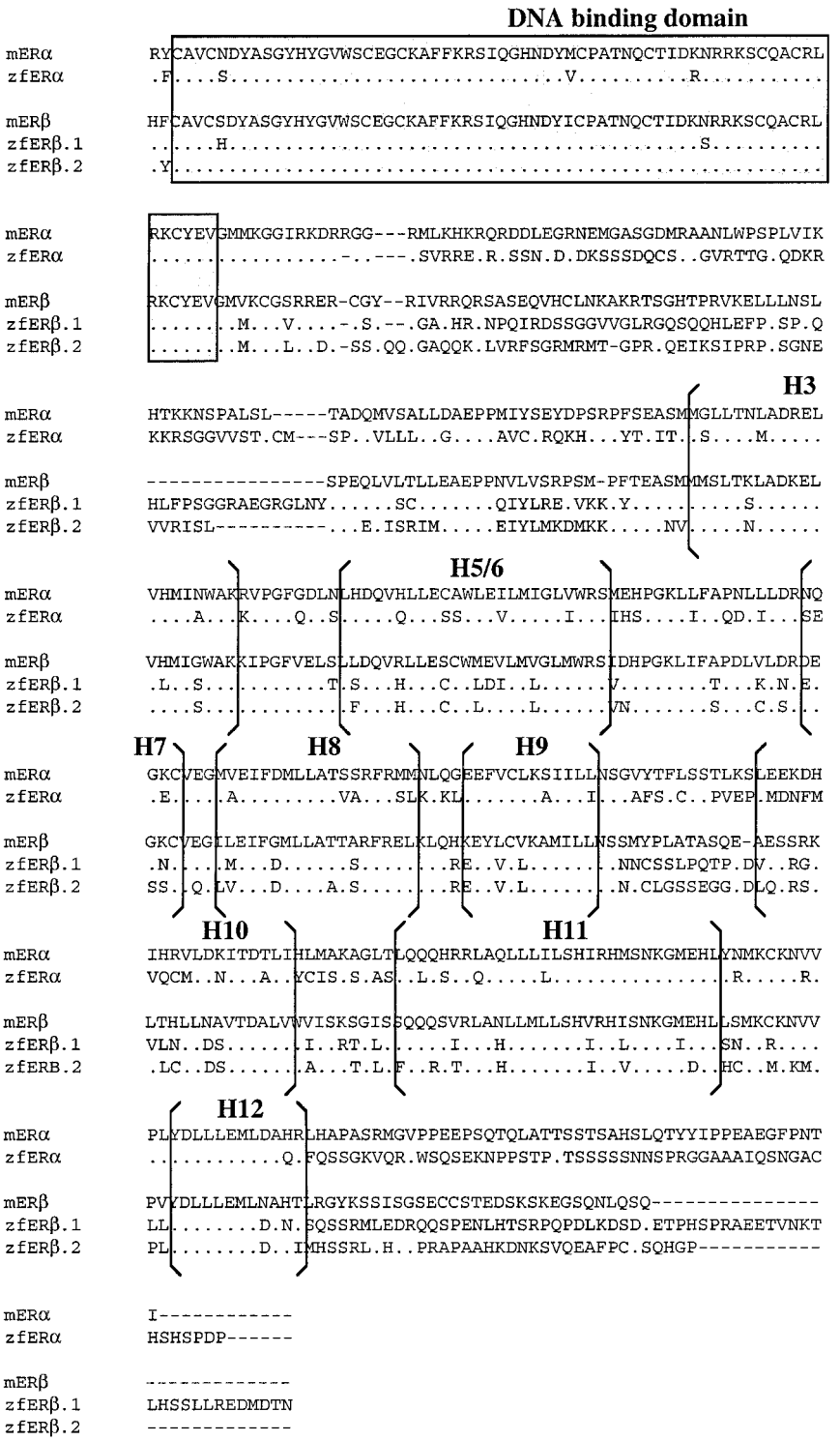

Figure 1 Alignment of mouse and zebrafish ER sequences. Mouse and zebrafish sequences were aligned using Clustal W with Seaview manual corrections. A dot represents identical amino acids using mouse sequence as a reference, whereas a dash symbolizes a gap. The DNA binding domain is boxed and the major helices (H) within the ligand binding domain appear within brackets.

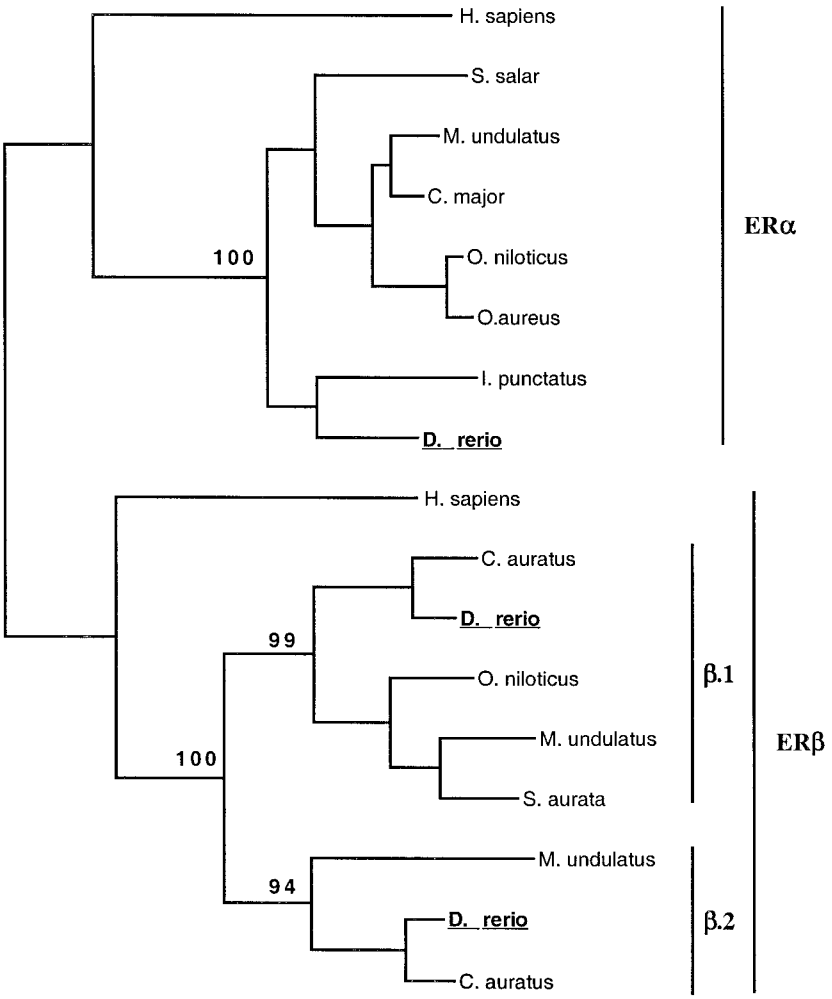

Figure 2 Phylogenetic tree of ERs. The tree was built using the neighbour-joining method, using 318 sites with global gap removal and performing 1000 bootstrap replicates. All bootstrap values were more than 90%. Only those bootstrap values that support grouping of all ERα genes, and clustering of ERβ ones in two subgroups, are shown. The Genbank accession numbers of the sequences used in the tree are: *Sparus aurata* (gilthead seabream): AF136980; *Micropogonias undulatus* (atlantic croaker): AF298183, AF298181, AF298182 (ERα, ERβ.1 and ERβ.2 respectively); *Carassius auratus* (goldfish): AF061269, AF177465 (ERβ.1 and ERβ.2 respectively); *Ictalurus punctatus* (channel catfish): AF061275; *Chrysophrys major* (red seabream): AB007453; *Oreochromis niloticus* (Nile tilapia): U75604, U75605 (ERα and ERβ respectively); *Salmo salar* (salmon): X89959; *Oreochromis aureus* (blue tilapia): X93557; *Homo sapiens* (human): M12674, AF051427 (ERα and ERβ respectively).

(10^{-8} M). The same levels of activation were achieved by diethylstilbestrol, regardless of the ER used. In contrast, 4OH-E_2, although acting as an activator of mERs, surprisingly had no effect on any of the zfERs. 4OH-Tamoxifen – a selective oestrogen receptor modulator – activated mERα but not mERβ, as already reported (Tremblay *et al.* 1997, Watanabe *et al.* 1997). However, tamoxifen was also unable to activate any of the zfERs. Lack of transactivation was also observed with 10^{-7} M 4OH-E_2 or tamoxifen (data not shown). The observed transactivations are due to

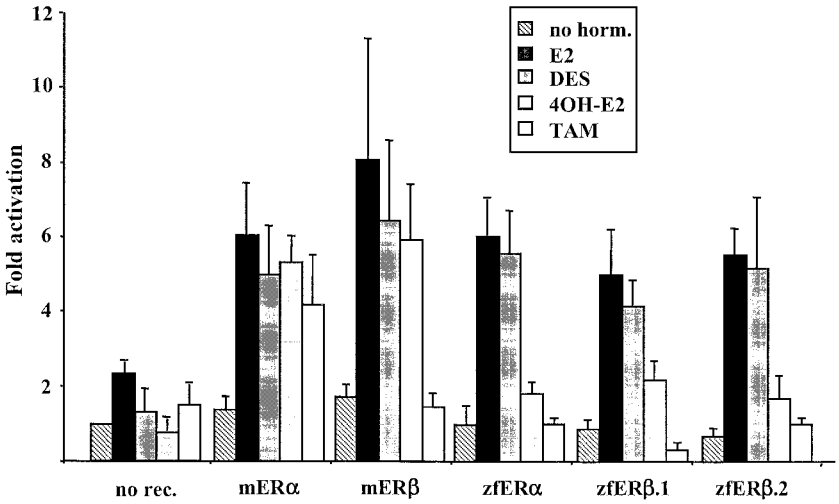

Figure 3 Transcriptional activities of the ERs. Rat osteosarcoma ROS17/2·8 cells were transiently cotransfected with 50 ng EREtkLuc reporter together with 50 ng ER-encoding plasmid, as indicated. Culture medium was supplemented with 10^{-8} M hormone (horm.) where indicated. Values are expressed as fold activation (and standard deviations) over the reporter transfected without ER and without hormone. E2, oestradiol; DES, diethylstilbestrol; TAM, tamoxifen.

the exogenously derived receptors, because the EREtkLuc, transfected alone, reacted only weakly to hormone treatment, suggesting that our cells only weakly express endogenous ERs. Our results indicate that the capacity of a given compound to activate the oestrogen receptor transcriptionally differs between zebrafish and mammals. This also suggests that it might not be appropriate to extrapolate an oestrogenic response from one species to another.

Expression of ERs during zebrafish development

As a next step in characterizing the zfERs, we attempted to determine the features of their expression during development. This was initially performed by *in situ* hybridization experiments in whole embryos. However, none of the zfERs displayed a discrete pattern of expression, at least during the first 4 days after fertilization. Indeed, ERβ.1 was expressed in a diffuse manner at a very low level throughout development, whereas ERα was undetectable using this procedure (data not shown). Therefore, we chose to use RNase protection assays (RPA) using RNAs extracted from pooled whole embryos at different stages of development. This has the advantage of enhancing potential signals and still provides temporal, though not spatial, information.

During early development (1–6 h after fertilization), only ERβ.2 was detected (Fig. 4), indicating that the expression of this gene is maternally inherited. Zygotic transition (begining of embryonic transcription) indeed only occurs around 4 h after fertilization. This is illustrated by the control of elongation factor 1 (EF1), also a maternally transmitted RNA, expression of which was dramatically enhanced at 6 h after fertilization (Delaunay *et al.* 2000). In contrast, expression of ERβ.2 was stable until this time of development and then collapsed between 6 and 12 h after fertilization. During the subsequent phases of development, ER expression was initially very low, and became augmented with time (Fig. 5). For instance, signals corresponding to ERα were very weak between 12 and 48 h after fertilization and underwent a dramatic enhancement around 72 h after fertilization. Though to a lesser extent, this was also true for ERβ.1 and ERβ.2, the corresponding signals for which were barely detectable before 48 h after fertilization. As a control, EF1 expression is stable during these phases of development. We conclude that *ER*

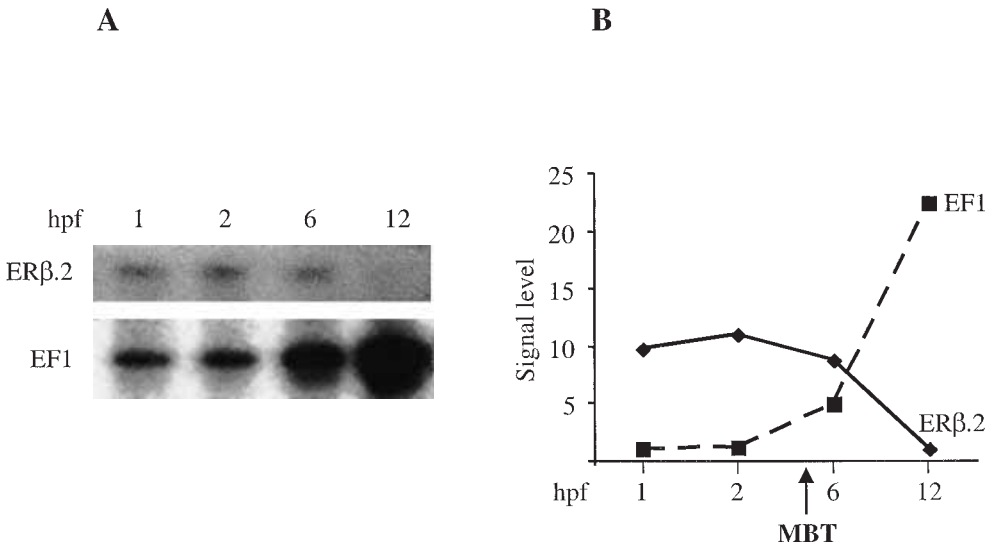

Figure 4 Early embryonic expression of zebrafish ERβ.2. (A) RNase protection assays. RNAs extracted at the indicated times after fertilization were protected by ERβ.2 and the corresponding EF1 probe. Different exposure times were used. (B) Quantification. Results are expressed relative to the amount of EF1 signal at 1 h after fertilization. The time of midblastula transition (MBT), when embryonic transcription starts, is indicated. hpf, hours post fertilization.

genes are poorly expressed during the early phases of zebrafish development, and suggest that they might not be necessary to these processes.

Discussion

In this paper, we describe the initial characterization of oestrogen receptors in the zebrafish (*Danio rerio*). Our work documents the existence of three ERs in this species, resulting from a duplication of the *ERβ* gene. We also show that the zfERs can react to oestrogen agonists in a different way as compared with their mammalian orthologues. Finally, we demonstrate three phases in the embryonic expression of ER.

Three ERs in zebrafish

Our phylogenetic analysis of ER proteins from fish and other vertebrates, including human, strongly supports two conclusions. First, the zebrafish ERα we have isolated is the orthologue of the human ERα. The characterization of only one ERα in each fish studied, with an ERα phylogeny reflecting that of the species, supports the experimental data in demonstrating that there is no second ERα in fish. Second, the other two ERs characterized in the zebrafish group had fish ERβ sequences, indicating that they represent a fish-specific duplication of *ERβ*. This is further supported by the grouping of various fish ERβ proteins with each zfERβ, including one of each ER for fish in which two ERβ proteins have also been reported (Hawkins *et al.* 2000). Thus it appears that the two other fish ERs should be named ERβ.1 and ERβ.2, and not ERβ and ERγ (Hawkins *et al.* 2000). Although their origin is debated (Amores *et al.* 1998, Robinson-Rechavi *et al.* 2001*b*), there is increasing evidence that duplicate genes are much more frequent in fish than in mammals (Robinson-Rechavi *et al.* 2001*a*). Thus there are, in zebrafish, three ERs instead of two, because *ERβ* is duplicated in euteleost fish.

ER differential transactivating properties

Oestrogens have been implicated either positively or negatively in a variety of diseases such as breast cancer and osteoporosis. This has led to the design of various molecules as therapeutic agents, now collectively referred to as selective oestrogen

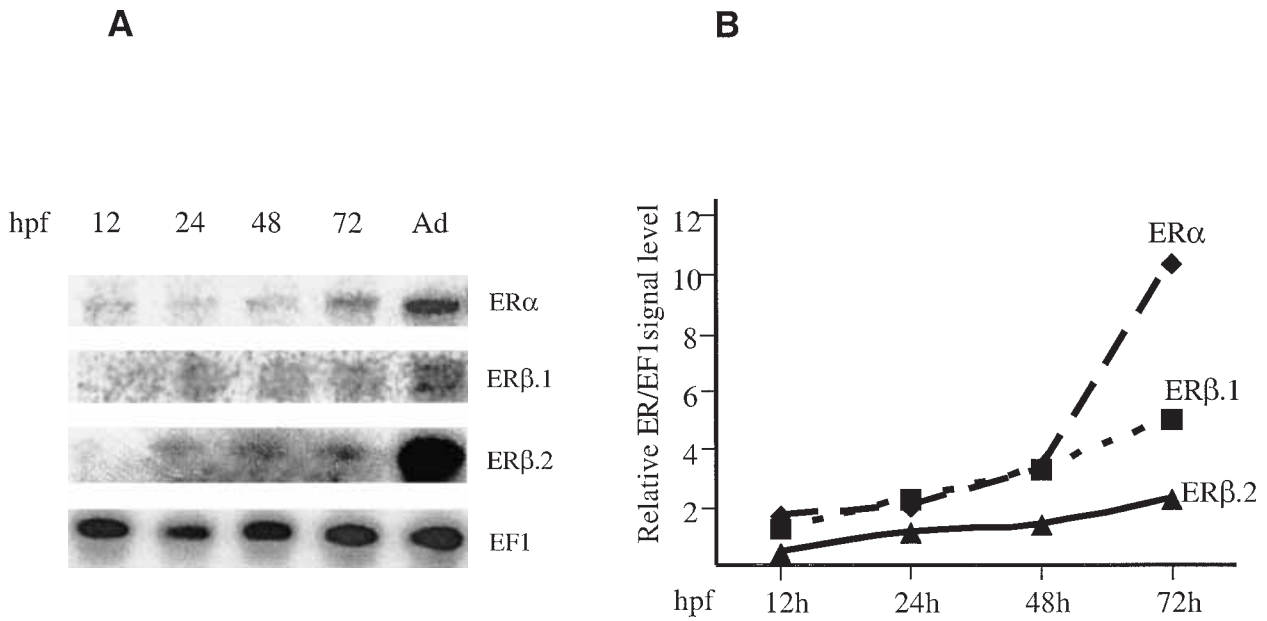

Figure 5 Late embryonic expression of zfERs. (A) RNase protection assays. RNAs extracted at the indicated times after fertilization were protected by ER and the corresponding EF1 probe. RNAs extracted from adult zebrafish (Ad) were used as a control. Different exposure times were used. Note that the ERβ.1 signal appears as two bands, which could represent alternative splice versions of the gene or, alternatively, could be due to persistent secondary structures. (B) Quantification. ER signals were plotted relative to the EF1 signal in each sample. hpf, hours post fertilization.

receptor modulators (SERMs), which display either agonistic or antagonistic activities according to the receptor, tissue/cell system and promoter concerned (reviewed in McDonnell 1999). Because of the ease of breeding, zebrafish may be presumed to be a convenient model for the *in vivo* evaluation of molecules that could affect development or physiology. As these molecules mostly act through the ERs, it was important to characterize the reactivity of the zfERs to some of these molecules, and to compare it with that observed for mammalian receptors.

All ERs tested have a comparable E_2 transcriptional response. This is also true for diethylstilboestrol, a synthetic oestrogen that has been widely used to prevent spontaneous abortions. In contrast, zfERs are not stimulated by 4OH-E_2, a catecholoestrogen, although this compound fully activates both mERs (Kuiper *et al.* 1997, our present results). This difference between mouse and zebrafish cannot be explained by sequence divergence in the ligand-binding pocket itself (as determined through crystallographic studies by Brzozowski *et al.* 1997), because all amino acids constituting it, including those in contact with the ligand, are conserved between zebrafish and mouse. However, it is still possible that 4OH-E_2 requires other amino acids for efficient binding. Alternatively, 4OH-E_2 may bind to zfERs but generate a receptor conformation different from that generated by E_2, and which is not recognized by the mammalian coactivators present in our cultured cells. 4OH-Tamoxifen is a SERM used in the treatment of breast cancer and osteoporosis (reviewed in Avioli 1999, Jordan & Morrow 1999) that activates ERα but not ERβ (Tremblay *et al.* 1997, Watanabe *et al.* 1997). It has been suggested that this compound requires both AF1 (the presence of which on ERβ is still questioned) and AF2 functions for full activation. The fact that the A/B domain where the AF1 function resides is not conserved between zebrafish and mouse ERs provides a likely explanation for the lack of activation capacity of 4OH-tamoxifen on zfERs.

Our results therefore show that zfERs behave in the same way as their mammalian counterparts for some oestrogenic ligands, and very differently for others.

Three phases of ER expression during zebrafish development

ERs are weakly expressed during zebrafish development and did not yield a discrete pattern of expression observable by *in situ* hybridization of whole embryos. However, three phases can be distinguished in ER expression. During the first, ERβ.2 is uniquely expressed in a maternally transmitted manner. The second is characterized by a near absence of ER expression. In the third, starting between 48 and 72 h after fertilization, all ERs, but predominantly ERα, begin to be expressed. During mouse embryonic development, ERα has been detected by RT-PCR in fertilized eggs and at the blastocyst stage (Hou & Gorski 1993). As detected by *in situ* hybridization techniques, ERs appear to be expressed relatively late, starting with ERα at E9·5 in the heart atrium (Lemmen *et al.* 1999). In mammals, there are no reports indicating a maternally inherited expression of ERβ, which could thus be specific to only one of the duplicated genes present in fish. The absence of ERs during long periods of development in zebrafish also suggests that ERs are not required for development. In agreement with this, mice knockouts for one or both *ER* genes survive throughout development, although multiple defects are reported that mainly affect reproductive functions (Lubahn *et al.* 1993, Krege *et al.* 1998, reviewed in Couse & Korach 1999, Couse *et al.* 1999, Dupont *et al.* 2000).

Our expression data suggest that E_2 signalling might be efficiently transduced in a time-dependent manner – that is, only when an ER is expressed. As no embryonic transcription is believed to take place before 4 h after fertilization (the onset of zygotic transition), ERβ.2, as the unique ER isotype expressed, could transmit an oestrogen signal from 4 h after fertilization to 8–12 h after fertilization, after which time no ER expression can be detected. However, this requires the presence of a ligand produced by the embryo during this period. In this respect, it has recently been reported that an isotype of the aromatase gene (cyp19 – the key enzyme in oestrogen production) is also expressed in zebrafish in a maternally inherited manner (Kishida & Callard 2001), which renders likely the *in situ* production of oestrogen before 12 h after fertilization. Aromatase expression becomes undetectable at 12 h after fertilization and remains absent until 48 h after fertilization, when both isotypes begin to be expressed. This time point corresponds to the onset of ER embryonic expression. It is therefore likely that the zebrafish embryo produces both ligand and receptor within the same window of time, provided that a source of androgens, the substrate of aromatase, exists. Ligand and receptor production are likely to be coordinated, as both aromatase and ERα expression have been reported to be under the control of oestrogens (Berkenstam *et al.* 1989, Pakdel *et al.* 1989). Indeed, E_2 treatment of zebrafish embryos results in an increase in aromatase expression (Kishida & Callard 2001). However, this stimulation could be visualized only at 48 h after fertilization, even when E_2 was supplemented as early as 2 h after fertilization. This suggests that the embryo is unable to transduce oestrogen signalling before 48 h after fertilization, which is in agreement with the absence of ER expression reported in the present paper. This is further illustrated by the fact that treating zebrafish embryos with E_2 or the anti-oestrogen, ICI 164,384 (10^{-8} M for 72 h starting at 1 h after fertilization in both cases) did not lead to an increase in ER expression, and did not have any obvious morphological effect (data not shown).

In summary, zfERs can react to oestrogenic compounds in a different way from their mammalian counterparts, including a total lack of transcriptional response to molecules that are powerful activators of mERs. In contrast, ERs present a lack of expression during most of the developmental period in zebrafish. Overall, this does not favour *Danio rerio* as a general model in which to detect the presence of endocrine disruptors acting on oestrogen receptors. One should therefore be aware that results obtained in zebrafish will be difficult to extrapolate to mammals.

Acknowledgements

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