Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERα, ERβ1 and ERβ2

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Abstract

We have previously cloned and characterized three estrogen receptors (ER) in the zebrafish (zfERα, zfERβ1 and zfERβ2). We have also shown that they are functional *in vitro* and exhibit a distinct expression pattern, although partially overlapping, in the brain of zebrafish. In this paper, we have shown that the hepatic expression of these zfER genes responds differently to estradiol (E2). In fact, a 48-h direct exposure of zebrafish to E2 resulted in a strong stimulation of zfERα gene expression while zfERβ1 gene expression was markedly reduced and zfERβ2 remained virtually unchanged. To establish the potential implication of each zfER in the E2 upregulation of the zfERα gene, the promoter region of this gene was isolated and characterized. Transfection experiments with promoter–luciferase reporter constructs together with different zfER expression vectors were carried out in different cell contexts. The data showed that *in vivo* E2 upregulation of the zfERα gene requires ERα itself and a conserved transcription unit sequence including at least an imperfect estrogen-responsive element (ERE) and an AP-1/ERE half site at the proximal transcription initiation site. Interestingly, although in the presence of E2 zfERα was the most potent at inducing the expression of its own gene, the effect of E2 mediated by zfERβ2 represented 50% of the zfERα activity. In contrast, zfERβ1 was unable to upregulate the zfERα gene whereas this receptor form was able to tightly bind E2 and activate a reporter plasmid containing a consensus ERE. Altogether, these results indicated that the two ERβ forms recently characterized in teleost fish could have partially distinct and not redundant functions.

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Introduction

In all vertebrates, estradiol (E2) is involved in numerous physiological processes during development and adult life. These effects are mediated by members of the nuclear receptor family, the estrogen receptors ERα and ERβ. These proteins classically regulate the expression of E2 target genes by direct binding with a specific palindromic DNA sequence called the estrogen-responsive element (ERE: AGGTCAnnnTGACCT) which permits recruitment of cofactors necessary for transcription (Zilliacus *et al.* 1995, Klinge 2000). Moreover, an important number of E2-sensitive genes, which do not contain ERE but do contain other cis-elements such as AP-1 or Sp1, have been described. It was recently proved that, in this case, ERs can regulate the transcription by direct protein–protein interactions with the AP-1 or Sp1 transcription factors (Paech *et al.* 1997, Webb *et al.* 1999, Saville *et al.* 2000). Although the mammalian ERs share the same modular organization, recent data showed that they may have different transcriptional capacities on E2 target genes (Kuiper *et al.* 1997, Tremblay *et al.* 1997). Differences between ER subtypes in relative ligand-binding affinity have been described. A range of natural and synthetic agonists or antagonists induced distinct conformational changes in the tertiary structure of the ERs which induced differential cofactor recruitment. In consequence, the effects of E2 at the transcriptional level depend on the promoter, the presence of cell-specific factors and the ER subtype, which

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could explain in part the pleiotropic effects of E2 at the physiological level.

In addition to natural hormones or effectors, including growth factors, several classes of environmental contaminants could also interact with ERs and modulate the expression of E2 target genes. These latter molecules, usually referred to as endocrine disrupters, are susceptible to impairing the reproductive success in all classes of vertebrates (Petit *et al.* 1997, Tyler *et al.* 1998, Le Guevel & Pakdel 2001, McLachlan 2001, Jobling *et al.* 2003).

Although in mammals only two ER subtypes have been characterized (Green *et al.* 1986, Kuiper *et al.* 1996, Mosselman *et al.* 1996), we and others have recently reported the existence of three ER forms in fish species: ERα, ERβ1 and ERβ2 (Hawkins *et al.* 2000, Ma *et al.* 2000, Menuet *et al.* 2002). These receptors are generated from three distinct genes, are able to bind E2 with high affinity and activate, with approximately the same fold induction, a reporter gene under the control of a consensus ERE (Bardet *et al.* 2002, Lassiter *et al.* 2002, Menuet *et al.* 2002). These results clearly demonstrated that these new ERβ forms were functional ligand-dependent transcription factors. However, *in vivo*, the majority of E2 target genes present imperfect ERE sequences or complex organizations with, notably, the presence of ½ERE close to the ERE. In consequence, it is necessary to investigate the respective transcriptional enhancement capacities of the ERβ forms on an endogenous target gene.

In oviparous species, in addition to the hypothalamic–pituitary–gonad axis, the liver is an important E2 target organ, in which the synthesis of the yolk protein vitellogenin by hepatocyte cells is strictly under the control of E2. The vitellogenin production which is crucial for embryo development and reproduction success is tightly coupled to a substantial E2-dependent upregulation of ERα gene expression. In chicken, *Xenopus* and trout, E2 treatment increases the ERα mRNA and protein accumulation in the liver (Pakdel *et al.* 1991, Ninomiya *et al.* 1992, Lee *et al.* 1995). This induction occurs at the transcriptional and post-transcriptional levels (Flouriot *et al.* 1996*a*).

Here, we have shown that zebrafish (zf) ERβ1 and zfERβ2 mRNAs are co-expressed with zfERα in the liver and that E2 treatment differentially modifies the expression levels of these receptor transcripts. To investigate whether or not zfERβ1 and zfERβ2 are involved in hepatic vitellogenesis and therefore in hepatic zfERα regulation, the zfERα promoter region was isolated and characterized. The data showed that, in contrast to zfERβ1, zfERβ2 was able to significantly stimulate the expression of the zfERα gene in the presence of E2. Moreover, expression of zfERβ1 was strongly downregulated by *in vivo* E2 treatment. This, added to the fact that *in vitro* data demonstrated that zfERβ1 is not directly implicated in the E2 upregulation of zfERα, suggests diverging functions for zfERβ1 and zfERβ2 in the liver.

Materials and methods

Animals

In this study, mature zebrafish (*Danio rerio*), raised in our facilities, were anesthetized on ice. Hormonal treatments consisted of exposure of fish for 48 h to 10^{-8} M 17β-estradiol (E2) or to a solvent control (0·1% ethanol) before anesthesia. The liver was removed by dissection and total RNA was extracted using the Trizol method according to the manufacturer's instructions (Gibco-BRL, Eggenstein, Germany). The liver RNA samples were enriched in poly$(A)^+$-RNA by the oligotex mRNA mini kit (Qiagen, Courtaboeuf, France).

Northern blot assay

Northern blot experiments were performed according to the previously published protocol (Thomas 1980). Poly$(A)^+$-RNA (1·5 µg) from E2-treated or untreated zebrafish liver was denatured at 65 °C for 10 min in formamide–formaldehyde solution, separated on agarose–formaldehyde gel and transferred onto a nylon membrane (Hybond-N; Amersham, Uppsala, Sweden). RNA were fixed by u.v. exposure (254 nm for 2 min) and by baking at 80 °C for 2 h. The membrane was prehybridized for 5–6 h, hybridized overnight with the probe labeled with [α-^{32}P]dCTP corresponding to the coding region of each zfER cDNA or to acidic ribosomal phosphoprotein (PO) cDNA as control. The membrane was then washed four times with 2 × SSC, 0·1% SDS solution at room temperature for 5 min and three times with 0·2 × SSC, 0·1% SDS solution at 55 °C for 30 min and exposed to biomax film.

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After hybridization with the zfERα probe, the membrane was kept at −80 °C for 1 month to decrease the radioactivity and stripped (incubation for 2 h at 65 °C with 75% formamide, 10 mM NaH_2PO_4 solution) in order to rehybridize with the zfERβ2 probe. The same procedure was used for PO and zfERβ1 probes.

Primer extension analysis

The probe synthesis was carried out according to the protocol described previously (Flouriot *et al.* 1996*b*). Briefly, a biotinylated single-stranded DNA template was used to prepare a labeled probe by extension from a specific primer by the T7 DNA polymerase in the presence of [α-^{32}P]dCTP. To generate the probe, the vector containing the zfERα cDNA was used to obtain biotinylated PCR product (346 bp) with a biotinylated primer 1 (5′-atgtaccctaaggaggagcacagcg-3′) beginning at the ATG of the exon 2 and a non-biotinylated primer 2 (5′-cctgctgagaggacaccaca-3′). After purification, the biotinylated PCR product was bound to streptavidin-coated magnetic beads (Dynal, Great Neck, NY, USA) and non-biotinylated single-strand DNA was eluted in 0·1 M NaOH. The labeled probe (320 bp) was obtained by extension in the presence of [α-^{32}P]dCTP from internal primer 3 of exon 3 (5′-tggctcagatacggggacag-3′). The probes were then eluted with an alkaline solution and purified on a 4% denaturing polyacrylamide–urea gel. Approximately 2 × 10^5 c.p.m. of single-strand probe were coprecipitated with 30 μg RNA (RNA from E2-treated zebrafish liver or yeast total RNA as control) and resuspended in 20 μl hybridization buffer. The templates were denaturated at 80 °C for 10 min and incubated at 50 °C overnight. After an ethanol precipitation of RNA/probe hybrids, reverse transcription was carried out at 42 °C for 1 h with 50 U Expand reverse transcriptase (Boehringer Mannheim, Mannheim, Germany). RNA matrix was digested at 37 °C for 30 min with RNase A and EDTA (0·1 M). After purification and denaturation, the samples were separated through a 4% denaturing polyacrylamide–urea gel.

Cloning of zfERα promoter

To clone the zfERα promoter, we generated a genomic DNA fragment corresponding to exon 1 (+15), intron 1 and exon 2 (+426). This zebrafish genomic DNA fragment (1181 bp) was amplified by PCR with a forward primer within exon 1 (5′-AGTCAGAGATACATCAGTGAGAG-3′) and a reverse primer within exon 2 (5′-GCGCTGTG CTCCTCCTTAG-3′). This fragment was subcloned and sequenced.

The zebrafish genomic library inserted in λgt10 EMBL 3 SP6/T7 vector was obtained from Clontech (Palo Alto, CA, USA). Recombinants phages (5 × 10^5) were screened with a ^{32}P-labeled probe corresponding to exon 1, intron 1 and exon 2 (corresponding to the 1181 bp genomic DNA fragment). A positive clone, λ2021, containing a 7 kb genomic fragment was purified after two to three rounds of screening. The fragment was subcloned into the BamHI site of Bluescribe vector (Stratagene, La Jolla, CA, USA) and the promoter region restricted to 1·8 kb upstream of the initiation site was inserted into the KpnI/BglII site of pGL2-basic vector containing the luciferase reporter gene. This construct, named PA-1·8 kb, was sequenced on both strands by the PRISM ready reaction big dye terminator cycle sequencing protocol (PE Biosystems, Courtaboeuf, France) and the putative transcription factor binding sites were analysed by the MatInspector program (Quandt *et al.* 1995).

Plasmid constructions and site-directed mutagenesis

PA-0·3 kb construct was obtained from the PA-1·8 kb vector by deletion of the 1500 bp upstream of the AP4 box using the Nco1 restriction site. The PA-0·23 kb, PA-ERE, PA-EREm1 and PA-AP-1:½ERE constructs, corresponding to various lengths of the 5′ flanking sequence of the zfERα gene, were obtained by PCR from the PA-1·8 kb vector. The forward primers (A, B, C, D) and the reverse primer (Rev) contained the BamHI or SmaI restriction site and are described in Table 1. PCR products were subcloned in SmaI/BglII sites of the pGL2-basic vector (Promega, Madison, WI, USA). The QuickChange site-directed mutagenesis kit from Stratagene was used for ½ERE and 3′ ERE half-site mutations of the PA-0·23 kbm and PA-EREm2 constructs. The primers used are also described in Table 1. Finally, each construct was sequenced on both strands by the PRISM ready reaction big dye terminator cycle sequencing protocol.

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Table 1 Oligonucleotides used for plasmid constructions and site-directed mutagenesis

	Constructs	Sequences
Primers		
A	PA-0·23 kb	*TCCCCCGGG*ATCAAGCGGTGACCTCCTAT
B	PA-ERE	*TCCCCCGGG*CTGGTTGCCATGACCTGCT
C	PA-EREm1	*TCCCCCGGGC*T**aa**TTGCCATGACCTGCTC
D	PA-AP1:½ERE	*TCCCCCGGGG*CTCTGAGAAGTGACCGTCAG
Rev	All	*CGCGGATCCG*TTCACTCCTCTGATGTTTTAC
QC-0·23up	PA-0·23 kbm	GGGATCAAGCGGTGA**aa**TCCTATCTCTTGTTTACCTGG
QC-0·23do	PA-0·23 kbm	CAAGAGATAGGA**tt**TCACCGCTTGATCCCGGGGG
QC-EREup	PA-EREm2	GGGCTGGTTGCCATGA**aa**TGCTCTGAGAAGTGACC
QC-EREdo	PA-EREm2	CTCAGAGCAT**tt**CATGGCAACCAGCCCGGGG

Italic letters correspond to enzyme restriction site SmaI and BamHI. Responsive elements are underlined. Nucleotide mutations are noted by small bold letters.

Cell culture and transfection experiments

CHO, Hela, and HepG2 cells were maintained at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 5% fetal calf serum (FCS; Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin, 100 mg/ml streptomycin and 25 mg/ml amphotericin (Sigma). CHO and Hela cells were seeded in 24-well plates (5×10^5 cells/well) or in six-well plates (30×10^4 cells/well) for HepG2 cells. After 24 h, the medium was replaced by fresh phenol red-free DMEM-F12 containing 2·5% charcoal/dextran FCS and ethanol (0·1%) with or without E2 (10^{-8} M). Cells were transfected with plasmid using FuGENE 6 reagent as indicated by the manufacturer (Boehringer Mannheim). The DNA templates for CHO and Hela transfections contained 25 ng expression vector (with or without the coding region of each zfER), 150 ng luciferase reporter gene vector and 50 ng internal β-galactosidase control vector. For HepG2 transfection assays, the DNA templates contained four times more vector. The luciferase activities were assayed 36 h later using a luciferase assay system (Promega). The β-galactosidase activity was used to normalize transfection efficiency in all experiments.

Results

E2 upregulates zfERα mRNA expression but not zfERβ forms

Twenty-four adult zebrafish were divided into two groups and treated for 48 h either with 10^{-8} M E2 or the vehicle (ethanol 0·1%). The livers from each group were mixed and poly(A)$^+$-RNA was prepared. Figure 1 shows the results of Northern blot experiments using each zfER probe. Hybridization with zfERα probe showed a single band of 4·8 kb which was clearly upregulated after E2 treatment. The zfERβ1 probe revealed a strong signal band at 4 kb in the liver of control animals. Interestingly, this mRNA was markedly downregulated after E2

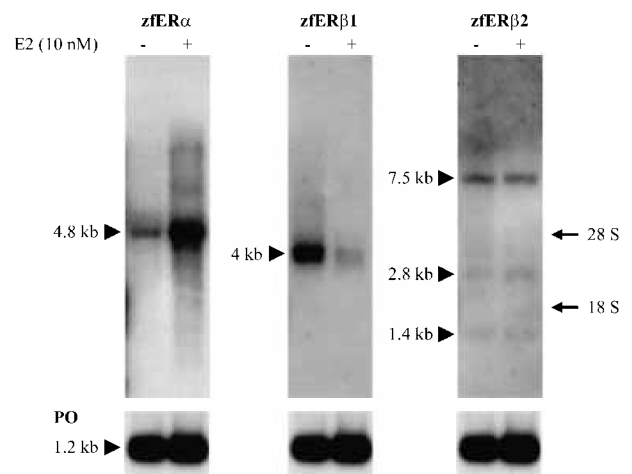

Figure 1 Northern blot analysis of ER mRNAs in the liver of untreated and E2-treated zebrafish. Poly(A)$^+$-RNA (1·5 µg), from the liver of zebrafish exposed for 48 h to ethanol (–) or 10 nM E2 (+), was separated on a denaturing formaldehyde–agarose gel and transferred to a nylon membrane. This membrane was successively hybridized under stringent conditions with ^{32}P-labeled zfERα, zfERβ1, zfERβ2 and PO probes prior to autoradiography. The size of zfER mRNAs was determined from standard markers and the position of ribosomal RNA stained by methylene blue is indicated on the right.

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treatment. Hybridization with the zfERβ2 probe showed a more complex expression pattern. A major signal was detected at 7·5 kb and two lower bands were also observed at 2·8 kb and 1·4 kb. The amounts of these mRNAs were not modified after E2 treatment.

Characterization of the promoter region and transcription initiation start site of the zfERα gene

To investigate the molecular mechanisms involved in the E2 upregulation of zfERα mRNA, we cloned the promoter region of the zfERα gene. A genomic DNA library was screened using a probe spanning exon 1 and exon 2. A sequence of 1·8 kb at the 5′ end of the zfERα gene was isolated and sequenced. First, to identify the transcription initiation site, a primer extension experiment on total RNA from liver was carried out (Fig. 2). The size of the major product of the primer extension assay was determined using the accompanying sequence ladder and was localized 139 bp upstream of the ATG of exon 2 (Fig. 2). Staining of other lower extension fragments was also detected and corresponded to minor initiation start sites.

In a second step, the presence of presumptive transcription factor DNA-binding sites was determined using computer analysis (Fig. 3). The identified promoter sequence was composed of 1745 bp. The major initiation start site is represented by an arrow. The promoter has no TATA or CAAT box, but presents an Inr sequence at − 50 bp (Javahery *et al.* 1994). Two C-Ets-1 sites at − 1625 and +8bp and two C/EBP-β sites at − 1280 and − 150 bp were found. Four GATA potential binding sites were also located (three GATA-1 sites at − 1744, − 785 and + 17 bp; GATA-2 or 3 at − 1321 bp). Brn2 and SREBP potential binding sites were identified at − 1708 and − 1265 bp respectively. Three AP-1 potential binding sites, which could include a ½ERE, were located at − 850, − 129 and − 80 bp. In the proximal part of the promoter region, an imperfect ERE was found at − 104 bp. AP-1 and AP-4 sites were also located at − 605 and at − 200 bp.

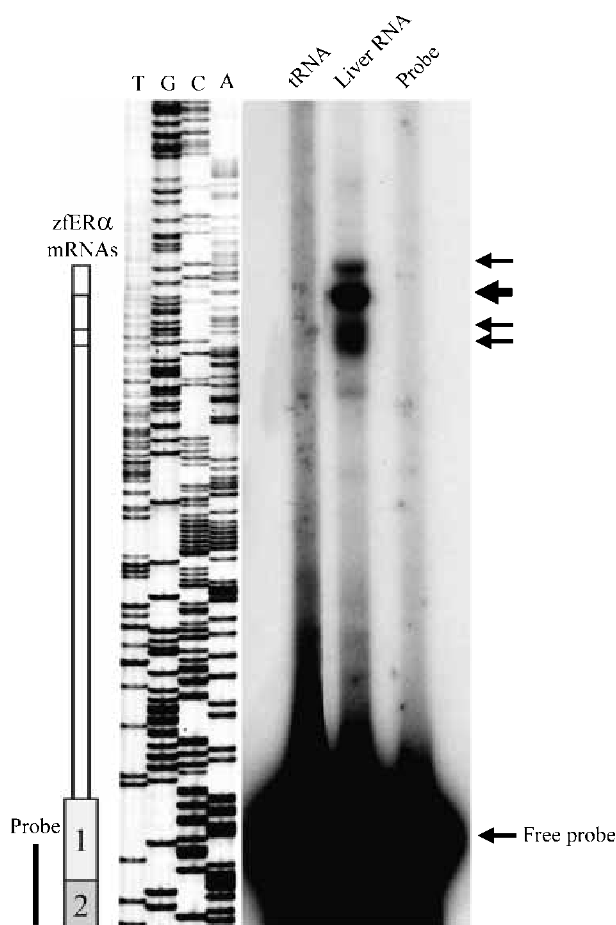

Figure 2 Identification of the transcription initiation start sites of the zfERα gene.
Labeled probe, extending from exon 2 to exon 3, was hybridized with 30 µg total RNA (tRNA) from E2-treated zebrafish liver or with yeast tRNA as control. The major extension product signal is noted by a thicker arrow and the lesser stainings by the other arrows. The extension product sizes were determined using the accompanying sequence ladder. The differently initiated mRNA are schematically represented on the left.

E2-dependent upregulation of the zfERα gene uses an imperfect ERE

To determine if the transcription rate of the zfERα gene was altered directly by ERs under E2 treatment, the zfERα gene promoter (PA-1·8 kb) was used as the reporter gene and was cotransfected with the zfERα expression vector into CHO cells. After 36 h of E2 treatment (10^{-8} M), a significant E2-dependent induction (approximately 10-fold) of the PA-1·8 kb reporter was observed (Fig. 4). Because this result showed that the zfERα gene was induced by zfERα itself, we next examined whether DNA potential sites, particularly the imperfect ERE described above at − 104 bp by computer analysis, could be involved. To address

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-1745 CTCGAGGATA TTACGAGCTC AGGGCTCTCT CCCGGGACAG CATGCCAAAT ACGCTTTATT GATTATCAGC TAAGTGTGAA
        GATA-1                                    Brn2
-1665 CTCTTGAAAG CTTATTAATG AGCATGGAAA CAGGCCATAG TCAGGACGAT GTGTACTCGC ACTGAAACTG CTCGTTCTAT
                                                C-Ets-1
-1585 TTTGAAAATT GAATAAAATA TGGGAAGATT TCTTTTGTTT TTTTTTTTTA ACTTAATTCC ACTGCTTATT TCTGTATAAA

-1505 TCTATTAATA CATTTTCTCT TCCCAAATCA TACAAATAAC AAATTGTCAA AGTTTCTCTG ATGCATTTTA TAGGATTTTT

-1425 TTTTAGTTCT GTGTACACAC ACACACACAC ACACACACAC GTATGTATTT ATGTATATAT CTATGTATTT GCAAGGAGCT

-1345 TATTGTTCTA TCTGTTCTAG AGAGTAAGAG ATAAGAGTAA AGAACCCCAC AAAGCACAAA TCATTAATGT TCTGCAATTA
                            GATA-2 and 3                                  C/EBP
-1265 TCACGTGAAC GCTATCTTTG TGTGTTTGGT GTCAGTTGAG GACTCTGGCC AAGCTGTTTT TTTTTGGAAA TCAAGGCCAT
      SREBP
-1185 GTTTTTGGTC TTTGAACTGT ACTAGTTCTA GCTCTGTTCT GGTTAAGCGT AGCTCAAGCA TACACTAAAA ATGCACTGTT

-1105 TTAAATAAAT AATAGTGTTG TATATCTAAA ATTGTTTTTC TAAGAACCCT GCAGGAGCAG ATTGAGACTT AATTGAGCCA

-1005 AAAGGACACA GACATCTGCT TGCATAAAGG CTATTAATAC AAAATGCTCT ATTTAGGAAA AGAACATTAC TATTTTAGAT

-945  TTGTAAGCTG CAAAACATGA ATACACACAT CAAAATGAGC CATTAGCAGT AATTATATTT GAGGAAAGAA TTGTGTTTTT

-865  TTTTATTAAA ACGGCTAATG ACCAATAAAA AAAAACTTGT GATCATTAGA AATTCTGTTT TTACCACCAC TATGTTCAAT
                      ½ERE AP-1
-785  ATATGATATA TATTTTTAAA TGATTTACAA TATATTTTTA CTGTATTTAT TTGACAGTAA AAACCAATTA AATATATTTA
        GATA-1
-705  AAACCATTTA ATGTGAAACA GTATTAAAAT GTAAATGTTT TTCTATTTTG ATTTTTTTTC AAATATTTTA TTAGTCACAA

-625  GAATATATAT TAATATGCAT ATCTGACAGC AAACGTAAAG TCAAATAATT ATCAATGTCT ACTCAGTTAT TTGAAATATT
                              AP-1
-545  TATTTCAATG CAAACGTTTT CACTGCCTTT TCTAATCAAA TCAATTCTTT TACTCAAATC TAATCAACTC TTGCTGAAAA

-465  AAAGTGTCAG TTTATTTTAT TGTTTCAACA GTACTGTATA TTTAAATCCA TAGTCAGTCT AATGAAGTTT CCCTAATCAA

-385  ACAAAAAGCA CAACACCCAC ACATGTTCCA TTTCTGTGTA AAATAGCTGC CATTACAGAG AAATGATTAT AATCAAACAG

-305  ATTTGTTTTG TCCCTAGAGA GCTGAACAGC GTCTCCTTCC CTTTTTAACC TCCTGCAAGC ATCTTCCAAC GAATTTAATT

-225  ACTCCTTTGA CATATAATTT CCCATGGCAG CAGCATGTAA AGTGGTTTCA CAGCGCATCA CCTGTAAAAC TCAAAGGTTT
                                  AP-4
-145  TGGCAAGTGA ATCAAGCGGT GACCTCCTAT CTCTTGTTTA CCTGGTTGCC ATGACCTGCT CTGAGAAGTG ACCGTCAGTA
      C/EBP             ½ERE  AP-1                ERE                         ½ERE  AP-1
-65   TCGACCTGGC TGTCTTTAAT TTGTTTACCT CCTCTTTTTT TAAGCCAGAG ACACAGACAG AGAAAGTAAG GACACAGGAA
                       Inr                                                 +1     C-Ets-1
+15   GTCAGAGATA CATCAGTGAG AGAGAGAAAG CATCCAGCCT GTAATGGGAC TCAAGTAAAA CATCAGAGGA GTGAACATTT
        GATA-1
+95   TGGTAAGGAT GCTGGAAAGA ATTCAGCAAC CAATTTTTCA GCTTTTGTTT TTGTAGTAAT GTAATTCCAT GTGTCTTTCA
      CTCTAGCGTT TTGTAATCGA GCATTGTTTC TATTATTAGT GCTTTCAGTT GAGTTTCCTG TCCTGTCGGT TTCCTGTTTT
      ATAGAAAAGC ACACCTTTCA GTTGGGTTTA TCCTTTCTTA CACTGCAAAT AATAATGCTT TTTGTCTTGT GTCTAGTCCA
      AATACCTAAC AATTATCTAA AAAAATGATG TAAAAATACT GTTTTTGTGT TGTTTTGTCC CTTAAAAATA ATCTGCCAAT
      GAAGTGGGTA AATAAATCTT AATTTCGGGT TGAAATAAGA TCATTTTAAA ATCATTTTTG TTGTTTGACA TAAAATCTCC
      CTCAATTTTG ACATTATTTC TGTTTCAAAG TAAAACAAAA CAATTTTTTT TACTTGTCTA GAAAATGCTT CCTGAATGCT
      CTTTCCTTCC AAAAAAAAAA AAAAAAAAAG CAGCTTCAAG AAGTCAGAAA TGCATTTCTT TGCAGTGTAA ATGTAATGCA
      TAAGACATTT GGGGAAAGAA GAACAATGAG GGGGAAAAGA TGGGATAAAA GAAAGGCAAA AGAATCAAAA TGAAAATGTG
      TTTACAGAGG GAAAGACAGG AAGAAAGAGA AAGAAAAGCA GTCTCACGTA TCTTAGAAAA ACATCATACT TTGTCCTCTC
      AGCCCAAGGG GGACTGTAGG AGAGGAAGGT AGTTTTTTTT TAGGAGGAGT AGGCAGAGCA GCGAGAGAGT GAAGCGGAAG
+137  CGTAGCAGAA CAAGGGGGAG AGAGTTTGAG AAAGACTGTG GGAGGTGGAA AAAAAGAAGC CTGAGCTTAA AAAAGACAGA

+207  AACAGTCTTT TGCTTATGAA CAGATAGTAC CCTAAGTCAT GCTTGCTGCG TGTGTGTTTC CCATGGTGAT GTCTGGAGGG

+287  CAGACCAGCG GAGAGGCTGT TGGTGCCAGG CAGCGACGCA GGACCAGCCC GAGTCCTGAG AGAGAAGCCC TGGAGGAACC
                                         *
+367  TTCCTCGCCA CCTGCCGCCC ACAAACTCTC ACCCATGTAC CCTAAGGAGG AGCACAGCGC
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Figure 3 Sequence analysis of the 5′ end region of the zfERα gene. The putative DNA-binding sites for several transcription factors were determined with the MatInspector program (Quandt *et al.* 1995) and are boxed. The major initiation start site is noted by an arrow. Exon 1 is identified by bold letters and the italic letters correspond to intron 1. The underlined sequence corresponds to exon 2. The potential ATG codon is noted by an asterisk.

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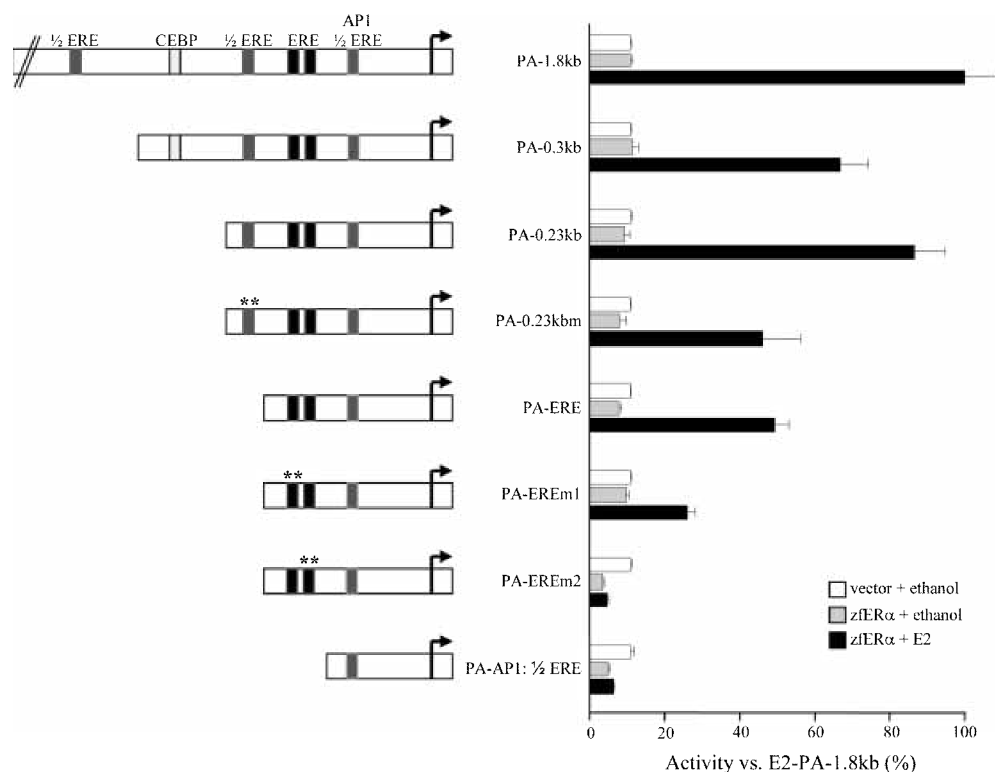

Figure 4 A complex unit containing ½ERE–ERE is involved in the E2-dependent upregulation of the zfERα gene. E2-treated or untreated CHO cells were cotransfected with an expression vector (empty or containing the coding region of zfERα) and several constructs of the zfERα gene promoter described at the left. Mutations on the half site and imperfect ERE are indicated by asterisks. On the right, data are expressed as percentage of activity versus the E2 induction (±S.E.M.) of PA-1·8 kb. Each experiment was repeated at least twice in triplicate.

this question, we compared E2 induction of several luciferase constructs versus the E2 induction of PA-1·8 kb.

Figure 4 shows that, compared with the PA-1·8 kb construct, the PA-0·3 kb vector was less inducible by zfERα (65% of E2-PA-1·8 kb) indicating that several binding sites, notably the CEPB-β and AP-1:½ERE, located between −1280 and −850 bp, could be involved in the E2-dependent zfERα gene induction. Curiously, E2 induction of the PA-0·23 kb construct was higher (85% of E2-PA-1·8 kb) than PA-0·3 kb (65% of E2-PA-1·8 kb), suggesting that a silencer element, unidentified by computer analysis, could exist between −205 and −135 bp.

Compared with PA-0·23 kb, E2 induction was significantly diminished when the AP-1:½ERE located at −129 bp was mutated (PA-0·23 kbm) or entirely deleted (PA-ERE), demonstrating that this half site is involved in the E2-dependent induction of the zfERα gene (Fig. 4). To investigate the role of the imperfect ERE (−104 bp), two different mutations were performed. Figure 4 shows that the stimulatory effect of E2 on the PA-ERE promoter was reduced from 50% to 25% of E2-PA-1·8 kb activity by mutation of the ERE in the 5′ half site (PA-EREm1). Interestingly, this induction was completely abolished with the mutation in the 3′ half site (PA-EREm2). Moreover, the deletion of this ERE, PA-AP-1:½ERE construct, also showed no obvious induction. These data confirmed the importance of this imperfect ERE for the positive regulation of the zfERα gene. The comparison of the zfERα promoter region with the proximal promoter of several estrogen-sensitive fish genes highlights a complex unit containing a ½ERE close to an ERE presenting the same structural organization (Fig. 5).

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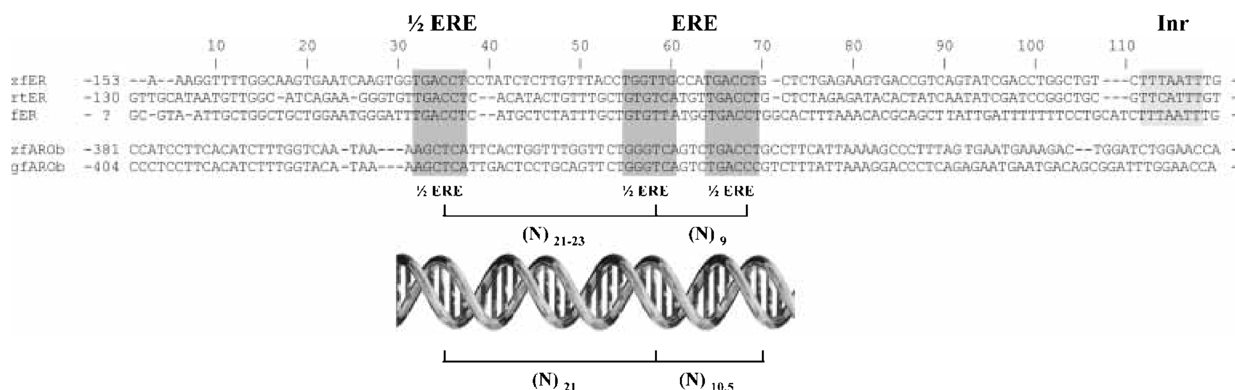

Figure 5 Alignment of the proximal promoters from some estrogen-sensitive fish genes.
Proximal promoters of the ERα and brain aromatase genes from several fish species (zf, zebrafish; gf, goldfish; rt, rainbow trout) were aligned with the GeneJockey II program. Putative EREs, as well as Inr sequences, are shaded. bp Positions were calculated from the transcriptional start site when it had been determined. The relative position of the three ½EREs on the DNA double helix is schematically illustrated below.

zfERβ2 but not zfERβ1 upregulates the zfERα gene

In order to investigate the E2-dependent transcriptional capacity of the two zfERβs on the zfERα gene, each expression vector (empty or containing the different coding region of each zfER) was cotransfected with PA-1·8 kb into CHO (Fig. 6A), Hela (Fig. 6B) and HepG2 cells (Fig. 6C). E2 stimulation of the reporter gene mediated by zfERα reached 10-fold in CHO, 6·5-fold in Hela and 5-fold in HepG2. When using the zfERβ2 expression vector, the E2 stimulation of the reporter gene was 50–60% lower with 3- to 4-fold induction in CHO and in Hela cells and 2·5-fold in HepG2 cells. Surprisingly, irrespective of the cell line used, no obvious E2 induction of the zfERα promoter was observed when using the zfERβ1 expression vector. Nevertheless, this form clearly induced the ERE-TK-luc construct under the same experimental conditions (data not shown) (Menuet *et al.* 2002).

Discussion

The egg yolk protein vitellogenin can be activated *de novo* from a totally silent state within hepatocytes by estrogens. In oviparous species, this process of hepatic vitellogenin is tightly coupled to a clear E2-dependent upregulation of ERα gene expression (Pakdel *et al.* 1991, Flouriot *et al.* 1996*a*). We have recently cloned and characterized two zfERβ forms which co-express with zfERα in the liver of zebrafish (Menuet *et al.* 2002). In this study, we have investigated the responsiveness of these zfER

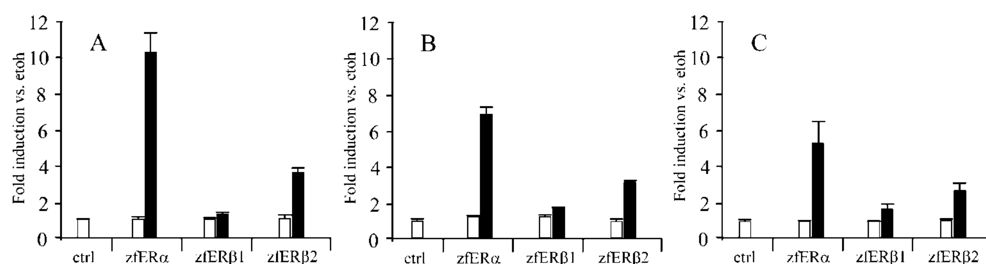

Figure 6 zfERα and zfERβ2, but not zfERβ1, induce the zfERα gene promoter. (A) CHO, (B) Hela and (C) HepG2 cells were cotransfected with different expression vectors (control (ctrl), zfERα, zfERβ1 or zfERβ2) and the PA-1·8 kb reporter gene. Cells were treated with 10 nM E2 (solid bars) or 0·1% ethanol as control (open bars). The histograms show the mean of fold induction versus ethanol (etoh) (±S.E.M.) for each reporter gene. Each experiment was repeated at least twice in triplicate.

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genes to *in vivo* E2 treatment and studied the potential effects of these zfERβ forms on zfERα gene regulation.

Nothern blot analysis of zebrafish liver revealed one single major band of 4·8 kb for zfERα, one band of 4 kb for zfERβ1 and three bands of 7 kb, 2·8 kb and 1·4 kb for zfERβ2. Since the entire coding region of zfERβ2 extends over 1·8 kb (Menuet *et al.* 2002), one can assume that the 1·4 kb transcript would generate a truncated zfERβ2 protein isoform. Similarly, small transcripts for ERβs were also found in the liver of other teleost fish (Xia *et al.* 2000, Socorro *et al.* 2000). It would therefore be interesting to investigate whether or not these transcripts could generate truncated protein and to seek their potential roles.

Interestingly, exposure of zebrafish to E2 showed a differential response of zfER genes. Our data confirmed that in the zebrafish, as in other oviparous species, the expression of the zfERα gene is robustly stimulated by E2 treatment *in vivo*. In contrast and surprisingly, the expression level of zfERβ1 was strongly reduced whereas the level of zfERβ2 mRNA was either not affected or very slightly affected. At the present time, the transcriptional and/or post-transcriptional effects of E2 treatment on these messengers remain to be determined. However, the present results may indicate that, during the reproductive cycle, when E2 levels fluctuate, the zfERβ forms could have different patterns of expression, suggesting a distinct implication on hepatic functions, notably on vitellogenesis.

To investigate the effect of zfERβs on this function, we analysed *in vitro* their potential actions on the regulation of the zfERα gene expression known to be essential for vitellogenin production. First, a zebrafish genomic DNA library was screened and a 7 kb DNA fragment was isolated. This fragment contained 1·8 kb of the promoter region and the first exons and introns of the zfERα gene. Interestingly, this genomic fragment showed a similar organization and structure to that of the corresponding fragment in the rainbow trout ERα gene. In fact, two classes of ERα mRNAs could be generated from the trout ERα gene by an alternative splicing and promoter usage (Leroux *et al.* 1993, Pakdel *et al.* 2000). These transcripts encode two functional ERα isoforms with different estrogen dependencies. The major hepatic isoform, rtERα short, was initiated from the ATG located in exon 2, while a minor isoform, rtERα long, was raised from an in-frame ATG initiator codon located in an intronic sequence of intron 1 (called exon 2a) which can be differentially spliced (Pakdel *et al.* 2000). As expected, our primer extension analysis showed that the major zfERα isoform corresponded with the classical short isoform issued from transcripts containing exon 1/exon 2. However, the presence of an additional in-frame ATG initiator codon in intron 1 (or exon 2a) located at the same position as in the rtERα gene suggested that a zfERα long isoform also exists in zebrafish.

From the genomic DNA, we have isolated 1·8 kb of the 5′ end of zfERα gene and linked it to the luciferase reporter gene. Cotransfection analysis of this promoter construct with the zfERα expression vector in different cell lines showed a clear induction of the reporter gene in the presence of E2. These results confirmed the E2 stimulation of the zebrafish ERα gene *in vivo* and demonstrated that E2 upregulation of the ERα gene by ERα itself is likely widespread among oviparous vertebrates (Pakdel *et al.* 1991, Ninomiya *et al.* 1992, Le Drean *et al.* 1995, Lee *et al.* 1995). Mutations and deletions of this promoter region showed clearly that the ½ERE (−127 bp) and the imperfect ERE (−104 bp) were largely involved in the E2-dependent induction of the zfERα gene. The comparison of the proximal promoter region of some fish estrogen-sensitive genes (ERα and brain aromatase) showed the conserved presence of ½ERE and ERE, separated by 21–23 bp (center-to-center). This number corresponds to two helix turns, indicating that these two elements are on the same side of the DNA (Fig. 5). This organization could stabilize receptor binding and enhance E2 induction. However, for the zfERα gene, we cannot exclude that the ½ERE located at −850 bp and −80 bp could also contribute to the E2 responsiveness. Moreover, computer analysis revealed that the ½EREs are included in an AP-1-like site. Numerous studies showed that ERs are able to modulate promoter activity by interacting with different DNA-bound transcription factors such as Jun and Fos which bind specifically to the AP-1 element (Paech *et al.* 1997, Webb *et al.* 1999). In this regard, more investigations will be necessary to determine if AP-1-like elements are also involved in the E2-dependent induction of the zfERα gene.

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To investigate the ERβ effects on the zfERα promoter, transfection experiments were carried out in several cell lines including CHO, Hela and HepG2. Curiously, the two ERβ forms were characterized by differential impacts on the E2-dependent induction of the zfERα construct. In fact, although clear inductions were obtained with zfERα, zfERβ2 seemed to be less effective (about 40–50% of zfERα activity). In contrast, whatever the cell line used, zfERβ1 showed very low, if any, transcriptional activity on the zfERα promoter. The molecular reasons for this low transcriptional activity are still unknown. However, these data contrast with previous results showing that zfERβ1 is able to induce reporter genes under the control of a consensus ERE in the same way as zfERα and zfERβ2 (Bardet *et al.* 2002, Menuet *et al.* 2002). Besides the fact that the ERE of the zfERα gene is imperfect with three mutations, it is possible that the inefficiency of zfERβ1 to induce zfERα promoter is due to its incapacity to recognize the zfERα ERE with high affinity. Gel mobility shift assays carried out with labeled oligonucleotides containing this imperfect ERE sequence did not allow us to obtain information about the *in vitro* DNA-binding capacity of these different zfERs. We were limited by the low sensitivity of this technique due to the high degree of mutation in this ERE sequence. Nevertheless, our assays performed in parallel with a consensus ERE showed low, but detectable, specific complexes, with higher intensity for zfERα than both zfERβs (data not shown). Similar to our finding, previous studies have reported that mammalian ERβs have lower transcriptional activity than ERα (Tremblay *et al.* 1997, Cowley & Parker 1999). At the present time, it is difficult to know which one of the zfERβs functions like the mammalian ERβ. This will necessitate a full comparison using a series of endogenous E2-sensitive genes. Moreover, these experiments may provide information about potential EREs that could favor ERβ transcriptional activity preferentially.

Although numerous studies have demonstrated that in oviparous species ERα gene regulation involves several nuclear factors including ERα itself, COUP-TFI (Lazennec *et al.* 1997, Petit *et al.* 1999, Métivier *et al.* 2002), C/EBPβ and the glucocorticoid receptor (Lethimonier *et al.* 2000, 2002), the present study has revealed that the zfERβ forms, and more probably zfERβ2, could also be largely involved. Interestingly, zfERβ2 has been characterized by a better affinity for E2 than the other forms (Menuet *et al.* 2002). Furthermore, the present data revealed that zfERβ2 expression is unmodified by E2 and that it is able to induce zfERα promoter activity significantly. Consequently, it is tempting to speculate that, *in vivo*, zfERβ2 could be involved in the maintenance of zfERα gene expression when the E2 levels are low during the reproductive cycle. Moreover, the fact that, in contrast with zfERα, zfERβ1 expression is downregulated by E2 treatment and unable to significantly induce zfERα promoter activity *in vitro*, suggests that this zfER form is likely not involved *in vivo* in zfERα gene regulation and in the vitellogenesis process.

In conclusion, this study has shown for the first time that the two ERβ forms that have emerged in teleost species are differentially regulated by E2 in the liver and characterized by distinct transcriptional activity on an endogenous gene such as zfERα. These data may also suggest that each ERβ form has different physiological functions that remain to be elucidated. Moreover, the isolation of the zfERα promoter and the demonstration of its sensitivity to estrogen provide new molecular tools in the zebrafish, a species commonly used to analyze the impact of endocrine disrupters in a whole vertebrate model organism.

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