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Multiple Structurally Distinct ER α mRNA Variants in Zebrafish are Differentially Expressed by Tissue Type, Stage of Development and Estrogen Exposure

Kellie A. Cotter^a, Anya Yershov^a, Apolonia Novillo^{a,b}, and Gloria V. Callard^{a,*}

^aBoston University Department of Biology, 5 Cummington Mall, Boston, MA 02215, USA

Abstract

It is well established that estrogen-like environmental chemicals interact with the ligand-binding site of estrogen receptors (ER) to disrupt transcriptional control of estrogen responsive targets. Here we investigate the possibility that estrogens also impact splicing decisions on estrogen responsive genes, such as that encoding ER α itself. Targeted PCR cloning was applied to identify six ER α mRNA variants in zebrafish. Sequencing revealed alternate use of transcription and translation start sites, multiple exon deletions, intron retention and alternate polyadenylation. As determined by quantitative (q)PCR, N-terminal mRNA variants predicting long (ER α_L) and short (ER α_S) isoforms were differentially expressed by tissue-type, sex, stage of development and estrogen exposure. Whereas ER α_L mRNA was diffusely distributed in liver, brain, heart, eye, and gonads, ER α_S mRNA was preferentially expressed in liver (female > male) and ovary. Neither ER α_L nor ER α_S transcripts varied significantly during development, but 17 β -estradiol selectively increased accumulation of ER α_S mRNA (~170-fold by 120 hpf), an effect mimicked by bisphenol-A and diethylstilbestrol. Significantly, a C-truncated variant (ER α_S -Cx) lacking most of the ligand binding and AF-2 domains was transcribed exclusively from the short isoform promoter and was similar to ER α_S in its tissue-, stage- and estrogen inducible expression. These results support the idea that promoter choice and alternative splicing of the *esr1* gene of zebrafish are part of the autoregulatory mechanism by which estrogen modulates subsequent ER α expression, and further suggest that environmental estrogens could exert some of their toxic effects by altering the relative abundance of structurally and functionally distinct ER α isoforms.

Keywords

estrogen receptor alpha; mRNA variants; zebrafish; alternative splicing; endocrine disruption

1. Introduction

Epidemiological, wildlife, and laboratory studies indicate that defects of development, reproduction, and physiology can be ascribed to hormonally active agents in the environment (Bergman, 2013; Diamanti-Kandarakis et al., 2009). Endocrine disrupting

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*Corresponding author: gvc@bu.edu (617-353-8980).

^bPresent Address: Departamento de Ciencias Biomedicas, Universidad Europea de Madrid, Calle Tajo, s/n, 28670 Villaviciosa de Odón, Spain

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chemicals (EDC) are rarely toxic, but, even at low doses and transient exposures, have the potential to induce lifelong and multigenerational defects by perturbing essential receptor-mediated hormone actions during critical periods of development. The extent of the problem and the ultimate risk for humans and animals requires a comprehensive understanding of the molecular mechanisms of EDC action. Among the identified and suspected EDC, a structurally diverse group (termed xenoestrogens, XE), are estrogen-like in their bioactivity by virtue of their ability to bind to estrogen receptors (ER). Canonical ERs are ligand-activated transcription factors that interact with DNA responsive elements in the promoters of target genes to activate or repress transcription (Heldring et al., 2007). Not surprisingly, our present understanding of the molecular mechanisms of XE action is centered on transcriptional activity, as measured by perturbations in the amount, timing, or location of estrogen regulated gene products. However, transcription is only one step in the actual production of functional proteins. Alternative promoter usage and splicing generate many structurally and functionally different mRNA variants and proteins from a single transcription unit, and are widely used to diversify and fine tune gene expression by tissue-/cell-type, stage of development, hormonal status and physiological condition (Hughes, 2006; Kalsotra and Cooper, 2011; Kelemen et al., 2013; Kornblihtt et al., 2013; Nilsen and Graveley, 2010). Moreover, promoter choice and splicing are mechanistically coupled to transcription and co-regulated by the same tissue-specific factors (including steroid receptors and co-activators) acting in cis and trans on target gene promoters (Braunschweig et al., 2013; Masuhiro et al., 2005; Meng et al., 2007; Montes et al., 2012; Moore and Proudfoot, 2009; Zhang et al., 2003). In theory, therefore, estrogen-like chemicals and any other EDC capable of activating ER to regulate transcription have the potential to control both promoter choice and alternative splicing of the targeted pre-mRNA, thereby determining not only the quantity but also the nature of the expressed gene product.

To address this hypothesis we chose to investigate alternative splicing of ER α in zebrafish. In contrast to mammalian species, which have two ERs (ER α , - β), zebrafish and other teleost fish have three ERs (ER α , - β a, - β b) (Bardet et al., 2002; Hawkins et al., 2000; Menuet et al., 2002). It is well established that the gene encoding ER α is estrogen responsive and a direct target of hormonal estrogen action (for review see, Nelson and Habibi, 2013). Like other members of the steroid receptor superfamily, ER α is a large modular protein with multiple conserved functional domains (A – F): the constitutively active, activation function 1 (AF-1, A/B) at the N-terminus; the DNA- (DBD, D) and ligand-binding (LBD, E) domains; and the ligand dependent AF-2 in the LBD domain (E/F) (Krust, 1986). In humans and mice, the eight coding exons of the ER α pre-mRNA are alternatively spliced to produce multiple mRNA variants and ER α isoforms, some of which lack one or more functional domains (Herynk and Fuqua, 2004; Ishii et al., 2011; Schreihöfer et al., 2002; Taylor et al., 2010). Additionally, the gene encoding ER α (*ESR1*) in mammalian species has four (mouse) or eight (human) alternative promoters/untranslated first exons, in addition to alternate 3'-untranslated regions (UTRs) and poly-adenylation sites (Ishii and Sakuma, 2011; Kobayashi et al., 2011; Kos et al., 2000; Kos et al., 2001). These are used in a tissue-specific manner where they affect mRNA expression, translation or stability (Kos et al., 2002).

Consistent with studies in rodents and humans, alternatively spliced ER α variants have been identified in several fish species and some are orthologous to mammalian forms: for example, blue tilapia (Tan et al., 1996); rainbow trout (Pakdel et al., 2000); channel catfish (Xia et al., 1999; Patino et al., 2000); mangrove rivulus (Seo et al., 2006); killifish (Greytak and Callard, 2007); sea bream (Pinto et al., 2012); for additional references, see Table 3. In the rainbow trout, alternative use of multiple transcription and translation start sites results in both an N-truncated, short ER α isoform lacking the A domain and a full-length, long isoform, which differ significantly in their tissue distribution and transactivating properties

(Menuet et al., 2001; Metivier et al., 2000; Pakdel et al., 2000; also see Discussion). Considering that ER α is at the apex of an entire estrogen response network, dysregulation of promoter choice or pre-mRNA processing of this gene by environmental chemicals is positioned to trigger system-wide effects.

The availability of a sequenced zebrafish genome makes it possible to apply a targeted PCR cloning approach to carry out a comprehensive survey of ER α splice variants in this species, and to determine expression patterns and effects of estrogen using quantitative (q)PCR. Here, we report the identification of six alternatively spliced ER α variants produced by the use of multiple transcription and translation start sites, exon deletions, and intronic sequence retention. Two N-terminal mRNA variants predicting a full-length long and an N-truncated short ER α isoform, as in rainbow trout, were differentially expressed by tissue-type, sex, stage of development, and estradiol or XE exposure. A C-terminal truncated variant was transcribed exclusively from the promoter/first exon that produces the short ER α mRNA, and was similarly regulated during development, in different tissues, and in response to estrogens. Our results support the view that hormonal estrogens regulate promoter choice and splicing on estrogen responsive genes, and that this mechanism of estrogen action can be appropriated by XE to exert at least some of their endocrine disrupting effects.

2. Materials and Methods

2.1 Zebrafish and Treatments

Wild type adult male and female zebrafish (*Danio rerio*) were obtained from Ekk Will Waterlife Resources (Gibson, FL) and maintained in 30 gallon aquaria at 28 °C on a 14:10 light-dark (LD) cycle, as previously described (Kishida and Callard, 2001). Fertilized eggs were collected after natural spawning, washed and distributed into 20 × 100 mm culture plates (Fisher Scientific) containing embryo medium (120 – 150 eggs per 50 ml medium; 0.006% Instant Ocean in deionized water, Aquarium Systems, Mentor, OH). Embryos were allowed to develop at 28 °C on a 14:10 LD cycle and collected at timed intervals post-fertilization, as specified in figure legends. Unfertilized eggs were obtained by stripping gravid females. Eggs/embryos/larvae were pooled (~25–30 per sample) by treatment group and immediately frozen on dry ice for storage at –70 °C.

For treatment experiments, embryos were exposed to 17 β -estradiol (E2; 0.1 μ M; Sigma, St Louis, MO), bisphenol A (BPA; 10 μ M; Aldrich, Milwaukee, WI), diethylstilbestrol (DES; 0.1 μ M; Sigma), or 2,3,7,8, tetrachlorodibenzo-p-dioxin (TCDD; 1 nM; Ultra Scientific, N. Kingstown, RI) beginning at 24 hpf and continuing for the period specified in figure legends. This protocol is based on earlier dose-response studies in our laboratory (Kishida et al., 2001; Kishida and Callard, 2001; Sawyer et al., 2006; Griffin et al., 2013). Chemicals were prepared as stock solutions in dimethyl sulfoxide (DMSO; final concentration, 0.0006% in embryo medium). Control embryos received DMSO alone. Medium and chemicals were replaced daily. Mortality, hatching, and morphological defects were routinely monitored.

Tissues were collected from reproductively active males and females from our breeding colony. Fish were anesthetized in 0.035% ethyl 3-amino-benzoate methanesulfonate salt (MS-222, Sigma) and then decapitated. Tissues were pooled by sex (2–4 fish per pool), quick-frozen on dry ice, and stored at –70 °C.

2.2 RNA Extraction and Reverse Transcription

Frozen adult tissues or pooled eggs/embryos/larvae were homogenized in Tri Reagent (Sigma) and total RNA was extracted according to the manufacturer's instructions. Extracts were treated with DNase I (Promega Corp., Madison, WI) to minimize gDNA

contamination. Concentration and quality of RNA was determined spectrophotometrically and confirmed by gel electrophoresis. Reverse transcription of 3 or 5 µg of total RNA (20 µl reaction volume) was performed using oligo(dT) priming and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

2.3 Oligonucleotides

Oligonucleotides used for PCR, 3'-RACE, Northern blot analysis and qPCR are listed in Table 1. The gene-specific primers used for cloning the ERα variant transcripts were designed using the online Primer III program. Primer design was aided initially by *in silico* analysis of the zebrafish *esr1* gene (Ensembl.org, #ENSDARG00000004111) and the previously cloned zebrafish (Genbank #: AF349412) and rainbow trout (Genbank #: AJ242741 and NM_001124349) ERα cDNAs and subsequently by our newly sequenced cDNAs. Primer sets for qPCR were tested for specificity using zebrafish liver cDNA as a template and standard PCR to verify production of a single band of the predicted size. Primers for β-actin (*bactin1*; the positive control for PCR) and elongation factor 1-α (*elfa*; the normalizer for qPCR) are also shown in Table 1 and were previously published (Chen et al., 2005; Keegan et al., 2002) and verified for use under the conditions in our laboratory (McCurley and Callard, 2008).

2.4 PCR and 3'RACE

Routinely, liver cDNA was used as the template for initial cloning of ERα mRNA variants. Synthesized cDNA was amplified using exon-specific primers (2 mM each) and GoTaq Flexi polymerase (Promega, Madison, WI) under the following conditions: 94 °C for 5 minutes; 40 cycles of 94 °C for 30 seconds, 58 °C for 45 seconds, and 72 °C for 1 min/kb; and 72 °C for 10 minutes. Where indicated, a nested primer strategy (reamplification of an aliquot of the initial PCR reaction with a second, internal gene specific primer) was applied to maximize the yield of low abundance transcripts and to increase the specificity of the amplification.

Additional variants of the ERα mRNA were identified using 3'RACE (System for Rapid Amplification of cDNA Ends; Invitrogen) with the kit-supplied adapter primer and a nested pair of gene specific forward primers located in exon 5. A "touch-down" strategy was used as follows: 94 °C for 3 minutes; 5 cycles of 94 °C for 30 seconds, and 72 °C for 3 minutes; 5 cycles of 94 °C for 30 seconds, and 70 °C for 3 minutes; 20 cycles of 94 °C for 30 seconds, 68 °C for 30 seconds, and 72 °C for 3 minutes; and 72 °C for 10 minutes. Where semi-quantitative RT-PCR was used to estimate selected transcripts, the PCR conditions were the same as in the targeted cloning approach.

2.5 Cloning and sequence analysis

The ERα cDNA products were size separated on a 2% agarose gel, and the bands extracted using the MinElute Gel Extraction Kit (Qiagen, Hilden, Germany; 10 µl final volume). An aliquot (3 µl) of purified product was ligated into pGEM®-T Easy vector (Promega) and cloned using α-Select Gold Efficiency competent cells (Bioline, Taunton, MA). Six colonies were cultured and plasmid DNA was isolated using a Wizard® SV Minipreps DNA Purification System (Promega). Each sample was sequenced (Macrogen; Seoul, South Korea) in two directions using plasmid specific primers. Sequences were analyzed using online software: VecScreen (<http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html>), CAP3 (<http://pbil.univ-lyon1.fr/cap3.php>), and BLAST (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>). Zebrafish *esr1* gene sequence information from Ensembl (ENSDARG00000004111; www.ensembl.org) was used for further analysis. Predicted amino acid sequences of the zebrafish ERα cDNAs cloned in this laboratory were aligned

with previously cloned ER sequences using Clustal W 2.1 and a Gonnet series scoring matrix as described previously (Greytak et al., 2005).

2.6 Northern blot analysis

The Totally RNA kit (Ambion, Inc. Austin, TX) was used to extract RNA from adult brain, ovary, and liver. Total RNA (25 µg) was size-separated on a 1% formaldehyde agarose gel and transferred to a nylon membrane (BrightStar-Plus; Ambion, Inc). RNA loading and integrity was monitored by ethidium bromide staining of ribosomal RNA. The hybridization probe used to detect the ER α mRNA was a riboprobe derived from the previously cloned ER α cDNA (Genbank #: AF349412) by PCR using primer set 11/12 (Table 1), and was designed to target sequence spanning exons 2b and 3, which is common to both long and short transcripts. The sub-cloned PCR product was linearized with ApaI, and transcribed and labeled with [32 P]UTP using the Strip-EZ RNA kit (Ambion, Inc). The membrane was hybridized overnight at 65 °C in ULTRAhyb (Ambion, Inc.) containing the riboprobe at a concentration of 3 X 10⁶ cpm/ml. The final stringent wash was 0.1 X SSC, 0.1% SDS before exposure to film (Biomax MR; Kodak, Rochester NY). Transcript sizes were compared to an RNA ladder (Life Technologies, Inc).

2.7 Quantitative (q)PCR Analysis

As previously described (McCurley and Callard, 2008), real-time qPCR analysis was performed using an ABI PRISM 7900 HT (Applied Biosystems, Foster City, CA) instrument with SYBR green fluorescent labeling. Amplifications were run in triplicate for each cDNA sample in optically clear 384-well plates. Cycling parameters were as follows: 50 °C for 2 minutes, 95 °C for 10 minutes; 40 cycles of 95 °C for 15 seconds, 60 °C for 1 minute. Data was exported to Q-gene (Simon, 2003), adjusted for amplification efficiency, and normalized to *elfa* as previously described (McCurley and Callard, 2008). Results were expressed as mean normalized expression (MNE $\pm$ SEM) of at least three independent biological replicates per experimental condition (see figure legends).

2.8 Statistics

Statistical analysis by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was performed using the Sigma-Stat 3.5 package (Aspire Software, Leesburg, VA). Student's standardized t-test was performed where specified using the online QuickCalcs calculator (GraphPad software, La Jolla, CA). See figure legends for details. Significance was set at $P = 0.05$.

3. Results

3.1 Identification of short (ER α_S) and long (ER α_L) N-terminal ER α variants

Previous research in the rainbow trout described two potential ER α isoforms, rtER α_L (long isoform) and an N-truncated rtER α_S (short isoform), which are generated by alternative use of transcription and translation start sites (Pakdel et al., 2000; see Introduction). In zebrafish, the previously cloned ER α cDNA corresponds to the transcript encoding the rainbow trout short isoform and is predicted to be lacking the A functional domain (Genbank: AB037185; Menuet et al., 2002). Our *in silico* analysis of the *esr1* gene sequence between exons 1 and 2 in the revised zebrafish genome database (Ensembl.org, #ENSDARG00000004111), in comparison with the rainbow trout long ER α sequence, suggested the possibility of two N-terminal variants in zebrafish, encoding both a full-length long isoform of ER α and the previously identified short isoform. To test this hypothesis, we first confirmed the size and sequence of the zebrafish short ER α cDNA (ER α_S) using end-to-end PCR amplification of liver cDNA with primer set #1/2 (see Table 1) positioned in untranslated regions of exons 1

and 9. To amplify the putative long ER α cDNA, we used the genomic DNA sequence in the zebrafish database to design a series of forward primers in the 5'-end of our presumed extension of exon 2, and paired each with the same exon 9 reverse primer (#2). As predicted, one of the forward primers (#3) successfully amplified a 2082 bp cDNA product, which differed from ER α_S at the N-terminus and, like rainbow trout, could result from alternate use and splicing of two putative transcription start sites and untranslated first exons (termed TSS I and II in exons 1 and 2, respectively; Figure 1, a/b). The newly identified long ER α cDNA (ER α_L) begins at TSSII in exon 2 and is lacking exon 1, which is 821 bp further upstream. By contrast, the previously identified ER α_S initiates at TSSI in exon 1, which is then spliced to an internal segment of exon 2 via a second consensus acceptor site. A translation start site (ATGII) in the 5' end of exon 2 (termed 2a) of ER α_L predicts a longer ER α protein that contains both the A and B domains (Figure 2). Alternatively, ER α_S , which splices out the ATGII in exon 2a, would be translated from ATGI in the 3' end of exon 2 (termed 2b), and thus ER α_S would be lacking 52 amino acids at the N-terminus compromising the A domain (Figure 2). The zebrafish ER α_S and ER α_L cDNAs described in this report have been entered into Genbank as accession numbers AF349412 and EU079275, respectively (Table 2).

As shown in Supplementary Figure 1, when the amino acid sequences encoded by the ER α_S and ER α_L zebrafish cDNAs cloned in the present study are aligned with the previously reported ER α peptides of rainbow trout and the full-length human ER α , there is a high degree of overall sequence conservation. Zebrafish ER α_L shared 62% and 48% overall amino acid identity with the rainbow trout ER α_L and the human ER α , respectively, and the ligand- and DNA-binding domains are even more highly conserved when compared to rainbow trout (respectively, 93% and 77% identity) and humans (respectively, 94% and 64% identity). While the extended A domains of the zebrafish and rainbow trout ER α_L are similar in length (44 vs 45 amino acids) they have only 18% overall sequence identity within this region.

To determine the occurrence of both ER α_L and ER α_S in fish species, we surveyed GenBank for all complete ER α mRNA sequences cloned from teleost fish, and classified them as either short or long. We also utilized *in silico* analysis of fish species with sequenced genomes available in Ensembl to identify additional putative short and long ER α variants. As shown in Table 3, 12 of the 50 species surveyed (including rainbow trout and zebrafish) have both ER α_L and ER α_S , whereas the remaining species have either ER α_L or ER α_S . The wide taxonomic distribution of those with both isoforms suggests that it is likely a conserved characteristic of all fishes.

To further identify conserved features of the extended N-terminus, all available A domain sequences were aligned. Although similar in length (41 to 53 amino acids), there is little apparent sequence conservation (Supplementary Figure 2). However, the majority of the A domains contain at least partial conservation of the LLxxL and ELE residues (Supplementary Figure 2, bold lettering), which were demonstrated to be important to the functional ability of the A domain to suppress the ligand-independent activity of AF-1 (Metivier et al., 2002). Additionally, using the MEME algorithm, which identifies common motifs in a set of protein sequences without direct alignment (Bailey et al., 2009), six overrepresented motifs were identified, dividing the A domain into four distinct regions. Motifs 2 and 4 are present in all 31 of the species, Motif 3B in 24, Motif 1A in 19, Motif 1B in 6, and Motif 3A in only 3 species. The significance of these motifs remains to be determined (aside from the residues mentioned above); however MEME has been used successfully to identify important functional and structural motifs (Bailey et al., 1997), and we assume that at least some of our conserved residues may prove to be significant regions of the A domain. Further phylogenetic analysis based on alignment of the A domains shows

that the sequences segregate according to the known taxonomic order, and again, that species which have both ER α_S and ER α_L are represented at all levels of phylogeny (Supplementary Figure 3).

3.2 Identification of a C-truncated variant (ER α_S -Cx)

To identify possible C-terminal ER α variants, 3'RACE was applied using nested forward primers (#10 and 11) positioned in exon 5. In addition to the expected product (~2300 bp), a single smaller (~1700 bp) product was identified. Sequencing showed that this variant was completely lacking exon 9 but retained 84 bp of intron 8 and a poly-A tail (Figure 1, f). Although the poly-A sequence could be an artifact of the oligo(dT) primer used to reverse transcribe the RNA, or the adapter primer used in the RACE reaction, its presence is consistent with a poly-adenylation signal hexamer (Beaudoing et al., 2000) located 22 nt upstream. This C-truncated ER α cDNA (termed ER α -Cx to correspond to terminology in killifish and human) was entered into GenBank as accession #EU079276. *In silico* translation of the ER α -Cx cDNA revealed an in-frame stop codon within the intronic sequence, thus predicting a C-truncated ER α protein with a new 20 amino acid region instead of the 64 amino acids normally found at the C-terminus (Figure 2; Supplementary Figure 1; Table 2). If translated, the resultant protein would be missing helices 11 and 12 of the LBD and AF-2 regions, and the entire F domain, which has been shown to play a role in the differential response to agonists and antagonists (Skafar and Zhao, 2008).

To determine which transcription start site/untranslated first exon is associated with the C-truncated transcript, PCR amplification was undertaken using a reverse primer in the retained intronic sequence (#5) paired with either a ER α_S -specific primer in exon 1 (#1) or a ER α_L -specific primer in exon 2a (#3). As control, a forward primer positioned in exon 2b (#4), which would amplify both N-terminal variants, was paired with primer #5. As shown in Supplementary Figure 4A and B, a product band of the correct size was generated exclusively from the primer in exon 1, indicating it is a product of TSSI and a variant of the short transcript (Figure 1, a). Therefore, the predicted ER α_S -Cx protein is missing the A domain as well as part of the E and the entire F domains (Figure 2; Table 2; Supplementary Figure 1). The absence of a product band with the exon 2a primer specific for the long transcript (Supplementary Figure 4B) reinforces the view that ER α_S -Cx is transcribed from TSSI and that retention of the intron 8 sequence is not an artifact of genomic contamination.

3.3 Identification of ER α variants with deleted internal exons

Targeted PCR cloning was applied to identify possible alternative splicing events affecting internal exons. First, the N-terminus was examined using nested reverse primers in exon 5 (#7 and 8) paired with a forward primer either in exon 1 (#1; ER α_S -specific) or in exon 2a (#3, ER α_L -specific). This strategy identified two additional variants, each with substantial deletions in the mRNA sequence. The first variant, was amplified using the ER α_S -specific primer (#1) and was missing exons 2b and 3 in their entirety (termed ER $\alpha_S\Delta$ 2b-3; Genbank: HM641841), (Figure 1, c). The initial in-frame start codon of this transcript is positioned at the beginning of exon 5; therefore, the predicted protein translated by this sequence would be lacking the A, B, and C regions (AF-1, DBD/dimerization domains) (Figure 2; Table 2; Supplementary Figure 1). A second variant, ER $\alpha_L\Delta$ 2b-5 (GenBank: JX457331) was amplified with the ER α_L -specific primer (#3) and was missing exons 2b, 3, 4, and most of 5 (Figure 1, d). Although this variant retains the original start codon in exon 2a, *in silico* translation indicates a frame-shift that would result in a premature stop codon shortly downstream, predicting that a protein translated from this transcript would be only 18 amino acids in length (Table 2).

To target exonic deletions in the LBD and AF-2 coding regions, forward nested primers in exon 5 (#10 and 11) were paired with a reverse primer in the untranslated region of exon 9 (#2). Only one variant was amplified, which was missing a region from the beginning of exon 7 through exon 8 and most of the coding region of exon 9 (termed ER α Δ 7-9; Genbank: HM641842) (Figure 1, e). The putative protein translated from this variant would be lacking most of the LBD and AF-2 regions (Figure 2; Table 2; Supplementary Figure 1).

3.4 Northern blot analysis

To determine the size, number and relative abundance of ER α mRNAs in different zebrafish tissues, a 384-bp riboprobe was generated from a region of the cloned cDNA that is common to both ER α_S and ER α_L (exons 2b/3) and used for northern blot analysis of brain, ovarian and liver RNA. As shown in Figure 3, a total of seven mRNAs hybridized to the ER α probe under high stringency conditions. Three predominant mRNAs in ovary were approximately 4.1, 3.8 and 2.4 kb in length, but only the 3.8 and 2.4 kb mRNAs were represented in liver, and only the 2.4 kb mRNA in brain. Additional minor mRNA species were seen in liver (1.2 and 1.0 kb) and ovary (8.5 and 7.0 kb). These results indicate that multiple ER α mRNAs are produced in a tissue-specific manner in zebrafish, possibly corresponding with some of our identified ER α mRNA variants.

3.5 Differential expression of short, long and total ER α mRNAs in adult tissues

Alternative promoter use and splicing of many different genes is known to be tissue specific (see Introduction/Discussion). We applied real time quantitative qPCR analysis to determine the expressed levels of ER α_S and ER α_L in male and female liver, ovary, testis, brain, eye, and heart. Forward primers (#Q1 and Q3) targeted the 5' exons that are exclusive to either ER α_S or ER α_L (exons 1 or 2a, respectively), while the reverse primer (#Q2) targeted exon 2b, which is common to both forms (Table 1; Figure 1). As control, a primer pair in exon 5 (#Q5 and Q6) was used to measure both mRNAs simultaneously and all other ER α splice variants containing exon 5 (termed "total" ER α mRNA).

Mean normalized expression of ER α_S was ~500-fold higher in female liver than in brain, eye and heart and ~50-fold higher than in male liver, ovary and testis (Figure 4A). By contrast, expressed levels of ER α_L were more diffuse across tissue types and, although levels in female liver were about 5-fold higher than in male liver, the difference was not significant (Figure 4B). Comparison between panels A and B reveals that tissue specific expression of ER α_L is generally higher than ER α_S , with the exception of female liver in which both isoforms have similar levels of expression. As with ER α_L , total ER α mRNA (Figure 4C) had a diffuse tissue distribution, but levels in female liver were significantly higher than those in all other tissue types, including male liver. This difference is due in part to exceptionally high levels of ER α_S in female liver, whereas the ER α_L mRNA predominated in the majority of tissues. These results indicate that choice of transcription start site/first exon usage on the zebrafish *esr1* gene is regulated by tissue specific factors.

3.6 Differential expression of short, long and total ER α mRNAs during development and in response to estrogen

It is well established that alternative promoter use is developmentally programmed and estrogen regulated (see Introduction). To determine patterns of expression during zebrafish development and the effects of estrogen, embryos were analyzed by qPCR at intervals between 24 and 120 hpf, in the presence of E2 (0.1 μ M) or vehicle (DMSO) alone, using the same qPCR primers described in section 3.5. Neither ER α_S (Figure 5A) nor ER α_L (Figure 5B) varied significantly by stage in the absence of added E2, but total ER α mRNA (Figure 5C) increased progressively during development, such that levels by 120 hpf were ~5-fold higher than those at 24 hpf. Additionally, when compared to ER α_L , expression of ER α_S was

very low in DMSO controls. As shown in Figure 5A, however, E2 exposure dramatically upregulated ER α_S , such that expression at 120 hpf was 170-fold higher ($P < 0.01$) than in DMSO controls. Interestingly, the estrogen effect was minimal before 72 hpf, but cumulative thereafter, perhaps reflecting maturation of the estrogen response system or morphogenesis of estrogen responsive organs such as the liver (Tao & Peng, 2009). By contrast, ER α_L was unaffected by estrogen exposure (Figure 5B). Thus, the E2 induced increase in total ER α mRNA, to levels 8-fold higher than DMSO treated controls at 120 hpf (Figure 5C, $P < 0.001$), is due entirely to the increase in ER α_S . These results indicate that ER α_S , when compared to ER α_L , is expressed at relatively low levels throughout development but is selectively upregulated by E2.

To determine whether environmental and synthetic estrogens mimic effects of hormonal estrogen in zebrafish embryos, we used the same treatment paradigm (24 to 96 hpf) to test bisphenol-A (BPA, 10 μ M) and diethylstilbestrol (DES, 0.1 μ M). As shown by qPCR analysis with the primers described in section 3.5 (Figure 6), BPA and DES mimicked E2 by selectively inducing ER α_S but not ER α_L , thereby increasing total ER α mRNA. Whereas the effects of BPA, a relatively weak estrogen, were modest (2- and 8-fold upregulation of total ER α and ER α_S , respectively), the effects of DES, a potent estrogen, were robust (12-fold and 150-fold upregulation of total ER α and ER α_S). By contrast, embryos/larvae treated with 2,3,7,8, tetrachlorodibenzo-p-dioxin (TCDD; 1 nM) showed no significant changes in expression of ER α_L , ER α_S , or total ER α mRNAs (data not shown). Taken together, these results suggest that TSSII is the default promoter during development resulting in the preferential production of ER α_L , and further indicate that estrogens redirect and upregulate transcription selectively on the TSSI promoter thereby selectively increasing expression of ER α_S .

3.7 Regulated expression of the truncated ER α_S -Cx mRNA and other ER α variants

Due to the small size of the retained intron 8 segment of the C-truncated variant and the difficulty of designing primers for deletion variants, we were unable to design suitable specific qPCR primers for the other ER α variants. Therefore, to estimate expressed levels of the additional variant ER α transcripts by tissue type, stage of development and estrogen exposure we compared patterns of expression on agarose gels after semiquantitative PCR with either variant specific primers (ER α_S -Cx, #4/5), or primers that measured both full-length transcripts and exon deletion variants, which were then identifiable by transcript size: ER α_S /ER $\alpha_S\Delta 2b-3$ (#1/6); ER α_L /ER $\alpha_L\Delta 2b-5$ (#3/8); total ER α /ER $\alpha\Delta 7-9$ (#9/10) (Table 1). Although differences in product size and amplification efficiencies preclude direct quantitative comparison between different amplicons, results provide an estimate of tissue distribution, developmental programming and E2-inducible changes in a given transcript. As shown in Figure 7A, semi-quantitative PCR confirmed results of qPCR analysis by showing that all transcripts are most highly expressed in liver, in particular ER α_S , ER $\alpha_S\Delta 2b-3$, and ER α_S -Cx. Co-expression of these three transcripts is unsurprising given their common use of the TSSI promoter. Additionally, the three internal exon deletion variants ($\Delta 2b-3$, $\Delta 2b-5$, and $\Delta 7-9$) are expressed at appreciably lower levels than the other transcripts.

Interestingly, the three internal deletion variants were below detectable limits of the assay during embryonic development or estrogen treatment (data not shown). Nonetheless, Figure 7B shows that ER α_L , ER α_S and ER α_S -Cx are present at high levels in unfertilized eggs but then decrease between 2 and 12 hpf. This pattern indicates maternal transfer after synthesis in mature oocytes or surrounding follicle cells, followed by degradation post-fertilization. The marked increases in accumulated levels of the ER α_L , ER α_S and ER α_S -Cx between 12 and 24 hpf are interpreted as due to the onset of embryonic transcription of the *esr1* gene and indicate that both TSSI and TSSII promoters are activated at this time. Comparison of

Figure 7B (PCR) with Figure 5 (qPCR) confirms that accumulated levels of ER α mRNAs and the use of the two transcription start sites are relatively constant between 24 and 96 hpf. Figure 7C shows that exposure to E2 dramatically upregulates the ER α_S , ER α_S -Cx, and total ER α mRNAs but has no effect on ER α_L , reinforcing the view that estrogen specifically targets transcription from the TSSI promoter, and that promoter choice impacts downstream splicing decisions. Lastly, as demonstrated in Supplementary Figure 4C, these effects of E2 are mimicked by estrogen-like chemicals, as both DES and BPA also upregulate expression of ER α_S -Cx.

4. Discussion

It is well established that estrogen signaling in mammalian species is accomplished by two genetically distinct ERs (α and β), but the functional significance of multiple alternatively spliced isoforms of each ER subtype is not entirely clear. The presence of one or more splice variants of ERs in frogs (Claret et al., 1994), birds (Griffin et al., 1999), and teleost fish (see Introduction), suggests that alternative processing of ER pre-mRNAs is phylogenetically conserved regulatory mechanism with adaptive value. In this study, we applied RACE and a targeted PCR cloning approach to characterize the ER α splice variants in zebrafish. We report three types of alternative splicing: (1) alternative promoter/first exon usage, (2) intron retention with alternate poly-adenylation, and (3) one or more exon deletions. Six alternatively spliced transcripts of ER α in zebrafish result from one or a combination of these processes, including a newly identified full-length (long) cDNA (ER α_L), confirmation of the previously identified N-truncated (short) cDNA (ER α_S), a C-truncated variant originating exclusively from the short isoform promoter (ER α_S -Cx), and variants with exon deletions (ER $\alpha_S\Delta 2b-3$; ER $\alpha_L\Delta 2b-5$; ER $\alpha\Delta 7-9$). As determined by qualitative and quantitative PCR analysis, ER α_L , ER α_S , and ER α_S -Cx are differentially expressed by tissue type, sex and stage of development, implying cell-specific regulation. Only the variants originating from the ER α_S promoter (ER α_S , ER α_S -Cx) are estrogen-inducible and therefore part of the well-established estrogen-driven autoregulatory loop in teleosts. Comparison of the identified zebrafish variants with ER α sequences from a taxonomically diverse array of teleosts identifies a subset of overrepresented motifs of potential functional significance within the A-domain of the extended ER α_L form and, more importantly, predicts that processes and factors generating the ER α_L and ER α_S isoforms are a general characteristic of fishes.

4.1 Alternative promoter/first exon usage

All fish ER α s initially cloned, including the zebrafish ER α , were lacking the A domain when aligned with mammalian orthologs (Menuet et al., 2002; Muñoz-Cueto et al., 1999; Pakdel et al., 1989; Tan et al., 1996; Todo et al., 1996; Touhata et al., 1999; Xia et al., 1999); however, subsequent studies in rainbow trout (Pakdel et al., 2000) and channel catfish (Patino et al., 2000) identified a second N-terminal extended ER α transcript that encoded a sequence similar in size to the mammalian A domain. Further analysis by S1 nuclease, primer extension, and RT-PCR experiments in rainbow trout determined that long and short isoforms, (termed ER α_S and ER α_L , respectively) originate from two transcriptional start sites, the second of which was spliced out in ER α_L , and resulted in translation from two different start codons. When expressed in yeast, both isoforms had similar ligand binding affinity and specificity but differed in their ability to activate a reporter gene construct. Whereas ER α_L was strictly ligand-dependent, ER α_S had some constitutive (ligand-independent) activity. It was surmised, therefore, that the rainbow trout A domain functions to suppress the ligand-independent activity of the AF-1 region in the B domain (Pakdel et al., 2000), and further experiments identified an 11 amino acid sequence in the A domain that is critical for this function (Metivier et al., 2000; Metivier et al., 2002).

Informed by the rainbow trout study and additional sequence in the zebrafish genome database, we successfully amplified two ER α transcripts, one corresponding to the previously reported N-truncated form and a new transcript encoding a zebrafish long isoform (ER α_L). As this transcript includes all domains previously identified in mammals, and is the ortholog of the rainbow trout long form (Menuet et al., 2002), we have designated ER α_L the full-length zebrafish ER α transcript. Comparison of the cloned mRNAs with zebrafish genomic sequence suggests that the N-terminal variants result from multiple transcription start sites (TSSI and TSSII) and ATG codons (I and II), as in rainbow trout. The predicted ER α_L of zebrafish has 44 additional amino acids at the N-terminus, which is comparable in size to the A domain of the rainbow trout (45 aa) and channel catfish (37 aa) long isoforms (Pakdel et al., 2000; Patino et al., 2000) and is within the size range of the A domains of all publically available teleostean long ER α isoforms (36 to 48 aa; Table 3). Despite little overall sequence identity, the distribution of the A domains on a phylogenetic tree reflects established taxonomic relationships, suggesting that residue substitutions are due mainly to species divergence (Supplementary Figure 3). Nonetheless, as shown in Supplementary Figure 2, computational analysis using an uninformed discovery algorithm (MEME) identifies motifs of yet to be determined significance within the teleostean A domains, and also recognizes some of the eleven amino acids previously found to suppress AF-1 of the B domain in rainbow trout (Metivier et al, 2000; Metivier et al 2002).

A survey of GenBank and Ensembl shows that ER α mRNA sequences are available for 50 fish species, of which 12 species have both long and short isoforms, the remainder having one or the other (see Table 3). Those species in which both isoforms have been identified are distributed across taxa rather than segregating in any one fish group (Supplementary Figure 3), suggesting that a purposeful search for the corresponding full-length (long) or N-truncated (short) ER α isoforms, where they are presently missing, will show that production of two N-terminal ER α variants is a general characteristic of fishes.

Human ER α mRNAs are transcribed from at least seven different promoters, some with multiple untranslated exons, and all splicing to the same position within exon 1 (Kos et al., 2001). Additionally, several N-truncated isoforms have been identified due to skipping of exon 1, including one missing the A and B domains. For example, the ER α_{46} isoform in humans is lacking the first coding exon (exon 2 in zebrafish), and as a result is missing the first 173 amino acids compromising the A and B protein domains (Flouriot et al., 2000). Studies on this variant have indicated that the lack of an AF-1 domain results in a receptor that acts as a dominant negative in the presence of full-length ER α . In humans, ER α promoter choice differs by tissue type (Brand et al., 2002; Flouriot et al., 1998; Kobayashi et al., 2011), and in normal and cancerous cells *in vivo* and *in vitro* (Amaral et al., 2009; Grandien et al., 1995; Hayashi, et al., 1997; Sogon et al., 2007). Thus, use of alternative promoters to produce ER α isoforms appears to be a widely used and flexibly implemented mechanism from fish to humans.

4.2 Intron retention

Through 3' RACE, we identified a C-truncated ER α variant with 84 bp of intronic sequence retained after exon 8 and a poly-A tail directly after the insertion, and further, showed that this intron retention alternative splicing event is specific to transcripts that originate from the promoter for exon 1 (ER α_S -Cx). This is consistent with previous studies reporting that transcription initiation, splicing, and poly-adenylation are interrelated processes that collaborate in gene regulation, and that promoter choice can control far downstream splicing events (Auboeuf et al., 2002; Montes et al., 2012; Moore and Proudfoot, 2009). *In silico* translation of ER α_S -Cx indicates that the retained intron contains an in-frame stop codon, and the putative protein would be lacking some of the LBD and AF-2 regions, implying a

deficiency in ligand binding and estrogen mediated transactivational activity in its own right. However, this protein would also lack the AF-1 suppression function of the A domain (due to transcription from exon 1) and thus could have ligand-independent activity. A comparable insertion (perhaps intronic) after exon 8 was seen previously in channel catfish (Patino et al., 2000) and it also contained a stop codon after 10 amino acids of novel protein sequence. No naturally occurring variant missing just the last coding exon (exon 9) has been reported in a mammalian species; however, artificial constructs of human ER α in which the F domain has been deleted or mutated demonstrate altered transcriptional activity in response to ER agonists and antagonists often in a cell-specific manner (Skafar and Zhao, 2008). Additionally, zebrafish ER α _S-Cx may be functionally similar to more severe C-terminal deletions of the human ER α (see discussion of ER α Δ 7-9 below).

4.3 Exon deletion

Three multi-exon deletion variants of ER α were cloned. An exon 2b-3 deletion variant was amplified with ER α _S specific primers (ER α _S Δ 2b-3), and is similar to two variants missing exon 2 (exon 3 in zebrafish) recently identified in sea bream (Pinto, 2012). The closest in frame start codon after the deletion is positioned at the beginning of exon 5, which predicts a protein lacking not only the A domain but also the first 214 amino acids of the AF-1 and DBD (B and C) domains, while retaining the full LBD/AF-2 region. Similar exon 3 deletion ER α variants in mammals (exon 4 in zebrafish) affecting the DBD, were unable to bind DNA, but did bind estrogen, and were found to inhibit estrogen-dependent reporter gene activation when co-transfected with wild-type ER α , suggesting that they function as a “buffer” that reduces effective concentrations of E2 (Bollig and Miksicek, 2000; Pasqualini et al., 2001; Varayoud et al., 2005).

A second deletion variant lacking exons 2b, 3, 4 and part of 5 (ER α _L Δ 2b-5) was amplified from the N-terminus with ER α _L specific primers. Although translation of this variant would start at ATG I in exon 2a, the deletion results in a frame shift and a premature stop codon after only 18 amino acids. We assume that this very small protein would be non-functional and degraded.

The third exon deletion variant identified in this study (ER α Δ 7-9) was obtained from the C-terminus using nested targeted primers; thus the transcription start site is unknown. Lacking exons 7, 8, and 9, it is missing significant portions of the E and F domains and in that respect resembles the zebrafish ER α _S-Cx variant described above. However, it does retain both the DBD and part of the dimerization region, suggesting that this protein, if translated, could dimerize with full-length ER α proteins. Similar N-terminal deletion variants missing either exons 6–8 or 8–9 have been identified in killifish (Greytak and Callard, 2007; Cotter, et al., 2011). A comparable ER Δ E7 variant in humans (exon 8 in zebrafish) has been shown to have weak DNA binding ability, a complete failure to bind ligand, and acts as a dominant negative receptor (Bollig and Miksicek, 2000; Garcia Pedrero et al., 2003).

4.4 Differential expression of ER α _L, ER α _S, ER α _S-Cx, and other ER α variant mRNAs

To obtain insight into factors regulating promoter choice and splicing decisions, and the possible functional significance of the different ER α isoforms, we first designed isoform-specific primers to measure ER α _S and ER α _L mRNA levels by qPCR in different tissues, developmental stages and after hormone or XE exposure. As a control, we used primers targeting sequences common to both ER α _L and ER α _S, designated “total” ER α . Both ER α _L and ER α _S are detectable in all tissue types, but the distribution patterns and relative abundance of each isoform differ. Whereas ER α _L mRNA is diffusely distributed in brain, eye, heart, liver and gonads, ER α _S is expressed at much higher levels in female liver than in all other tissue types and is relatively high in male liver and ovary. The same pattern is seen

with ER α_S -Cx, and ER $\alpha_S\Delta 2b-3$, variants transcribed from the short isoform promoter: namely, expression predominates in liver and ovary. In the earlier trout study, both ER α_L and ER α_S were isolated from liver but subsequent studies using northern blot analysis, *in situ* hybridization, and qualitative PCR failed to detect ER α_S in tissues other than liver (Menuet et al., 2001). The discrepancy with our findings is likely due to the much higher sensitivity and specificity of qPCR. Although other studies in fish cloned full-length mRNAs corresponding to either the long or the short ER α isoforms (see Table 3 for references), expression analyses utilized primers located downstream in the coding region, and thus would recognize both isoforms, similar to our “total” primers. One exception is in yellow perch, where primers were specific to the cloned short ER α isoform and, consistent with our results, expression was found to be greatest in the female liver and ovary (Lynn et al., 2008).

Similar to tissue distributions, here we show that ER α_L is greater than ER α_S mRNA at all time points between 24 and 120 hpf of development. Following treatment with E2, however, ER α_S increases progressively to levels 170-fold higher than controls, while ER α_L remains unchanged. Effects of E2 treatment are similar, in direction and amount, when PCR products of ER α_S -Cx, the other variant transcribed from the short promoter, are compared to amplicons of ER α_S and ER α_L on agarose gels, supporting the conclusion that estrogens specifically and robustly activate transcription from the short but not the long form promoter. Moreover, we show that BPA and DES mimic the effects of hormonal estrogen by selective induction of the ER α_S and ER α_S -Cx transcripts, which provides strong support for the idea that any chemical that interacts with nuclear receptors on the promoter of a transcription target gene also has the potential to alter promoter choice and splicing decisions (see Introduction).

In the earlier trout study, the authors reported that both ER α_L and ER α_S are estrogen inducible, but this conclusion was inferred from an S1 nuclease mapping assay that detected both isoforms in the liver of estradiol pre-treated but not in control fish (Pakdel et al., 2000). Whether there are true differences in the estrogen inducibility of short and long ER α isoforms when trout and zebrafish are compared, or whether discrepant results are due to the method of analysis remains to be determined. It is relevant, however, that the sequence immediately upstream of exon 1 in zebrafish contains an imperfect ERE and an ERE/AP-1 half site, both of which are important for estrogen-dependent induction of ER α in transfection experiments using luciferase reporters (Menuet et al., 2004). By contrast, inspection of the zebrafish genomic sequence (Ensembl) upstream of exon 2a reveals no consensus ERE sites. Taken together, our findings are consistent with two main conclusions: (1) the zebrafish *esr1* gene has two different promoters, both of which are constitutively active but differ in their level of activity during development in embryos/larvae and in most tissue types in adult fish (ER α_L > ER α_S); and (2) the promoter transcribing the short isoform is selectively activated in the liver of adults (female > male), perhaps reflecting sex differences in circulating estrogen, and in developing zebrafish in response to hormonal estrogen and xenoestrogens.

Although further studies are required to determine whether the identified variants are translated and, if so, how they differ functionally in relation to each other and to ER β and β *in vitro* and *in vivo*, a plausible hypothesis is that promoter switching is a mechanism that matches ER α isoform production to low versus high estrogen contexts, thereby optimizing response characteristics with the physiological status of the organism. Thus, when endogenous estrogen levels are low (during early embryogenesis or in reproductively inactive fish) expression of the short isoform would be limited and estrogen signaling mediated by the strictly ligand-dependent long isoform. On the other hand, when endogenous estrogen levels increase (during reproductive recrudescence in adults, or

gonadal sex differentiation in larvae), the estrogen-inducible short isoform promoter would be activated, dramatically increasing expression of the ER α_S selectively. ER α_S not only has a degree of ligand-independent activity but also has higher transactivating activity than ER α_L at a given ligand concentration, at least in rainbow trout (Pakdel et al., 2000). Together with the autoregulatory loop by which estrogens drive ever-increasing levels of ER α_S transcription, estrogen responsiveness would be further amplified by the intrinsic physicochemical properties of the ER α_S , as compared to the ER α_L . The proposed scenario would be especially advantageous in liver where rapid increases and sustained production of yolk protein normally occurs seasonally, at least in females. If so, it is worth considering whether the ER α_S -Cx variant, which increases concomitantly with ER α_S in response to estrogen, and is expressed at comparable levels to other ER α variants, could serve as a negative regulator of this positive feedback loop, by virtue of its potential as a dominant negative (see section 4.3 above).

N-terminal variants of the glucocorticoid receptor α contribute to the resistance to glucocorticoids (Gross et al., 2011). Interestingly, a subset of ER α variants have been implicated in the acquisition of estrogen resistance states in human breast cancer cells *in vivo* and *in vitro* (Barone et al., 2010; Herynk and Fuqua, 2004; Rao et al., 2011). In the cloning of the killifish ERs, identification of multiple ER α variants has led to the hypothesis that alternative splicing plays a role in acquired resistance to an estrogenic environment (Cotter et al., 2011; Greytak and Callard, 2007; Greytak et al., 2010). In addition, post-translational modifications at the protein-level have been implicated in resistance to high levels of 17- α -ethinylestradiol in killifish (Doyle et al., 2013).

Although the present study focused exclusively on ER α variants, numerous ER β variants have been reported in humans (Herynk and Fuqua, 2004) and have also been described in fishes: goldfish (Tchoudakova et al., 1998); killifish (Greytak et al., 2007); common sole (Caviola et al., 2007); sea bream (Pinto et al., 2006); and sea bass (unpublished, Genbank #AJ489525). Thus, to fully understand ER biology in fishes it will be necessary to document alternative promoter use and splicing of all three *esr* genes. Zebrafish have many technical advantages, in particular the use of morpholinos to target specific translation or splice sites (Griffin et al., 2013), for addressing the actions and interactions of ER alpha and the two betas and their splice variants in modulating downstream estrogen responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AF	activation function
BPA	bisphenol A
DBD	DNA binding domain
DES	diethylstilbestrol

DMSO	dimethyl sulfoxide
E2	17 β -estradiol
EDC	endocrine disrupting chemicals
ER	estrogen receptor
hpf	hours post fertilization
LBD	ligand binding domain
qPCR	quantitative polymerase chain reaction
RACE	rapid amplification of cDNA ends
TCDD	tetrachlorodibenzo-p-dioxin
TSS	transcription start site
XE	xenoestrogens

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HIGHLIGHTS

- A targeted PCR cloning approach identified six alternatively spliced ER α mRNAs in zebrafish
- Two N-terminal mRNA variants were differentially expressed by tissue-type, sex, stage of development and estrogen exposure
- The “short” N-terminal variant was preferentially expressed in response to estrogen
- A C-terminal truncated variant was expressed in a similar pattern to the short variant

Figure 1. Alternative promoter/first exon use and splicing (a–f, circled) of the zebrafish *esr1* gene, as determined by sequence analysis of cloned ERα mRNAs

See text and Table 2 for GenBank Accession numbers. Two transcription start sites (TSI and TSII) and alternate first exons (1 and 2a) were identified. A transcript beginning at TSI in exon 1 was spliced to an acceptor site in exon 2 (a), resulting in a short transcript (3437 nt) lacking exon 2a and predicting translation from ATGI in exon 2b. Transcription from TSII in exon 2a (b) resulted in a long transcript (3498 nt) lacking exon 1 and predicting translation from ATGII in exon 2a. Additional alternative splicing events (c – e) resulted in mRNAs with the deletion of two or more coding exons or retention of 84 bp of intronic sequence (hatched bar) with a premature termination codon and a poly-A tail (f). Splicing events c and f were associated exclusively with the short transcript (TSI/exon 1) whereas splicing event d was associated exclusively with the long transcript (TSII/exon 2b). Splicing event e was identified through targeted PCR cloning at the 3'-end, but its 5'-end was not determined. Primer placements are indicated with forward and reverse arrows and correspond to the numbering system used in Table 1. The six functional domains (A–F) of the mammalian ERα protein as defined by (Krust, 1986) are shown below the corresponding exons. Although the *esr1* gene in all vertebrates has 8 coding exons, the first coding exon is termed exon 2 in zebrafish and rainbow trout (Menuet et al., 2004; Pakdel et al., 2000) but exon 1 in mouse and human (Ponglikitmongkol, 1988; White et al., 1987).

Figure 2. Multiple zebrafish ERα isoforms, as predicted by translation of the identified mRNA variants

Lower case letters in parenthesis (a–f) are keyed to the splicing events shown in Figure 1. The long transcript (ERα_L, b) was produced from TSII in exon 2a, and translation was predicted from ATGII resulting in a protein of 621 aa containing all six functional domains. The short transcript (ERα_S, a) was produced from TSI in exon 1 which was spliced to exon 2b, and was predicted to use ATGI resulting in a protein of 569 aa lacking the A domain. An mRNA variant produced exclusively from TSI/exon 1 (ERα_S-Cx, a/f) lacked all of exon 9 but retained a portion of intron 8 that encoded an additional 20 aa before a stop codon (unshaded region). A deletion variant produced exclusively from TSI/exon1 (ERα_SΔ2b-3, a/c) lacked coding exons 2b and 3 and was predicted to utilize a downstream ATG in exon 5. This variant was identified by targeted PCR cloning at the 5'-end, and its C-terminal sequence was not identified. A final deletion variant identified by targeted PCR cloning at the 3'-end lacked all of exon 8 and most of exons 7 and 9 (ERαΔ7-9, e) but its transcription start site/first exon were not identified. The key to the functional domains of the ERα protein as defined by (Krust, 1986) in human and by (Pakdel et al., 2000) in rainbow trout is shown at the bottom of the figure (see text). Characteristics of the variant mRNAs and predicted proteins are summarized in Table 2.

Figure 3. Number and size of ER α mRNAs in zebrafish, as determined by Northern blot analysis Total RNA (25 μ g/lane) from zebrafish female brain (B), liver (L) and ovary (O) was size separated and hybridized with a [32 P]-labeled ER α riboprobe. Band sizes and positions relative to an RNA ladder are shown to the right of the panel. Ethidium bromide staining of ribosomal RNA verified RNA loading volume and integrity.

Figure 4. Tissue-specific expression of (A) ERα_S, (B) ERα_L and (C) total ERα transcripts in zebrafish, as determined by qPCR analysis

Tissues from male and female zebrafish were pooled by sex and tissue type (2–4 fish per pool) and analyzed using primers specific for ERα_S (panel A) or ERα_L (panel B), and as control a third set of primers targeting sequence in exon 5 common to both ERα_S and ERα_L (and any additional variants containing exon 5; total, panel C) (Table 1). Results are expressed as mean normalized expression (MNE) $\pm$ SEM of 2 (male liver, female liver, testes, ovaries) or 4 (brain, eye, heart of mixed sex) independent pools per tissue type. Analysis by one-way ANOVA separately for each mRNA species showed significant differences by tissue type: ERα_S ($F = 170.5$; $P < 0.001$); ERα_L ($F = 4.614$; $P = 0.012$); total ERα ($F = 22.993$; $P < 0.001$) mRNA. Letters indicate which tissue samples differed significantly ($P < 0.05$) within an mRNA subtype as determined by Tukey's multiple comparisons test. Note the logarithmic scale of the y-axis.

Figure 5. Developmental expression of (A) ERα_S (B) ERα_L and (C) total ERα transcripts in zebrafish embryos/larvae, and effects of E2, as measured by qPCR analysis
 Embryos were treated with vehicle alone (DMSO) or 0.1 μM estradiol (E2) beginning at 24 hpf and collected for qPCR analysis at 48, 72, 96 or 120 hpf. Results are expressed as mean normalized expression (MNE) ± SEM of 3 independent embryo/larva pools per time point and treatment (25–30 individuals per pool). One-way ANOVA of DMSO treated embryos showed no change in the levels of either the ERα_S (A) ($F = 0.219$; $P = 0.921$) or ERα_L (B) ($F = 1.248$; $P = 0.352$) by stage of development, but there was a significant increase in total (C) ERα mRNA ($F = 3.625$; $P = 0.045$). One-way ANOVA of E2 treated embryos showed significant changes in ERα_S (A) ($F = 16.183$; $P < 0.001$) and total (C) ERα mRNAs ($F = 115.412$; $P < 0.001$), but levels of ERα_L (B) ($F = 3.431$; $P = 0.052$) did not change. Asterisks indicate time points where there is a significant difference in expression between the DMSO treated and E2 treated embryos at a given time point as determined by t-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Note the logarithmic scale of the y-axis.

Figure 6. Effects of bisphenol-A (BPA) and diethylstilbestrol (DES) on expressed levels of ERα_S, ERα_L and total ERα transcripts in zebrafish embryos/larvae, as measured by qPCR analysis Embryos were treated with vehicle alone (DMSO), 0.1 μM diethylstilbestrol (DES), or 10 μM bisphenol A (BPA) beginning at 24 hpf and then collected for qPCR analysis at 120 hpf. Results are expressed as mean fold change over respective DMSO controls $\pm$ SEM of 3 independent embryo/larva pools per treatment group (25–30 individuals per pool). Asterisks indicate a significant effect of treatment on a given mRNA species when compared to respective DMSO controls by t-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Note the logarithmic scale of the y-axis.

Figure 7. Tissue distribution (A), developmental changes (B) and estrogen regulation (C) of ERα_S-Cx and other ERα mRNA variants, in comparison to ERα_S, ERα_L and total ERα transcripts, as determined by PCR analysis

RNA was prepared from different tissues of adult fish, developing embryos, or embryos/larvae treated with DMSO or 0.1 μM E2 beginning at 24 hpf and then collected at 96 hpf for RT-PCR analysis, as described in legends to Figures 4 and 5. Separate aliquots of reverse transcribed template were amplified with primer sets specific for ERα_L (#3/8), ERα_S (#1/6), total ERα mRNAs (#10/11), and ERα_S-Cx (#4/5) to obtain 1184, 1165, 1049 and 1448 bp products, respectively (Table 1). The first three primer sets also allowed for the amplification of internal exon deletion variants within those regions (panel A only), which were identified by their amplicon size: respectively, ERα_LΔ2b-5 (174 bp), ERα_SΔ2b-3 (510 bp), and ERαΔ7-9 (545 bp). Reverse transcribed cDNA was also amplified with β-actin primers (actin) to demonstrate equal loading. All primer sets were run simultaneously at 40 cycles and under the same PCR conditions. Aliquots of the PCR reactions were size separated by agarose gel electrophoresis and ethidium bromide (EtBr) stained. In panels B

and C all samples within one primer set were run on a single gel and cropping allowed for the exclusion of intermediary lanes that were not relevant to this publication

Table 1

Oligonucleotide primer sequences.

	Primer	Exon Position	Nucleotide Position	Sequence 5'-3'
PCR	1 (F)	Exon 1	1–29*	GATACATCAGTGAGAGAGAGAAAGCATCC
	2 (R)	Exon 9	2061–2082	CTCTTTCGAATGTCTCGGTTTC
	3 (F)	Exon 2a	1–22	TTTGAGAAAGACTGTGGGAGGT
	4 (F)	Exon 2b	299–324	AACTACCTAGATGGAGCTTATGAGTA
	5 (R)	Intron 8		AGTGTAATTTAGTTATGTGTGTGTGA
	6 (R)	Exon 5	1204–1228	GATCATGTGGACGAGTTCTTTGTCA
	7 (R)	Exon 5	973–997	ATCACTGGTGTCTTGTCTCGATCT
	8 (R)	Exon 5	1162–1184	ACATCATGGTGATCTCGGTGTAG
	9 (R)	Exon 9	2163–2188	TCAGCTTCCACAAAACCGAGCGAAA
	Actin (F)			CGAGCAGGAGATGGGAACC
	Actin (R)			CAACGGAAACGCTCATTGC
3'-RACE	10 (F)	Exon 5	1141–1167	ACGACAGAAACACAGCCGGCCCTACAC
	11 (F)	Exon 5	1173–1199	TCACCATGATGTCCCTGCTCACCAACA
Northern Blot	12 (F)	Exon 2b	283–303	CATCAGCTCCTCTGTCAACTA
	13 (R)	Exon 3	646–666	GCTCTGGATCCAGTGTACAAC
qPCR	Q1 (F)	Exon 1	1–29*	GATACATCAGTGAGAGAGAGAAAGCATCC
	Q2 (R)	Exon 2b	93–109*	R-TCGCTGCCTGGCACCAA
	Q3 (F)	Exon 2a	6–27	F-GAAAGACTGTGGGAGGTGGAAA
	Q4 (R)	Exon 2a	87–106	R- CGCAGCAAGCATGAGTTAGG
	Q5 (F)	Exon 5	1119–1136	F- CTGAGCCACCCGCTGTCT
	Q6 (R)	Exon 5	1197–1215	R- GACGAGTTCTTTGTGAGCCATGT
	elfa (F)			F-CTTCTCAGGCTGACTGTGC
	elfa (R)			R- CCGCTAGCATTACCCTCC

Numbering of nucleotide position corresponds to ERα_L (GenBank: EU079275) except where indicated by asterisk (*) numbering corresponds to position in ERα_S (GenBank: AF349412)

Table 2

Summary of cloned zebrafish ER α mRNA variants.

Genbank Accession #	mRNA variant	Splicing event(s) *	Transcript (nt) **	Predicted protein (aa) **	Affected domain(s)
AF349412	ER α_S	(b)	3439	569	A
EU079275	ER α_L	n/a	3502	613	none
EU079276	ER α_S -Cx	(b)(f)	1746	505	A, E, F
HM641841	ER $\alpha_S\Delta 2b-3$	(b)(c)	(2784)	(353)	A, B, C
IX457331	ER $\alpha_L\Delta 2b-5$	(d)	(2492)	(18)	No protein
HM641842	ER $\alpha\Delta 7-9$	(e)	(2935/2998) ***	(401/445) ***	E

* See Figure 1

** Numbers in parenthesis are based on partial cDNAs and assume a single splicing event per transcript and predicted protein

*** Alternate sizes are predicted from the short or the long 5'end

Table 3

Summary of publically available short and long ER α mRNAs in teleostean species. Species in which both the short and long isoforms are present, as indicated by cDNAs or *in silico* analysis of the genome, are designated in bold. Splice variants other than the short and long isoforms were not included in this survey.

Scientific Name	Common Name	Short Isoform			Long Isoform		
		Genbank #	Size (aa)	Citation	Genbank #	Size (aa)	Citation
<i>Acanthogobius flavimanus</i>	Yellowfin Goby	AB290321	564	(Ito et al., 2007)			
<i>Acanthopagrus schlegelii</i>	Black Porgy	AY074780	583	(Huang et al., 2002)			
<i>Acheilognathus yamatsutae</i>	Korean Striped Bitterling	JF275853	568	*			
<i>Acipenser schrenckii</i>	Amur Sturgeon	AB435631	523	(Katsu et al., 2008)			
<i>Atracosteus tropicus</i>	Tropical Gar				AB435634	581	(Katsu et al., 2008)
<i>Candidia barbata</i>	Lake Candidus Dace	AJ487687	565	*			
<i>Carassius auratus auratus</i>	Goldfish	AY055725	564	(Choi and Habibi, 2003)			
<i>Carassius auratus pengze</i>	Pengze Crucian Carp	JX440380	564	*			
<i>Cirrhinus molitorella</i>	Mud Carp				GU391263	549	*
<i>Clarias gariepinus</i>	North African Catfish				X84743	620	*
<i>Cyprinodon variegatus</i>	Sheepshead Minnow				N/A	542	(Karels and Brouwer, 2003)
<i>Cyprinus carpio</i>	Common Carp				AB334722	604	*
<i>Danio rerio</i>	Zebrafish	AF349412	569	**	EU079275	613	**
<i>Dicentrarchus labrax</i>	European Seabass				AJ505009	639	(Halm et al., 2004)
<i>Epinephelus coioides</i>	Orange Spotted Grouper	GU721076	574	(Chen et al., 2011)			
<i>Fundulus heteroclitus</i>	Killifish	AY571785	573	(Greytak and Callard, 2007)	HM641843	620	*
<i>Gambusia affinis</i>	Western Mosquitofish				AB295655	621	(Katsu et al., 2007)
<i>Gasterosteus aculeatus</i>	Three-Spined Stickleback	AB330740	552	*	***	623	*
<i>Gobiocypris rarus</i>	Chinese Rare Minnow				HM045496	598	(Wang et al., 2011)
<i>Halichoeres tenuispinis</i>	Chinese Wrasse	AY305026	574	*			
<i>Haplochromis burtoni</i>	Burton's Mouthbrooder	AY422089	582	*			
<i>Ictalurus punctatus</i>	Channel Catfish	AF061275	581	(Xia et al., 1999)	AF253505	617	(Patino et al., 2000)
<i>Kryptolebias marmoratus</i>	Mangrove Rivulus	AB251458	580	(Orlando et al., 2006)	DQ339108	648	(Seo et al., 2006)
<i>Lepomis macrochirus</i>	Bluegill	EF191017	582	(Sumida and Saito, 2008)			

Scientific Name	Common Name	Short Isoform			Long Isoform		
		Genbank #	Size (aa)	Citation	Genbank #	Size (aa)	Citation
<i>Melanotaenia flaviatilis</i>	Murray River Rainbowfish				GU319956	610	(Woods et al., 2010)
<i>Micropterus salmoides</i>	Largemouth Bass				AF253062	627	(Sabo-Attwood et al., 2004)
<i>Odontesthes bonariensis</i>	Argentinian Silverside				EU284021	602	*
<i>Oncorhynchus masou</i>	Cherry Salmon				AY520443	620	*
<i>Oncorhynchus mykiss</i>	Rainbow Trout	AJ242741	577	(Pakdel et al., 2000)	AJ242740	622	(Pakdel et al., 2000)
<i>Oreochromis niloticus</i>	Nile Tilapia	TNU75604	585	(Chang et al., 1999)	***	632	*
<i>Oryzias javanicus</i>	Marine Medaka				AY917147	642	(Yu et al., 2006)
<i>Oryzias latipes</i>	Medaka	***	585	*	***	629	*
<i>Paralichthys olivaceus</i>	Japanese Flounder	AB070629	578	*			
<i>Paranisgurnus dabryanus</i>	Loach				JN981144	608	(Zhang et al., 2012)
<i>Perca flavescens</i>	Yellow Perch	DQ984124	576	(Lynn et al., 2008)			
<i>Pimephales promelas</i>	Fathead Minnow	AY77528	560	(Wilson, 2004)	EF191016	602	(Sumida and Saito, 2008)
<i>Poecilia reticulata</i>	Guppy				AB621910	621	*
<i>Pseudolabrus japonicus</i>	Wrasse	DQ298133	526	*			
<i>Rutilus rutilus</i>	Roach Minnow	AB190289	559	(Katsu et al., 2007)			
<i>Salmo salar</i>	Atlantic Salmon				NM001123592	623	(Rogers et al., 2000)
<i>Sebastes schlegelii</i>	Korean Rockfish				FJ594994	624	*
<i>Sparus aurata</i>	Gilthead Seabream	AF136979	581	*			
<i>Spinibarbus denticulatus</i>	Chinese Phoenix Barb	DQ504306	578	(Zhu et al., 2008)			
<i>Takifugu rubripes</i>	Pufferfish	***	507	*	***	554	*
<i>Tanichthys albonubes</i>	White Cloud Mtn Minnow				EU035309	607	*
<i>Tetraodon nigroviridis</i>	Green Spotted Pufferfish	***	578	*	***	625	*
<i>Varicorhinus barbatulus</i>	Taiwan Shoveljaw Carp				AJ547632	612	(Fu et al., 2007)
<i>Xiphophorus maculatus</i>	Southern Platyfish	***	580	*	***	627	*
<i>Zoarces viviparus</i>	Viviparous Eelpout	AY223902	570	(Andreassen et al., 2003)			

* Unpublished

** This publication

*** In silico prediction based on the genomic database (Ensembl.org)