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Estrogen receptors in *Xenopus*: duplicate genes, splice variants, and tissue-specific expression

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Abstract

The estrogenic steroid hormones, acting primarily through the nuclear estrogen receptors ER α and ER β , regulate sexual differentiation in a wide variety of vertebrates. In the frog *Xenopus laevis*, estrogen regulates the strength of vocal neuromuscular synapses and contributes to the physiological basis of sexually differentiated songs. To understand the mechanisms by which estrogen produces these effects, we have characterized the ERs of *X. laevis* and their expression in laryngeal muscle and other tissues. We found a remarkable molecular diversity in the estrogen receptor population within individuals. First, we have identified two distinct ER α genes, xLER α 1 and xLER α 2, which represent, to our knowledge, the first discovery of retained duplicates of the ER α gene in any species. These two genes are highly conserved at the amino acid level but have distinct nucleotide sequences; moreover, ER α 2 has no N-terminal domain. Cloning of ER α and ER β in the related species *Xenopus tropicalis* and phylogenetic analysis indicate that the two xLER α loci were generated by a duplication specific to the *X. laevis* lineage—most likely the genome duplication that led to a doubling of the *X. laevis* chromosome number about 30 million years ago. The primary ER expressed in *X. laevis* laryngeal muscle is the novel gene xLER α 2; ER α 1 is primarily expressed in liver, forebrain, and oviduct. Alternatively spliced transcripts of both xLER α 1 and xLER α 2 are also expressed in a tissue-specific manner. We propose that complementary spatial expression of these two genes and their alternatively spliced transcripts contributes to their conservation over such a long period of time, consistent with the subfunctionalization model for evolution after gene duplication.

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1. Introduction

The gonadal hormone estradiol plays an essential role in vertebrate development and sexual differentiation. The role of the estrogen receptors (ERs), a class of ligand-activated transcription factors, in tissue responses to the hormone has been studied intensively in mice using gene inactivation (reviewed in Hewitt and Korach, 2002). Functional estrogen receptors are essential for development and adult function of the mammary glands, uterus, and testes, for fertility, and for bone growth. Estrogen can thus exert pleiotropic effects on a wide variety of tissues, and regulation can be specific to developmental stage. In birds and anurans, estrogen is

also responsible for yolk deposition; the molecular events that underlie vitellogenin synthesis by the liver have been extensively investigated in the South African clawed frog, *Xenopus laevis* (cf., Dodson and Shapiro, 1994). The estrogen receptor thought responsible (Westley and Knowland, 1978) has been cloned from *X. laevis* liver (Weiler et al., 1987). As in other vertebrates, the *X. laevis* oviduct is an estrogen target tissue (reviewed in Kelley, 1996; see also Marsh and Tata, 1987); estrogen also accumulates in specific brain regions (Morrell et al., 1975), notably the ventral forebrain. The strength of neuromuscular synapses within the frog vocal organ is also known to be estrogen regulated (Tobias et al., 1995; Wu et al., 2001), but ERs are not expressed in laryngeal motor neurons (Morrell et al., 1975); it is not known whether *Xenopus* laryngeal muscle or oviduct expresses the ER. Here we examine splice variants of ERs and their tissue specificity in forebrain, oviduct, and laryngeal muscle.

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Three genes for estrogen receptors have been identified: α and β in tetrapods and α , β , and γ in teleosts (Hawkins et al., 2000; Thornton, 2001; the ER γ arose as a duplication of the ER β locus in an event specific to the teleost lineage). In mammals, ER α and ER β are expressed in partially overlapping domains and have been shown to exist as a variety of alternatively spliced isoforms (Denger et al., 2001a; Lewandowski et al., 2002). ER α and ER β are both estrogen-activated transcriptional activators on estrogen response elements; on AP-1 elements, however, ER α 's trans-activating function can be opposed by ER β (Paech et al., 1997). Moreover, some ER α and ER β splice variants can also inhibit ER activation functions (Inoue et al., 2000; Ogawa et al., 1998). In combination with specific co-activators, tissue-specific expression of various forms of the ER may contribute to the diverse phenotypic effects of estrogen (McDonnell et al., 2002).

The markedly polyploid genus, *Xenopus*, is believed to have arisen through genome-wide duplication events triggered by hybridization and endoreduplication (Kobel et al., 1996). The only known diploid member of the Xenopodinae is *Xenopus (Silurana) tropicalis*. *X. laevis*, a pseudotetraploid, is anticipated to have as many as two ER α and two ER β genes, all expressed in a variety of isoforms. To gain insight into the evolution of estrogen action, we have cloned ER isoforms in *X. laevis* and *X. tropicalis* and subjected their sequences to phylogenetic analysis.

2. Materials and methods

2.1. Animals

Adult female *X. laevis* were purchased from *Xenopus* I (Ann Arbor, MI, USA) or from Nasco (Ft. Atkinson, WI, USA). *X. tropicalis* are maintained as a colony in the laboratory from stock of Nigerian origin (courtesy of R. Grainger and M. Kirschner). Animals were maintained, in polycarbonate tanks containing dechlorinated water, on a 12:12 h light:dark cycle (lights on at

08:00 and off at 20:00), at 20 °C for *X. laevis* (26 °C for *X. tropicalis*) and fed frog brittle (*X. laevis*; Nasco) or salmon pellets (*X. tropicalis*; Zeigler) three times a week.

2.2. RT-PCR and primers

Oviducts, brains, larynges, and livers from *X. laevis* and *X. tropicalis* were dissected from animals anaesthetized with MS-222. All tissues were frozen in powdered dry ice immediately after dissection. Total RNAs from frozen tissues were prepared with RNazol B (TEL-TEST, Friendswood, Texas) based on the manufacturer's manual. Reverse transcriptions were conducted with a Superscript Preamplification System for First Strand cDNA Synthesis (Life Technologies; currently Invitrogen, Carlsbad, California) using LB1 (see below for sequence) as the primer. The temperature for the reaction was either 42 or 50 °C. For high temperature reverse transcription, RetroAmp RT DNA polymerase from Epicentre (Madison, WI) was used. The annealing temperature was 50 °C while reverse transcription progressed at 70 °C for 10 min. All PCRs were performed with a Perkin Elmer DNA Thermal Cycler 480. Hot start PCRs were achieved by adding the *Taq* polymerase at high temperature after denaturing the DNA template at 94 °C for 1 min. A typical PCR cycle is 94 °C for 30 s, annealing temperature for 30 s and then 72 °C for designed time length. Typical PCRs have 35 cycles. Sequences of the primers used in this study and shown in Fig. 1 are as follows:

dxlER-LB1: 5' CAT YTC NAR NAR NAR RTC
RTA 3'
dxlER-LB2: 5' ARR TGY TCC ATI CCY TTR TT 3'
dxlER-LB3: 5' AVI ARC ATR TCR AAD ATY TC 3'
dxlER-DB1: 5' ATI TGY CCI GCI ACI AAY CA 3'
dxlER-e0a: 5' TGT TGC TGT GGG AAT GGA T 3'
dxlER-e0b: 5' ACT GCA AAA GAA GAC AAC
ACA T 3'
dxlER-e1a: 5' TAT GAC CAT GCC CCT ACC 3'
dxlER-e1b: 5' GGG CAC GAC TTA TGA CTT TG 3'
dxlER-e1c: 5' AAC AAT GTG CCT CCA AGT C 3'

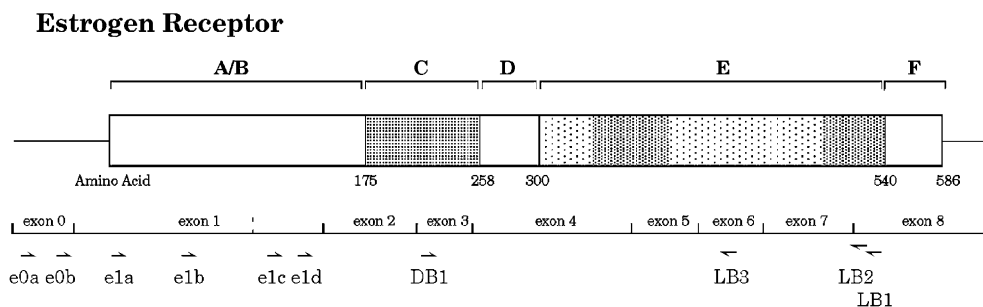


Fig. 1. A schematic diagram of the *Xenopus* estrogen receptor and the relation between the ER molecule and primers used in this study. The rectangular boxes represent the domain structure (A/B,C,D,E,F) and number of amino acid residues of the liver ER based on Weiler et al. (1987). The hatched boxes denote the regions that are highly conserved within ERs of different species; the denser the hatch, the higher the conservation of a region. Small arrows mark the approximate regions from which PCR primers were designed.

dxIER-e1d: 5' GCC AGC AAG TTC CAT ACT ATT 3'

Authenticity of PCR products was verified by using BLAST (Altschul et al., 1990) to search the GenBank database for exact or near-exact hits.

2.3. Rapid amplification of cDNA ends

3'-rapid amplification of cDNA ends (RACE) was performed based on the method described by Frohman, 1993. An oligonucleotide dQ_T that consists of 17 nucleotides of oligo(dT) preceded by a unique 35-base oligonucleotide sequence was used as a primer in a reverse transcription reaction. PCR was then performed on the first-strand cDNA with primers corresponding to the unique sequence in dQ_T (dQ₀ and dQ₁) and ER-specific forward primers (dxIER-3PR1: 5' CAT GAC TCA ACC AAA CCA 3' and dxIER-3PR2: GCT TCT TCT CAT TCT TTC AC 3'). The ER-specific primers were designed using cDNA sequences that were shared by xIER α 1 and xIER α 2. Two rounds of PCR utilizing nested primers amplify enough cDNA to be visualized in an agarose gel.

A RACE variation with single-strand ligation of an oligonucleotide to ss-cDNA ends (Edwards et al., 1991) was used for the amplification of the 5'-end of ER transcripts. Briefly, first-strand synthesis was performed with primer LB1. An oligonucleotide, dQ35 (5' pCCA TTG AGC TCG AGT CTT CTC CAA CTG CTC ACC ATT TTT TddA 3') which had a unique sequence, a phosphorylated 5' terminal and a blocked 3'-end was ligated to the first-strand cDNA by T4 RNA ligase (NEB, Beverly, MA). After removing the free oligonucleotide with a QIAquick Nucleotide Removal Kit (Qiagen, Santa Clarita, CA), the first-strand cDNA was used as template for PCR with ER-specific primers (LB1 and dxIER-5PR1: 5' AGT CGA CAG GCT TGG CAG 3') and primers for the unique sequence of dQ35 (dQ5: 5' ATG GTG AGC AGT TGG AGA 3' and dQ4.6: 5' TGA GCT GTT GGA GAA GAC 3').

BLAST searches using the *Xenopus* sequences as queries indicated that our PCR products were not due to contamination by any other known template.

2.4. Phylogeny reconstruction

Amino acid sequences of 21 estrogen receptor sequences, chosen for broad taxonomic distribution, along with 8 other steroid and estrogen-related receptors as outgroups, were retrieved from the Genpept database (NCBI, 2002; for accessions, see Fig. 5). The unalignable N-terminal portions of these sequences and the *Xenopus* sequences generated in this study were removed, and the remaining portions of the sequences were aligned to each other using ClustalX 1.82 (Thompson et al., 1997) at gap/change cost = 10, using the empirical Gonnett replacement probability matrix.

Phylogenetic inference was performed in two ways. First, we applied the parsimony criterion in PAUP*4.0b10 (Swofford, 2002), using a stepmatrix that weights amino acid changes by the inverse of their probability in the Gonnett matrix (Thornton, 2001), and a heuristic strategy of 100 random terminal additions followed by TBR branch swapping. Bootstrap analysis used 100 bootstrap replicates and 10 random additions with TBR. Second, we used Bayesian Markov Chain Monte Carlo analysis as implemented in MrBayes v3.0b (Huelsenbeck and Ronquist, 2001). Three independent analyses were conducted, each using the Jones protein replacement matrix, a 4-category gamma distribution of variable rates among sites, and 4 heated Markov chains run for 500,000 generations, the first 100,000 of which were discarded as burn-in, with tree sampling every 100 generations; posterior probabilities were calculated over the 12,000 trees saved from these analyses. All trees were rooted on steroid and estrogen-related receptors that are closely related to the clade of ERs (Thornton and DeSalle, 2000). Percent amino acid similarity, adjusted for gaps, was calculated using PAUP*.

3. Results

3.1. Isolation of two ER α genes in *X. laevis*

To examine ER expression in *X. laevis* tissues, we used PCR with nested degenerate primers (DB1, LB1, LB2, and LB3) predicted to anneal to the highly conserved DNA-binding and ligand-binding domains of different ER isoforms (ER α and ER β) of different species. The locations of the primers with respect to a previously cloned *X. laevis* ER (Weiler et al., 1987) are shown in Fig. 1. A product of the predicted size (620 bp) was amplified from oviduct, laryngeal muscle and forebrain (Fig. 2). This product was cloned from laryngeal muscle and from oviduct and two ER sequences were obtained (for details see Fig. 3). The sequences reveal unique restriction digest sites for each ER. A unique *Bam*HI site was present in one sequence (xIER α 1; see below for nomenclature) while a unique *Apa*LI site was present in the other (xIER α 2). Digestion of the 620 bp cDNA with *Apa*LI results in two products, a 380 and a 240 bp fragment, present in all tissues (Fig. 2). Digestion with *Bam*HI results in fragments of 438 and 182 bp. These are present in forebrain and oviduct; laryngeal muscle expresses little (Animal 1) or none (Animal 2) of these products (Fig. 2). These data suggest that at least two ER isoforms are present in *X. laevis* and that expression is tissue-specific.

Fig. 3 compares the two oviduct sequences (xIER α 1 and xIER α 2) to that obtained from liver (Weiler et al., 1987). xIER α 1 matches the previously published xIER α sequence from liver while the second sequence, xIER α 2,

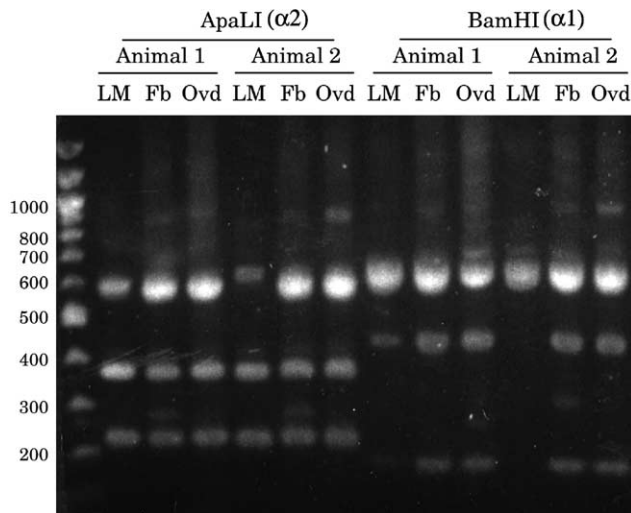


Fig. 2. Restriction fragment size polymorphism demonstrates the expression of two ER isoforms in *X. laevis* tissues. A 620 bp cDNA fragment is amplified from all tissues; this fragment represents undigested ER. $x\text{IER}\alpha 1$ can be digested by *Bam*HI and $x\text{IER}\alpha 2$ can be digested by *Apa*LI. The results illustrate that while forebrain and oviduct express both $x\text{IER}\alpha 1$ and $x\text{IER}\alpha 2$, laryngeal muscle expresses primarily $x\text{IER}\alpha 2$; Animal 1 also expresses a low level of $x\text{IER}\alpha 1$.

is novel. We have called the second sequence $x\text{IER}\alpha 2$ because it is phylogenetically classified as an $\text{ER}\alpha$ -type gene (see below) and because it represents a second locus that is distinct from $x\text{IER}\alpha 1$, rather than a polymorphism or splice variant. This conclusion is based on several lines of evidence, described in detail in the following sections. First, the sequences of $x\text{IER}\alpha 1$ and $x\text{IER}\alpha 2$ are significantly diverged from each other, and the degree of dissimilarity is on the order expected from two different estrogen receptor genes. Second, the scattered distribution of these differences along the length of the sequences cannot be explained by alternative splicing. Third, the sequences have different spatial expression patterns, a result expected if they are transcribed from different genes but not if they are alleles of the same locus. Finally, both sequences have multiple alternatively spliced transcripts at the 5'-end, and some splice forms are associated with only one of the ER sequences.

The sequences of $x\text{IER}\alpha 1$ and $x\text{IER}\alpha 2$ are conserved but not identical, with 95% identical nucleotides and a predicted amino acid identity of 97%. At first and second codon positions combined, the sequences are 99% identical, but at the third position they are only 88% identical, indicating that the genes have been conserved by stabilizing selection and serve some function related to fitness. The DNA and ligand-binding domains, which are unambiguously shared by $x\text{IER}\alpha 1$ and $x\text{IER}\alpha 2$, differ at 4.3% of nucleotide positions. Splice variants should be identical in a shared region, so these data rule out alternative splicing as the origin of $x\text{IER}\alpha 1$ and $x\text{IER}\alpha 2$. Predicted amino acid differences

between $x\text{IER}\alpha 1$ and $x\text{IER}\alpha 2$ are concentrated in the hinge and ligand-binding portions of the sequence, where differences between other pairs of ER orthologs and paralogs are most common (e.g., Hawkins et al., 2000).

The number of substitutions makes it very unlikely that the two sequences are alleles of one locus. $\text{ER}\alpha$ alleles in humans are generally single nucleotide polymorphisms and result in a nucleotide difference measure of $\sim 0.06\%$, some 75 times lower than that observed here (e.g., Schuur and Weigel, 2000; Wang and Miksicek, 1994). The degree of divergence between $x\text{IER}\alpha 1$ and $x\text{IER}\alpha 2$ is consistent, however, with the two sequences representing two loci generated by a gene duplication event tens of millions of years ago. The $\text{ER}\alpha$ genes of rat and mouse, for example, which arose from a common ancestor ~ 33 million years ago (Nei et al., 2001), are 94.7% identical, about the same as the two $x\text{IER}\alpha$ genes. Similarly, the $\text{ER}\beta$ and $\text{ER}\gamma$ genes of the teleost fish, the Atlantic croaker, which diverged from each other in a duplication event ~ 300 –450 million years ago (Hawkins et al., 2000; Taylor et al., 2001), are 71% identical, indicating a roughly similar rate of divergence among ER loci.

3.2. ER isolation from *X. tropicalis* and phylogenetic analysis

Xenopus laevis is a pseudotetraploid species that has retained, in many instances, diverged copies of duplicated genes (Hughes and Hughes, 1993). To understand the origin of the $x\text{IER}$ genes, and to determine whether they are properly classified as α or β , we also isolated and sequenced ERs from the related species, *X. tropicalis*, the only diploid species in the genus. We examined the phylogenetic relationship between *laevis* and *tropicalis* ERs and the steroid receptors of a variety of vertebrate species.

Using degenerate PCR with the same set of primers as for *X. laevis*, followed by RACE, we isolated two distinct estrogen receptor genes in *X. tropicalis* (Fig. 4). In contrast to the two *X. laevis* ERs, the amino acid sequences of the two *X. tropicalis* ERs are only 48% identical to each other. The amino acid sequence of one of the *X. tropicalis* ERs, which we have called $\text{xtER}\alpha$, is 96 and 97% identical to the $x\text{IER}\alpha 1$ and the novel $x\text{IER}\alpha 2$. The other is 78% identical to the $\text{ER}\beta$ of quail but is no more than 51% identical to any $\text{ER}\alpha$ sequences used in this analysis; thus, we have called this sequence $\text{xtER}\beta$.

Phylogenetic analysis of the *X. laevis* and *X. tropicalis* ERs in the context of other published ER sequences (Fig. 5) indicate that the two *X. laevis* ER genes are both of the $\text{ER}\alpha$ class and that the *X. tropicalis* PCR products include one $\text{ER}\alpha$ and one $\text{ER}\beta$ ortholog. The phylogeny shows that the gene duplication that generated the two

Liver	MTMPLPNKTTGVTF FLHQIQSSELET TLTRPPLK i SLERPLGEMYVENNRTG	50
xER1	MTMPLPNKTTGVTF FLHQIQSSELET TLTRPPLK t SLERPLGEMYVENNRTG	50
xER2	-----	0
Liver	IFNYPEGTTYDFAAAAAAPVYSSASLSYAASSETFGSSSLTGLHTLNNVPP	100
xER1	IFNYPEGTTYDFAAAAAAPVYSSASLSYAASSETFGSSSLTGLHTLNNVPP	100
xER2	-----	0
Liver	SPVVFL La ki PQLSPFIHHHGQQVPYYLESEQGTFAVREAAPPTFYR SSSD	150
xER1	SPVVFL qt -PQLSPFIHHHGQQVPYYLESEQGTFAVREAAPPTFYR SSSD	149
xER2	-----	0
Liver	NRRQSGRERMSSANDKGPPSMEST K ETRYCAVCSDYASGYHYGVWSCEGC	200
xER1	NRRQSGRERMSSANDKGPPSMEST K ETRYCAVCSDYASGYHYGVWSCEGC	199
xER2	-----MSSANDKGPPSMEST K ETRYCAVCSDYASGYHYGVWSCEGC	41
	RDAH	
Liver	KAFFKRSIQ GH NDYMC PATN Q CTIDKNRRKSCQACRLRKC Y EVGMMKGGI	250
xER1	KAFFKRSIQ GH NDYMC PATN Q CTIDKNRRKSCQACRLRKC Y EVGMMKGGI	249
xER2	KAFFKRSIQ GH NDYMC PATN Q CTIDKNRRKSCQACRLRKC Y EVGMMKGGI	91
	C S	
Liver	RKDRRG GR LLKHK R QKEE Q EQKNDV D PSEIRTASIWVNPSVKSMKLS P V L	300
xER1	RKDRRG GR LLKHK R QKEE Q EQKNDV D PSEIRTASIWVNPSVKSMKLS P V L	299
xER2	RKDRRG GR LLKHK Q QKEE P EQKNDV N PSEIRTASIWVNPSVKSMKLS P V L	141
	R E C Y	
Liver	SLTAEQLISALMEAE a PIVYSEHDS TKPLSEASMMTLLTNLAD r ELVHMI	350
xER1	SLTAEQLISALMEAE p PIVYSEHDS TKPLSEASMMTLLTNLAD k ELVHMI	349
xER2	SLTAEQLISALMEAE p PIVYSEHDS TKPLSEASMMTLLTNLAD k ELVHMI	191
	T S	
Liver	NWAKRVPGFVDLTLHDQVHLLCAWLEILMVGLIWR S VEHP G KLSFAPNL	400
xER1	NWAKRVPGFVDLTLHDQVHLLCAWLEILMVGLIWR S VEHP G KLSFAPNL	399
xER2	NWAKRVPGFVDLTLHDQVHLLCAWLEILMVGLIWR S VEHP E KLSFAPNL	241
	S C V D	
Liver	LLDRNQGR C VEGLVEIFDMLVTTATFR M RL R GEEFICLKS IILLNS G V	450
xER1	LLDRNQGR C VEGLVEIFDMLVTTATFR M RL R GEEFICLKS IILLNS G V	449
xER2	LLDRNQGR C VEGLVEIFDMLVTTATFR M RL H GEEFICLKS IILLNS G V	291
	D E E HK A SM	
Liver	YTFLSSTLESLEDTDLIHIILDKIIDTLVHFMAK S GLSLQQQ R RLAQLL	500
xER1	YTFLSSTLESLEDTDLIHIILDKIIDTLVHFMAK S GLSLQQQ R RLAQLL	499
xER2	YTFLSSTLESLEDTDLIHIILDKIIDTLVHFMAK S GLSLQQQ R RLAQLL	341
	P A W S	
Liver	LILSHIRHMS N KGMEHLYSMKCKNVVPLYD L LLEMLDAHR I HTPKDKTTT	550
xER1	LILSHIRHMS N KGMEHLYSMKCKNVVPLYD L LLEMLDAHR I HTPKDKTTT	549
xER2	LILSHIRHMS N KGMEHLYSMKCKNVVPLYD L LLEMLDAHR I HTPKDKTTT	391
	L N	
Liver	QEEDSRSP P TTTVNGASPC L Q P YY T NTEEVSLQSTV	586
xER1	QEEDSRSP P TTTVNGASPC L Q P YY T NTEEVSLQSTV	585
xER2	QEEDSRSP L STTVNGASPC L Q F Y K NTEEVSLQSTV	427

Fig. 3. Comparison between amino acid sequences of *X. laevis* ER α 1, ER α 2, and liver ER. The rectangular boxes around sequences indicate regions of high similarity between ERs from other species; these regions correspond to the boxes shown in Fig. 1. The ER cloned from male liver was reported by Weiler et al. (1987) and has a sequence very similar to ER α 1, which can be cloned from both *X. laevis* oviduct and brain. The amino acid residues that differ between liver ER and ER α 1 are indicated in bold lower case italics. Sequence differences between ER α 1 (red) and ER α 2 (green) are indicated. Differences between ER α 's 1 and 2 occur in all domains, but are more frequent in the hinge and the C-terminal regions. The region of ER α 2 represented by dashes indicates that no amino acid sequences have been identified in this region. Highly conserved amino acid residues in ER β that are different from ER α , and have different properties, are shown in blue at the bottom of the aligned xIER sequences.

ER α s in *X. laevis* occurred after the divergence of the *laevis* lineage from the *tropicalis* lineage, an estimated 30–110 million years ago (Graff, 1996). This conclusion is supported by phylogenies inferred using both maximum parsimony and Bayesian Markov Chain Monte Carlo methods. The existence of a *laevis*-specific ER α duplicate is consistent with the pseudotetraploid nature

of *X. laevis*, which has 36 chromosomes, as compared to the diploid number (20) in *X. tropicalis* (Kobel et al., 1996). *X. tropicalis* is therefore not predicted to contain a second ER α ortholog. *X. laevis* is predicted to contain an ortholog of ER β , the origin of which predates the tetrapod-actinopterygian divergence (Thornton, 2001); however, we have not been able to isolate an ER β

Quail β	mafcs p ammnyniasnfgdsesav r qt s ps l lwsapgh l splthc q l	50
Trop β	-----	0
Trop α	mlffclfiimlsipvasvfi-----	20
xLERα1	mtmplpnkttgvtflhqigsseletltrpplkislerplgemyvennr t g	50
Quail β	s l lyae q pk s pwce a r p le v lp v s r et l kr k t n g s dct s pi a s n pg---	97
Trop β	-----	0
Trop α	-----AAAA P V Y S S A S L S Y A A S S E T F G S S S L T G L H T L N N V P P	58
xLERα1	ifn y pe g tt y dfAAAA P V Y S S A S L S Y A A S S E T F G S S S L T G L H T L N N V P P	100
Quail β	-----	97
Trop β	-----	0
Trop α	SPV V FL Q T P Q L SP F I H H H Q Q V P Y Y LE S E Q G T F A V R E A A P T F Y R S S D N	108
xLERα1	SPV V FL Q T P Q L SP F I H H H Q Q V P Y Y LE S E Q G T F A V R E A A P T F Y R S S D N	150
Quail β	-----SK R D A H F C A V C S D Y A S G Y H Y G V W S C E G C K	126
Trop β	-----Y H Y G V W S C E G C K	12
Trop α	RR Q S G R E R M T S A N D K G P P S M E S T K E T R Y C A V C S D Y A S G Y H Y G V W S C E G C K	158
xLERα1	RR Q S G R E R M S S A N D K G P P S M E S T K E T R Y C A V C S D Y A S G Y H Y G V W S C E G C K	200
Quail β	A F F K R S I Q G H N D Y I C P A T N Q C T I D K N R R K S C Q A C R L R K C Y E V G M M K C G S R	176
Trop β	A F F K R S I Q G H N D Y I C P A T N Q C T I D K N R R K S C Q A C R L R K C F E V G M M K C G T R	62
Trop α	A F F K R S I Q G H N D Y M C P A T N Q C T I D K N R R K S C Q A C R L R K C Y E V G M M K G G I R	208
xLERα1	A F F K R S I Q G H N D Y M C P A T N Q C T I D K N R R K S C Q A C R L R K C Y E V G M M K G G I R	250
Quail β	R E R C G Y R I L R R H R N S E D ---CM G K T K K Y N E A A T R V K E I L L S T V S P E Q F V L	223
Trop β	R E R C G Y R I V R H R R H S D D Q m h C L A K N K L T D N I Q R V K E I S A S S L G P E Q F V L	112
Trop α	K D R R G G R M L K H K R Q K E E Q E Q K N D V T P S E I R T S I W V N P S V K S M K L S P V L S	258
xLERα1	K D R R G G R M L K H K R Q K E E Q E Q K N D V D P S E I R T A S I W V N P S V K S M K L S P V L S	300
Quail β	T L L E A E P-----P N V-L V S R P S K P F T E A S M M M S L T K L A D K E L V H M I G	264
Trop β	I I S D A E P-----P N V M L M N R L C K P F T E A S M M M S L T K L A D K E L V H M I G	154
Trop α	L T A E Q L I S A L M E A E P P I V Y S E H D S T K P L S E A S M M T L L T N L A D K E L V H M I N	308
xLERα1	L T A E Q L I S A L M E A E P P I V Y S E H D S T K P L S E A S M M T L L T N L A D K E L V H M I N	350
Quail β	W A K K I P G F I D L S Y D Q V R L L E S C W L E V L M I G L M W R S I D H P G K L I F A P D L V	314
Trop β	W A K K I P G F V E L S Y D Q V R L L E S C W L E V L M M G L M W R S I D H P G K L L F A P D L T	204
Trop α	W A K R V P G F V D L T L H D Q V H L L E C A W L E I L M V G L I W R S V E H P G K L S F A P N L L	358
xLERα1	W A K R V P G F V D L T L H D Q V H L L E C A W L E I L M V G L I W R S V E H P G K L S F A P N L L	400
Quail β	L D R D E G K C V E G I L E I F D M L L A M T S R F R E L K L Q H K E Y L C V K A M I L L N S S M F	364
Trop β	L D R D E G K C V E G I L E I F D M L L A T T S R L R E L K L Q H R E F L C L K V M I L L N S S H M F	254
Trop α	L D R N Q G R C V E G L V E I F D M L V T T A T R F R M M R L R G E E F I C L K S I I L L N S G V Y	408
xLERα1	L D R N Q G R C V E G L V E I F D M L V T T A T R F R M M R L R G E E F I C L K S I I L L N S G V Y	450
Quail β	P L ---S A E E P E S N R K L H H L L N V V T D L V W V I A K S G I P S Q Q Q T T R L A N L L M	411
Trop β	P L T S -S E E E S S R K L H H L L N T V T D L V W V I A K S G I P F R Q Q S T R L A N L L M	303
Trop α	T F L S T L E S L E D T D L I H I I L D K I I D T L V H F M A K S G L S L Q Q Q R R L A Q L L L	458
xLERα1	T F L S T L E S L E D T D L I H I I L D K I I D T L V H F M A K S G L S L Q Q Q R R L A Q L L L	500
Quail β	L L S H V R H A S N K G M E H L L S M K C K N V V P V Y D L L L E M L N A H T L R G Q R K S P V T H	461
Trop β	L L S H V R H A S N K G M E H L L S M K C K N V V P V Y D L L L E M L N A H T L R D H R K S T L A I	353
Trop α	I L S H I R H M S N K G M E H L Y S M K C K N V V P L Y D L L L E M L D A H R M H T P K D K A T A Q	508
xLERα1	I L S H I R H M S N K G M E H L Y S M K C K N V V P L Y D L L L E M L D A H R I H T P K D K T T T Q	550
Quail β	P D F e g v sh f g v -----	472
Trop β	S N Y s k a e e a r d a g g q t q-----	372
Trop α	E E D S R S P L T T T A N G A S P C L Q P F Y T S T E E V S L Q S T V	543
xLERα1	E E D S R S P P T T T V N G A S P C L Q P Y Y T N T E E V S L Q S T V	585

Fig. 4. Comparison of the amino acid sequences of ERα and ERβ of *X. tropicalis* and ERα1 of *X. laevis*. The sequence of the quail (*Coturnix*) ERβ, the most closely related ERβ in the database, is included for comparison. Only a partial sequence for *X. tropicalis* ERβ is available; other ER sequences were aligned to this fragment. Amino acid residues in blue are those conserved in ERβ but not ERα of different species and have properties different from the conserved amino acid residues in ERα. These residues are also highlighted in blue in Fig. 3. Amino acid residues in green are those found in beta which are different from, but have similar properties to, those found in alpha.

transcript despite extensive degenerate PCR experiments on multiple tissues. The lack of a recoverable ERβ from *X. laevis* may be due to a unique loss of this gene in the

X. laevis lineage, to restricted expression, or to extreme sequence divergence that may foil a degenerate PCR strategy.

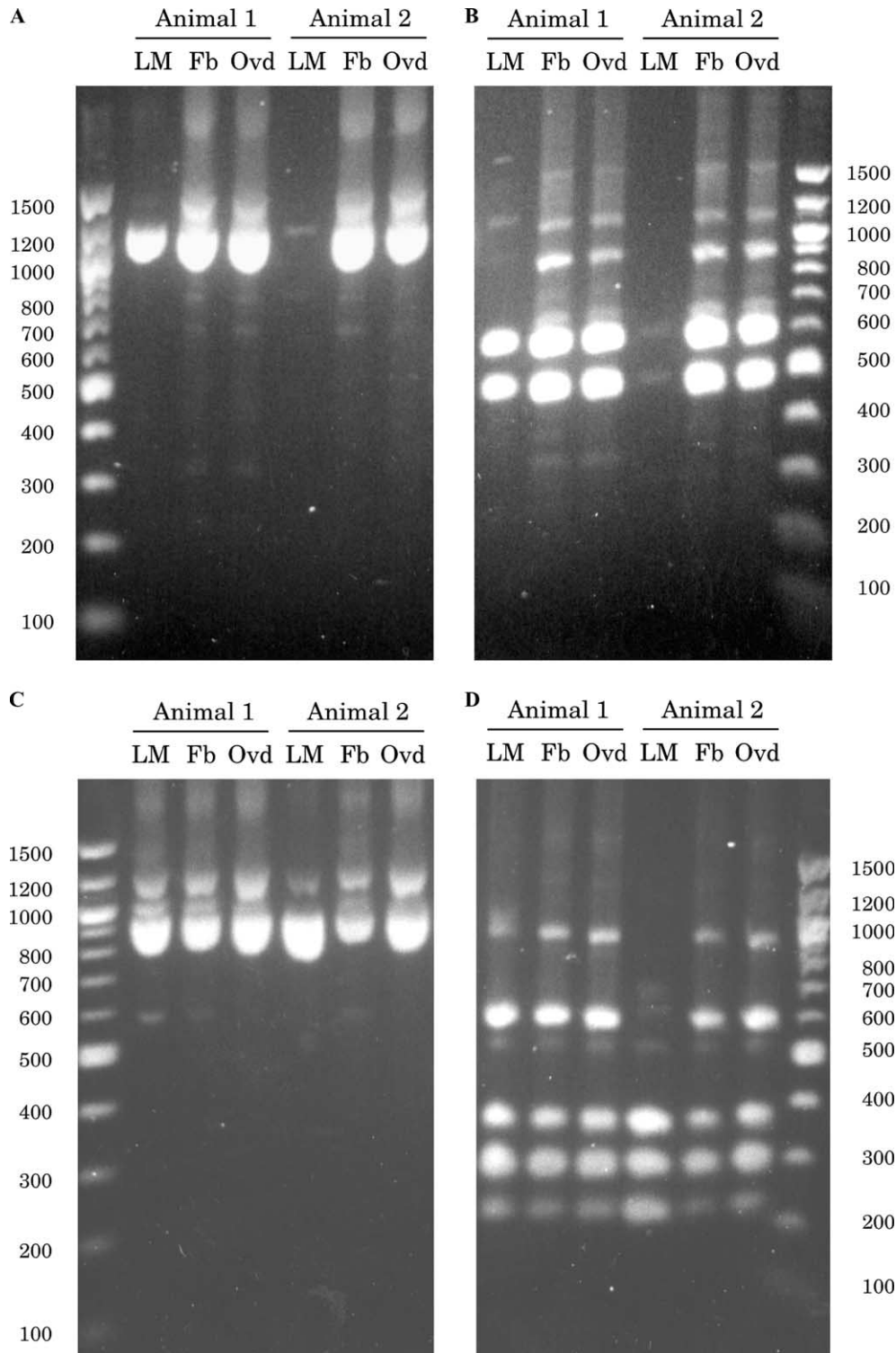


Fig. 6. Tissue-specific expression and detection of exon 1 (A and B) and exon 1' (C and D) xIER α isoforms. (A) RT-PCR with primers from the 5'-end of exon 1 (e1a and e1b) revealed a single product of 1092 bp present in all tissues except laryngeal muscle from Animal 2. (B) ApaLI digestion illustrates that the PCR product originates from xIER α 1. ApaLI cuts the 1092 bp fragment from xIER α 1 into fragments of 600 and 492 bp; the predicted sizes of ApaLI digestion of ER α 2 would be, instead, 492, 376, and 224 bp. In all tissues, amplified cDNAs are from xIER α 1. (C) RT-PCR with primers e1c and e1d reveals cDNAs of the expected size, 904 bp, for exon 1' are amplified in all tissues. (D) ApaLI digestion of xIER α 2 results in fragments of 376, 304, and 224 bp; all tissues express these fragments. These data indicate that exon 1' is associated with the xIER α 2 gene. ApaLI digestion also results in fragments from xIER α 1 (600 and 304 bp). However the production of an xIER α 1 fragment with this set of primers does not imply that xIER α 1 is also expressed as an exon 1' variant since this portion is common to both exon 1 and 1'.

suggesting that xLER α 2 from different tissues includes only the 3' portion of exon 1.

A similar approach was used to determine the relation of exon 1-skipped (exon Δ 1) transcripts to xLER isoforms (Fig. 7). cDNAs were amplified using primers matching the sequences of exon 0 (e0a, e0b) and LB3 (see Fig. 1), and the products were restriction digested. Products of the predicted sizes for full-length xLER α 1 (1363 bp) and exon Δ 1 xLER α 1 or xLER α 2 (860 bp) transcripts are observed (Fig. 7A). When these bands were digested, fragments characteristic of both xLER α 1 and xLER α 2 transcripts were detected, indicating that both loci express exon 1-skipped transcripts (Fig. 7B). The xLER α 1 Δ 1 transcript was detected in forebrain and oviduct, but not laryngeal muscle, and the xLER α 2 Δ 1 transcript was identified at low levels in all tissues except laryngeal muscle from Animal 2.

No fragments of the expected size for a transcript in which exon 0 is spliced to exon 1' in xLER α 2 were detected (Fig. 7B). Since laryngeal muscle contains predominantly xLER α 2, we conclude that most transcripts of xLER α 2 do not contain exon 0. This conclusion is supported by the low abundance of xLER α 2 digestion products in PCR products amplified using exon 0 primers. Those transcripts of xLER α 2 that do contain exon 0 are predominantly composed of exon 0 spliced directly to exon 2. These results suggest that the region we have called exon 1' is not an additional exon with its own unique splicing boundaries but is merely the transcribed

portion of a diverged xLER α 2 exon 1. Since exon 1' is unique to xLER α 2, and no upstream exon has been detected in xLER α 2 transcripts with exon 1', it is possible that a new transcription start site (perhaps inside exon 1, just upstream of exon 1') is acquired in the xLER α 2 locus.

The first exon (exon 1') of xLER α 2 includes only 225 bp 5' to the region encoding the DNA-binding domain. The translation start site used by xLER α 1 is thus not available to xLER α 2. Two downstream possibilities are the methionines in positions that align with M170 and M159 of xLER α 1. A strong context for a start codon is RNNatgG (R is a purine) while an adequate context is RNNatgY or YNNatgG (Y is a pyrimidine). The sequences around the start codon of M159 and M170 are CGCatgT and TCCatgG, respectively; M170 is thus a stronger candidate. From the conceptual translation, the size of the protein translated from M170 would be 47.5 kDa while that from M159 would be 48.6 kDa. Since the sizes of the conceptual translated products from other exon-skipped mRNAs are 15.6, 61.5, 53.6, 40.8, 60.9, and 58.9 kDa for xLER α 1 Δ 2, Δ 3, Δ 4, Δ 5, Δ 6, and Δ 7, respectively, an origin of this protein from other exon-use transcripts of xLER α 1 or 2 is unlikely.

In summary, the expression of xLER α splice variants is tissue-specific. For xLER α 1, forebrain and oviduct contain both full-length and exon Δ 1 transcripts. The small quantity of xLER α 1 expressed in some laryngeal muscles contains only the full-length transcript. Most xLER α 2 transcripts lack exon 0 and the 5' part of exon 1;

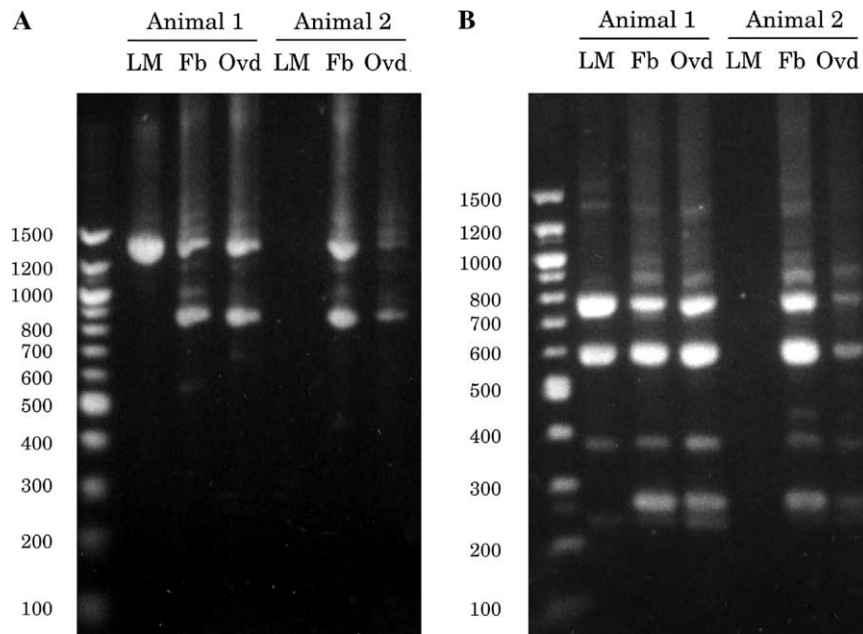


Fig. 7. ER isoform and tissue-specificity; detection of exon 1-skipped transcripts and the presence of exon 0. (A) A 1363 bp fragment, expected for full length xLER α 1 is found in all tissues except laryngeal muscle from Animal 2. A second product, of 860 bp, is expected from exon 1 skipped transcripts; this product is apparent only in forebrain and oviduct. (B) Digestion of the 1363 bp product from xLER α 1 would produce fragments of 763 and 600 bp. Both are present in all tissues except laryngeal muscle from Animal 2. For an xLER α 2 transcript with exon 0 spliced to exon 1', a 477 bp fragment is predicted. The absence of this band suggests that all the xLER α 2 transcripts that contain exon 0 also skip exon 1'. The 253 bp fragment is from digestion of the 860 bp exon 1'-skipped transcript of xLER α 2 and exon 1-skipped transcript of xLER α 1.

instead, this isoform has exon 1' spliced to exon 2. All tissues express this xLER α 2 transcript and it is the predominant isoform in laryngeal muscle. xLER α 2 also contains a transcript in which exon 0 is spliced to exon 2; this cDNA is present in low amounts in all tissues except laryngeal muscle from Animal 2.

4. Discussion

Our finding that *X. laevis* has two ER α genes represents the first discovery of a retained ER α duplicate. A large number of genes that exist in single copies in mammals are duplicated in *X. laevis*, apparently due to a genome duplication that occurred at least 30 million years ago and multiplied the haploid chromosome number vis-à-vis *X. tropicalis* (Bisbee et al., 1977).

If the ER α duplicates in *X. laevis* were generated by this ancient genome doubling, how and why have they been retained for such a long time? The classic model for evolution after gene duplication (Ohno, 1970) argues that one duplicate will be functionally redundant and must either evolve a novel function that is subject to stabilizing selection or become a pseudogene relatively rapidly. Lynch and Conery (2000) found that degeneration of duplicates into pseudogenes does occur frequently and quickly, but Hughes (1994) found that a surprisingly large number of duplicated genes have been retained in *X. laevis*. The very high conservation of sequence in the DNA-binding and ligand-binding domains between the two *X. laevis* ER α duplicates indicates that both ER α 1 and ER α 2 have remained under strong selection in this species.

An alternative explanation for the persistence of these two ER α s is the subfunctionalization hypothesis (Force et al., 1999), which suggests that the functions of an ancestral gene are partitioned among duplicate genes by complementary loss of tissue-specific expression. One duplicate gene is expressed in a portion of the tissues in which the ancestral gene was expressed, and the other is expressed in the remaining tissues. Selection to maintain the ancestral functions of the single gene conserves both copies. Our finding that *X. laevis* ER α 1 and ER α 2 have unique expression domains and high sequence conservation corroborates this hypothesis, as does the loss in ER α 2 of most of the N-terminal domain, including the transactivation function AF-1, which has regulatory properties distinct from those of the AF-2 found in the conserved ligand-binding domain (Paech et al., 1997). In teleosts, subfunctionalization also best explains the retention of ER β duplicates, which have similar biochemical characteristics but are expressed in non-overlapping tissues (Hawkins et al., 2000).

The results of this study indicate that ER isoform expression is tissue specific. Both xLER α 1 and xLER α 2 are expressed at high levels in forebrain and oviduct. In

contrast, ER α 2 is the predominant transcript in laryngeal muscle. Whether ER serves separate functions in these tissues is not known. Estrogen controls the efficacy of synaptic transmission at the laryngeal neuromuscular junction (Tobias et al., 1995; Wu et al., 2001). Because the laryngeal motor neuron does not express ER (Morrell et al., 1975), we have speculated that estrogen action is due to a retrograde signal from laryngeal muscle. Western analysis and immunocytochemistry results indicate that laryngeal muscle expresses an ER protein, and the molecular weight of that protein is the same as that predicted for the translated product of the xLER α 2 transcript (Wu et al., 2003).

Estrogenic effects are largely dependent on the nature of the receptor. For example, the classical estrogen receptor (ER α), a 66 kDa protein which includes all canonical domains of the steroid hormone receptor superfamily, functions as a transcriptional activator after binding to estrogen (Green et al., 1986; Gronemeyer, 1991; Krust et al., 1986; Kumar et al., 1987; Maxwell et al., 1987; White et al., 1987). In contrast, ERs missing exons 3 or 5 act as dominant negatives with respect to the full-length ER (Bollig and Miksicek, 2000; Dotzlaw et al., 1992). In addition, a number of N-terminal truncated ER isoforms have been described, and these may also have specific functions. An ER protein of 61 kDa expressed in the liver of oviparous species including chicken, *X. laevis* and rainbow trout (Claret et al., 1994; Griffin et al., 1999), as well as in mammalian anterior pituitary and basal hypothalamus (Griffin et al., 2001), has a partial transactivating or repressing activity in the absence of ligand. A smaller 46 kDa ER protein, lacking exon 1 and expressed in human tissues, may play a role in cellular proliferation (Denger et al., 2001b; Flouriot et al., 2000).

We show here that alternatively spliced ER isoforms are expressed in estrogen target tissues in *X. laevis*. The tissue-specific expression of ER α genes and isoforms provides the potential for very flexible regulation of target tissues by estrogen in *X. laevis*. Exactly what form this regulation takes, and whether some isoforms have dominant negative roles, remain to be determined.

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