Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts

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This paper describes three distinct estrogen receptor (ER) subtypes: ERα, ERβ, and a unique type, ERγ, cloned from a teleost fish, the Atlantic croaker *Micropogonias undulatus*; the first identification of a third type of classical ER in vertebrate species. Phylogenetic analysis shows that ERγ arose through gene duplication from ERβ early in the teleost lineage and indicates that ERγ is present in other teleosts, although it has not been recognized as such. The Atlantic croaker ERγ shows amino acid differences in regions important for ligand binding and receptor activation that are conserved in all other ERγs. The three ER subtypes are genetically distinct and have different distribution patterns in Atlantic croaker tissues. In addition, ERβ and ERγ fusion proteins can each bind estradiol-17β with high affinity. The presence of three functional ERs in one species expands the role of ER multiplicity in estrogen signaling systems and provides a unique opportunity to investigate the dynamics and mechanisms of ER evolution.

Despite the diversity of estrogen target tissues and effects, the actions of estrogens were long thought to be mediated through a single ligand-activated transcription factor, estrogen receptor α (ERα). This idea was challenged by the discovery of a second form of the estrogen receptor, ERβ, in rat prostate (1). ERβ differs from ERα in its ligand binding affinities to both natural and synthetic estrogens, tissue distributions, and trans-activation properties (2, 3). ERβ has been found in many vertebrate species (4, 5) and has provided an explanation for the seemingly contradictory actions of estrogens in certain target tissues (6). However, some estrogenic effects cannot be attributed to either ERα or ERβ (7), which suggests vertebrates may possess additional ER subtypes. Recently, an estrogen binding protein, pER, was identified, but this binding moiety is immunochemically, structurally, and functionally distinct from classical steroid hormone receptors (8). The current paper describes three distinct estrogen receptors identified in a single species: ERα, ERβ, and a previously unrecognized form, ERγ. The cDNAs encoding these receptors were discovered in the Atlantic croaker *Micropogonias undulatus*, a well studied teleost model of vertebrate reproductive endocrinology and toxicology (9). The tissue distributions of the three ERs, the binding characteristics of their fusion proteins, and their phylogenetic relationships to other ERs were investigated. The presence of three ERs in one species gives new insight into the evolution of ER genes and suggests that estrogenic action may be more complex than previously thought.

Materials and Methods

Animal Care and Tissue Collection. Tissues for cDNA libraries and *in situ* hybridization were obtained from adult female Atlantic croaker collected and maintained as described previously (10). Gonadally regressing fish were injected intraperitoneally with estradiol-17β (E2) at a concentration (1 mg/kg) that results in high physiological levels of E2 and up-regulation of ER and plasma vitellogenin levels in fishes (11–13). At 48 h later, fish were anesthetized in a seawater:phenoxyethanol bath (1:2,000) and quickly killed by severing the spinal cord. Tissues were immediately frozen in liquid N_2 and stored at $-80°C$. For Northern analyses, adult, sexually mature croaker (≈ 150 g) were captured with gill nets from the Port Aransas, TX, ship channel during the fall spawning migration, transported to the lab in a live well, and killed within 2 h. Tissues were collected as described above.

Cloning. Total liver RNA was extracted (14) to isolate mRNA (Promega PolyATract System). Reverse transcription (RT) was performed with 630 ng of liver mRNA, 13.5 pmol of primer ERr1, 5′-RTGNSCRTCNAGCATYTC-3′, and 200 units Super-script II RNase H-Reverse Transcriptase (Life Technologies, Grand Island, NY). Five microliters of the RT reaction was added to a first-round PCR containing degenerate primers ERf1 (50 pmol), 5′-TAYGGNGTNTGGWSNTGY-3′ and ERr1 (30 pmol). Five microliters of this PCR was used in a second-round PCR using nested primers ERf2, 5′-ATGTGYCCNGCNAC-NAAYCARTG-3′, and ERr2, 5′-NGTNGCNARNARCATRT-CRAADAT-3′ (50 and 60 pmol, respectively). PCR products from both rounds were cloned into the pCRII vector (Invitrogen) and sequenced (The University of Texas Institute for Cellular and Molecular Biology DNA Analysis Core Facility). A croaker ovarian cDNA library (Stratagene) was screened by PCR using primers derived from the nucleotide sequences of clones 1.2, 7.10, and 2.1. (OLIGO 4.02 PRIMER ANALYSIS software, National Biosciences, Plymouth, MN). Primers used were 1.2f, 5′-ACCTGGAGCACAGAACAGC-3′; 1.2r, 5′-CCTGGGCA-GTGAATGGAT; 7.10f, 5′-TAAATGCTACGAAGTGGG-CAT; 7.10r, 5′-AACGGCTTCTTCAGGTCCT-3′; 2.1f, 5′-AA-ACTACAGGAGCCCGCAGATGAG-3′; and 2.1r, 5′-TCTCCACATCAGTCCGACCATC-3′. The library was screened as described in ref. 15, and PCR-positive inserts were sequenced by the University of Chicago Cancer Research Center DNA Sequencing Facility. 3′-Anchored rapid amplification of cDNA ends (3′ RACE) (Life Technologies) was performed by using E2-injected female liver mRNA and nested forward primers:

BIOCHEMISTRY

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acERγ ---------- ---------- ----MAVASS PEKD-QPLLQ LQKVDSSRVG GQVLSPTLSS S--------- LETSQPICIT SPYTDLGHDF P----TIPFY SPTIFSYAG- PSISDCT---  78
acERα ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------PTS--   3
acERβ ---------- ---------- ----..SSPG LDPHPL.M.. ..E.G..K.S ERPR..G.LP AVYS---PPL GMD.HTV..P .....SS.EY NHSHGPLT.. ..SVL..SR- .P.TNSPS--  90

acERγ SVHQSLNPSL FWPSRGHMGS PIPLHHS--- ---QHGQPIQ SPWVEISPLD NVLKTKQDGA SLPLAVVPVR HKSARRRSQE S-----EEAV VTSGGKTDLH YCAVCHDYAS GYHYGVWSCE 187
acERα PLVFVPS--- -S.RLSPFMH .PSH...--- ---YLETTS  T.VYRS.VSS SQQQLS---- -----REDQC GTSDDSYSVE GSGAGAGAGA GGFEMAKEMR F....S.... ..D....... 101
acERβ .LCP..S..A ....HN.P-T MPS.TLHCPE SIVYNEPSPH A..L.SKAHS INASSSS--- ------IIGC N..LVK..E. GVEDMNSSLC SSAV..A.M. F.......... 200

acERγ GCKAFFKRSI QRDNEYICPA TNECTIDKNR RKSCQACRLR KCYEVGMTKC GMRKERGNYR SPQMRRMTRL TSQ--GRTDS SSVLTGSAVV SLNAPQP--- ---------- --SALT SEQL 290
acERα .......... .GH.D.M... .Q....R... ......Q...M .G .V..D.--G. VLRRDLRRTG ..DKASKDLE HRTAPPQDRR KHSSSSSSAG -----GGGKS SIIGMS PD.V 214
acERβ .......... .GH.D..... ..Q....... ....... M.. .V.R..CS.. GARH..GGLQ PRDPT..GLV RVG.GSR.QR H.HLEA.LTP LAPILQAKHV HL..MS P.EF 320

acERγ IERLMEAEPP EIYLMKDMKK PLTEAKVMMS LTNLADKELV HMITWAKKIP GFVELGLLDQ VHLLECCWLE VLMVGLMWRS VDHPGKLVFS PDLSLSREEG SCVQGFAEIF DMLLAATSRV 410
acERα LLL.QG.... VLCSRQKLSR .Y..VTM.TL ..SM...... ...A....L. ..LQ.S.H.. .Q...SS... ...I..I... IHC....I.A Q..I.D.S. D..E.M.... .....T...F 334
acERβ .S.I.D.... .....E.L.. .F...SM.... ........ F.S...... ......S.A. IN..K.... I.L...... ...FK.N... Q..E.IM.... .....G...F 440

acERγ RELKLQREEY VCLKAMILLN SNMCLSSS-- ----ESSSKL LRLLDAVTDA LVSAIGKTVL SFRQGYTRLA HLLMLLSHIR HVSNKGMDHL HCMKMKNMVP LYDLLLEMLD AHIMHS- SRL 523
acERα .M...KT..F .....I.... .GAFSFCTGT MEPLHDGAAV QNM..TI... .IHH.SQSGC .AQ..SR.Q. .L....... .M....E.. YS...C..K. .......... .RI.RP D.P 454
acERβ .......... .....L.T..PQT AEEL..RN... ...S.I. ..W..S.MG. TTQ..TL..G ..T....... ...... ST..R..V.L V......... .NTSSGG SQP 560

acERγ PRRSPEQEPE DQADAPAPP- -------HSS G-------SG PSYTWTPSSS EGAGEPQ--- ---------- ---------- ---------- ---------- ---------- ---  565
acERα AESWSQADR. PPY---TTSN NNNSSSSSGG .DG---GP.S AGSGSG.RVN HESL-----S RAPTPVLQYG GPRSDCTHIL ---------- ---------- ---------- ---  523
acERβ SSSPSSETYS ..HQYPQ..S HLHPGSEQTT ADHAIVPPL. .ADDPILDGH LDAMPL.SSP PFQSLVVPHM DTNDYIHPEQ WSLGTGDAAP SVEPTDYITT ERVVMETALV TQP  674
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Fig. 1. The deduced amino acid sequences of the three croaker cDNAs [Atlantic croaker ER α (acERα), Atlantic croaker ER β (acERβ), Atlantic croaker ER γ (acERγ)] indicating diagnostic features of ER genes. Croaker sequences were aligned with 33 other ERs by using CLUSTALX (16). Amino acids that diagnose the clade of ERγs (those that changed on the branch leading to this group of receptors on the phylogeny in Fig. 2 and thereafter were conserved in all known members) are red. The DBD is underlined and the LBD is bracketed. The transactivation function region, TAF-2, which is involved in ligand-dependent transactivation, is underlined (22). Amino acids at the receptor dimerization interface are noted with ($\blacklozenge$) (23). Positions that contact ligands in mammalian ERs are noted with an asterisk (24, 25). The amino acids in the LBD known to be critical for E2 binding are noted with arrows (26). Sequence notation includes alignment gaps (–) and identity with croaker ER gamma (.).

5′-GAAGAGGAAGAATGTGGTGC-3′, 5′-TACGACCTC-CTCCTGGAAAT-3′. 1.2-derived and 7.10-derived clones were joined into contiguous clones by RT-PCR of E2-stimulated female liver and ovary mRNA, respectively, and primers ERαf, 5′-GGCCTACCAGTCCTCTTGTT-3′; ERαr, 5′-AATC-CCCTTTCACTGTTGTG-3′; ERβf, 5′-CTGCCTGACATG-GCCTCCTCTC-3′; and ERβr, 5′-CGCGGTGGAAATCCAT-TAAGGT-3′.

Phylogenetic Analysis. The entire lengths of the sequences were used for alignment with CLUSTAL X 1.5 (16) and for phylogenetic analysis with PAUP*, Version 4.0b2 (17). Sequences were aligned by using an elision technique that avoids the arbitrary choice of a single gap-change cost by aligning at several values within a defined window (a geometric progression of 2, 4, 8, and 16) and then eliding the resulting alignments into a master data matrix (46 sequences by 2,857 characters, of which 2,180 were parsimony-informative) (18). In phylogenetic analysis, uninformative characters were excluded, gaps were treated as missing, and a heuristic search strategy of random addition followed by tree bisection and reconnection branch swapping was repeated 1,000 times. Decay indices were calculated by using AUTODECAY (19) as above (20 repetitions). Decay indices and tree lengths were divided by 4 to reflect the use of a 4-fold elision matrix. Bootstrap values were calculated by PAUP*, using 100 bootstrap replicates with heuristic search strategy as above times 20. Because the elision method can artificially inflate bootstrap values, the bootstrap analysis was done with one of the alignments (gap:change cost = 16), for which the most parsimonious phylogeny was the same as that for the elision matrix. Phylograms were drawn with all characters included. Tree statistics were calculated with parsimony-uninformative characters excluded.

Corrected pairwise proportions of amino acid differences were calculated [K = (1 − ln$D$), where D is the proportion of observed differences, gaps excluded] for all full-length sequences in each group paired with the closest available outgroup (mouse ERβ for the ERβ/ERγ comparison; *Xenopus* ERα for the teleost/tetrapod comparisons). Mean relative rates for each group were calculated, and departure of their ratios from unity was evaluated by using a two-tailed t test assuming unequal variances.

Amino acid evolution was reconstructed on the phylogeny by using parsimony-based algorithms in PAUP*, with the ACCTRANS setting. Amino acids that changed on the branch leading to the last common ancestor of all ERγs and were conserved thereafter in all members of the clade were considered diagnostic synapomorphies.

Northern Hybridization. mRNA was extracted from frozen tissue with magnetic (oligo)dTs as per manufacturer's instructions (Straight As; Novagen). Only samples with spectrophotometric OD$_{260}$:OD$_{280}$ ratios greater than 1.7 were used in further analyses. mRNA was mixed with loading dye and ethidium bromide (20 μg/μl), and loaded onto formaldehyde gels in triplicate. Gels were run with buffers and protocols provided (Northern Max Kit, Ambion, Austin, TX). After electrophoresis, mRNA integrity was checked on a UV transilluminator by visualizing residual rRNA bands. Gels were blotted onto nylon membranes (Bright Star; Ambion) and crosslinked by baking at 80°C for 30 min. Blots were hybridized overnight with a [^{32}P]UTP-labeled (Amersham Pharmacia) riboprobe generated from a subclone of each ER cDNA: acERα, amino acids 1–81; acERβ, amino acids 443–552; acERγ, amino acids 266–406 (Fig. 1). After washing, Northern membranes were exposed to a phosphorimaging screen for 12–36 h and then scanned with a phosphorimager (Molecular Dynamics). Images were analyzed by proprietary IMAGEQUANT software Version 1.11. The presence of a hybridized transcript was defined as a band peak with a pixel intensity greater than two times background. To determine relative abundance, only samples on the same blot and hybridized with the same probe were compared. Triplicate blots were hybridized independently with one ER probe, then stripped and rehybridized with another to ensure that differences in mRNA transcript abundance were not caused by loading or blotting variation.

***In Situ* Hybridization.** Brains from E2-stimulated female croaker were frozen (−80°C), embedded in OCT compound (Tissutek, Durham, NC), and cut into 20-μm sections in a cryostat (Frigocut; Reichert-Jung). Consecutive sections were transferred to alternate slides to localize the ER subtypes relative to each other in the same brain area. [^{35}S]CTP-labeled antisense riboprobes were transcribed from subclones of each ER cDNA: acERα, amino acids 177–523; acERβ and acERγ, same as for Northern blots (Fig. 1). *In situ* hybridizations were conducted as described previously (20). Three controls were performed to confirm that the antisense riboprobe
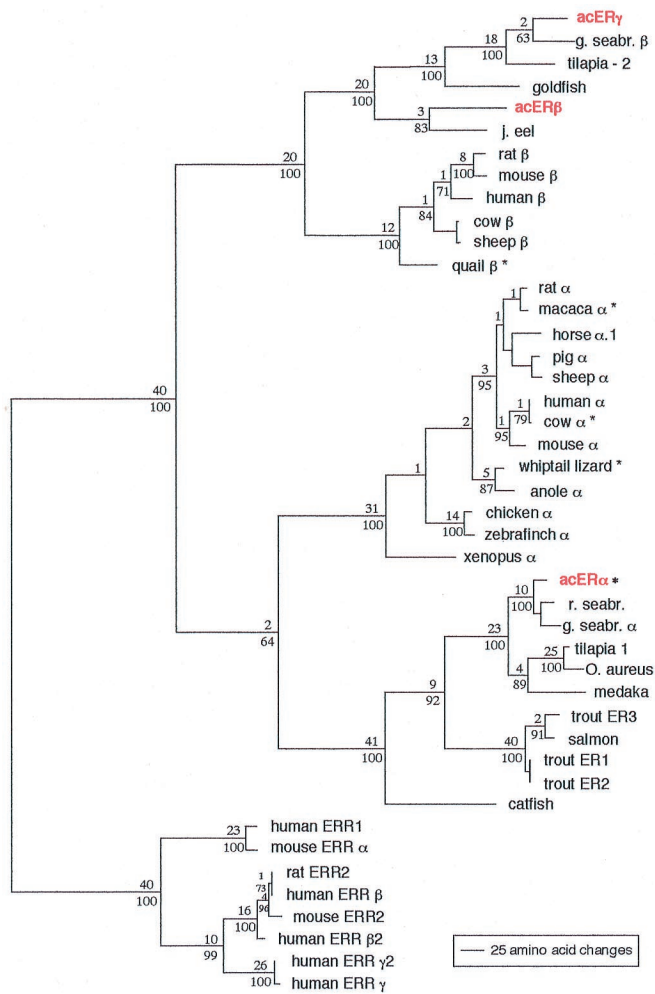
Fig. 2. Phylogenetic analysis of ER sequences including Atlantic croaker (ac) ER cDNAs acERα, acERβ, and acERγ. This tree shows three clades of paralogous ERs, demonstrating the existence of three distinct ER types: ERα, ERβ, and ERγ. ERαs and ERβs are present in both teleosts and tetrapods, whereas ERγs are present only in teleosts and are closely related to ERβs. Each clade is well supported, as shown by high bootstrap values (below each branch) and Bremer supports (above each branch), which express the degree of character support for each clade as the number of extra amino acid changes in the most parsimonious tree that does not contain the clade as compared with the most parsimonious tree that does (27). The tree was rooted by using the estrogen-related receptors (ERR) as the out-group. Branch lengths are proportional to the number of amino acid changes on the branch. Partial sequences (*) may have artificially short branches. Tree length, 3,090 amino acid changes; consistency index, 0.7714; retention index, 0.8797. GenBank accession nos.: *anolis* ER, AAC64412.1; catfish ER, AAC69548.1; chicken ER, 625329; cow ERα, P49884; cow ERβ, AAD24432.1; acERα, AF298183; acERβ, AF298181; acERγ, AF298182; gilthead seabream (g. seabr.) ERβ, AAD31033.1; g. seabr. ERα, AF136979_1; goldfish ER, AF061269_1; human ERR γ, NP001429.1; horse ERα.1, AAD17316.1; human ERα, P03372; human ERR1, P11474; human ERβ, BAA24953.1; human ERR β, NP 004443.1; human ERR β2, AAC99409.1; human ERR γ2, AAC99410.1; Japanese eel ER, BAA19851.1; macaca ERα, P49886; *medaka* ER, P50241; mouse ERR2, S58087; mouse ERα, P19785; mouse ERβ, AAB51132.1; mouse ERR α, AAB51250.1; *Oreochromis aureus* ER, P50240; pig ERα, Q29040; quail ERβ, AAC36463.2; rat ERα, CAA43411.1; rat ERβ, AAC52602.1; red seabream (r. seabr.) ER, O42132; salmon ER, P50242; sheep ERβ, AAD10826.1; sheep ERα, Z49257.1; *tilapia* ER2, AAD00246.1; *tilapia* ER1, AAD00245.1; trout ER1, CAB45139.1; trout ER2, CAB45140.1; trout ER3, P16058; whiptail lizard ERα, AAB35739.1; *Xenopus* ERα, 625330; and zebrafinch ERα, AAB81108.1.

hybridized specifically to ER mRNA: hybridization with labeled sense probe only, hybridization with labeled antisense in the presence of an excess of cold antisense probe, and digestion of the

tissue with RNase before adding labeled antisense probe. None of these treatments resulted in detectable riboprobe binding.

Saturation Binding Analysis. Fusion proteins were created to use in binding studies by subcloning acERβ and acERγ cDNAs into the pT7 Blue-2 expression vector (Novagen). To create the acERγ fusion protein, the entire coding region of acERγ was subcloned by using an *Avr*II restriction site 63 nt upstream from the start codon and a *Not*I site in the pBluescript SK(−) multicloning site. The acERβ fusion protein was constructed by subcloning most of the DNA-binding domain (DBD), hinge, and ligand-binding domain (LBD) (amino acids 217–552, Fig. 1), using sequence-specific primers incorporating restriction sites: forward 5′-CAG[CTCGAG]GCGGCTTATGTGCCCTGCC-3′ [*Xho*I] and reverse 5′-CA[TCTAGA]CAGCTTGTGGGCATCAAG-CATCT-3′ [*Xba*I]. These constructs were translated *in vitro* with the TNT-Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's directions, and the protein products were prepared for E2 saturation binding assays. Briefly, the products of the translation reaction were diluted (4:150) in assay buffer [20 mM Hepes, 150 mM NaCl, 10% (wt/vol) glycerol, 1.5 mM EDTA, 6 mM monothioglycerol, 10 mM Na_2MoO_4, 1 mM PMSF, and 0.04 trypsin inhibitor units/ml aprotinin, pH 7.5], and 150-μl aliquots of the mixture were placed into reaction tubes. Total binding was measured by the addition of 50 μl of $[2,4,6,7-^3H(N)]$-estradiol [72 Ci/mmol (1 Ci = 37 GBq), New England Nuclear] dissolved in assay buffer for final reaction concentrations ranging from 0.25–6.0 nM. Nonspecific binding was measured as above in a parallel set of reaction tubes containing 200-fold excess diethylstilbestrol. All reaction mixtures were incubated for 8 h at 4°C. Bound estradiol was separated from free by filtration through a hydrophilic mixed cellulose ester membrane filter with a high protein-binding capacity and 0.22-μm pore size (Millipore, no. GSWP02400), then washed immediately with 12.5 ml of buffer. Bound radioactive estradiol trapped on the filter was counted in a liquid scintillation counter. Specific binding was calculated as the difference between total and nonspecific binding. Scatchard plots (21) of specific binding were used to estimate dissociation constants (K_d).

Results

Cloning. RT-PCR of Atlantic croaker liver mRNA using nested degenerate ER primers revealed two clones from PCR round 1 [clone 1.2, amino acids 117–456 (Fig. 1, acERα), and clone 7.10, amino acids 217–552 (Fig. 1, acERβ)] and one clone from PCR round 2 [clone 2.1, amino acids 204–407 (Fig. 1, acERγ)] with >71% overall similarity to mammalian ERs. Two longer, overlapping clones corresponding to clone 1.2 [COA20, amino acids 1–376, and COO2, amino acids 156–523 and 823 nt of 3′ untranslated region (UTR) (Fig. 1, acERα)] and clone 7.10 [COO3: amino acids 133–457, and COA5, amino acids 1–417 and 274 nt of 5′ UTR) (Fig. 1, acERβ)] were obtained from a croaker ovarian cDNA library (Stratagene). The 3′ end corresponding to 7.10 (amino acids 553–674 and 53 nt of 3′ UTR) was obtained by 3′ RACE. Two full-length cDNA clones, encompassing all 1.2-derived and 7.10-derived sequences were isolated by RT-PCR from croaker liver and ovary mRNA and designated clone 1.2d and clone 7.10d, respectively. A clone that encompassed an entire ORF (COA7, amino acids 1–565, 575 nt of the 3′ UTR, and 1,548 nt of the 5′ UTR) was obtained from the ovarian cDNA library that corresponded to clone 2.1 and was designated clone 2.1d.

Clone 1.2d was most similar to rat and human ERα (≥94/64% identity in the DBD/LBD domains), whereas clone 7.10d was most similar to the β subtype (≥97/66% identity). We designated these cDNAs Atlantic croaker ERα (acERα) and Atlantic croaker ERβ (acERβ), respectively. Clone 2.1d also resembled
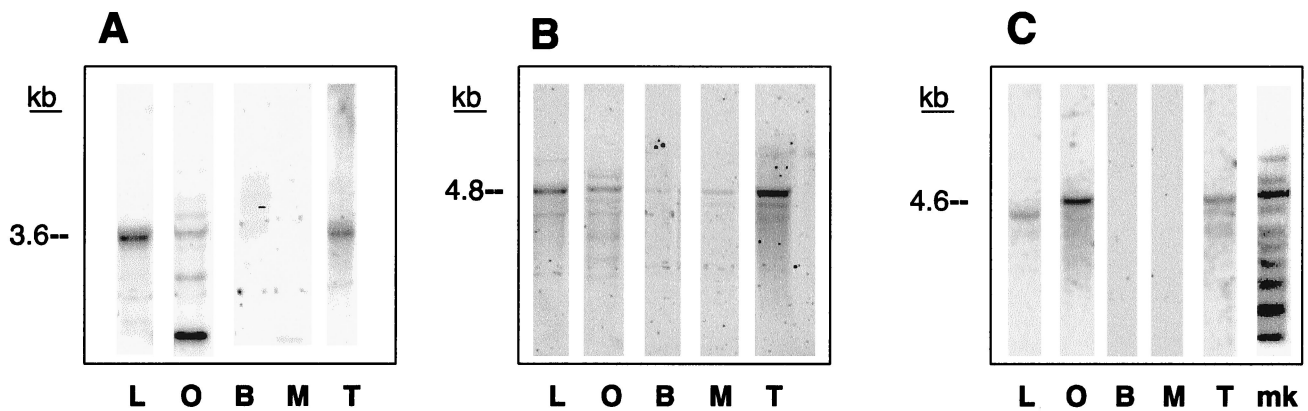
Fig. 3. Northern blot analysis of ER mRNAs in tissues of mature female (*n* = 3 for α and γ; *n* = 2 for β) and mature male (*n* = 2, testes only) Atlantic croaker. L, liver; O, ovary; B, brain; M, muscle; T, testes. Amounts of mRNA loaded per tissue type: liver, ovary, testes = 3 μg per lane; brain = 2 μg per lane; muscle = 1.3 μg per lane. Lanes shown are one of three loads of mRNA from the same fish. (*A*) Atlantic croaker ERα (acERα). (*B*) Atlantic croaker ERβ (acERβ). (*C*) Atlantic croaker ERγ (acERγ). The size (kb) of each transcript is indicated. RNA size markers (mk) are shown (Millennium Markers, Ambion).

mammalian ERs, but was more divergent than acERα or acERβ (≥91/57% identity). We designated this cDNA Atlantic croaker ERγ (acERγ). All three cDNA sequences possess diagnostic features of ERs, including amino acids involved in receptor transactivation (22), dimerization (23), and ligand binding (24–26) (Fig. 1). acERγ is clearly not an alternatively spliced isoform of the acERβ gene because there are amino acid differences throughout its coding regions (Fig. 1), and nucleotide silent substitutions are common where amino acids are identical (data not shown).

Phylogenetic Analysis. The deduced amino acid sequences of the croaker cDNAs, along with all other available ER sequences, were used to infer a phylogeny of ER subtypes (Fig. 2). This analysis indicates there are three major groups of ERs: The acERα clusters with ERαs, whereas acERβ and acERγ are members of two separate groups of receptors that are more closely related to each other and to previously identified ERβs than to ERαs. These groupings suggest that the ERγs result from duplication of the ERβ gene, which probably occurred after the divergence of ray-finned and lobe-finned fishes, but before the radiation of major teleost classes. ERs from three other teleost species cluster with acERγ: gilthead seabream ERβ, tilapia ER-2 (ERβ), and goldfish ER (Fig. 2), revealing that ERγ has been discovered in three other teleost species but has not been recognized as such.

Patterns of amino acid divergence indicate that, after duplication of the ancestral ERβ gene, the coding sequences of ERγs accumulated novel mutations at a greater rate than paralogous ERβs do. The parsimony reconstruction in Fig. 2 shows there were 45% more amino acid replacements on the branch leading from this duplication to acERγ than on the branch leading to acERβ. Further, relative-rate tests indicate that ERγ amino acid sequences diverged an average of 15% more rapidly after the duplication event than did teleost ERβ sequences (*P* < 0.01). Teleost ERβs did not diverge significantly faster than did ERβs in the tetrapod lineage, where no duplication has been found (*P* > 0.15).

Twenty-two amino acid changes and two short deletions have been conserved among all known ERγ sequences and therefore "diagnose" the ERγ clade (Fig. 1). Several of these conserved characters are at or adjacent to amino acid positions that participate in ligand binding and receptor dimerization in mammalian ERs, or they are in regions involved in transactivation (22–26). For example, the diagnostic amino acids Met 499 and

Phe 396 are in positions that directly contact ligands in mammals, whereas His 495 and Val 317 are immediately adjacent to ligand-contacting regions (25, 26) (Fig. 1, acERγ). Tyr 471 and Leu 435 are immediately adjacent to regions involved in receptor dimerization (23), and Ile 517 is located within the ligand-dependent transactivation function region, TAF-2 (22).

Northern Hybridization Analysis/Tissue Distribution. Northern hybridization reveals that in wild, unmanipulated animals with natural levels of E2, the 3.6-kb mRNA transcript corresponding to acERα is abundant in the liver, less abundant in testes, and barely detectable in ovary and brain (Fig. 3*A*). Interestingly, an alternate transcript less than 1 kb is the dominant form of acERα detected in the ovary and also is detected at low levels in muscle. The acERβ transcript (4.8 kb) is abundant in liver and testes, less abundant in ovary, barely detectable in muscle, and less than two times background in brain by using this technique (Fig. 3*B*). In contrast to acERα and acERβ, the 4.6-kb transcript encoding acERγ is abundant in the ovary and undetectable in the liver. Expression in testes is also high, whereas expression in brain and muscle is low or undetectable (Fig. 3*C*). The smaller, faint band detected in liver is probably crossreactivity with high levels of acERα mRNA.

***In Situ* Hybridization/Hypothalamic Distribution.** acERα, acERβ, and acERγ are expressed in discrete regions of the preoptic nucleus (28) of the croaker hypothalamus (Fig. 4). *In situ* hybridization in E2-stimulated mature female croaker showed that acERα expression is distributed throughout the preoptic nucleus, whereas acERβ expression is localized to the third ventricle wall (Fig. 4 *B* and *C*). In contrast, acERγ is expressed lateral to the third ventricle in the suprachiasmatic nucleus (Fig. 4*D*).

Saturation Binding Analysis. The ability of acERβ and acERγ fusion proteins to bind radiolabeled E2 was determined by saturation-binding analyses. Saturation analyses indicated the presence of high-affinity binding with K_ds of 1.38 nM and 1.16 nM, respectively (Fig. 5). These values are similar to the affinity of ER for E2 in croaker liver and testicular tissue reported previously (10).

Discussion

This study demonstrates the presence of three distinct and functional estrogen receptor subtypes (acERα, acERβ, and
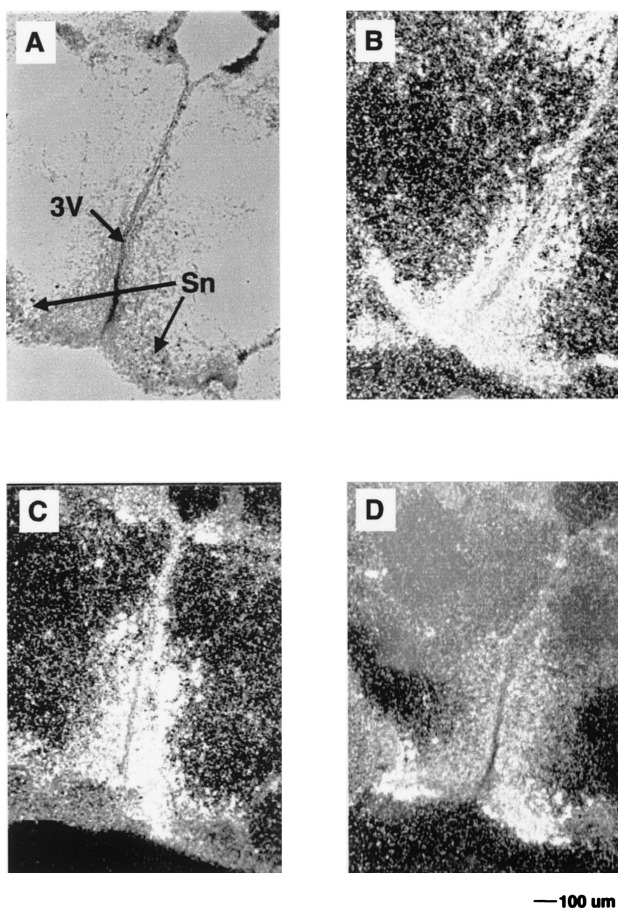
Fig. 4. Photomicrographs showing differential ER subtype mRNA distribution in the female Atlantic croaker hypothalamus through *in situ* hybridization. Sections shown are adjacent transverse cryosections hybridized with a [^{35}S]CTP-labeled antisense riboprobe complementary to each ER mRNA. Specific binding to mRNA by the riboprobes is seen in the dark-field images (*B*, *C*, and *D*) as bright white dots (silver grains) clustered over cells, and in the bright-field image (*A*) as small black dots. (*A*) The corresponding bright-field image of the section shown in *D*. (*B*) acERα mRNA distribution throughout the preoptic nucleus. (*C*) acERβ mRNA localization along the third ventricle wall (3V). (*D*) acERγ mRNA distribution lateral to the third ventricle in the suprachiasmatic nucleus (Sn).

Fig. 5. Scatchard plots of acERβ (*A*) and acERγ (*B*) translation products showing saturable binding to radiolabeled E2. Specific binding (*Inset*) was calculated as the difference between total and nonspecific binding. Unprogrammed reticulocyte lysate exhibited no specific binding (data not shown).

acERγ) in a teleost fish, the Atlantic croaker. The results also show that the three ER subtypes are present in natural populations of Atlantic croaker and are differentially expressed in reproductive tissues that are known targets of estrogen action. Furthermore, acERβ and acERγ fusion proteins bind E2 with similar affinities as ERs in croaker tissues (10). Phylogenetic analysis suggests that ERγ is present in other teleosts but was erroneously named ERβ because of lack of evidence for all three ER subtypes in any one species, and shows that ERγ resulted from duplication of ERβ early in the teleost lineage. The ERγ gene may have been created in a gene-specific duplication of ERβ or in a genome duplication event, as has been suggested based on the presence of duplicate members of other gene families in teleost genomes (29).

Once a gene duplicates, the classical model predicts that the resulting redundancy will release one of the descendant paralogs from selective constraints. This release allows the duplicate to degenerate into a nonfunctional pseudogene while the other copy retains the original functions. In rare cases, the duplicate may take on novel functions that constrain further sequence divergence, a process called neofunctionalization (30). Alterna-
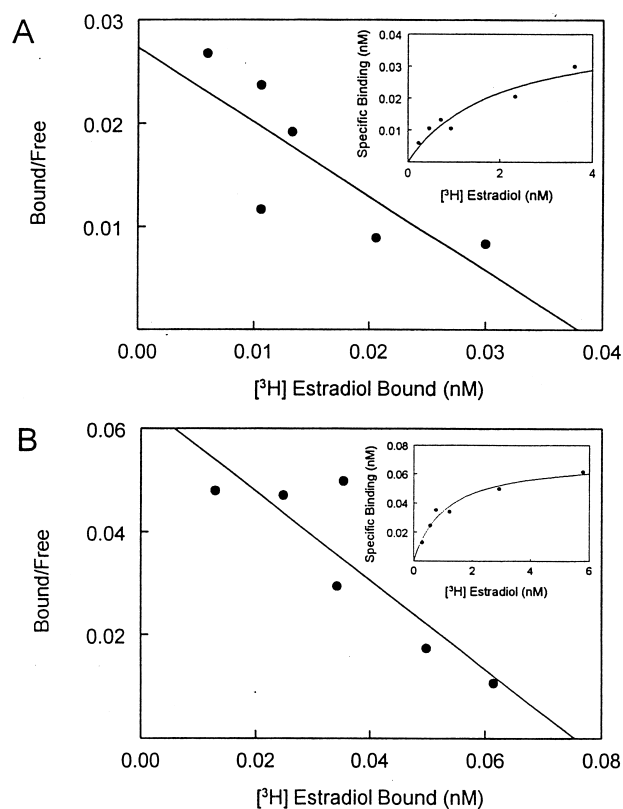
tively, multiple functions of the ancestral gene may be allocated between the two copies (subfunctionalization) (31). The existence of three paralogous genes in one species allows us to evaluate these scenarios. Remarkable conservation of sequence in the DBD (91–98%) and LBD (92–99%) among all pairs of ERγs clearly indicates that duplication of the ERβ gene at least 150 million years ago has not resulted in the creation of a pseudogene.

The patterns of amino acid divergence indicated by relative-rate tests and branch lengths in Fig. 2 are not consistent with subfunctionalization of ancestral ERβ's biochemical functions among the duplicated ERβ and ERγ. The subfunctionalization hypothesis predicts that after a gene duplication, both descendant genes will continue to be subject to strong negative selection, leading to slow rates of sequence change in both genes. The rapid sequence divergence at the base of the ERγ lineage followed by a much slower rate of change within the clade of ERγs instead suggests that one or more novel functions emerged that imposed strong selective constraints on additional sequence divergence. The 22 diagnostic amino acids identified in the ERγs are located in regions known to be important to receptor–ligand interactions, receptor transactivation, and dimerization. Although the significance of the amino acid changes in ERγs is currently unknown, diagnostic residues in other steroid receptors have been shown genetically and structurally to be required for the specific functions that distinguish receptors from each other (32). Thus, altered functions, such as different affinities for specific estrogens or the capacity to interact with different transcriptional coactivators or corepressors, may impose unique selective constraints on further divergence of ERγs.

ERα and ERβ are differentially distributed in the hypothalamus of rats, suggesting distinct functions for the two subtypes in the neuroendocrine control of reproductive physiology and behavior (33). The overall pattern of ER mRNA distribution in the brain of fish parallels that of mammals (34), but the distribution of ERα and ERβ subtypes in the fish hypothalamus has not been previously determined. The present results show, through *in situ* hybridization, that acERα, -β, and -γ mRNAs have different patterns of expression in the croaker hypothalamus. Their unique distributions within this region of the brain suggests that each ER gene product may serve distinct neuroendocrine roles. The differences in tissue distributions of acER α, β, and γ transcripts in estrogen target tissues suggest that they have different functions in these tissues as well. The differential expression of these receptors in tissues that are estrogen-responsive in nonteleost vertebrates (i.e., ovary, testis, and liver) supports the hypothesis that subfunctionalization has occurred by partitioning spatial domains of receptor expression among duplicated ERs. It remains possible, however, that acERβ and acERγ have taken on novel cell-type-specific functions within these tissues. A detailed comparison of the distribution, regulation, and functions of ER types throughout the life cycle of croaker and nonteleost taxa may suggest which of the functions of ERγ are evolutionary novelties and which are the result of partitioning ancestral ERβ's functions to specific cell types.

ERs are perhaps the most pervasively important and best understood ligand-activated transcription factors in vertebrates. The identification of three functional ERs in a well characterized physiological model provides a unique opportunity to investigate the partitioning of functions among ER subtypes and the dynamics by which they evolved. The existence of ERγ in teleost fish illustrates the importance of gene duplication in the evolution of steroid hormone receptors. It is conceivable that at some point in evolution, an independent duplication of ERα or ERβ has also occurred in other vertebrates to create a novel paralog analogous to the teleost ERγ. Recent studies suggest that some genomic actions of estrogen cannot be attributed to either ERα or ERβ. For example, E2 continues to protect against vascular injury in both ERα and ERβ knockout mice, suggesting either redundant functions for ERα and ERβ, or the presence of a third receptor (7). Studies with the recently developed ERα/ERβ knockout mouse (35) may provide an indication of whether a third ER also exists in mammals.

Environmental estrogens have long been known to have deleterious effects in wildlife, including fish (36). The high levels of ERγ expression in tissues such as the testes, whose functions are particularly susceptible to interference by environmental estrogens (10, 36), suggest ERγ may mediate some of the endocrine disrupting effects of these compounds, including the widespread feminization of male fish in rivers receiving substantial inputs of sewage (37).

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