Sequence analysis and expression of the P450 aromatase and estrogen receptor genes in the *Xenopus* ovary

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Abstract

Recent studies point to a key role for the estrogen synthesizing enzyme P450 aromatase (P450 arom) in ovary determination in fish, birds and reptiles. It is unclear whether estrogen synthesis is important in sex determination of *Xenopus* gonad. To determine whether the aromatase gene is transcribed in the gonads of *Xenopus* tadpoles during the sex determination, we cloned a P450 arom cDNA and examined the level of P450 arom and estrogen receptor (ER) gene expression in association with estrogen activity. cDNA clones for P450 arom were isolated from a *Xenopus* ovarian cDNA library. There was an open reading frame (ORF) of 1500 bp from the ATG start to TAA stop codons encoding 500 predicted amino acids. cDNAs for P450 arom have previously been cloned from various vertebrates. The homology between the *Xenopus* P450 aromatase and the human P450 arom was higher. The expression of the P450 arom gene was mainly limited to reproductive organs. To determine the beginning of estrogen activity in gonads of embryos, expression of the aromatase and ER gene was also examined by RQ-RT-PCR. Both *Xenopus* aromatase and ER mRNA was detected at stage 51 in gonads. These observations are consistent with estrogens having a key role in ovarian development in various other vertebrates. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Estrogen synthesis; Gonads; P450 aromatase

1. Introduction

P450 aromatase catalyzes the conversion of androgen to estrogen, which is a rate-limiting step in estrogen biosynthesis [1]. cDNA clones encoding aromatase have been isolated from human [2], rat [3], bovine [4], chicken [5], rabbit [6], catfish [7] and tilapia [8]. The aromatase activity in cell is closely related to the level of the very labile aromatase mRNA species, suggesting that transcriptional control is the main mechanism of regulation of enzyme activity [9]. Recent studies have shown that the regulation of P450 arom gene expression is important because estrogens are considered to play a role in sex differentiation. Treatment of embryos or larvae with sex steroid hormones have led to sex

reversal in fish [10], reptiles [11,12] and birds [13,14]. A major role of estrogen and aromatase in ovary differentiation has also been confirmed by the use of aromatase inhibitors [15–19]. In contrast to the well-defined physiological functions of P450 arom and estrogens of fish, birds and reptiles [20–22], very little is known about the molecular characteristics and mechanisms of the regulation of transcription of the P450 arom gene in *Xenopus* ovaries [23]. In the present study, we characterized a full-length cDNA of the *Xenopus* ovary aromatase gene and compared it with vertebrates.

Estrogen exerts its biological effects through a specific nuclear receptor protein, which functions as ligand activated transcription factor, and a *Xenopus* estrogen receptor (ER) cDNA has been cloned [24]. Therefore, we examined the level of P450 arom and ER mRNA during development of embryos by real-time quantitative polymerase chain reaction after reverse transcription (RQ-RT-PCR) [25].

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2. Materials and methods

2.1. Isolation of RNA and cDNA synthesis

Total RNA was isolated from adult ovary, testis, liver, kidney and heart of *Xenopus* and gonads with the attached mesonephros of *Xenopus* tadpoles at various stages with RNAzol B (Cinna/Biotecx Laboratories, Inc.). The total RNA of gonads was used for RQ-RT-PCR. Poly (A) RNA of the adult ovary was isolated using an oligo (dT) column. A total of 3 µg poly (A) RNA was used for first- and second-strand cDNA synthesis (Stratagene). The double-stranded cDNA was ligated into λZAP II and packaged with Gigapack-Gold (Stratagene) according to the manufacturer's instructions.

2.2. RACE and sequence analysis

The sequence of a *Xenopus* P450 arom partial length cDNA (Miyata et al., 1999) was used to design primers for Rapid Amplification of cDNA Ends (RACE) [26] and the cDNA library was used as template. For 5′-RACE, semi-nested PCR was performed using primer pairs T3 and arom-R1 for the first PCR and T3 and arom-R2 for the second PCR. Primers were as follows, T3, 5′-AATTAACCCTCACTAAAGGG-3′; arom-R1, 5′-GCAGCTATTAGCATTTCCAGAATA-C-3′; arom-R2, 5′-ACATTCTCAGCTGTTAGAT-CTCC-3′. For 3′-RACE, semi-nested PCR was performed using primer pairs T7 and arom-F1 for the first PCR and T7 and arom-F2 for the second PCR. Primers were as follows, T7, 5′-GTAATACGACTCACTAT-AGGGC-3′; arom-F1, 5′-TTCTGAGAAGCTGGAT-GAGGATATG-3′; arom-F2, 5′-CATCAGAACTCAT-ATTTGCCC-3′. Reaction mixtures of 50 µl contained EX Taq PCR buffer, 2 mM MgCl$_2$, 0.2 mM dNTP, 0.2 µM primers, 1.25 U EX Taq (Takara Shuzo Co., Ltd., Japan) and 200 ng of cDNA for the first PCR and 1 µl of the first PCR product for the second PCR. Three-step PCR amplification was performed according to the following schedule; 93°C for 1 min, 30 cycles at 93°C for 40 s, 50°C for 40 s, and 72°C for 1.5 min, then 72°C for 10 min. PCR products were analyzed on a 2% agarose gel. A 1000-bp (5′-RACE) and 1800-bp (3′-RACE) cDNA fragment were detected and these fragments were cut out and extracted with GeneClean (BIO 101 Inc., CA, USA). The cDNA fragments were ligated with pGEM-T Easy Vector (Promega, Co., WI, USA) and transfected into *Escherichia coli* (DH5 α) (Toyobo Co., Ltd., Tokyo, Japan). Six clones, for each RACE reaction, were isolated and plasmid DNAs were extracted with the alkaline mini preparation method. The sequences of cDNAs were analyzed with an ABI PRISM 310 sequencing analyzer (Perkin–Elmer Applied Biosystems, CA, USA) using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer Applied Biosystems). Consensus sequences were determined by comparing the sequences of the six clones.

2.3. RQ-RT-PCR

RQ-RT-PCR was carried out using the following primers, P450 arom forward primer, 5′-CCCT-CATTTCTTATGGCCGTTT-3′ (198–219) and P450 arom reverse primer, 5′-CGCCATTAATCCAGACT-CTCAC-3′ (278–299); ER forward primer, 5′-GCA-CTTTCGCAGTAAGG-3′ (457–471) and ER reverse primer, 5′-CGGGACACATCATATAGTCATTAT (694-715). Using these primer sets, we obtained a single DNA fragment for each set of primer, which had the predicted sequence (data not shown). Real-time information was obtained using an integrated system for thermal cycling, real-time fluorescence detection and subsequent analysis (Perkin–Elmer ABI PRISM 7700 Sequence Detector System). For the reporter fluorescent dye, SYBR Green I was used in a SYBR Green PCR Reagents Kit (Perkin–Elmer Applied Biosystems). Reaction mixtures of 50 µl contained the SYBR Green PCR buffer, 3 mM MgCl$_2$, dNTP's (0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, and 0.6 mM dUTP), 0.2 µM primers, 1.25 U AmpliTaq Gold (PE biosystems), 1 U uracil-*N*-glucosidase (UNG) and cDNA obtained from 200 ng total RNA. The two-step amplification protocol consisted of a 2-min incubation step at 50°C (digestion of PCR product contaminants by UNG), 10 min at 95°C (inactivation of UNG, denaturation of target DNA, and activation of Ampli-Taq Gold), followed by target amplifications via 50 cycles of 15 s at 95°C and 1 min at 57°C. The measurements were performed in triplicate.

3. Results

3.1. Nucleotide and deduced amino acid sequence of P450 arom cDNA

The sequence of the 2191-bp containing complete open reading frame (ORF) of the P450 arom cDNA is shown in Fig. 1. Despite similarities in the coding regions of P450 arom among various species, the sizes of the transcripts differed markedly. Two transcripts of 3.4 and 2.9 kb in size were observed in human tissues [6] and there are three rat transcripts of 3.3, 2.6 and 1.9 kb [3]. The large size of transcripts appears to result from the presence of a long 3′-untranslated region, which could have a role in the stability of transcripts. This cDNA appears to be the same length as the cDNA insert of catfish P450 arom (2102 bp). The first initiation codon was 32 bp from the 5′-end, with an ORF of

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        10        20        30        40        50        60        70        80        90       100       110
GCACGAGGCAGGGCCTTGACAAGCCGTGAATATGGAAGCCTTGAATCCAGTGCAGTATAACATCACAGAAGCTGTTCCCACTCTGGCACCTGCCACTACTCTTTCTCTGC
            *             M  E  A  L  N  P  V  Q  Y  N  I  T  E  A  V  P  T  L  A  P  A  T  T  L  S  L  L

       120       130       140       150       160       170       180       190       200       210       220
TGCTCTTCATTTTTGTGCTCATCATTCTATGGAATCAAGAGGAGACATCTCTGATACCAGGCCCAGCTTATTGCATGGGACTCGGGCCCCTCATTTCTTATGGCCGTTTT
 L  F  I  F  V  L  I  I  L  W  N  Q  E  E  T  S  L  I  P  G  P  A  Y  C  M  G  L  G  P  L  I  S  Y  G  R  F

       230       240       250       260       270       280       290       300       310       320       330
CTACTGACAGGAATTGGCAAAGCAGCAAATTACTACAACAACATGTATGGAGAATTTGTGAGAGTCTGGATTAATGGCGAGGAAACACTGATTATCAGCAAATCTTCAGC
 L  L  T  G  I  G  K  A  A  N  Y  Y  N  N  M  Y  G  E  F  V  R  V  W  I  N  G  E  E  T  L  I  I  S  K  S  S  A

       340       350       360       370       380       390       400       410       420       430       440
AACATTTCACATCATGAAACACAGCCATTATGTCTCACGCTTTGGAAGCAAGCTAGGGCTACAGTGCATTGGCATGAATGAAAATGGCATCATATTCAATAGCAACCCAT
 T  F  H  I  M  K  H  S  H  Y  V  S  R  F  G  S  K  L  G  L  Q  C  I  G  M  N  E  N  G  I  I  F  N  S  N  P  S

       450       460       470       480       490       500       510       520       530       540       550
CTCTATGGAAGGTCATTCGGCCATACTTCATCAGAGCTTTGTCTGGTCCAGGACTTATGCAAACAACAGAAAACTGTATAAGATCTACAAATCACTACCTGGATAACCTG
 L  W  K  V  I  R  P  Y  F  I  R  A  L  S  G  P  G  L  M  Q  T  T  E  N  C  I  R  S  T  N  H  Y  L  D  N  L

       560       570       580       590       600       610       620       630       640       650       660
AGTAATGTTACAAATGAACTGGGAAATGTAGATGTCCTTAAGCTAATGAGGCTTATTATGTTAGATACATCAAACAATCTCTTCCTAAGGATACCCTTAGATGAAAGTGA
 S  N  V  T  N  E  L  G  N  V  D  V  L  K  L  M  R  L  I  M  L  D  T  S  N  N  L  F  L  R  I  P  L  D  E  S  E

       670       680       690       700       710       720       730       740       750       760       770
AATTGTTCTTAAGATCCAGAAATACTTTGACGCCTGGCAGGCTCTGCTTCTGAAACCAGACATCTTCTTTAAAATTTCCTGGCTGTACAAGAAATATGAAAAATCAGCAA
 I  V  L  K  I  Q  K  Y  F  D  A  W  Q  A  L  L  L  K  P  D  I  F  F  K  I  S  W  L  Y  K  K  Y  E  K  S  A  N

       780       790       800       810       820       830       840       850       860       870       880
ATGATCTGAAGGAAGCTATTGAACTTCTCATTGAACAGAAAAGACAGAAACTCTCAAGTTCTGAGAAGCTGGATGAGGATATGGATTTTTCATCAGAACTCATATTTGCC
 D  L  K  E  A  I  E  L  L  I  E  Q  K  R  Q  K  L  S  S  S  E  K  L  D  E  D  M  D  F  S  S  E  L  I  F  A

       890       900       910       920       930       940       950       960       970       980       990
CAGAATCATGGAGATCTAACAGCTGAGAATGTCAATCAGTGTATTCTGGAAATGCTAATAGCTGCTCCCGATACCATGTCTGTATCTCTCTTCTTCATGTTAGTTCTGAT
 Q  N  H  G  D  L  T  A  E  N  V  N  Q  C  I  L  E  M  L  I  A  A  P  D  T  M  S  V  S  L  F  F  M  L  V  L  I

      1000      1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
TGCTCAACACCCAAAGATAGAAGAAGGAATAATGAATGAAATGGATAAAGTTATTGGTAACCGGGATGTAGAGAGCAATGACATTCCAAATCTTAAAATTCTGGAGAGCT
 A  Q  H  P  K  I  E  E  G  I  M  N  E  M  D  K  V  I  G  N  R  D  V  E  S  N  D  I  P  N  L  K  I  L  E  S  F

      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200      1210
TTATTTATGAAAGCATGAGGTACCAACCAGTGGTAGACCTGGTTATGCGCAAGGCTCTGGAGGATGATATCATTGATGGTTACTATGTGAAGAAAGGCACTAACATCATT
 I  Y  E  S  M  R  Y  Q  P  V  V  D  L  V  M  R  K  A  L  E  D  D  I  I  D  G  Y  Y  V  K  K  G  T  N  I  I

      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
TTAAACTTGGGGCGCATGCACAAAATTGTATACTTTCCAAAACCGAACGAGTTCACCTTGGAAAATTTTGAAAAGACGGTTCCATATCGTTACTTCCAGCCCTTTGGCTC
 L  N  L  G  R  M  H  K  I  V  Y  F  P  K  P  N  E  F  T  L  E  N  F  E  K  T  V  P  Y  R  Y  F  Q  P  F  G  S

      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430
TGGTCCACGTGCATGTGCCGGGAAGTACATAGCCATGGTGATGATGAAAGTCATTCTGGTTACTCTTCTCAAGAGGTACAAAGTGCAGACATTGAGAGGAAGATGCCTGG
 G  P  R  A  C  A  G  K  Y  I  A  M  V  M  M  K  V  I  L  V  T  L  L  K  R  Y  K  V  Q  T  L  R  G  R  C  L  E

      1440      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540
AGAATATCCAAAATAACAATGATTTGTCCATGCACCCTGATGAAAGTCAACCTTCCTTAGAGATGATCTTCATTCCTAAAAACACAGCAGAGTTCAAACTGTAACAGTGA
 N  I  Q  N  N  N  D  L  S  M  H  P  D  E  S  Q  P  S  L  E  M  I  F  I  P  K  N  T  A  E  F  K  L  *

      1550      1560      1570      1580      1590      1600      1610      1620      1630      1640      1650
ATATAATCCCATATATGTCAACTCTGGAAATTACAAATGTTATTAGCATAGAGTTGGTGACAGTGTTGGTCTGTGTTAAACTTTGATTTAACCGCTGCTCTTGTTTGCTC

      1660      1670      1680      1690      1700      1710      1720      1730      1740      1750      1760
TTTGTAATTATCAGTCACTAAAAAATAGTGCAACAATCATAACTGTCAGGGTTTTCAGTTTTTCTACTGTTAATGTTGGAAAATGACATTCAGCAGCTTTAAAGGATGAGT

      1770      1780      1790      1800      1810      1820      1830      1840      1850      1860      1870
AAAACCAACAAATGAAAATGTCCTAAAGGGGTTTTTAACAGAATTGCCATTCAAGCAGTTTATAAAAAGCTTGATATCTACATTTGAAAATACTTGTTTTTCTGGTAAGGC

      1880      1890      1900      1910      1920      1930      1940      1950      1960      1970      1980
TTCGGGGAGCAGTTTGTTAATATGTATTATCCAAGGCCCCATTAAATACAGTAGAGTGGGCAGAACATTATTTAGACACAAACCAAAGATGGTTGTGCATGACAGGGCTT

      1990      2000      2010      2020      2030      2040      2050      2060      2070      2080      2090
TGATTAGAAGAAAACATTTTAATTGAATTTAAGAATAGATACAAATATGCCAGGTCAAACTAATCATTTTATTTTTTTTTGTGTCACTGTTTTTTAAAGGCTGTGCTTTA

      2100      2110      2120      2130      2140      2150      2160      2170      2180      2190      2200
TACTCCTGTCCAGTACAGTGATATTTTTATTGTTTATATTTATCAGTATTATTGATAATAAATATGCCTATATTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide and deduced amino acid sequence of the P450 arom of *Xenopus* ovary. The asterisks upstream and downstream of the protein coding region indicate the stop codons. The ORF of 1500 bp encodes a protein of 500 residues. There is a putative polyadenylation signal (AATAAA underline) 14 bases from the first A of the poly A tract.

1500 nucleotides encoding a putative 500 amino acid residues to the TAA stop codon at 1532 bp. This ORF was slightly shorter than the human and bovine forms (504 residues) and longer than chicken (496 residues). The 3′-untranslated region of *Xenopus* P450 arom was 659 bp long. The polyadenylation signal, AATAAA, was 14 bases from the first A of the poly A tract.

3.2. Comparison of amino acid sequences of P450 arom

We compared the amino acid sequence derived from the cDNA of *Xenopus* P450 arom with derived amino acid sequences from other P450 arom isoforms identified in several species (Fig. 2). P450 arom from *Xenopus* shared over 71% amino acid identity with that of human P450 arom, and 61% nucleotide identity with that of human P450 arom, which was greater than any of the other species sequenced to date [2]. Amino acid identities of the *Xenopus* P450 arom with the human, rabbit, chicken, bovine, rat, catfish and tilapia P450 arom are 71, 71, 70, 68, 67, 53, and 53%, respectively.

The first 22 amino acids have no obvious hydrophobic or amphipathic stretch. The region that can be described as hydrophobic lies between amino acids 23 and 37, and is believed to comprise a membrane-anchoring domain. However, the amino acid sequence of *Xenopus* P450 arom does not have very high identity with that of human P450 arom. There are four long stretches of conserved residues among all species. An α-helix-(residues 288–322), Ozol's peptide (residues 347–370), an aromatic region (residues 405–416) and a heme-binding region (residues 429–453) were defined and showed a higher degree of identity. The helix region for *Xenopus* P450 arom has 32 of 35 amino acid match with the human sequence [2]. The P450 Ozol peptide region, which is presumed to be involved in steroid substrate binding, of *Xenopus* versus human shows identity at 20 of 23 residues [2]. The aromatic region of *Xenopus* P450 arom shows 100% identity (12 residues) with the human sequence. The heme-binding region shows identity at 22 of 25 residues [2].

3.3. P450 arom mRNA expression in various Xenopus tissues.

Total RNA fractions were prepared from various tissues in adult *Xenopus* and aromatase expression was determined by RQ-RT-PCR (Fig. 3). Aromatase was either expressed markedly or at extremely low levels in these tissues. In adult *Xenopus*, aromatase was expressed in the ovary and testis. Extremely low levels of aromatase mRNA was found in kidney, heart and liver.
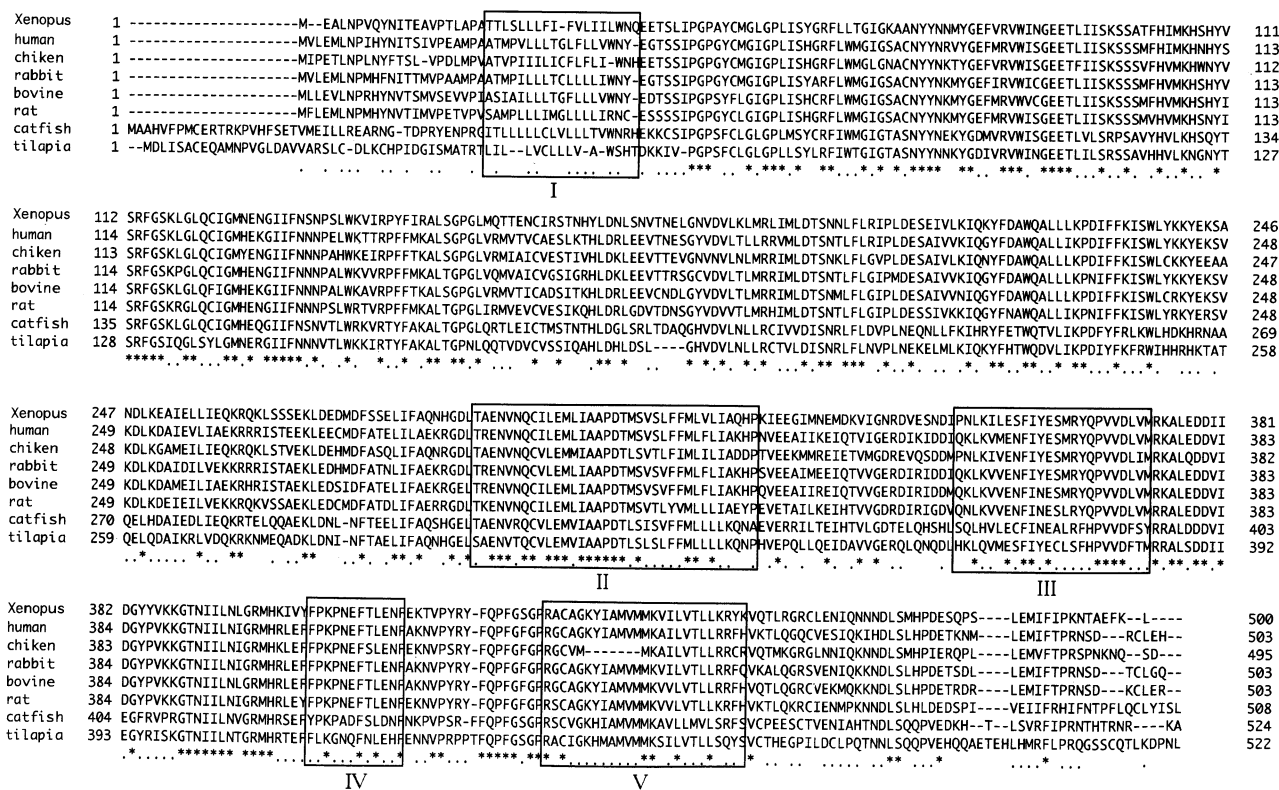

Fig. 2. Alignment of deduced amino acid sequences of *Xenopus*, human, rat, bovine, chicken, catfish and tilapia P450 arom. The identical and similar amino acid residues are denoted by asterisks and dots. Gaps were introduced to improve alignment (−). Roman numerals indicate the membrane-anchoring (I), Helix (II), Ozol's peptide (III), aromatic (IV) and heme-binding (V) regions.
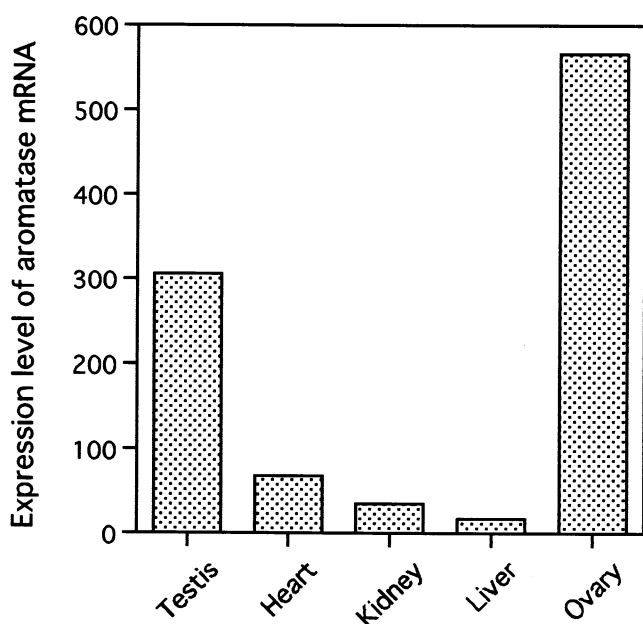
Fig. 3. Expression level of aromatase mRNA in various tissues of *Xenopus*. Total RNA was obtained from various tissues. Expression levels of aromatase mRNA were determined by real-time quantitative RT-PCR. Measurements were performed in triplicated.

4. Discussion

Estrogen is implicated in sex differentiation of fish, avians and reptiles. We cloned a cDNA for aromatase, which is essential for estrogen synthesis, from *Xenopus* ovaries. The predicted amino acid sequence of the aromatase showed high identity with that of human [2].

Xenopus tadpoles are capable of steroidogenic activity in the interrenal region after stage 47, but not in the gonads between stages 50 and 52 [28,29]. Steroid metabolism and production in the gonads of *Rana catesbeiana* also occur after gonadal differentiation [30]. Furthermore, an ER cDNA in *Xenopus* has been cloned and expression was detected in the gonads at stage 55 [31]. Previous studies indicate that the first morphological signs of ovarian and testicular differentiation in *Xenopus* embryos appears between stages 50 and 53 [32], that is, before transcription of ER. In *Xenopus*, it is unlikely that estrogen plays a role in sex determination, although estrogen may be involved in normal gonadal differentiation. However, administration of estradiol to *Xenopus* causes embryos to become female [27,33,34]. If estrogen is responsible for inducing sex differentiation, then estrogen and ER must be produced prior to or during sex differentiation. However, because

In reproductive organs, the highest level of expression was found in ovary.

3.4. P450 arom and ER mRNA expression in tadpole gonads

We previously reported that the sensitivity to exogenous estradiol occurred at stage 50–52 [27]. Sexual differentiation is a sequential and orderly process, and the step that is sensitive to exogenous estradiol in the sex determination cascade is probably important during the natural development of tadpoles. We determined the stage of initiation of transcription of P450 arom and ER gene in the natural sex determination of *Xenopus* by RQ-RT-PCR.

Aromatase transcripts were detected in gonads from stage 51, but not at stage 50. Expression was low at stage 51, increased slightly at stage 52, and then decreased slightly during stages 53 and 54 (Fig. 4). At stage 56, aromatase expression increased markedly. ER transcripts were also detected in the ovary at embryonic stage 51 (Fig. 5). The low levels of ER mRNA continued during stages 51–55. Thereafter, ER mRNA in gonads increased markedly. The profile of ER gene transcription was the same as that of the aromatase gene.
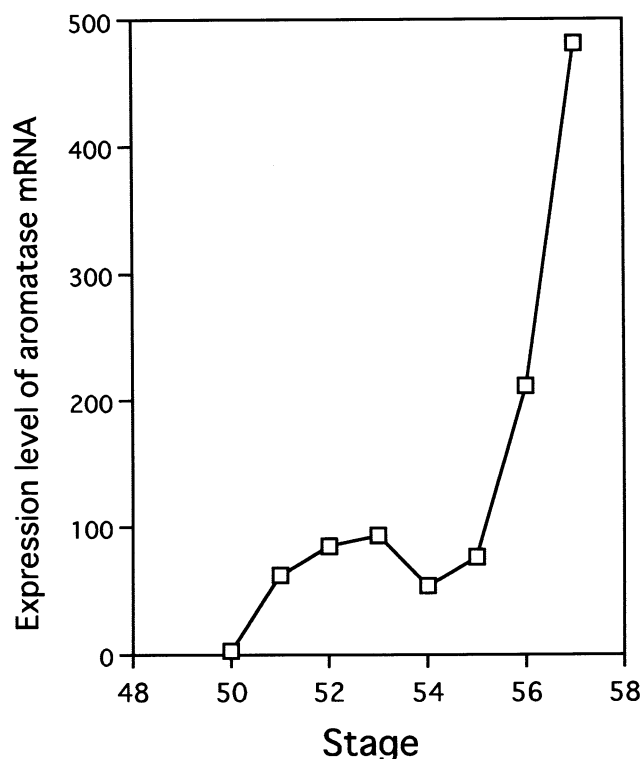

Fig. 4. Expression level of aromatase mRNA in *Xenopus* gonads at various differentiation stages. Total RNA was obtained from gonads at various stages of differentiation. Expression levels of aromatase mRNA were determined by real-time quantitative RT-PCR. Measurements were performed in triplicated.
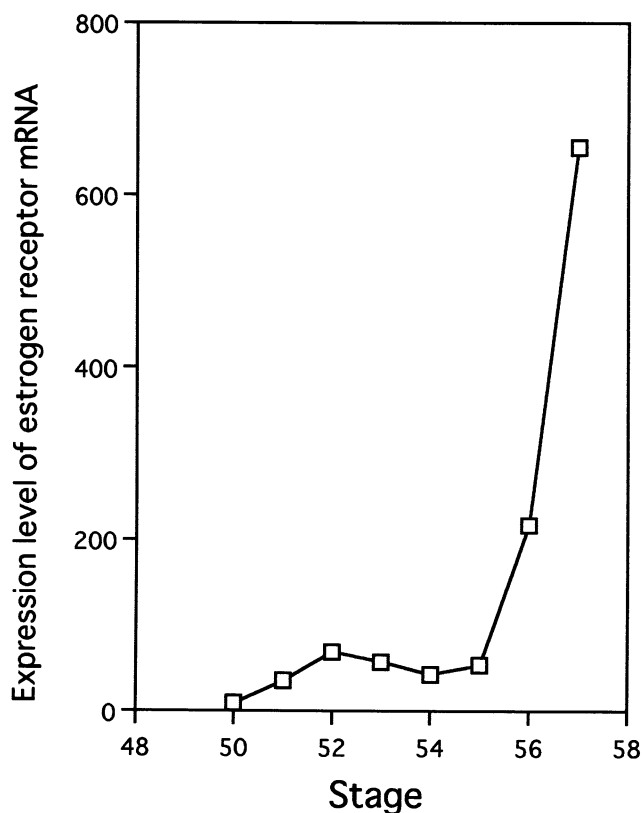
Fig. 5. Expression level of ER mRNA in *Xenopus* gonads at various differentiation stages. Total RNA was obtained from gonads at various stages of differentiation. Expression levels of aromatase mRNA were determined by real-time quantitative RT-PCR. Measurements were performed in triplicate.

a very small amount of estrogen is sufficient for ovarian differentiation, only a very small amount of steroidogenic enzyme may be required. Therefore, estrogen synthesis might not be detected by ordinary methods. We decided to determine the stage of initiation of transcription of P450 arom and ER in *Xenopus* sexual differentiation by highly sensitive RQ-RT-PCR. We detected both aromatase and ER transcripts in the gonad during sex differentiation at low levels at stages 51 and 52. These findings suggest that estrogen involvement in sex differentiation in *Xenopus* gonads is mediated by the ER. The factors controlling the initiation of aromatase or ER gene expression in the sex differentiation of the *Xenopus* ovary are not known. In studies of mammals, it has been reported that two critical regulatory proteins, Sry and SF-1, can bind to aromatase promoter elements in vitro [35–37]. Homologous regulatory proteins, which control aromatase gene, may exist in ovary during sex differentiation of *Xenopus*.

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