Pergamon

0960-0760(95)00172-7

J. Steroid Biochem. Molec. Biol. Vol. 55, No. 2, pp. 261–269, 1995

0960-0760/95 $9.50 + 0.00

Reptilian Sex Steroid Receptors: Amplification, Sequence and Expression Analysis

Larry J. Young,* John Godwin, Mike Grammer, Manfred Gahr† and David Crews

Institute of Reproductive Biology, Department of Zoology, University of Texas at Austin, Austin, TX 78712, U.S.A.

Sex steroid hormones secreted by the gonads play a central role in the reproduction of all vertebrates. In addition to direct effects on gametogenesis, sex steroid hormones are important in sexual development, brain organization, and sexual behavior. The actions of sex steroid hormones are mediated primarily by ligand-dependent transcription factors, or receptors which bind to specific sequences of the DNA and alter the transcription rates of nearby genes. We have used the polymerase chain reaction to amplify cDNA fragments of the estrogen receptor, progesterone receptor and androgen receptor from the unisexual whiptail lizard, *Cnemidophorus uniparens*. The lizard steroid hormone receptors share a high degree of sequence homology to the steroid hormone receptors of other vertebrates. Ribonuclease protection assays demonstrate that both estrogen receptor mRNA and progesterone receptor mRNA are increased in the oviduct during vitellogenesis and after estrogen treatment. This report demonstrates the utility of the polymerase chain reaction to generate species specific probes for comparative molecular studies and provides the first report of cDNA sequences for reptilian steroid hormone receptors.

J. Steroid Biochem. Molec. Biol., Vol. 55, No. 2, pp. 261–269, 1995

INTRODUCTION

Sex steroid hormones secreted by the gonads play a central role in the reproduction of all vertebrates. In addition to direct effects on gametogenesis, sex steroid hormones are important in sexual development, brain organization, and sexual behavior. The actions of sex steroid hormones are mediated primarily by ligand-dependent transcription factors which bind to specific sequences of the DNA and alter the transcription rates of nearby genes [1–5]. These receptors are members of an ancient superfamily of genes that evolved from a common ancestral gene prior to the divergence of the vertebrate taxa. Steroid receptor cDNA's have now been cloned in several species representing diverse vertebrate groups (see references for Fig. 5). The sequences of these genes reveal a common structural organization and very strong sequence homology in certain functional domains across species. This highly conserved nature of certain sex steroid receptor domains, especially of those coding the steroid- and DNA-binding portions of the proteins, facilitates the cloning of these genes for use in molecular studies in unconventional animal models.

Comparative studies in endocrinology and neuroendocrinology are useful for identifying generalities as well as novelties in sex steroid hormone action. Our laboratory has focused on the reproductive physiology and behavior of reptiles, particularly *Cnemidophorus uniparens*, a unisexual species of whiptail lizard inhabiting the desert grasslands of Arizona and New Mexico. Our interest in the effects of sex steroid hormones on the reproductive biology of whiptail lizards prompted us to clone fragments of the sex steroid receptors from lizard tissue.

Based on sequence information available from other species, we used the polymerase chain reaction (PCR) to amplify fragments of the estrogen receptor (ER), progesterone receptor (PR), and the androgen receptor (AR) from *C. uniparens*. Sequence analysis indicates a high degree of sequence homology between the reptil-

*Correspondence to L. J. Young at: Department of Psychiatry, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.
†Current Address: Max Planck Institut fur Verhaltensphysiologie, D-8130 Seewiesen, Starnberg, Germany.
Received 21 Apr. 1995; accepted 17 Jul. 1995.

ian receptor genes and those of other vertebrates. The ER and PR clones were then used to generate probes to analyze ER-mRNA and PR-mRNA expression and regulation in whiptail oviduct. These clones have also proven useful in the analysis of steroid receptor expression and regulation in the lizard brain.

The sequences provided here are the first sex steroid receptor sequence data to be published for reptiles and should be useful for other investigators attempting to generate homologous probes for comparative endocrine studies. These results also demonstrate the utility of the relatively simple PCR method for generation of these probes for examining sex steroid receptor expression.

MATERIALS AND METHODS

Animals

Adult *C. uniparens* were captured near Portal, Arizona (U.S.A.), transported to the University of Texas, and housed in environmental chambers as previously described [6]. All animals were housed in groups of four in 29 gallon aquaria. Each aquarium was supplied with a heat lamp, water dish, a small board for basking and cover and sand as substrate. Animals were fed crickets or meal worms three times per week. Some of the animals used in the gene regulation study were ovariectomized as previously described [6], allowed to recover for 1 week, injected with 0.5 μg of 17β-estradiol 3-benzoate (Sigma) in 10 μl of Steroid Suspension Vehicle (SSV; National Cancer Institute, Bethesda MD), or 10 μl of SSV without steroid, and tissue was taken 24 h after treatment. All animals were killed by rapid decapitation. Dissected tissue was frozen immediately on dry ice and stored at −80°C until use.

RNA extraction and cDNA synthesis

Total RNA was extracted from oviduct and kidney tissue using a variation of the procedure described by Chomczynski and Sacchi [7]. Briefly, approx. 100 mg of oviduct or kidney tissue was homogenized with a polytron in 10 vol (v/w) of denaturation solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, 0.5% *N*-lauroylsarcosine). After homogenization, 1/10th vol of 2 M sodium acetate (pH 4.0), and an equal vol of a mixture of water-saturated phenol–chloroform–isoamyl alcohol added and vortexed as described by Chomczynski and Sacchi [7]. After 15 min incubation on ice to precipitate the DNA, the mixture was centrifuged at 10,000 *g* and the aqueous phase removed and precipitated in 100% isopropanol. The pellet was then resuspended in 300 μl of denaturation solution, extracted with buffered phenol–chloroform–isoamyl (25:24:1) and reprecipitated in 100% isopropanol. The resulting pellet was washed for 15 min in 75% ethanol at room temperature to remove any residual guanidinium salts and, finally, resuspended in 100 μl of RNase-free water. The RNA was quantified using spectrophotometry and the integrity of the sample assessed by visual inspection on a denaturing agarose gel.

Complementary DNA for ER and PR amplification was synthesized from the 10 μg RNA extracted from oviduct tissue using random hexamer primers and

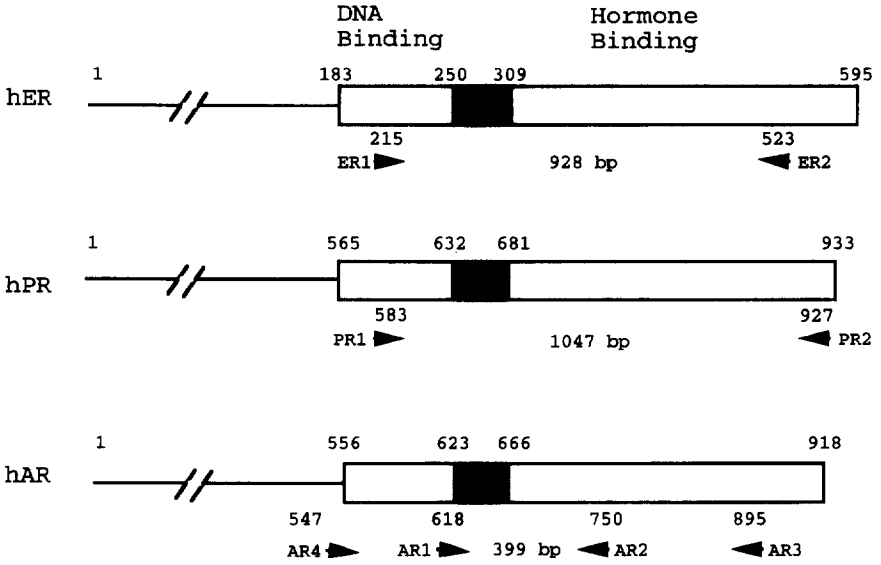

Fig. 1. Schematic illustrating the positions of the primers used to amplify the whiptail estrogen receptor, progesterone receptor and androgen receptor cDNA fragments. The structures of the human estrogen receptor (hER), progesterone receptor (hPR) and androgen receptor (hAR) are illustrated to demonstrate the relative position of the primer sequences used to amplify the lizard sequences. The numbers positioned above and below the human receptor structures are amino acid sequence positions for the human estrogen receptor [8], progesterone receptor [12] and androgen receptor [14].

```
1    GlyHisAsnAspTyrMetCysProAlaThrAsnGlnCysThrIleAspLysAsnArgArg
     GGGCACAACGACTATATGTGTCCTGCTACCAACCAATGCACCATTGACAAGAATAGGAGA

61   LysSerCysGlnAlaCysArgLeuArgLysCysTyrGluValGlyMetMetLysGlyGly
     AAAAGCTGCCAGGCTTGCAGGCTTCGCAAATGCTATGAAGTTGGAATGATGAAAGGTGGA

121  IleArgLysAspArgArgGlyGlyArgIleLeuLysHisLysArgGlnArgGluGluHis
     ATTCGGAAAGATCGCAGAGGTGGCCGAATACTGAAACATAAGCGGCAGAGAGAAGAACAT

181  AspAsnArgAsnAlaGlyAlaIleValGluArgArgSerProAsnLeuTrpProSerPro
     GATAACAGGAATGCAGGGGCTATAGTTGAGAGAAGGAGCCCCAATCTTTGGCCAAGTCCT

241  LeuMetIleThrHisAsnLysLysAsnSerProAlaLeuSerLeuThrAlaAspGlnIle
     CTAATGATCACACACAACAAAAAGAATAGCCCAGCTCTGTCTCTGACTGCCGATCAGATT

301  ValSerAlaLeuLeuGluAlaGluProProValValTyrSerGluTyrAspProSerArg
     GTTAGTGCCTTGCTAGAAGCTGAGCCACCTGTCGTTTATTCCGAGTATGATCCTAGCAGG

361  ProPheSerGluAlaSerMetMetThrLeuLeuThrAsnLeuAlaAspArgGluLeuVal
     CCTTTCAGTGAAGCTTCTATGATGACGCTGTTGACCAACCTCGCTGACCGAGAACTGGTG

421  HisMetIleAsnTrpAlaLysArgValProGlyPheValAspLeuSerLeuHisAspGln
     CACATGATCAACTGGGCCAAAAGAGTTCCAGGGTTTGTGGATTTATCACTCCATGATCAG

481  ValHisLeuLeuGluCysAlaTrpLeuGluIleLeuMetIleGlyLeuValTrpArgSer
     GTCCATCTACTGGAATGTGCCTGGTTAGAGATACTGATGATTGGATTAGTGTGGAGATCA

541  ValGluHisProGlyLysLeuLeuPheAlaProAsnLeuLeuLeuAspArgAsnGlnGly
     GTGGAACATCCAGGAAAGCTACTGTTTGCTCCTAACCTACTCTTGGACAGGAATCAAGGA

601  LysCysValGluGlyPheValGluIlePheAspMetLeuLeuAlaThrSerSerArgPhe
     AAGTGCGTTGAGGGTTTTGTGGAAATATTTGACATGCTGCTGGCTACTTCTTCTCGCTTT

661  ArgMetMetAsnValGlnGlyGluGluPheValCysLeuLysSerIleIleLeuLeuAsn
     CGAATGATGAATGTCCAAGGGGAAGAATTTGTGTGCCTTAAATCCATCATCCTACTCAAT

721  SerGlyIleTyrThrPheLeuSerSerThrLeuLysSerLeuGluGluLysAspHisIle
     TCTGGTATCTATACATTTCTTTCCAGCACTTTAAAGTCATTGGAAGAAAAGGACCATATC

781  HisArgValLeuAspLysIleIleAspThrLeuLeuHisLeuMetAlaLysSerGlyLeu
     CATCGTGTTCTGGACAAAATCATTGACACTTTGTTGCATTTGATGGCCAAGTCAGGCCTC

841  SerLeuGlnGlnGlnHisArgArgLeuAlaGlnLeuLeuLeuIleLeuSerHisPheArg
     TCTTTGCAGCAGCAGCATAGACGGTTGGCTCAGCTCCTTCTCATTCTTTCCCACTTTAGG

901  HisMetSerAsnLysGlyMet
     CACATGAGCAACAAAGGCATGGA
```

Fig. 2. Nucleotide sequence and predicted amino acid sequence of the whiptail estrogen receptor PCR fragment (LYCUER 1). The sequences with a dotted underline at the beginning and end of sequence are the primers. The single solid underlined sequence is an EcoR I sight and the double underlined sequence is the Hind III site used to subclone the receptor to aid in sequencing and to produce the ribonuclease protection assay probe.

M-MuLV reverse transcriptase using the First Strand Synthesis Kit (Strategene). Complementary DNA for the AR amplification was synthesized from 9.2 μg of RNA extracted from kidney tissue using the AR3 primer (below) and M-MuLV reverse transcriptase using the Superscript first strand synthesis kit (Gibco BRL).

Polymerase Chain Reaction (PCR)

Primer sequences used for PCR amplification were chosen on the basis of sequence homology comparisons between published amino acid sequences for each receptor: ER—human [8], chicken [9], rat [10]; PR—rabbit [11], chicken [12], human [13]; and AR—rat and

```
     CysGlySerCysLysValPhePheLysArgAlaMetGluGlyGlnHisAsnTyrLeu
1    CCTGTGGAAGCTGTAAAGTCTTCTTCAAGAGGGCAATGGAAGGACAGCATAACTACTTA

     CysAlaGlyArgAsnAspCysIleValAspLysIleArgArgLysAsnCysProAlaCys
60   TGTGCTGGGCGGAATGATTGCATTGTGGATAAAATTCGTCGGAAGAACTGTCCTGCGTGT

     ArgLeuArgLysCysCysGlnAlaValMetValLeuGlyGlyArgLysPheLysLysPhe
120  CGATTAAGGAAGTGCTGCCAAGCTGTTATGGTGCTGGGAGGTCGCAAATTTAAGAAGTTT

     AsnLysValLysValLeuArgAlaLeuAspValValAlaLeuGlnGlnProThrValLeu
180  AATAAAGTCAAAGTTCTACGCGCGTTGGATGTTGTGGCACTCCAACAGCCCACAGTCCTT

     ProAsnGluHisGlnThrLeuValGlnArgLeuSerTyrSerProThrGlnAspValGln
240  CCCAATGAACACCAAACCTTGGTACAGAGGCTGTCTTATTCTCCGACTCAAGATGTTCAA

     PheIleProProLeuIleSerIleLeuGlnSerIleGluProGluValValTyrAlaGly
300  TTTATTCCTCCGCTGATCAGCATCTTGCAAAGCATCGAGCCAGAAGTGGTCTATGCAGGT

     TyrAspAsnThrGlnProGluThrProSerIleLeuLeuThrSerLeuAsnGlyLeuCys
360  TATGACAACACGCAACCAGAGACTCCAAGCATTTTGTTGACCAGCCTCAATCAGTTGTGT

     GluArgGlnLeuLeuCysValValLysTrpSerLysSerLeuProGlyPheArgAsnLeu
420  GAAAGGCAACTTCTCTGTGTAGTCAAGTGGTCCAAATCGTTGCCAGGATTTCGGAATTTG

     HisIleAspAspGlnIleThrLeuIleGlnTyrSerTrpMetAsnLeuMetValPheAla
480  CATATTGACGATCAGATAACCCTTATCCAATATTCATGGATGAACTTAATGGTCTTTGCC

     MetAlaTrpArgSerTyrLysHisValSerGlyGlnMetLeuTyrPheAlaProAspLeu
540  ATGGCCTGGAGATCTTACAAGCATGTCAGTGGCCAGATGCTGTATTTTGCACCTGATCTA

     IleLeuAsnGluAspGlyIleArgGlnLysMetLysGluSerSerPheTyrSerLeuCys
600  ATATTAAATGAGGATGGAATAAGACAGAAGATGAAAGAATCATCGTTCTACTCACTATGC

     LeuSerMetTrpArgIleProGlnGluPheValLysLeuGlnLeuThrAlaGluGluPhe
660  TTGTCCATGTGGCGGATACCACAAGAGTTTGTCAAATTACAACTAACCGCTGAAGAGTTC

     LeuCysMetLysAlaLeuLeuLeuLeuSerThrIleProLeuGluGlyLeuArgSerGln
720  CTTTGCATGAAGGCCTTGCTTCTTCTAAGCACAATACCGTTGGAAGGTCTCAGAAGCCAA

     GlyGlnPheAspGluMetArgSerSerTyrIleArgGluLeuValLysAlaIleGlyLeu
780  GGCCAGTTTGATGAAATGAGATCAAGTTACATTCGAGAACTAGTCAAAGCCATTGGGTTG

     ArgAlaLysGlyValValAlaSerSerGlnArgPheTyrGlnLeuThrLysLeuMetAsp
840  CGGGCGAAGGGAGTTGTGGCTAGCTCTCAACGTTTCTACCAGCTGACAAAACTGATGGAC

     SerMetHisAspLeuValLysGlnLeuHisLeuPheCysLeuAsnThrPheLeuGlnSer
900  TCCATGCATGATCTTGTGAAACAACTCCATCTGTTCTGTTTGAACACATTTCTCCAGTCC

     ArgAlaLeuCysIleGluPheProGluMetMetSerGluValIleCysAlaGlnLeuPro
960  CGGGCTTTGTGCATTGAATTTCCAGAGATGATGTCAGAAGTAATCTGTGCGCAACTTCCC

      GlnAsnProAlaGlyMetValLys
1020 AAAATCCCGGCAGGGATGGTGAAACC
```

Fig. 3. Nucleotide sequence and predicted amino acid sequence of whiptail progesterone receptor PCR fragment (LYCUPR 3). The sequences with a dotted underline at the beginning and end of sequence are the primers. The solid underline is the Hinc II site used to subclone the receptor to produce the ribonuclease protection assy probe. The double underlined amino acids are residues which appear to be an insertion since they are not found in human, rat or chicken PR sequences.

human [14]. The position and orientation of the primers are illustrated in Fig. 1. The primers used to amplify ER and AR were degenerate primers. However, the primers used to amplify PR were not degenerate primers since analysis of the cDNA sequences of human, chicken and rabbit indicated that the regions used to generate the primers were 100% conserved at the nucleotide level. The primer sequences are as follows: ER1, 5′-GG(AGT) CA(CT) AA(CT) GA(TC) TA(TC) ATG TG-3′; ER2, 5′-TCC AT(GT) CC(CT) TT(AG) TT(AG) CTC AT-3′, PR1, 5′-CCT GTG GAA GCT GTA AAG TCT TC-3′; PR2, 5′-GGT TTC ACC ATC CCT GCC A-3′; AR1, 5′-TG(TC) TA(TC) GA(AG) GCI GGI ATG AC-3′; AR2, 5′-CCA (IC)CC CAT NGC (AG)AA NAC CAT-3′; AR3, 5′-GCC ATC AT(CT) TCI GG(AG) AA(GA)-3′; AR4, 5′-CC(IC) AU(UCA) GA(UC) UA(UC) UA(UC) UU(UC) CC-3′ where bases in parentheses denote degenerate positions, I represents inosine and N represents a degenerate position containing all four nucleotide possibilities. These primers were purchased from the Midland Certified Reagent Company (Midland, TX).

For the amplification of the ER fragment, optimum conditions for PCR amplification was determined to be 2 mM $MgCl_2$, 0.2 mM dNTP, and 20 pmol primer per 100 μl reaction. Each sample was overlain with 100 μl of mineral oil to minimize evaporation. One-tenth of the cDNA synthesis reaction was used in each PCR sample. Prior to the addition of Taq polymerase, the sample was denatured at 92°C for 5 min. then cooled to 50°C to allow annealing of the primers. Five units of Taq polymerase (Promega) was added and the samples were carried through the following thermal cycle for 30 cycles: 3 min extension at 72°C, 1 min denaturation at 92°C and 1 min at 50°C for primer annealing.

Amplification of the PR cDNA fragment was performed as described for ER with the exception of the thermal cycle which was as follows: 3 min extension at 72°C, 30 s denaturation at 94°C and 1 min at 54°C for primer annealing. The AR cDNA fragment was amplified using a nested primer strategy with primers AR4 and AR3 and one tenth of the cDNA reaction (1 μl) being used in a first round PCR. One 1 μl of this first-round PCR reaction was then used in a second round amplification using primers AR1 and AR2. The PCR conditions were as for ER except that 4 mM $MgCl_2$ was used, the denaturation step was at 94°C, and primer annealing was at 53°C. Analysis of the PCR products on agarose gels revealed bands of the appropriate size fragments.

Cloning and sequencing

The PCR amplified ER cDNA fragment was blunt-end cloned into the Sma I site of the pGEM 7f+ (Promega) to generate a 928 bp clone (LYCUER 1). The PCR amplified PR and AR fragments were cloned into the pCRII plasmid supplied in the TA Cloning Kit (Invitrogen), resulting in a 1047 bp PR clone (LYCUPR 3) and a 399 bp AR clone (JGCUAR 1). This cloning strategy proved much more efficient than the blunt end cloning used for the ER fragment. In order to aid in sequencing, LYCUER 1 and LYCUPR 3 were subcloned utilizing internal restriction enzyme sites. The clones were sequenced using the Sequenase 2.0 Kit (USB). The sequences were analyzed and the predicted amino acid sequences were produced with the aid of the computerized sequence analysis program (Microgenie, Beckman).

```
     CysTyrGluAlaGlyMetThrLeuGlyAlaArgLysLeuLysLysLeuGlyAsnLeuLys
1    TGTTACGAGGCGGGGATGACGCTTGGAGCCCGCAAGTTGAAGAAGCTTGGCAACCTGAAG

     MetGlnGlyGluGlyGluAlaAlaGlyProSerSerProThrGluGluGlnAlaProLys
61   ATCCAGGAGGAAGGGGAGGCTGCTGGCCCCTCCAGCCCCACCGAAGAGCAGGCGCCCAAG

     LeuThrValSerHisValAspSerLeuGluCysGlnProIlePheLeuAsnValLeuGlu
121  TTGACCGTGTCACACGTGGACAGCCTAGAATGCCAGCCCATCTTCCTCAACGTCCTGGAA

     AlaIleGluProGlyValValCysAlaGlyTyrAspAsnAsnGlnProAspSerPheAla
181  GCCATCGAGCCTGGCGTGGTTTGTGCGGGATACGACAACAACCAGCCGGATTCCTTCGCC

     ThrLeuLeuThrSerLeuAsnGluLeuGlyGluArgGlnLeuValHisValValLysTrp
241  ACGCTCCTGACCAGCTTGAACGAGCTTGGCGAGAGACAGTTGGTCCACGTCGTCAAATGG

     AlaLysAlaLeuProGlyPheArgAsnLeuHisValAspAspGlnMetAlaIleIleGln
301  GCGAAAGCTTTGCCAGGGTTCCGCAACTTGCATGTGGATGACCAGATCGCCATAATTCAG

     TyrSerTrpMetGlyLeuMetValPheAlaMetGlyTrp
361  TACTCCTGGATGGGCCTGATGGTCTTTGCTATGGGCTGG
```

Fig. 4. Nucleotide sequence and predicted amino acid sequence of the whiptail androgen receptor (JGCUAR 1). The sequences with a dotted underline at the beginning and end of sequence are the primers.

Ribonuclease protection assay

Tissue from two sets of animals were analyzed. The ovariectomized, vitellogenic and postovulatory tissues were collected to analyze changes in mRNA expression as a function of ovarian condition while the ovariectomized + 0 μg or ovariectomized + 0.5 μg EB tissues were collected to determine the effects of estrogen on mRNA expression (see Fig. 6). Since the tissues from the two groups were processed separately, comparisons should only be made within the groups. Ribonuclease protection assays (RPA) were performed

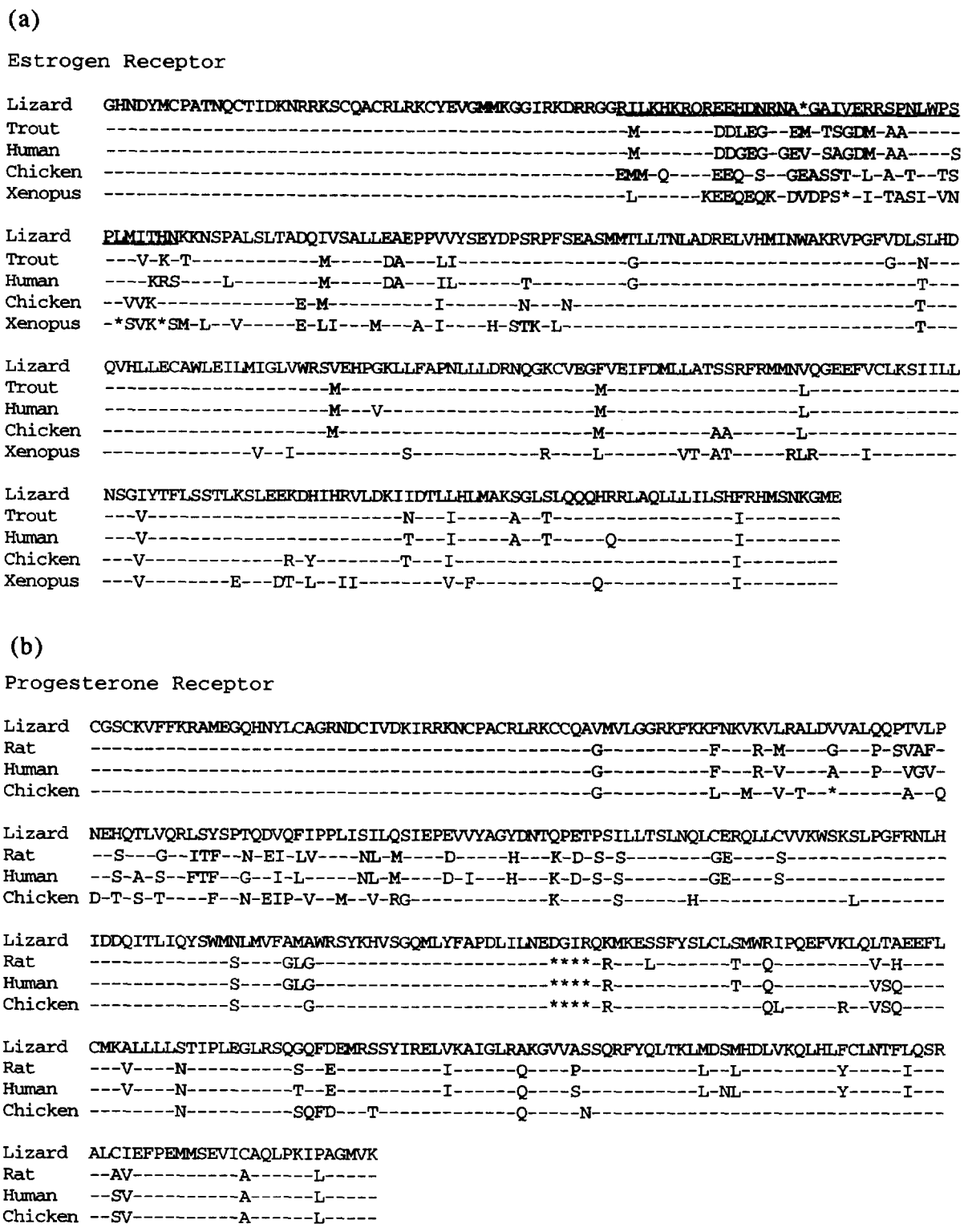

Fig. 5(a and b)—*legend opposite.*

(c)

Androgen receptor

```
Lizard  CYEAGMTLGARKLKKLGNLKMQGEGEAAGPS***SPTEEQAPKLTVSHVDSLECQPIFLNVLEAIEPGVVCAGYDNNQPDSFA
Rat     ----------------------L-E---NSSAG***----DPSQ-M----IEGY----------------------H---------
Human   ----------------------L------SSTT***-----TTQ------IEGY----------------------H---------
Canary  ----------------------A-DDI-G-SS-***----------VMTRI-GY----------------------H--S-----S
Xenopus ----------------------A-E-LDWIFSAGRR-SKELSPGMGIPQLLE--S--------------V-----H---------

Lizard  TLLTSLNELGERQLVHVVKWAKALPGFRNLHVDDQMAIIQYSWMGLMVFAMGW
Rat     A--S-------------------------------V---------------
Human   A--S-------------------------------V---------------
Canary  N---------------------------------S---------------
Xenopus L--S-----------------N----------S---T-------
```

Fig. 5. Amino acid sequence homology of the lizard (a) estrogen receptor, (b) progesterone receptor and (c) androgen receptor with receptors of other species. The sequences given were taken from the Genbank data base and are referenced as follows: ER (trout, [28]; xenopus, [29]; chicken, [9]; human, [30]), PR (chicken, [12]; rat, [31]; human, [13]), and AR (rat, [32]; human, [33], Canary [34], Xenopus [Picard, Genbank accession #x58955].

using the RPA II kit (Ambion, Austin, TX). Total RNA was extracted from oviducal tissue as described above. 20 μg of total RNA were used for sample. 20 μg of yeast tRNA was used in the control to insure that probe digestion was complete. Tissue samples representing each treatment group were pooled from 4 individuals prior to extraction. Antisense ^{32}P-labeled RNA probes were produced by *in vitro* transcription using SP6 and T7 RNA polymerases from subclones generated from the ER and PR clones. The subclones were generated by cutting the original clones at the Hind III site for LYCUER 1 and Hinc II site for LYCUPR 3 to generate clones containing the 5′ end of each clone. Transcription of these clones produced probes 372 bp and 394 bp in length for ER and PR, respectively. The results of each RPA were analyzed on X-ray film and each assay was replicated at least 3 times with similar results.

RESULTS AND DISCUSSION

Sequence analysis

The nucleotide and deduced amino acid sequences for the lizard ER, PR and AR polymerase chain reaction products are presented in Figs 2–4. In each case the DNA-binding domain and the steroid-binding domain of the lizard sex steroid receptors share a high degree of sequence homology with other species (Fig. 5). The hinge sequences are less conserved. The most convincing demonstration that a putative sex steroid receptor cDNA encodes the functional protein is the cloning of the full-length transcript, followed *in vitro* translation and characterization of the ligand binding properties of the protein. While this was not performed in this study, several lines of evidence indicate that the sequences described do represent fragments of the genes encoding the lizard ER, PR, and

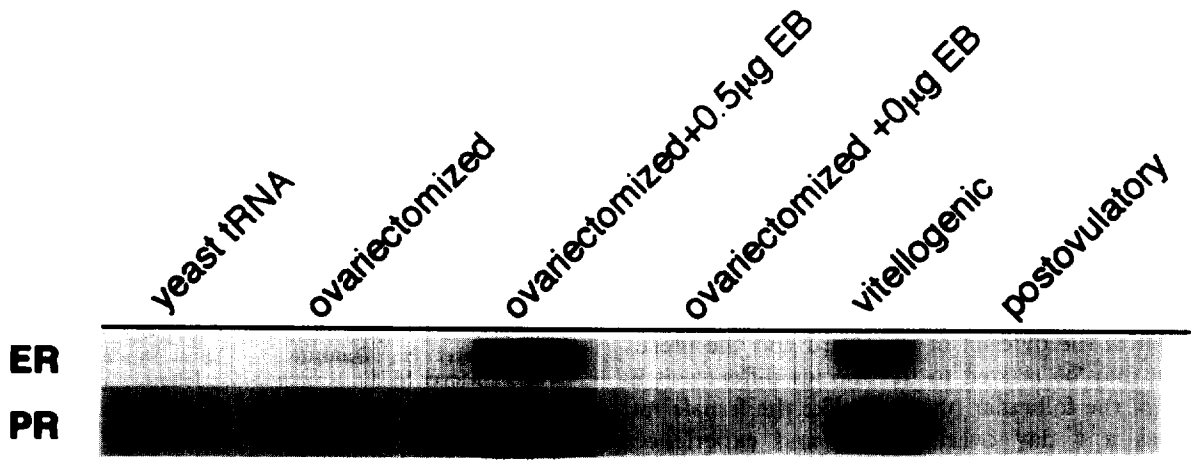

Fig. 6. Representative ribonuclease protection assay results for ER- and PR-mRNA in whiptail lizard oviduct. Hybridization of the probes with 20 μg of yeast tRNA produced no signal. For both ER- and PR-mRNA, expression was highest in animals with elevated levels of estrogen, i.e. ovariectomized +0.5 μg estradiol benzoate (EB) and vitellogenic animals.

AR respectively. The sequence homology of each putative receptor fragment with those described in other vertebrates is very high in the functionally important steroid- and DNA-binding domains. Second, the pattern of expression of these sequences in the whiptail lizard brain [15] matches the strongly-conserved distribution of steroid-concentrating cells in the lizard *Anolis carolinensis* [16] and other vertebrates [17] and distribution of ER-like immunoreactivity found in whiptail lizard brain with the H222 ER antibody (M. Gahr and D. Crews, unpublished data). Lastly, expression of the putative ER- and PR-mRNAs are regulated by estrogen in discrete areas of the brain [18] and oviduct (see results below).

The nucleotide and deduced amino acid sequence of a fragment of the androgen receptor from *C. inornatus*, a sexual ancestor of *C. uniparens* [19], has been described previously [20]. Interestingly, the androgen receptor sequence from the all-female, parthenogenically reproducing *C. uniparens* reported here shares 99% homology with that of *C. inornatus*, suggesting that AR function may be necessary for normal development and reproduction in females.

Regulation of estrogen receptor and progesterone receptor gene expression

In reptiles, as in mammals and birds, estrogen plays an important role in preparing the reproductive tract for reproduction [21]. In the whiptail lizard, ovulation is preceded by vitellogenesis, during which estrogen levels are elevated and oviducal mass increases approx. 5-fold [22]; this oviducal mass increase is estrogen dependent in lizards [21]. Analysis of mRNA expression indicates that both ER-mRNA and PR-mRNA in the oviduct are stimulated by estrogen. Ovariectomized or postovulatory animals had little ER-mRNA or PR-mRNA compared to estrogen treated or vitellogenic individuals (Fig. 6). While the upregulation of PR-mRNA by estradiol is consistent with that reported in other species [11, 23, 24], the upregulation of ER-mRNA by estrogen in the reproductive tract is opposite to that found in rat [25] or immature chickens [26]. However, our findings are supported by measurements of oviducal estrogen and progestin binding sites in ovariectomized estradiol-primed females of the lizard *Podarcis s. sicula* [27]. A single injection of estradiol increased *Podarcis* oviducal ER binding approx. 5-fold in cytosolic and nuclear fractions in 12–24 h and PR binding 2–3-fold in 12–48 h. The difference in the regulation of ER-mRNA expression in the oviduct of the lizard and the uterus of the rat may be related to the species differences in duration of the follicular phase. Unlike the female rat, which has a 4 day ovarian cycle and experiences elevated estrogen for a single day of that cycle, oviparous reptiles often experience elevated levels of estrogen for days or weeks while the eggs are yoking. Thus, it may be necessary to become increasingly sensitive rather than decreasingly sensitive to estrogen during the follicular phase in the reptile.

Our data represents the first report of the molecular cloning and sequence analysis of sex steroid receptor gene fragments in reptiles. These data not only provide valuable information on the sequence of the sex steroid receptor genes of a reptile, but also illustrate how species-specific molecular probes can be rapidly generated and used to examine tissue expression of these receptors. The primers and methodology presented here should be applicable to many vertebrate species. This is also the first analysis of ER and PR gene expression in the reproductive tract of reptile.

Acknowledgements—We wish to thank James Skipper and Judith Bergeron for the technical advice and assistance that they provided. This research was supported by an NSF Predoctoral Fellowship to LJY, an NIH individual NRSA (NS 09219) to JG, and NIMH Training Grant 18837, NIMH MERIT Award 41770, and NIMH Research Scientist Award 00135 to DC.

REFERENCES

1. Beato M.: Gene regulation by steroid hormones. *Cell* **56** (1989) 335–344.
2. Carson-Jurica M. A., Schrader W. T. and O'Malley B. W.: Steroid receptor family: Structure and functions. *Endocrine Rev.* **11** (1990) 201–220.
3. Evans R. M.: The steroid and thyroid hormone receptor superfamily. *Science* **240** (1988) 889–894.
4. Truss M. and Beato M.: Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. *Endocrine Rev.* **14** (1993) 459–479.
5. Laudet V., Hanni C., Coll J., Catzeflis F. and Strehelin D.: Evolution of the nuclear receptor gene family. *EMBO* **11** (1992) 1003–1013.
6. Wade J. and Crews D.: The effects of intracranial implantation of estrogen on receptivity in sexually and asexually reproducing female whiptail lizards, *Cnemidophorus uniparens* and *C. inornatus*. *Horm. Behav.* **25** (1991) 342–353.
7. Chomczynski P. and Sacchi N.: Single step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. *Analyt. Biochem.* **162** (1987) 156–159.
8. Green S., Walter P., Kumar V., Drust A., Bornet J-M., Argos P. and Chambon P.: Human estrogen receptor cDNA: sequence, expression, and homology to v-erb-A. *Nature* **320** (1986) 134–139.
9. Krust A., Green S., Argos P., Kumar V., Walter P., Bornert J-M. and Chambon P.: The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. *EMBO* **5** (1986) 891–897.
10. Koike S., Sakai M. and Muramatsu M.: Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucl. Acids Res.* **15** (1987) 2499–2513.
11. Loosfelt H., Atger M., Misrahi M., Guiochon-Mantel A., Meriel C., Logeat F., Benarous R. and Milgrom E.: Cloning and sequence analysis of rabbit progesterone-receptor complementary DNA. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 9045–9049.
12. Gronemeyer H., Toucotte B., Quirin-Skticker C., Bocquel M. T., Meyer M. E., Krozowksi Z., Jeltsch J. M., Lerouge T., Garnier J. M. and Chambron P.: The chicken progesterone receptor: sequence, expression and functional analysis. *EMBO J.* **6** (1987) 3985–3994.
13. Misrahi M., Atger M., d'Auriol L., Loosfelt H., Meriel C., Fridlansky F., Guiociion-Mantel A., Galibert F. and Milgrom E.: Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA. *Biochem. Biophys. Res. Commun.* **143** (1987) 740–748.

14. Chang C., Kokontis J. and Liao S.: Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. *Proc. Natn. Acad. Sci. U.S.A.* **85** (1988) 7211–7215.
15. Young L. J., Lopreato G., Horan K. and Crews D.: Cloning and *in situ* hybridization of estrogen receptor, progesterone receptor and androgen receptor expression in the brain of whiptail lizards (*Cnemidophorus uniparens* and *C. inornatus*). *J. Comp. Neurol.* **347** (1994) 288–300.
16. Morrell J., Crews D., Ballin A., Morgentaler A. and Pfaff D. W.: ^{3}H-estradiol, ^{3}H-testosterone, and ^{3}H-dihydrotestosterone localization in the brain of the lizard, *Anolis carolinensis*: an autoradiographic study. *J. Comp. Neurol.* **188** (1979) 201–224.
17. Pfaff D. W. and Schwartz-Giblin S.: Cellular and molecular mechanisms of female reproductive behaviors. In: *The Physiology of Reproduction. Volume 2. Second Edition* (Edited by E. Knobil and J. D. Neill). Raven Press, NY (1994) pp. 107–220.
18. Young L. J., Nag P. K. and Crews D.: Regulation of estrogen receptor messenger ribonucleic acid by estrogen in the brain of the whiptail lizard (*Cnemidophorus uniparens*). *J. Neuroendocr.* 7 (1995) 119–125.
19. Wright J. W.: Evolution of the lizards of the genus *Cnemidophorus*. In: *Biology of Whiptail Lizards* (Edited by J. W. Wright and L. J. Vitt). Oklahoma Museum of Natural History (1993) pp. 27–82.
20. Lopreoto G.: Cloning and sequencing of the androgen receptor from the little striped whiptail lizard, *Cnemidophorus inornatus*. Master's Dissertation, University of Texas at Austin (1994).
21. Fox H.: The urogenital system of reptiles. In: *Biology of the Reptilia, Vol. 6: Morphology E* (Edited by C. Gans and T. S. Parsons). Academic Press, NY (1977). pp. 1–157.
22. Moore M. C., Whittier J. M. and Crews D.: Sex steroid hormones during the ovarian cycle of an all-female, parthenogenetic lizard and their correlation with pseudosexual behavior. *Gen. Comp. Endocr.* **60** (1985) 144–153.
23. Conneely O. M., Sullivan W. P., Toft D. O., Birnbaumer M., Cook R. G., Maxwell B. L., Zarucki-Schulz T., Greene G. L., Schrader W. T. and O'Malley B. W.: Molecular cloning of the chicken progesterone receptor. *Science* **233** (1986) 767–770.
24. Tokarz R. R., Crews D. and McEwen B. S.: Estrogen-sensitive progestin binding sites in the brain and oviduct of the lizard, *Anolis carolinensis*. *Brain Res.* **220** (1981) 95–105.
25. Shupnik M. A., Gordon M. S. and Chin W. W.: Tissue specific regulation of rat estrogen receptor mRNA. *Molec. Endocr.* 3 (1989) 660–665.
26. Maxwell B. L., McDonnell D. P., Conneely O. M., Schulz T. Z., Greene G. L. and O'Malley B. W.: Structural organization and regulation of the chicken estrogen receptor. *Molec. Endocr.* 1 (1987) 25–35.
27. Paolucci M. and Fiore M. M. D.: Estrogen and progesterone receptors in lizard *Podarcis s. sicula* oviduct: seasonal distribution and hormonal dependence. *J. Exp. Zool.* **269** (1994) 432–441.
28. Pakdel F., Guellec C. L., Vaillant C., Roux M. G. L. and Valotaire Y.: Identification and estrogen induction of two estrogen receptors (ER) messenger ribonucleic acids in the rainbow trout liver: Sequence homology with other ERs. *Molec. Endocr.* 3 (1989) 44–51.
29. Weiler I. J., Lew D. and Shapiro D. J.: The *Xenopus laevis* estrogen receptor: sequence homology with human and avian receptors and identification of multiple estrogen receptor messenger ribonucleic-acids. *Molec. Endocr.* 1 (1987) 355–362.
30. Greene G. L., Gilna P., Waterfield M., Baker A., Hort Y. and Shine J.: Sequence and expression of human estrogen receptor complementary DNA. *Science* **231** (1986) 1150–1154.
31. Park-Sarge O. K. and Mayo K. E.: Regulation of progesterone receptor gene by gonadotropins and cyclic adenosine 3′,5′-monophosphate in rat granulosa cells. *Endocrinology* 134 (1994) 709–718.
32. Tan J., Joseph D. R., Quarmby V. E., Lubahn D. B., Sar M., French F. S. and Wilson E. M.: The rat androgen receptor: Primary structure, autoregulation of its messenger ribonucleic acid, and immunocytochemical localization of the receptor protein. *Molec. Endocr.* 2 (1988) 1276–1285.
33. Govindan M. V.: Specific region in hormone binding domain is essential for hormone binding and trans-activation by human androgen receptor. *Molec. Endocr.* 4 (1990) 417–427.
34. Nastiuk K. L. and Clayton D. F.: Seasonal and tissue-specific regulation of Canary androgen receptor messenger ribonucleic acid. *Endocrinology* 134 (1994) 640–649.