Characterization of Unique Truncated Prolactin Receptor Transcripts, Corresponding to the Intracellular Domain, in the Testis of the Sexually Mature Chicken*

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ABSTRACT

We have examined expression of the chicken PRL receptor (cPRLR) gene in different tissues of the chicken by Northern blot analysis. Most tissues examined (ovary, testis, oviduct, kidney, and fat) possess a prominent full-length (4.6-kb) cPRLR transcript. A larger (11.7-kb) transcript is also detected in ovary, oviduct, testis, and kidney after longer exposure. A unique pattern of cPRLR expression was found in the testis of sexually mature chickens, which have an unusually high abundance of three small transcripts (1.2, 1.7, and 2 kb) in addition to the 4.6-kb transcript found in other tissues. Three domain-specific complementary DNA (cDNA) probes were constructed that correspond to the first and second ligand-binding regions in the extracellular domain and the transmembrane-intracellular domain. With these probes, Northern blot analysis of polyadenylated RNA prepared from the testes of a mature (22-week-old) chicken indicates that the highly abundant (1.2- and 1.7-kb) and less abundant (2.0-kb) cPRLR transcripts in testis hybridize only to the intracellular domain probe.

Two types of truncated testis-specific cPRLR transcripts were identified using 5'-RACE (rapid amplification of cDNA ends) analysis of polyadenylated RNA from the testis of a 22-week-old chicken. The predominant truncated cDNA sequence contains the highly conserved box 1 motif [(+)box 1 cDNA] and diverges (at nucleotide 1396) from that of the cPRLR cDNA, just downstream of the transmembrane domain. The other truncated cDNA lacks the box 1 motif [(−)box 1 cDNA], which is replaced by 39 bases that could encode a hydrophobic N-terminus with a putative 5'-untranslated region of 131 bases. Young chickens predominantly express the full-length cPRLR messenger RNA (4.6 kb) in the testis. At the onset of sexual maturity, there is a dramatic increase in abundance of the testis-specific (+)box 1 transcript, whereas expression of the full-length cPRLR is depressed. The presence of truncated [(+) or (−)box 1] cPRLR transcripts in the sexually mature chicken testis suggests a complex mechanism of PRL action on gonadal function. (*Endocrinology* **140:** 1165–1174, 1999)

PRL IS THOUGHT to participate in a variety of physiological functions in birds, including reproduction, maternal behavior, osmoregulation (1, 2), fat metabolism (3, 4), and immunomodulation (5). The importance of PRL in the initiation of maternal behavior in columbid (dove and pigeon) and gallenaceous (chicken and turkey) birds has been well documented by extensive reviews (1, 6, 7). However, the role of PRL in sexual maturation, sperm production, and mating behavior of male birds is poorly understood. Although earlier studies show that exogenous PRL has a strong antigonadal action in sexually mature chickens (8, 9), turkeys (10), or Japanese quail (11), PRL appears to be required for normal gonadal development and for the photosexual response of chickens (12, 13) and turkeys (14–16). In male chickens and turkeys, plasma PRL levels are generally high for the first 2 or 3 weeks after hatching, decline during the juvenile period, increase with the onset of sexual maturity, and then decline in adults (17–19). Collectively, these studies show that PRL is required for normal sexual development, whereas hyperprolactinemia causes cessation of reproductive activity (*i.e.* incubation behavior, ovarian regression, and male impotence) in domestic fowl.

PRL exerts its effects by binding to its membrane-bound cell surface receptor (PRLR), which is a member of the cytokine/GH receptor gene superfamily. The GH/PRL receptor family appears to use a common activation pathway that involves ligand-induced receptor homodimerization and tyrosine phosphorylation of intermediates [*i.e.* Janus kinases (JAKs) and signal transducers and activators of transcription (STATs)] for signal transduction (see Ref. 20 for review). The primary structure of several mammalian PRLRs has been deduced from cloning of the complementary DNAs (cDNAs) (21–25). The PRLR consists of an extracellular ligand-binding domain, a single transmembrane domain, and an intracellular domain involved in signal transduction. Mammalian PRLR isoforms differ in length and composition of the intracellular domain (26), and it appears that different portions of the intracellular domain transduce different signals into cells (27).

Recently, the cDNA sequences of the chicken (28), pigeon (29), and turkey (30) PRLRs have been determined. The avian PRLRs are structurally different from mammalian PRLRs due to the presence of two conserved ligand-binding regions in the extracellular domain. Each repeat contains the essential regions for hormone binding, including the WSXWS motif. Mutational analysis of the pigeon PRLR has shown that the wild-type (containing both ligand-binding domains)

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and a mutant pPRLR (containing only the membrane-proximal domain) have equal ligand binding affinities (29). Although the extracellular domain of avian PRLRs is unusually large, tandem repeats of the extracellular domain are found among other members of this distinct gene family [*i.e.* interleukin-3 (31) and leptin (32, 33) receptors].

In the present study, we report the discovery and initial characterization of two truncated testis-specific chicken PRLR (cPRLR) transcripts, corresponding to the intracellular domain, which differ in either the presence (+) or absence (−) of the highly conserved proline-rich box 1 motif. The abundant expression of the truncated testis-specific (+) box 1 cPRLR isoform in the sexually mature chicken suggests a functional role in reproductive activity.

Materials and Methods

Animals and tissues

Single comb White Leghorn (egg-type) and broiler (meat-type) chickens were used in the present study. One-day-old male and female chicks were obtained from a commercial hatchery (Longnecker's Hatchery, Lancaster, PA), raised in a heated battery-brooder until 3 weeks of age, and transferred to wire cages within a controlled temperature room for the duration of the experiment. Tissue samples were collected, frozen immediately in liquid nitrogen, and stored at −70 C until used for isolation of RNA. Animals were handled and cared for in accordance with the principles and procedures outlined by the university's animal care and use committee.

Materials

Restriction enzymes were obtained from Promega Corp. (Madison, WI) and Life Technologies (Gaithersburg, MD). The Sequenase version 2.0 DNA sequencing kit was purchased from U.S. Biochemical Corp. (Cleveland, OH). Radiolabeled nucleotides were purchased from New England Nuclear (Boston, MA). Oligonucleotides (primers) were prepared by Bio-Synthesis, Inc. (Sanger, TX). A PolyATtract messenger RNA (mRNA) Isolation System was purchased from Promega Corp. (Madison, WI). PCR products were cloned into pCR 2.1 T-vector (Invitrogen, San Diego, CA). Superscript RNA PCR and 5'-RACE kits were purchased from Life Technologies. Components for the ribonuclease (RNase) protection assay were obtained from Ambion, Inc. (Austin, TX). A double antibody testosterone RIA kit was purchased from ICN Diagnostics (Costa Mesa, CA).

### Preparation of cPRLR cDNA probes

Three different probes, corresponding to the first ligand-binding region in the extracellular domain (*extra-1*), the second ligand-binding region (*extra-2*), and the transmembrane-intracellular domain (*intra*) of the cPRLR cDNA (28), were synthesized using RT-PCR. One microgram of polyadenylated [poly(A)] RNA from the ovary of a laying hen was reverse transcribed with random hexamers using 200 U Moloney murine leukemia virus reverse transcriptase according to the manufacturer's instructions for the SuperScript cDNA system (Life Technologies). Oligonucleotide primers corresponding to different regions of cPRLR were synthesized [primers A, B, C, D, E, and F correspond to nucleotides (nt) 70–84, 659–675, 730–753, 1231–1254, 1315–1337, and 2466–2496, respectively] and used in PCR amplification of specific regions in the cDNA (Fig. 1A). Each PCR cycle consisted of 1 min of denaturation at 94 C, 1 min of annealing at 55–60 C, and 1.5 min of extension at 72 C. After 30 cycles of PCR, the amplified DNA products were cloned into pCR2.1 T-vector and sequenced. Using this method, three domain-specific fragments were obtained that correspond to nt 70–675 (*extra-1*), nt 730-1254 (*extra-2*), and nt 1315–2496 (*intra*) of cPRLR cDNA. The three domain-specific cDNA fragments were used in Northern and Southern blot analyses.

Northern and Southern blot analyses

Total cellular RNA was isolated from various chicken tissues by the guanidine thiocyanate lysis method, followed by centrifugation through CsCl (34). Polyadenylated RNA was prepared from total cellular RNA using a magnetic mRNA isolation procedure (PolyATtract, Promega Corp.). Northern blots were performed as described previously (35). After the initial exposure to film, the blots were stripped and rehybridized to a chicken glyceraldehyde 3-phosphate dehydrogenase (cGAPDH) (36) cDNA probe to verify the integrity and consistent transfer of RNA.

Total chromosomal DNA was isolated from chicken liver, digested with restriction endonucleases (*Bam*HI, *Hin*dIII, *Eco*RI, and *Bgl*II), and subjected to Southern blot analysis using standard procedures (37).

FIG. 1. Schematic diagram of the cPRLR cDNA- and domain-specific probes (A). The *extra-1* probe, corresponding to the first tandem repeat, was amplified using primer A (nt 70–84) and primer B (nt 659–675). The second tandem repeat probe (*extra-2*) was amplified using primer C (nt 730–753) and primer D (nt 1231–1254). The *intra* probe, corresponding to the transmembrane (TM) and intracellular domains, was amplified using primer E (nt 1315–1337) and primer F (nt 2466–2496). B, Southern blot analysis of the cPRLR gene. Triplicate samples of genomic DNA (20 μg) were digested with the indicated restriction endonucleases, electrophoresed on an 0.8% agarose gel, and transferred to a nylon membrane. Each blot was hybridized to a domain-specific cPRLR probe. The migration of a DNA ladder is indicated on the *left*.
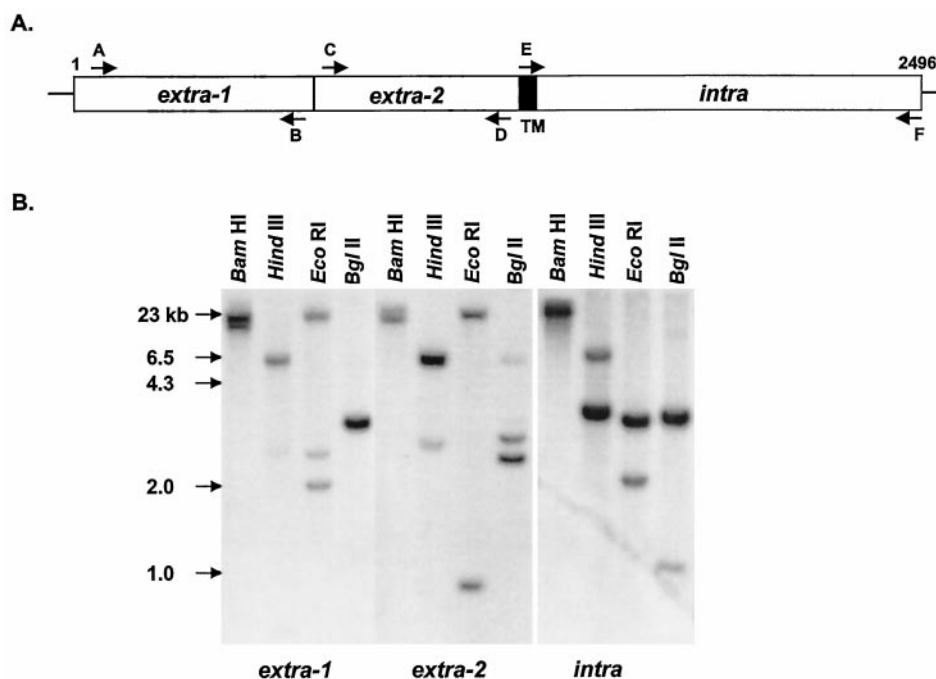
5'-Rapid amplification of cDNA ends (5'-RACE)

Two nested primers were designed based on the known sequence of the chicken cPRLR cDNA (28) and used for 5'-RACE analysis. The primers were designated gene-specific primer 1 (GSP1), which corresponds to nt 1901–1922, and GSP2, which corresponds to nt 1876–1892 (see Fig. 5A). First strand cDNA synthesis was carried out using GSP1 and poly(A) RNA from the testis of a 22-week-old Leghorn rooster. Purification and tailing of the cDNA were performed according to the manufacturer's protocols (Life Technologies). Amplification of dC-tailed cDNA was carried out using GSP2 and a poly(G) primer. PCR conditions were 94 C for 1 min, 55 C for 1 min, and 72 C for 1 min for 35 cycles, and extension at 72 for 5 min. A PCR product(s) of about 600 bp was obtained and directionally cloned into the TA vector (pCR2.1, Invitrogen). Seventeen clones with slightly different sized inserts (~600 bp) were selected for DNA sequencing.

RNase protection assay (RPA)

A RPA was developed to examine expression of different cPRLR transcripts in chicken tissue. The cPRLR riboprobe, complementary to nt 1245–1545 of the cPRLR cDNA (28), was designed to span the transmembrane domain and the box 1 motif. The riboprobe was amplified from total kidney RNA using PCR primers G (nt 1245–1266) and H (nt 1545–1515; see Fig. 5A). RNase digestion yields three protected fragments that correspond to the full-length cPRLR (296 bp), (+)box 1 (151 bp), and (−)box 1 (76 bp) transcripts. The conditions of the cPRLR RPA are similar to those that we recently described for the cGHR RPA (35, 38). Briefly, 35 μg total RNA, 20,000 cpm cPRLR riboprobe, and 20,000 cpm cGAPDH riboprobe (for normalization) were hybridized at 40 C for 16 h and digested with RNase T1 (0.25 U/μl). Protected fragments were separated by electrophoresis in a 6% polyacrylamide-8.3 M urea gel. After autoradiography, protected fragments were excised, and the radioactivity was measured in a β-scintillation counter. Duplicate samples of pooled RNA (35 μg) from testes of 22-week-old broiler chickens were included in each assay so that data could be pooled from different gels.

Results

Southern blot analysis

The domain-specific cDNA probes were used to examine cPRLR gene structure by Southern blot analysis (Fig. 1). The *Eco*RI- and *Bam*HI-digested DNA shows two bands of at least 20 kb that hybridize to the extracellular probes (*extra 1* and *extra 2*), whereas the *intra* probe hybridizes to two slightly larger *Bam*HI-digested fragments (>23 kb). The *intra* probe hybridizes to two smaller *Eco*RI fragments (2 and 3.5 kb). Based on these hybridization patterns and apparent sizes, there appears to be a single gene for the cPRLR that spans at least 40 kb, although this does not rule out the possibility of multiple, nearly identical genes.

Identification of cPRLR isoforms by Northern blot analysis

The expression of cPRLR transcripts in different tissues of adult female and male (9 months of age) Leghorn chickens was examined by Northern blot analysis. Total RNA from all tissues, except muscle and male liver, possesses a large 11.7-kb transcript, which probably represents a primary transcript of partially processed RNA (Fig. 2A). The large transcript was also evident in the female liver and in fat of both sexes after longer exposure. The 4.6-kb cPRLR transcript was found in all tissues that express the large 11.7-kb transcript, whereas the highest levels were found in the kidney, oviduct, and testis. A unique pattern of cPRLR gene expression was found in the testis, where there is an unusually high abundance of two smaller transcripts (1.2 and 1.7 kb). Northern blot analysis of poly(A)
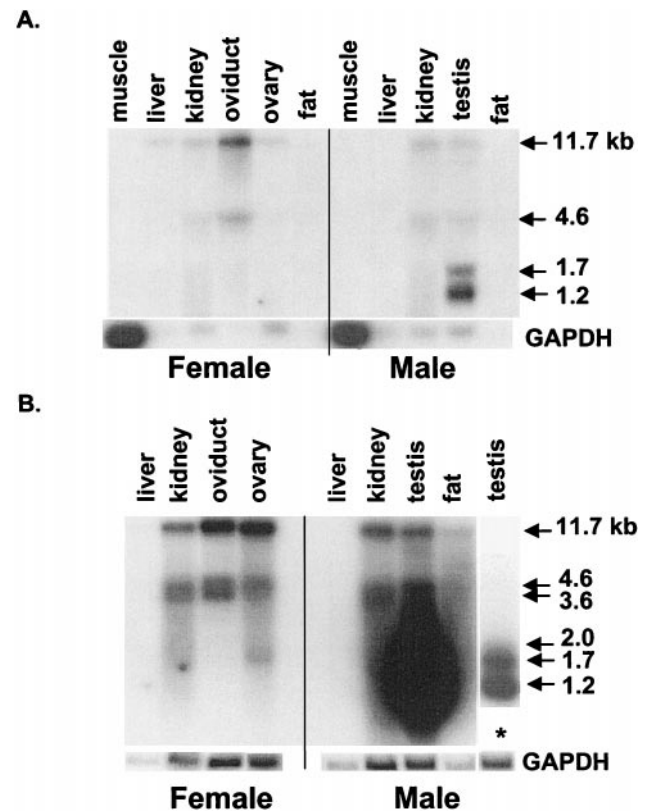

FIG. 2. Northern blot analysis of cPRLR gene expression in various tissues of the chicken (A). Thirty micrograms of total RNA prepared from different tissues of a Leghorn laying hen and a Leghorn male (9 months of age) were loaded into each lane. An equal mixture of the *extra 2* and *intra* probes was used for hybridization. The blot was exposed to film for 36 h at −75 C with intensifying screens. The blot was stripped and rehybridized to a cGAPDH probe to verify the integrity and transfer of RNA (4-h exposure). The sizes of major cPRLR transcripts, based on migration of a RNA ladder, are indicated on the *right*. Five micrograms of poly(A) RNA were prepared from these tissues (except muscle) and used for Northern blot analysis (B). The blot was exposed to film for 18 h at −75 C with intensifying screens. The blot was exposed again briefly (1.5 h) to show detail of the short cPRL transcripts found in the testis sample (indicated by the *asterisk*). The blot was then stripped and rehybridized to the cGAPDH probe (4-h exposure).

RNA prepared from these tissues gives better resolution of the multiple cPRLR transcripts expressed in the adult chicken (Fig. 2B). In addition to the 4.6-kb transcript, a slightly smaller (3.6-kb) cPRLR transcript was found in poly(A) RNA from the kidney, oviduct, and ovary, but not in female liver or male fat. Five cPRLR transcripts were found in poly(A) RNA from the testes (1.2, 1.7, 2.0, 4.6, and 11.7 kb). The two smaller testis-specific cPRLR transcripts (1.2 and 1.7 kb) were expressed in very high abundance, as evident by overexposure of the blot (after 18 h), whereas all three small transcripts (1.2, 1.7, and 2 kb) were visible in testicular poly(A) RNA after 1.5 h of exposure. Northern blot analysis of total RNA from testes of several adult domestic birds (pigeon, turkey, and Japanese quail) with *extra 2* and *intra* cPRLR cDNA probes showed a single transcript corresponding to the size of the full-length receptor (4.6 kb; data not shown).
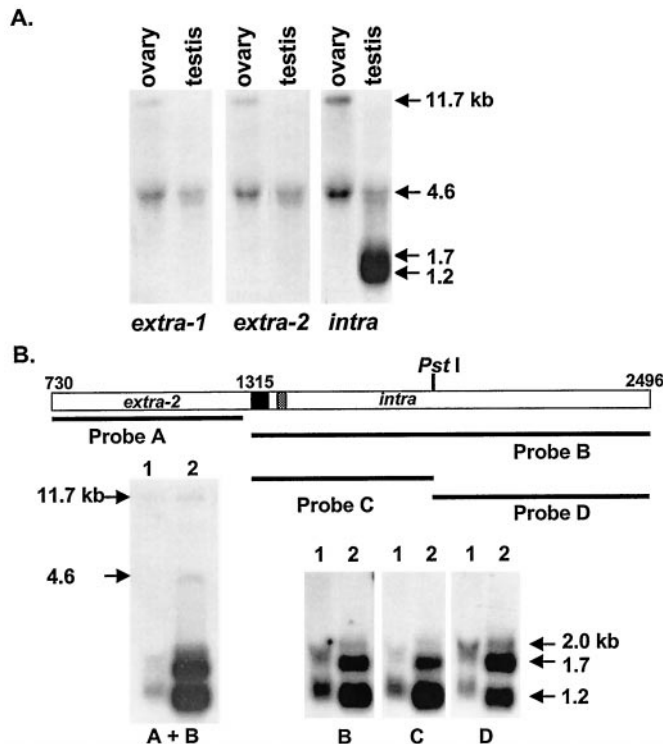
FIG. 3. Northern blot analysis of gonadal RNA using domain-specific cPRLR probes (A). Twenty-five micrograms of total RNA from ovary and testis were loaded into each lane of three identical gels. The tissues were obtained from adult female and male Leghorn chickens (22 wk of age). Blots were hybridized as indicated to *extra 1*, *extra 2*, or *intra* probes. The blots were exposed to film for 24 h at −75 C with intensifying screens. Northern blot analysis of testicular total and poly(A) RNA was performed using domain-specific cPRLR probes (B). Twenty-five micrograms of total (lane 1) or 5 μg of poly(A) RNA (lane 2) from the testis of a 22-week-old Leghorn male were loaded into each lane of replicate gels. Blots were hybridized to *extra 2* (probe A), *intra* (probe B), or *Pst*I-digested fragments (probe C or probe D) of the *intra* cDNA probe. The blots were exposed to film for 20 h at −75 C with intensifying screens.

Testis-specific cPRLR transcripts correspond to the intracellular domain

The domain-specific probes were used to determine which cPRLR domains are expressed in gonads of the chicken (Fig. 3A). The 4.6- and 11.7-kb transcripts in the ovary and the 4.6-kb transcript in the testis hybridize to all three probes. The highly abundant 1.2- and 1.7-kb transcripts in the testis are only detected by the *intra* probe.

In the poly(A) RNA samples (Fig. 3B), there is clearer resolution of two truncated cPRLR transcripts (1.2 and 1.7 kb) that are compressed in total RNA by high levels of 18S ribosomal RNA. Three minor cPRLR transcripts (2.0, 4.6, and 11.7 kb) are seen upon longer exposure in the poly(A) RNA sample from the testis of a 22-week-old chicken hybridized to a mixture (equal specific activity) of *extra 2* (probe A) and *intra* (C+D) probes. Hybridization of testicular poly(A) RNA to the *intra* probe (probe B), which contains the transmembrane and intracellular domains, showed equal intensity of the autoradiographic signal in the 1.2- and 1.7-kb transcripts (Fig. 3B). However, the membrane proximal *Pst*I-digested
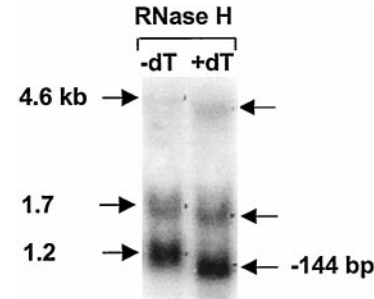

FIG. 4. Northern blot analysis to determine the poly(A) tail length of the cPRLR transcripts. Thirty-five micrograms of total RNA from the testis of a 22-week-old Leghorn chicken were incubated without (−dT) or with (+dT) oligo(deoxythymidine)₁₈ before RNase H digestion to remove the mRNA poly(A) tail. The blot was hybridized to a mixture of *extra 2* and *intra* probes. The blot was exposed to film for 48 h at −75 C with intensifying screens. The *arrows on the right* show the reduction in size (about 144 bp) after RNase H digestion of the oligo(deoxythymidine)-mRNA duplex.

fragment of the *intra* probe (probe C) hybridized more strongly to the 1.2-kb transcript. In contrast, the distal *Pst*I-digested fragment (probe D) showed stronger hybridization to the 1.7-kb transcript.

The major cPRLR transcripts found in the testis (1.2, 1.7, and 4.6 kb) were distinct and polyadenylated. The poly(A) tail length was measured to determine whether the difference in size of the truncated transcripts (1.2 and 1.7 kb) is due to differences in length of the poly(A) tail. The sizes of all three cPRLR transcripts (1.2, 1.7, and 4.6 kb) expressed in the testis were reduced by about 144 bp after RNase H digestion of the poly(A)-oligo(deoxythymidine) duplex (Fig. 4).

Structure of truncated testis-specific cPRLR transcripts

The primary structure of the 5′-end of the truncated testis-specific cPRLR transcripts was determined by 5′-RACE PCR analysis. Seventeen clones obtained from the initial 5′-RACE product(s) (~600 bp) were sequenced. Sixteen clones contained the box 1 motif and had a sequence overlap of 497 bases (nt 1396–1892) with the cPRLR cDNA (see Fig. 5A). The divergent point from the full-length cPRLR cDNA was the G at nt 1396, which lies 16 bases upstream of the highly conserved box 1 motif (nt 1410–1434). The cDNA sequence of these 16 clones revealed three types of testis-specific (+)box 1 transcripts that differ only in the length and composition of their putative 5′-untranslated regions (5′-UTRs; 144, 82, or 20 bases). They are referred to as (+)box 1-A, -B, and -C, respectively. The predominant type (10 of 16 clones) of transcript was 1180 bases long, including a unique 5′-end (82 bases) upstream from the in-frame methionine (M467). This common type is referred to as the truncated testis-specific (TTS) (+)box 1-B cPRLR cDNA[1] (Fig. 5B). The largest putative transcript, with a 144 base 5′-UTR, was named TTS (+)box 1-A[2] cPRLR cDNA (1242 bases). The first 81 bases in the 5′-end of the (+)box 1-A clones are identical to the unique 5′-UTR of the (+)box 1-B clones. However, the rest of this 5′-UTR contained a novel sequence of 62 bases that joins the

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[1] GenBank accession no. AF071026.
[2] GenBank accession no. AF072676

A.

B. (+) Box 1 cDNAs (5'-ends)

(+) Box 1-A 5'-UTR

-144
ac

attccactgatagaaatgaacaggattggccttagaattgagccctggggaacattgctagtaactggcca
ccagccagGTGTAGACCCATTCCCTGCAACCCTTGCAACTCTACAATTGAGTCAGTTCATCACCACTGTGA
▲

(+) Box 1-B cDNA (5'-end)

-82
acattccactg

atagaaatgaacaggattggccttagaattgagccctggggaacattgctagtaactggccaccagccacA
▲

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1                    *              *        *                        54
ATG ATA ACT TTT  ATC CTA CCG CCA GTT CCA GGA CCA  AAG ATA AAA GGC ATA GAT
 M   I   T   F    I   L   P   P   V   P   G   P    K   I   K   G   I   D

                                 *                                   108
ACA CAT CTA TTA GAG ACA GGG AAA TCT GAA GAA CTG TTG AGT GCT CTT GGT TGC
 T   H   L   L   E△  T   G   K   S   E   E   L   L   S   A   L   G   C▲
                                                                   Nco I
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(+) Box 1-C 5'-UTR

-20
ATTGGCGAATTTGCTAAGGA
▲

C. (-) Box 1 cDNA (5'-end)

-131
ATTCTCCACAGTCTCTTGCCA
TGCTGAAGAGACAAGGGCATAGCTGATCTATCATTCTCCTCCATTTCCTCCTTGCCAAACCTTAGAGGCCC
1   24
TTATTTGCAGAAGCTCCTCAGTCCTTCCATTTTAGAAAC ATG AAG TCT GGG ATT GTC TTC CTC
                                         M   K   S   G   I   V   F   L

                *   78
TGG AGG GAA CTC GGG ACA GGG AAA TCT GAA GAA CTG TTG AGT GCT CTT GGT TGC
 W   R   E   L   G▲ T   G   K   S   E   E   L   L   S   A   L   G   C▲

Fig. 5. A, Structure of the full-length and unique truncated testis-specific cPRLR cDNAs. The primary structures of the (+)- and (−)box 1 cPRLR transcripts were determined by sequencing PCR products from 5′-RACE. Two nested gene-specific primers (GSP) were based on the cPRLR cDNA sequence (28), where GSP1 corresponds to nt 1901–1922, and GSP2 corresponds to nt 1876–1892. The transmembrane domain is indicated by the *black box*, and the highly conserved proline-rich box 1 motif is indicated by the *stippled box*. The *Nco*I restriction enzyme site (nt 1506) is used as a reference point (*arrow*) for presenting the 5′-end sequence of each testis-specific cPRLR transcript. A 300-bp riboprobe used in a RPA was prepared by PCR using primers G (nt 1245–1266) and H (nt 1545–1515) and kidney total RNA as template (see Fig. 7). B and C, The cDNA sequence of the 5′-ends of two unique truncated testis-specific (TTS) cPRLR transcripts. Three different 5′-UTRs were found among the (+)box 1 TTS cPRLR cDNAs (B). The (+)box 1-A 5′-end was composed of 144 bases, the first 81 bases (in *lowercase letters*) are identical to those of the (+)box 1-B 5′-UTR, whereas the remaining 62 bases are unique to the (+)box 1-A 5′-end. The cDNA sequence of testis-specific truncated (+)box 1-B cPRLR (1179 bp) diverges from that of the cPRLR cDNA at nt 1396 (indicated by the *black triangle*). The *underlined* sequence is identical to that of the intracellular domain of the cPRLR cDNA. The box 1 motif is outlined by the *rectangle*, and base differences from the published cDNA sequence are indicated by the *asterisks*. The *black* (nt 1396) and *open* (E489) *triangles* mark the boundaries of the box 1 coding region. The cDNA sequence of the (+)box 1 transcripts differ only in length and composition of their 5′-UTRs (144, 82, or 20 bases). The cDNA sequence (C) of the (−)box 1 TTS cPRLR (1199 bp) diverges from that of the cPRLR cDNA at nt 1467 as indicated by the *black triangle*. The 5′-end of the (−)box 1 cDNA contains 170 bp of unique sequence.

in-frame methionine (M467) in the cPRLR cDNA. The shortest 5'-UTR sequence [TTS (+)box 1-C[3]] was composed of 20 unique bases. All 16 of the (+)box 1 clones contained a distinct 5'-UTR that joins an in-frame methionine (M467) codon found in the full-length cPRLR cDNA. If this in-frame ATG was used as the initiator methionine, the predicted (+)box 1 protein would be composed of 365 amino acids with an apparent molecular mass of 40,536 Da.

The first clone sequenced from the 5'-RACE products did not contain the highly conserved box 1 motif. The divergent point was the G (nt 1467), which lies 32 bases downstream of the box 1-coding region (Fig. 5C). The box 1-coding region was replaced by a unique 5'-end sequence, which contained an in-frame ATG that began 39 bases upstream of the divergence point (nt 1467). This putative transcript (1199 bases) contains 131 bases of unique 5'-UTR and is referred to as the TTS (−)box 1 cPRLR cDNA.[4] If translated, the (−)box 1 TTS cPRLR protein (355 amino acids; M_r = 39,542 Da) would have a novel N-terminus of 13 hydrophobic amino acids. The coding regions for the (+)box 1 and (−)box 1 TTS cPRLRs were amplified by RT-PCR from the first strand synthesis product used in 5'-RACE analysis by using a forward primer, corresponding to the first 36 bases of either (+)box 1 or (−)box cDNAs (see Fig. 5, B and C), and a common reverse primer (primer F; Fig. 1). The two distinct PCR products (1.1 and 1.2 kb) were cloned into pCR-II vector, and the sequence of the coding region was confirmed by primer walking.

The cDNA sequence within the box 1 coding region of all 16 clones from the 5'-RACE analysis as well as the riboprobe fragment that was amplified from kidney RNA (see below) differ from the published cDNA sequence of the cPRLR (28). Within the region presented in Fig. 5B, four base differences were found in the cDNA sequence, while only the base difference at nt 1463 (from G to C) changed the codon for the first amino acid in the highly conserved Box 1 motif from a methionine (M) to an isoleucine (I) residue.

Expression of truncated testis-specific cPRLR transcripts

Northern blot analysis using unique 5'-end cDNA probes. Three cDNA probes, which correspond to the unique 5'-ends of (+)box 1-A, box 1-B, and (−)box 1, were amplified from 5'-RACE clones (Fig. 5, B and C). These cDNA probes, which span the 5'-UTR and first 36 bases in the putative coding regions, were used in Northern blot analysis (Fig. 6). The TTS (+)box 1-A probe hybridizes to all three (1.2, 1.7, and 2.0 kb) cPRLR transcripts in poly(A) RNA isolated from mature chicken testes (Fig. 6A). The TTS (+)box 1-B 5'-end probe hybridizes strongly to the 1.2-kb transcript and with lower intensity to the 1.7- and 2.0-kb transcripts in testicular poly(A) RNA. In contrast, the TTS (−)box 1 cDNA probe shows very weak hybridization to all three transcripts in poly(A) RNA from the testes of a mature (22-week-old) Leghorn chicken.

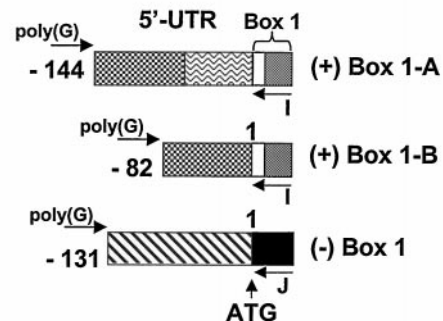[3] GenBank accession no. AF072677.
[4] GenBank accession no. AF051808.
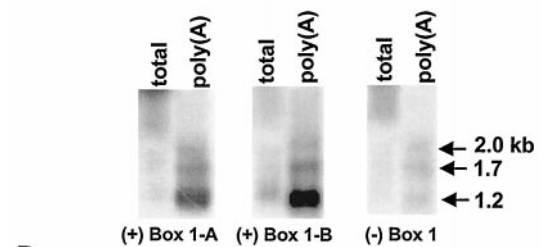
FIG. 6. Northern blot analysis of total and poly(A) RNA prepared from the testes of an adult male chicken (22 wk of age) using unique 5'-ends of the (+)box 1-A, (+)box 1-B, and (−)box 1 cDNAs (A). Twenty-five micrograms of total RNA or 5 μg poly(A) RNA were loaded on three replicate gels. The blots were hybridized to probes unique to the 5'-ends of (+)box 1-A (nt −144 to +36), (+)box 1-B (nt −82 to +36), or (−)box 1 (nt −131 to +36) cDNAs (B). The blots were exposed to film for 22 h at −75 C with intensifying screens. The unique 5'-end cDNAs were amplified from the 5'-RACE clones using a poly(G) primer and primer I [nt 1–36 of (+)box 1 cDNA] or primer J [nt 1–36 of (−)box 1 cDNA]. Unique 5'-UTR sequences are identified by different patterns. The first 81 bases in the 5'-UTR of (+)box 1-A and (+)box 1-B are identical as indicated by the same pattern. The box 1 motif is indicated by the *stippled box*. The *solid black box* denotes the unique 5'-coding region of the (−)box 1 cPRLR.

RPA of cPRLR transcripts in different tissue of male and female chickens

A riboprobe that spans the transmembrane domain and the box 1 motif was used in a RPA to examine expression of the full-length and truncated cPRLR transcripts in different tissues from sexually mature chickens (Fig. 7). This riboprobe protects fragments (296, 151, and 76 bp) that correspond to the full-length, TTS (+)box 1, and TTS (−)box 1 cPRLR transcripts in testes of both Leghorn and broiler chickens (Fig. 7A). A comparison of these cPRLR transcripts in the testis of a mature (22-week-old) with a juvenile male (5-week-old) chicken shows that the full-length cPRLR fragments are more abundant in the juvenile, whereas the TTS (+)box 1 fragments are more abundant in the adult. The (−)box 1 transcript appears to be a minor TTS cPRLR mRNA, although it is expressed at slightly higher levels in the adult than in the juvenile testis.

Consistent with the results of Northern blot analysis, the RPA shows that all tissues examined in the adult chicken express the full-length cPRLR, with the exceptions of breast muscle from both sexes and male liver (Fig. 7B). The greatest abundance of full-length cPRLR mRNA was found in kidney,
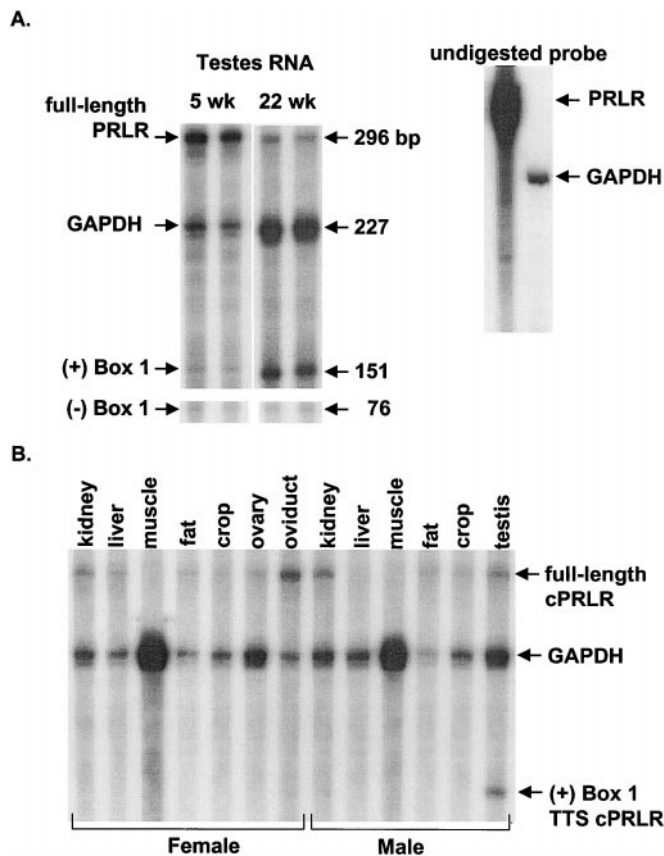
FIG. 7. RPA of cPRLR transcripts in total RNA from the testes of an immature (5-wk-old) and adult (22-wk-old) Leghorn chicken (A). Thirty-five micrograms of total RNA were hybridized to a mixture of cGAPDH and cGHR riboprobes and digested with RNase T1, and protected fragments were separated by electrophoresis on a 6% polyacrylamide-8.4 M urea gel. The gel was exposed to film for 16 h at −75 C with intensifying screens. Digestion of the hybridized cPRLR riboprobe (see Fig. 5A) yields three protected fragments, which correspond to the full-length (296 bp), (+)box 1 (151 bp), and (−)box 1 (76 bp) cPRLR transcripts. The 227 bp-protected fragment corresponds to cGAPDH, which is usually used for normalization of RNA in each sample. The *right panel* shows migration and sizes of undigested riboprobes. B, RPA of cPRLR transcripts in different tissues collected from an adult female and male Leghorn chickens (9 months of age). The conditions of the RPA were the same as described above.

oviduct, and testis compared with total RNA from other tissues. Furthermore, the truncated (+)box 1 cPRLR transcript was found only in testis.

Ontogeny of full-length and TTS cPRLR transcripts in the chicken.
The cPRLR RPA shows that the full-length cPRLR transcript (296-bp protected fragment) is expressed in high abundance in the testis of the immature chicken between 1–11 weeks of age (Fig. 8). After 13 weeks of age, the abundance of the full-length cPRLR transcript is greatly reduced. The abundance of the (+)box 1 fragment increases dramatically after 13 weeks of age, which marks the onset of sexual maturity, as indicated by the sharp rise in plasma testosterone levels. However, the (−)box 1 transcript appears to be a minor species and is expressed at very low abundance, even after 13 weeks of age.
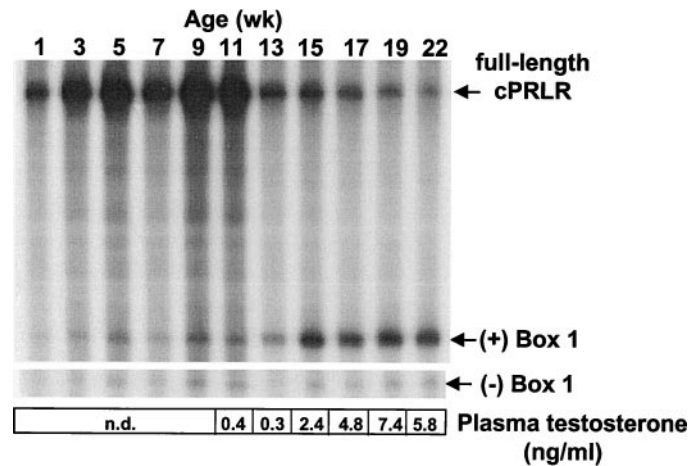

FIG. 8. Ontogeny of full-length and unique truncated testis-specific cPRLR transcripts in the chicken testis. The positions of the different protected fragments (see Fig. 7A) are indicated on the *right*. Plasma testosterone levels represent the mean of four birds at each age. Plasma testosterone levels below 0.1 ng/ml were undetectable (n.d.) by the RIA.

Discussion

In the present paper, we report the discovery of unique truncated cPRLR transcripts, which correspond to the entire intracellular domain, in the testis of the sexually mature chicken. Northern blot analysis shows three distinct truncated testis-specific cPRLR transcripts (1.2, 1.7, and 2 kb) in the adult testis. Two types of truncated testis-specific cPRLR mRNAs were identified that lack the extracellular and transmembrane domains. These truncated isoforms of the cPRLR differ from each other because of the presence or absence of the highly conserved box 1 motif at the N terminus [*i.e.* (+)box 1 or (−)box 1 TTS cPRLR]. At least five unique 5'-UTRs are found among the different cPRLR mRNAs expressed in the chicken testis (20, 82, 131, 144, or 222 bases). Multiple forms of unique 5'-UTR in murine PRLR mRNA result from alternative utilization of three first exons and alternative splicing of the second exon (39–41). Alternative promoters are used to transcribe multiple PRLR mRNAs from the same PRLR gene (40). One promoter (P-I) is gonad specific, and steroidogenic factor-1 is required as a transcriptional activator and for tissue-specific utilization of the alternative promoters. The onset of expression of the truncated testis-specific cPRLR transcripts accompanies the developmental increase in circulating androgen levels. The abundance of the (+)box 1 cPRLR transcript appears to be inversely related to that of the full-length cPRLR mRNA. Young chickens mainly express the full-length cPRLR in the testes until they reach 11 weeks of age. Thereafter, the abundance of the truncated testis-specific (+)box 1 cPRLR mRNA increases dramatically, which coincides with the sharp rise in plasma testosterone levels (17). In contrast, the (−)box 1 cPRLR transcript seems to be constitutively expressed at low levels throughout posthatching development.

Long, intermediate, and short forms of the mammalian PRLRs (23, 42, 43) have been characterized, all of which include the box 1 motif, but differ mainly in the length and sequence of the cytoplasmic domain (see Ref. 20 for review).

In the chicken testis, the extracellular and transmembrane coding regions in the full-length cPRLR have been spliced out, leaving the intracellular domain intact. There is a minor truncated isoform in which the box 1 exon is replaced by a short testis-specific exon encoding a hydrophobic N-terminus [*i.e.* the (−)box 1 cPRLR]. In rodents, the carboxyl terminus of the long PRLR isoform is encoded by exon 10, whereas multiple exon 11s encode several short PRLR isoforms (44). A different mechanism of alternative splicing is responsible for generation of long and short forms of the ovine and bovine PRLRs (44). The divergence point for generating the short forms of the mammalian PRLRs is E261, which marks the junction between exons 9 and 10. In ruminants, the short PRLR transcript results from insertion of 39 bases between exons 9 and 10, which contain two in-frame stop codons (44). In chickens, the divergence point for generating the TTS (−)box 1 cPRLR is E489 (equivalent to E261 in mammals), which also marks the boundary between exons 9 and 10. In the case of the (−)box 1 cPRLR transcript, there is substitution of 39 unique bases (which could encode a hydrophobic N-terminus) for the box 1-encoding exon. The 5'-RACE analysis shows that there are at least three different kinds of (+)box 1 cPRLR mRNAs that differ only in the length and composition of their putative 5'-UTRs (20, 82, or 144 bases). The divergence point for splicing of the unique 5'-UTRs in the three (+)box 1 cPRLR transcripts is the G (nt 1396), which is 14 bases upstream of the beginning of the highly conserved box 1 motif. The in-frame ATG (nt 1399–1401) could serve as the initiator methionine for translation of the (+)box 1 cPRLR protein (365 amino acids). In addition, there is a distinct testis-specific (−)box 1 exon (encoding 13 amino acids) that is substituted for the box 1 motif at the 5'-end of the truncated cPRLR mRNAs. Thus, the boundaries of the box 1 encoding exon (for 23 amino acids) in the cPRLR are marked by nt 1396 and nt 1467. The initiator methionine codons proposed for the (+)box 1 and (−)box 1 cPRLR isoforms have a reasonable fit with Kozak's (45) consensus sequence and are very similar to those found in all avian PRLR transcripts and the cGHR.

The box 1 motif is highly conserved among mammalian (20, 46–48) and avian (28–30, 49) GH and PRL receptors. The revised sequence of the box 1 region in the cPRLR cDNA shows that chicken, pigeon (29), and turkey (30) PRLRs have the same amino acid sequence for the box 1 motif (ILP-PVPGP). The four proline residues, particularly the last proline, are required for activation of JAK2 (50), whereas STAT5 activation (20) requires carboxyl-terminal phosphorylation of the PRLR (51). Mutation of the box 1 motif blocks JAK2 activation and transmission of the PRL signal (48, 50, 52). Chickens express truncated cPRLR mRNAs that could encode the entire intracellular domain, including the box 1 motif and the carboxyl-terminus. If translated, these putative cytoplasmic proteins could dampen cPRL signal transduction by acting as a sink for JAK2, or perhaps STAT5, or by forming either homo- or heterodimers. Although only the long form of the PRLR stimulates transcription of milk protein genes (21, 53), short forms of the PRLR block the PRL signal by forming heterodimers with the long form (52, 54). A mutant form of the rabbit PRLR, which is the membrane-anchored intracellular domain, exerts a dominant negative effect on PRL signal transduction when coexpressed with the wild-type PRLR (52). A cytoplasmic version of the long PRLR, generated by deletion of the signal peptide, is not membrane embedded, but it is able to activate JAK2 and STAT5 (52). Furthermore, this mutant cytoplasmic PRLR can rescue the PRL signal from a box 1-inactive PRLR expressed in cell culture. The role that truncated intercellular derivatives of the cPRLR play in reproductive function of the male chicken is presently unknown.

Avian PRLRs are unusually large (831 amino acids) compared with long forms of the mammalian PRLRs (591 amino acids) due to a tandem repeat of the extracellular ligand-binding domain (28–30). Southern blot analysis shows the presence of a single gene for the cPRLR that spans about 40 kb, whereas the larger mammalian PRLR gene spans 70 kb in the rat (26) and 100 kb in the human (55). Multiple transcripts of the cPRLR gene (3.6, 4.6, and 11.7 kb) are found by Northern blot analysis in different tissues (kidney, ovary, oviduct, testis, and fat) of adult chickens. The large cPRLR transcript (11.7 kb) found in most tissues is probably a primary transcript of incompletely processed RNA. As the cPRLR cDNA is approximately 2.8 kb (28), it is likely that the 4.6-kb transcript encodes the full-length cPRLR. An additional 3.6-kb cPRLR transcript was found in poly(A) RNA from kidney, oviduct, and ovary. No cPRLR transcripts were observed by Northern blot or RNase protection analysis in breast muscle of either sex or in male liver. However, cPRLR mRNA is expressed in the female liver, which is consistent with the detection of PRLR transcripts by RT-PCR in the liver of the female chicken (28, 56), pigeon (29), and turkey (30). Two PRLR transcripts of 3.3 and 7.5 kb were found in the chicken kidney and hypothalamus (56), whereas a 3-kb PRLR transcript was reported in poly(A) RNA from liver of the turkey hen (30). The liver of the female mouse has exceptionally high levels of the short form (291 amino acids) of the murine PRLR during late pregnancy (23, 43). Exogenous estrogen induces expression of the short PRLR transcript in liver of the rat (21, 43, 57–59). Although there is no true avian correlate to pregnancy, expression of cPRLR transcripts in the liver of the laying hen could be related to synthesis of the metabolic precursors required for egg production. The different sizes of the truncated testis-specific cPRLR transcripts could reflect heterogeneity of the 5'- and 3'-UTRs, as they have similar lengths of putative coding region (1068 or 1098 bp). Northern blot analysis with (+)box 1- or (−)box 1-specific cDNA probes and the RPA show that the truncated (+)box 1 cPRLR transcript is the major one found in the testis of the sexually mature chicken. No truncated transcripts were found in testicular RNA from several adult domestic birds (pigeon, turkey, and Japanese quail). The expression of the multiple transcripts of the cPRLR in different tissues of the chicken is consistent with the known actions of PRL in birds.

Receptor isoforms containing the intracellular domain, but no extracellular-transmembrane domain, have not been reported for any other member of the cytokine/GH receptor family. One member of this family, MPL, appears to be a protooncogene with thrombopoietin as a ligand (60, 61). The MPL virus encodes the v-*mpl* oncogene, which is an amino-terminal truncated form of MPL, and with infection there is

unregulated proliferation of target cells (62). In this protein, elimination of the extracellular domain uncouples ligand binding from signal transduction. This is analogous to the v-*erb*B oncogene, which corresponds to the intracellular domain of the epidermal growth factor receptor and is constitutively active (63) In the rat, PRL modulates Leydig cell function by altering expression of LH receptors (64), which affects steroidogenesis. However, it is clear that enhanced PRL secretion has antigonadal activity. This change from a gonadal growth factor to a factor causing gonadal regression is likely to involve changes in PRLR activity, as PRL is able to up- or down-regulate its receptor depending upon concentration and duration of exposure (26). Recent work has shown expression of long and short forms of PRLR in the testis of the rat (64) and red deer (65). The multiple cPRLR isoforms found in the testis could confer different roles in gonadal development and reproductive activity (66).

Generation of multiple transcripts of the cPRLR in the testis could reflect differential transcriptional initiation, alternative splicing, use of alternative polyadenylation sites, or any combination of these events. In rodents, multiple mRNA species are transcribed through use of tissue-specific promoters (40, 41). The gonad-specific promoter directs hormonally sensitive transcription of long and short forms of PRLR mRNA in ovary and testis. However, the multiple isoforms of the mammalian PRLR contain the extracellular ligand-binding domain, the transmembrane domain, and an intracellular domain of variable length and composition. As the truncated cPRLR transcripts found in the chicken testis lack sequences encoding the extracellular and transmembrane domains, the testis-specific promoter could reside in an intron just downstream of the transmembrane domain and be activated by the high levels of testosterone found in the mature testis. Alternatively, there could be extensive splicing of all exons encoding the extracellular and transmembrane domains, leaving the coding region of the intracellular domain intact. If full-length and truncated cPRLR mRNA levels reflect transcriptional activity, it appears that testosterone could divert transcription from production of the full-length cPRLR to formation of the truncated cPRLR transcripts. Even in the absence of translation, the truncated cPRLR transcripts could limit the effects of PRL on the mature testis. However, if the truncated transcripts are translated, they could function in a dominant negative fashion and interfere with ligand-mediated signaling through the JAK/STAT pathway. Of course, additional studies are required to determine whether the (+)box 1 and (−)box 1 cPRLR transcripts are translated and if they have any functional significance in PRL signaling. The expression of multiple PRLR isoforms within a target organ could provide a mechanism for tissue-specific regulation of PRL function (66).

Our study has clearly shown that unique truncated transcripts of the cPRLR, corresponding to the intracellular domain, are expressed in very high abundance in the testis of the sexually mature chicken. Analysis of the different testicular cPRLR isoforms is likely to provide new information about cPRLR gene structure and PRL regulation of reproductive activity in domestic birds.

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