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Two forms of the prolactin receptor messenger ribonucleic acid are present in ovine fetal liver and adult ovary

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Previous binding studies indicated that there is little to no specific prolactin binding in ovine fetal liver and adult ovary. Therefore, we sought to determine if ovine prolactin receptor (PRLR) mRNA is present in those tissues. Primers were designed from the bovine PRLR cDNA sequence for use in reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR analysis of ovine fetal liver total cellular RNA (tcRNA) isolated from days 60, 90, 105, 120 and 135 of gestation, and luteal tcRNA isolated from days 3, 7, 10, 13 and 16 of the estrous cycle revealed that PRLR mRNA was present in these tissues. However, two RT-PCR products were generated from both tissues. The two RT-PCR products did not differ between the two tissue sources in sequence, and were designated oPRLR-1 and oPRLR-2. Ovine PRLR-1 is 513 bp in length and is 96.4% identical to the bovine cDNA. Ovine PRLR-2 is identical to oPRLR-1 until nucleotide (nt) 420 at which point a 39 bp insertion occurs. This insertion occurs between Homology Boxes 1 and 2 within the cytoplasmic domain of the receptor, resulting in an 11 amino acid divergent sequence, followed by two stop codons. Ribonuclease-protection assay revealed that oPRLR-1 mRNA is the most abundant in these tissues. Our data indicate that two forms of oPRLR mRNA are present in fetal liver and adult ovary, and that one form (oPRLR-2) is predicted to encode a truncated PRLR.

Keywords: Prolactin; receptor; sheep; liver; ovary

Introduction

Growth hormone (GH) and prolactin (PRL) receptors are members of the cytokine receptor superfamily (Kelly *et al.*, 1991) and both exhibit large extracellular domains, a single transmembrane region and a cytoplasmic domain. The cDNA encoding the PRL receptor (PRLR) has been cloned in the mouse (Davis & Linzer, 1989), rat (Boutin *et al.*, 1988; Shirota *et al.*, 1990), rabbit (Edery *et al.*, 1989), human (Boutin *et al.*, 1989) and cow (Scott *et al.*, 1992). Recently, PRLR cDNAs have been reported for the chicken and pigeon which encode a non-perfect duplication of the ligand binding domain including two WSXWS amino acid motifs (Tanaka *et al.*, 1992; Chen & Horseman, 1994). This motif has been implicated in GH receptor dimerization (Duriez *et al.*, 1993) and PRLR signaling (Rozakis-Adcock & Kelly, 1992). Within the membrane-proximal region of the cytoplasmic domain of PRLRs and other members of this receptor family, lie two Homology Boxes. Homology Box 1 is a proline rich region, and recent evidence (DaSilva *et al.*, 1994) indicates that the Homology Box 1/Box 2 region is critical for phosphorylation and association of JAK2 tyrosine kinase. Four different cDNAs for the mouse PRLR have been reported (Davis & Linzer, 1989; Clarke & Linzer, 1993) and two distinct PRLRs exist in the rat (Boutin *et al.*, 1988; Shirota *et al.*, 1990). The nucleotide sequences encoding one of the rat PRLRs and three of the mouse PRLRs diverge in such a manner that translation of the receptors is truncated between Homology Box 1 and 2. Recently, DaSilva *et al.* (1994) demonstrated that deletion of Homology Box 2 impeded PRL induced signal transduction, raising into question the function of the short-forms of the rodent PRLRs. Expression of the various mouse and rat PRLR mRNAs appears to be tissue specific, and dependent on the physiological state of the tissue (Clarke & Linzer, 1993; Clarke *et al.*, 1993; Nagano & Kelly, 1994). The existence of multiple PRLR mRNAs, expressed in a time and tissue dependent manner, raises questions regarding cell specific responses to PRL.

The bovine PRLR mRNA is present in maternal and fetal liver, maternal endometrium and luteal tissue during gestation (Scott *et al.*, 1992). It has been proposed that bovine placental lactogen (bPL) binding in fetal liver and maternal endometrium may in part be accounted for by the bovine PRLR (Galosy *et al.*, 1991; Scott *et al.*, 1992). However, ovine placental lactogen (oPL) and ovine prolactin (oPRL) do not appear to compete for the same binding site in fetal liver tissue (Gluckman *et al.*, 1983; Freemark *et al.*, 1987). Furthermore, the amount of specific binding of oPRL to ovine luteal tissue is marginal (Bramley *et al.*, 1987a,b). With the reported variation in PRLR transcripts, it is possible that alternative splicing events or differential transcriptional start sites of the ovine PRLR gene could yield a receptor capable of binding oPL. Here we report the existence of ovine PRLR mRNA in fetal liver and adult luteal tissue, and that two distinct ovine PRLR mRNAs are expressed by these tissues, one of which encodes a truncated PRLR.

Results

Reverse transcriptase-polymerase chain reaction (RT-PCR), utilizing the primers depicted in Figure 1, was used to examine the existence of ovine PRLR mRNA in maternal and fetal tissues. Maternal liver total cellular RNA (tcRNA) gave a RT-PCR product of the expected size (513 bp), which hybridized to the bovine PRLR cDNA (Figure 2). A cDNA of the expected size which hybridized with the bovine PRLR cDNA was also generated from fetal liver tcRNA obtained on days 60, 90, 105, 120 and 135 of gestation (Figure 2) and from luteal tcRNA obtained on days 3, 7, 10, 13 and 16 of the estrous cycle (data not shown). On further analysis of RT-PCR products generated from fetal liver (data not shown) and adult luteal tcRNA (Figure 3), two RT-PCR products were detected. The two RT-PCR products were amplified from both day 105 fetal liver tcRNA and day 10 adult luteal tcRNA, subcloned and sequenced.

Nucleotide sequence analysis revealed that the two RT-PCR products did not differ in their respective sequences as a result of the tissue of origin. One RT-PCR product is designated oPRLR-1 and the other as oPRLR-2. The nucleotide sequence comparison of oPRLR-1 to the bovine PRLR cDNA is presented in Figure 4. Ovine PRLR-1 exhibited

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96.4% nucleotide sequence identity with the bovine receptor sequence, and was 471 bp long (minus the 5′ and 3′ primers generated from the bovine PRLR nucleotide sequence). Ovine PRLR-2 matched oPRLR-1 identically until nt 420 (Figure 4), at which point the sequence diverged for 39 bp. After this 39 bp insertion, the nucleotide sequence for both cDNAs again matched identically. The 39 bp insertion in oPRLR-2 is in the cytoplasmic domain, and upon translation should result in the insertion of 11 amino acids followed by two stop codons (Figure 5). Thus, translation of oPRLR-2 is predicted to result in a truncated form of the receptor, lacking Homology Box 2. The two ovine cDNAs differed from the predicted bovine PRLR amino acid sequence by only nine residues, before the branch point of the two ovine cDNAs. Two amino acid residues were different in the proposed transmembrane domain, and one in the cytoplasmic domain. Ovine PRLR-1 contained an additional amino acid substitution, when compared to the bovine PRLR cDNA, within the carboxy-terminal region of oPRLR-1. The amino acids predicted for the WSXWS motif were identical for the bovine and ovine PRLR cDNAs.

To determine which of the two oPRLR mRNAs may be the most abundant in fetal liver and adult luteal tissue, we examined both sources of tcRNA by ribonuclease-protection assay (RPA). Both oPRLR-1 and oPRLR-2 were transcribed into cRNAs and used in RPA analysis. We predicted that the oPRLR-1 cRNA would be protected by both forms of the mRNA, and that the oPRLR-2 cRNA would be partially digested if it hybridized to the mRNA from which the oPRLR-1 cDNA was generated. The RPAs indicated that

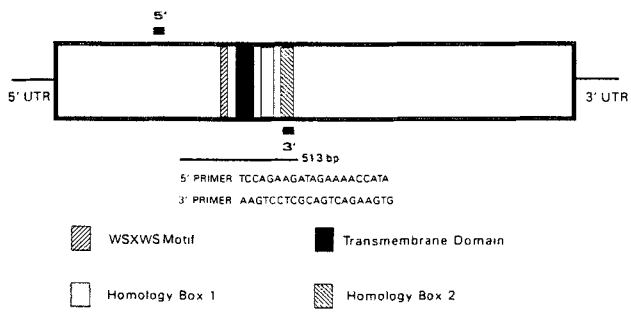

Figure 1 Schematic representation of PRLR mRNA. The 5′ (5′ UTR) and 3′ (3′ UTR) untranslated regions are depicted by straight lines. The coding region is represented by the large open box. Other features of this mRNA include the WSXWS motif in the extracellular region, the transmembrane domain coding region, and the membrane-proximal cytoplasmic Homology Boxes 1 and 2. The relative positions of the 5′ and 3′ PRLR primers are depicted by black rectangles. The 5′ primer recognition site resides 5′ to the WSXWS motif and the 3′ primer recognition site resides within Homology Box 2. These primers were predicted to generate a 513 bp RT-PCR product, and the specific primer sequences are provided below the figure

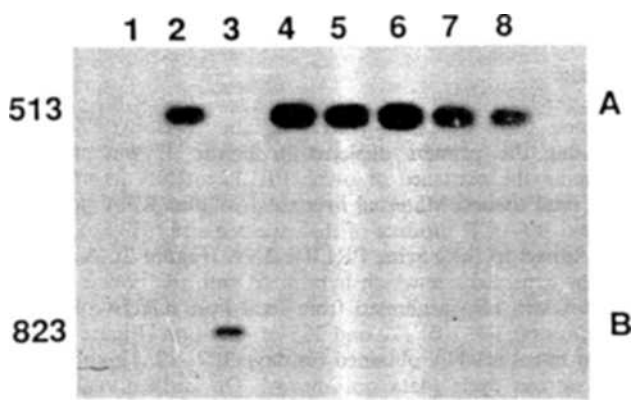

Figure 2 RT-PCR analysis of ovine fetal liver and adult liver total cellular RNA (tcRNA). Lane designations are: (1) PRLR primers in the absence of tcRNA, (2) PRLR primers with adult ovine liver tcRNA, (3) GHR primers with adult ovine liver tcRNA, (4) PRLR primers with day 60 fetal liver tcRNA, (5) PRLR primers with day 90 fetal liver tcRNA, (6) PRLR primers with day 105 fetal liver tcRNA, (7) PRLR primers with day 120 fetal liver tcRNA, and (8) PRLR primers with day 135 fetal liver tcRNA. Panel A was hybridized to ^{32}P-labeled bovine PRLR cDNA and panel B to ^{32}P-labeled bovine GHR cDNA. The numbers to the left provide the size of the RT-PCR products in bp

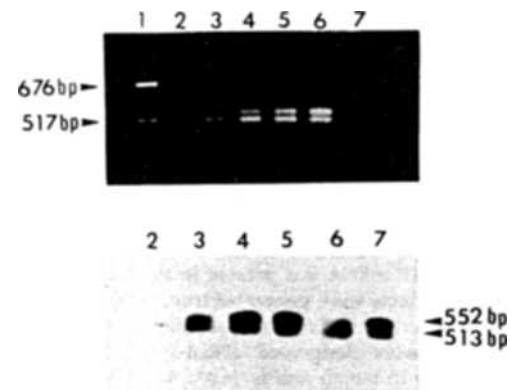

Figure 3 RT-PCR analysis of ovine luteal total cellular RNA (tcRNA). Lane designations are: (1) pGEM M$_r$ markers, (2) no tcRNA template, (3) day 3 luteal tcRNA, (4) day 7 luteal tcRNA, (5) day 10 luteal tcRNA, (6) day 13 luteal tcRNA, and (7) day 16 tcRNA. The RT-PCR reactions were separated on a 1.5% agarose gel and stained with EtBr (top panel), blotted onto nylon and hybridized with the ^{32}P-labeled bovine PRLR cDNA (bottom panel). Note that in each tcRNA sample (Lanes 2–7) that two RT-PCR products were generated

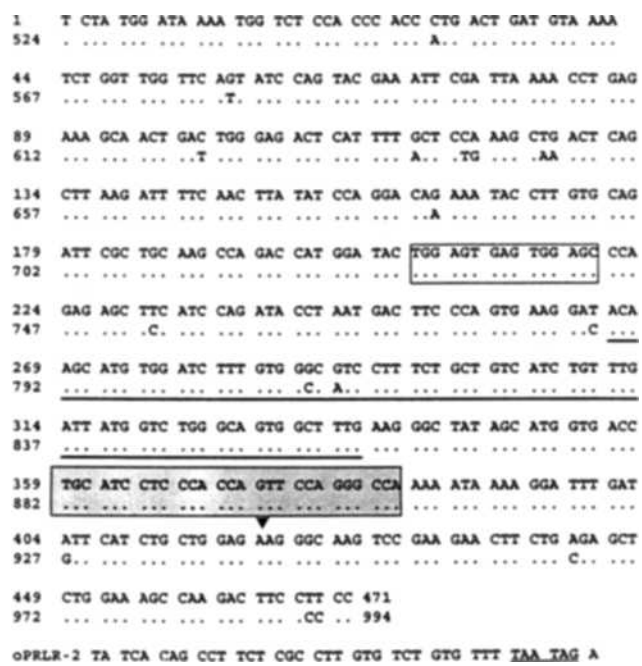

Figure 4 Nucleotide sequence comparison of oPRLR-1 (top) and the bovine PRLR cDNA (bottom). The numbering of the bovine PRLR is in accordance with Scott *et al.* (1992). The WSXWS motif coding region is enclosed by an open box, the predicted transmembrane domain coding region is underlined and Homology Box 1 is enclosed within the shaded box. The 39 bp insert of oPRLR-2 is given at the bottom and the inverted triangle marks its insertion site. The two in-frame stop codons within this insert are underlined

the mRNA from which the oPRLR-1 cDNA was generated was the most abundant oPRLR mRNA in both fetal liver tcRNA (data not shown) and adult luteal tcRNA (Figure 6).

Discussion

By using RT-PCR we have isolated partial cDNAs from two species of oPRLR mRNA which are present in both fetal liver and adult luteal tissue. The two RT-PCR products were subcloned, sequenced and shown to be similar to PRLR cDNAs reported for the rat (Boutin *et al.*, 1988), mouse (Davis & Linzer, 1989), human (Boutin *et al.*, 1989) and cow (Scott *et al.*, 1992). To our knowledge, this is the first report of two species of PRLR mRNA in a non-rodent species.

The highest degree of nucleotide sequence identity (96.4%) was observed between oPRLR-1 and the bovine PRLR cDNA (Scott *et al.*, 1992). Within the sequence of the oPRLR-1, excluding the sequence for the bovine receptor primers, 17 nucleotides differed from the bovine cDNA, resulting in 10 amino acid substitutions. Six of the predicted amino acid substitutions occurred in the extracellular domain, but none were contained within the WSXWS motif. This motif has been implicated in ligand/receptor interaction for the cytokine receptor family (Miyasaki *et al.*, 1991; Quelle *et al.*, 1992; Duriez *et al.*, 1993). In fact, mutations in this region inhibit biological activity of the chicken growth hormone receptor (Duriez *et al.*, 1993).

Ovine PRLR-2 is identical to oPRLR-1 until nucleotide 420, at which point an additional 39 nucleotides are inserted. These 39 nucleotides result in an 11 amino acid divergent sequence, followed by two in frame stop codons. Therefore, upon translation, this mRNA should encode a truncated PRLR. This insertion site (amino acid 263 of the bovine PRLR) occurred at the same point in the cytoplasmic domain as the reported variants of the mouse (Davis & Linzer, 1989) and rat (Boutin *et al.*, 1988) PRLRs. Hypothetically, an alternative splice site could exist in the intron 39 bp 5′ to the normal splice site. The nucleotide sequence of the ovine cDNAs were identical after the insertion, which is different from the variant PRLRs in the rat and mouse (Boutin *et al.*, 1988; Davis and Linzer, 1989). In rats and mice the nucleotide sequence 3′ to the splice site lacks similarity to the long forms of the receptor.

Regardless of the splicing events that may take place to generate the variant PRLR mRNAs, these mRNA in the mouse, sheep and rat, upon translation should generate PRLRs that truncate between Homology Box 1 and 2. Ali *et al.* (1992) demonstrated that the short-form of the rat PRLR did not stimulate the expression of a co-transfected β-casein/chloramphenicol acetyltransferase gene construct, whereas the long-form of the rat PRLR and the form found in Nb2 cells were capable of stimulating the expression of this reporter construct. Both the long-form (Boutin *et al.*, 1989) and the Nb2-form (Ali *et al.*, 1991) of the rat PRLR contain both Homology Box 1 and 2, and the short-form (Boutin *et al.*, 1988) contains only Homology Box 1. Prolactin induced proliferation of Nb2 cells requires the association and activation of the tyrosine kinase JAK2 (Lebrun *et al.*, 1994), and deletion of either Homology Box 2 or both Homology Box 1 and 2 disrupts the phosphorylation and association of JAK2 in response to PRL (DaSilva *et al.*, 1994). These recent results raise into question the functional significance of the short-form PRLRs, or at least raise the possibility that other signal transduction mechanisms may be coupled to the short-form PRLRs.

Ribonuclease-protection analysis indicated that, in both the fetal liver and adult ovary, the mRNA that lacks the 39 bp insert (oPRLR-1) is the most abundant. This mRNA is predicted to encode the full-length receptor, analogous to the one reported for the cow (Scott *et al.*, 1992). Since the cDNAs generated by RT-PCR do not contain the entire coding region, we can not rule out that the oPRLR-1 cRNA may be protected by other oPRLR mRNAs that diverge 3′ to this sequence. Furthermore, we can not rule out the possibility that sequence divergence exists 5′ to our cDNAs as

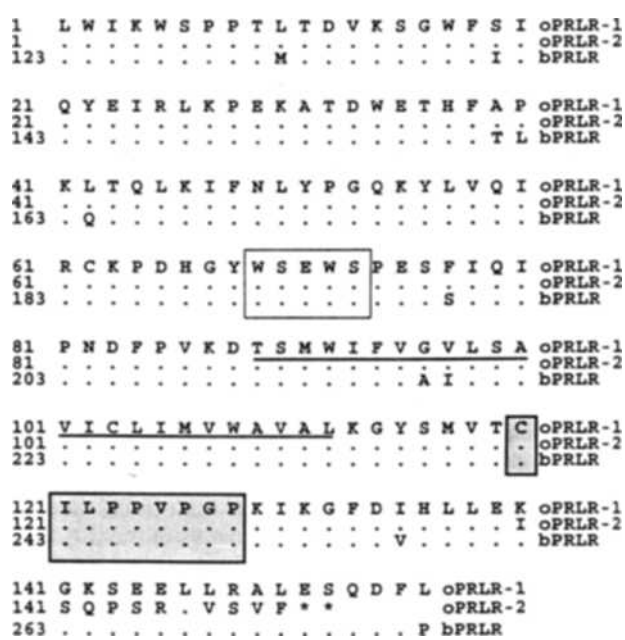

Figure 5 Amino acid comparison of oPRLR-1 (top), oPRLR-2 (middle) and the bovine PRLR (bPRLR, bottom). The WSXWX motif is enclosed by an open box, the predicted transmembrane region is underlined and Homology Box 1 is enclosed in the shaded boxes. The numbering of the bPRLR is according to Scott *et al.* (1992)

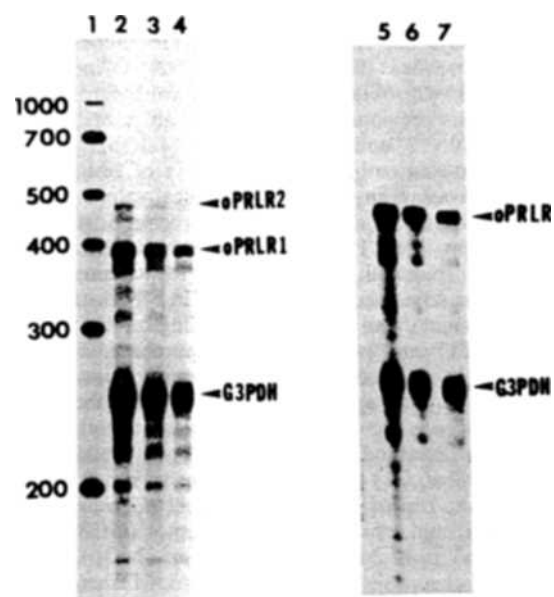

Figure 6 Ribonuclease-protection assay of 30 μg (lanes 2 and 5), 20 μg (lanes 3 and 6) and 10 μg (lanes 4 and 7) of day 10 ovine luteal total cellular RNA (tcRNA), using oPRLR-2 (lanes 2–4) and oPRLR-1 (lanes 5–7) as the cRNAs. An ovine cRNA for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was also used as an internal control to test tcRNA integrity. Note that in lanes 2–4 the cRNA is predominantly cleaved to a smaller size because the majority of the oPRLR mRNA does not possess the 39 bp insert found in oPRLR-2, but that in lanes 5–7 the oPRLR-1 cRNA is protected by both species of oPRLR mRNA. Lane 1 represents 5′-end labeled DNA molecular weight markers

occurs in chickens and pigeons (Tanaka *et al.*, 1992; Chen & Horseman, 1994), but no such variants of the extracellular domain of mammalian PRLRs have been reported. If we assume that oPRLR-1 is indeed just protecting mRNA encoding the long-form of the oPRLR, then our data is in agreement with what has been reported in the mouse and rat. In the mouse (Clarke & Linzer, 1993) and rat (Nagano & Kelly, 1994), the mRNA encoding the long-form PRLR predominates in the ovary, although variation in this pattern occurs if individual tissues within the ovary are examined (Clarke & Linzer, 1993; Clarke *et al.*, 1993). In contrast, in these species, the short-form PRLR predominates in the liver (Clarke & Linzer, 1993; Nagano & Kelly, 1994).

The oPRLR cDNAs were generated from two tissues that do not appear to readily bind oPRL (Gluckman *et al.*, 1983, Bramely *et al.*, 1987a,b, Freemark *et al.*, 1987). The lack of oPRL binding reported for these tissues may be misleading in that only unoccupied receptors were quantified. Furthermore, the disparity between binding of human GH and oPRL to ovine luteal membranes was attributed to selective inactivation of oPRL (Bramley *et al.* 1987b). In light of our data, the identification of PRLRs in these tissues needs to be revisited. Additionally, it can not be ruled out that oPL, or ovine analogs of the bovine prolactin-related proteins (Schuler *et al.*, 1991) are binding to one or more of the receptors encoded by these mRNA. What we can conclude is that sheep, like rodents, possess more than one mRNA encoding the PRLR, and it will be of interest to examine the binding affinities, ligand-specifities and functions of the PRLRs encoded by these mRNA.

Materials and methods

Reagents

All animals and tissues obtained were done so in accordance with animal care and use protocols 1041 and 1706 at the University of Missouri. Fetal livers were obtained by performing a mid-ventral laparotomy and hysterectomy on ewes ($n = 21$) at days 60, 90, 105, 120 and 135 of gestation as previously described (Kappes *et al.*, 1992). Ovine corpora lutea ($n = 24$) were obtained by mid-ventral laparotomy and ovariectomy as previously described (Smith *et al.*, 1994) on days 3, 7, 10, 13 and 16 postestrus. Maternal liver was obtained following euthanasia at 100 days of gestation. The tissues were washed in sterile physiological saline, frozen in liquid N_2 and stored at $-80°C$ until processed for tcRNA. The GeneAmp RNA PCR kit was purchased from Perkin-Elmer Cetus (Norwalk, CT) and the TA cloning kit was acquired from Invitrogen (San Diego, CA). Nick-translation kits were bought from Amersham (Arlington Heights, IL), and [α-^{32}P]dCTP and [α-^{32}P]UTP were purchased from New England Nuclear (Boston, MA). RNA transcription kits were purchased from Stratagene (LaJolla, CA) and the RPA II ribonuclease-protection assay kit was bought from Ambion (Austin, TX). Zetabind nylon membrane was obtained from CUNO (Meriden, CT) and the Sequenase version 2.0 kit was procured from United States Biochemical (Cleveland, OH). Bovine PRLR and growth hormone receptor (GHR) cDNAs were obtained from Monsanto (St. Louis, MO). All other reagents and chemicals were purchased from Fisher Scientific (St. Louis, MO).

RNA isolation

Total cellular RNA (tcRNA) was isolated from fetal liver, maternal liver and corpora lutea by methods previously described (Warren *et al.*, 1990, Smith *et al.*, 1994). Briefly, the tissue was homogenized in 4 M guanidinium thiocyanate (0.5% sodium N-lauroyl sarcosine, 25 mM Na-citrate/pH 7.0, 0.1% antifoam A and 0.1 M 2-mercaptoethanol) and centrifuged at 10 000 *g*. The supernate was collected and loaded on a 5.7 M CsCl (25 mM K-acetate/pH 5.5) cushion and centrifuged at 100 000 *g* for 20 h. The resulting RNA pellet was then resuspended in diethyl pyrocarbonate-treated double-distilled water, reprecipitated, washed with 80% EtOH, and resuspended at a concentration of 1 μg/μl.

Reverse transcriptase-polymerase chain reaction

Two μg of tcRNA samples were reverse transcribed using oligo-dT primers (2.5 μM) or a 3′ PRLR-specific primer (0.16 μM at 42°C for 1 h, and was heated at 99°C for 5 min to inactivate the recombinant Moloney Murine Leukemia Virus reverse transcriptase (M-MLVRT). The reaction mixture contained 2.5 U of M-MLVRT and 1 U of RNase inhibitor in 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-Cl (pH 8.3) and 1 mM of each dNTP in a final volume of 20 μl. The RT reactions for each sample were run in duplicate and pooled and 6 μl of the pooled RT reactions were subjected to the polymerase chain reaction (PCR). The reaction mixture contained the template, 0.16 μM of the 5′ and 3′ primers, and 0.4 U of Taq DNA polymerase in a final volume of 30 μl (2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-Cl/pH 8.3). The mixture was overlaid with 50 μl of mineral oil and subjected to PCR. The PCR reactions were conducted for 35 cycles at 94, 60 and 72°C for 1 min each. The PRLR 5′ (TCCAGAAGATAGAAAACCATA) and 3′ (AAGTCCTCGCAGTCAGAAGTG) primers were predicted to generate a 513 bp product beginning at nucleotide (nt) 503 (extracellular domain) and ending at nt 1015 (cytoplasmic domain) of the bovine PRLR cDNA (Scott *et al.*, 1992; Figure 1). The 3′ primer resides within the analogous sequence to Homology Box 2 (DaSilva *et al.*, 1994) of the rat and human PRLRs, and the 5′ primer resides upstream of the WSXWS motif. Additionally, primers for the ovine growth hormone receptor (GHR) were also used to evaluate specificity of hybridization. The 5′ (CAACTGGACTCTGCTGAACAT) and 3′ (TAGCAGGGGCAGCATCATTAG) ovine GHR primers generate a product that span from nt 648 to 1510 of the oGHR cDNA (Adams *et al.*, 1990).

Southern hybridizations

Each 30 μl PCR reaction was subjected to electrophoresis in a 1% or a 1.5% agarose gel. The gel was stained with ethidium bromide (EtBr; 1 μg/ml), photographed, and prepared for Southern blotting by incubation for 20 min in 0.5 N NaOH and 1.5 M NaCl and by neutralization in 0.5 M Tris-Cl/pH 8.0 and 1.5 M NaCl two times for 15 min. The gels were equilibrated in 1 × SSC (20 × = 3 M NaCl and 0.3 M Na-citrate) and capillary blotted onto Zetabind nylon membranes. Membranes were baked for 1 h at 80°C, prehybridized for 4 h in 6 × SSPE (20 × = 3 M NaCl, 0.21 M NaH_2PO_4 and 0.02 M EDTA/pH 7.0), 10 × Denhardt's solution (50 × = 1.0% Ficoll, 1.0% polyvinylpyrrolidone and 1.0% BSA), 50 μg/ml salmon sperm DNA, 50 μg/ml yeast tRNA, 0.5% SDS and 50% deionized formamide at 42°C. Hybridization in the presence of $\approx 1 \times 10^6$ c.p.m./ml of a ^{32}P-labeled bovine PRLR cDNA or GHR cDNA was conducted overnight at 42°C in 6 × SSPE, 0.5% SDS, 50 μg/ml salmon sperm DNA, 50 μg/ml yeast tRNA and 50% deionized formamide. Membranes were washed for 30 min each in 2 × SSC/0.1% SDS and 0.1X SSC/0.1% SDS at room temperature, followed by a 30 min wash in 0.1 × SSC/0.1% SDS at 65°C. The membranes were then exposed to autoradiographic film from 1 h to overnight. The RT-PCR products, amplified as described above, were subcloned with the TA Cloning Kit, and the resulting clones were subjected to dideoxy-nucleotide sequencing (Warren *et al.*, 1990; Smith *et al.*, 1994). Two different sizes of ovine PRLR cDNAs were obtained (oPRLR-1 and oPRLR-2) from both fetal liver and adult ovary tcRNA. The sequences of these cDNAs were compared to known PRLR cDNAs by the IBI Pustell sequence analysis software (New Haven, CT).

Ribonuclease protection assay

Complementary RNAs were generated by linearizing the plasmid DNA containing oPRLR-1, oPRLR-2 and ovine glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNAs, and incubating 1 μg in 40 mM Tris-Cl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 40 μM each of rGTP, rATP, rCTP and 4.0 μM rUTP, 30 mM DTT, 1 U RNase inhibitor, 125 μCi [α-^{32}P]UTP and 10 U of the appropriate RNA polymerase. Following the transcription reaction, the samples were treated with 10 U DNase, and the cRNAs were gel-purified from 5% acrylamide/8 M urea sequencing gels. The cRNAs (200 000 c.p.m. each) were co-precipitated with the appropriate tcRNA samples or yeast tRNA, and the resulting pellets resuspended in 20 μl of hybridization buffer (80% deionized formamide, 100 mM Na-citrate/pH 6.4, 300 mM Na-acetate/pH 6.4, 1.0 mM EDTA). The samples were denatured at 95°C for 3 min, and allowed to hybridize at 45°C for 18 h. Following hybridization, the samples were treated with RNase (1 U RNase A and 40 U RNase T) at 37°C for 30 min, and the reactions were inactivated and precipitated. The resulting pellets were resuspended and the protected fragments separated on a 5% acrylamide/8 M urea sequencing gel and visualized by autoradiography (overnight exposure).

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