Alternative splicing of the prolactin receptor gene generates a 1.7 kb RNA transcript that is linked to prolactin function in the red deer testis

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ABSTRACT

A cDNA encoding a putative non-membrane bound prolactin receptor was amplified by RT-PCR from red deer (Cervus elaphus) testis. Sequence analysis suggests that the testicular cDNA is generated by alternative splicing resulting in the deletion of exons 7 and 8, which code for: (a) the final 53 aa of the extracellular domain of the receptor including the fifth conserved cysteine residue and the WS × WS motif, (b) the entire transmembrane domain, (c) the first three cytoplasmic amino acid residues, and (d) two nucleotides of the fourth cytoplasmic amino acid codon. The resultant RNA would encode a putative protein of 174 aa due to a single bp frame shift and a premature stop codon. Northern blot analysis confirmed that the PCR-amplified cDNA is encoded by a specific 1.7 kb RNA transcript whereas the membrane bound receptor is encoded by transcripts of 3.5 and 2.5 kb. HPLC studies using media from 293 cells transfected with the 1.7 kb cDNA failed to detect any specific binding for prolactin. These data suggest that: (a) the deletion in the 1.7 kb transcript alters the structure of the prolactin binding domain in the putative protein encoded by the 1.7 kb transcript, and (b) alternative splicing of the prolactin receptor gene toward the 1.7 kb transcript is a means of down-regulating the expression of the full length prolactin receptor and hence may modify the role of prolactin in the testis of seasonally breeding mammals such as red deer. The sequence reported in this paper has been deposited in the Genbank/EMBL data base with accession number Y14753.

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INTRODUCTION

The pituitary hormone prolactin acts on a wide variety of tissues and exerts multiple effects (Nicoll et al. 1986) through membrane bound receptors which belong to the superfamily of cytokine receptors (Kelly et al. 1991). The prolactin receptor exists in several isoforms that differ primarily in the sequence and length of the cytoplasmic domain. In rodent species long and short variants of a single transmembrane receptor have been described (Boutin et al. 1988, Davis & Linzer 1989, Shirota et al. 1990). These are transcribed by alternative exon splicing of a single gene that is arranged into at least 11 exons (Kelly et al. 1991).

The existence of multiple forms of the receptor may explain the pleiotropic actions of prolactin in diverse species. The long form of the receptor is associated with lactogenic signal transduction and activates the transcription of β-lactoglobulin, β-casein (Lesueur et al. 1991) and the early immediate gene interferon regulatory factor 1 (O’Neal & Yu-Lee 1994) through a JAK/Stat pathway, whereas both the long and short forms are involved in transducing the mitogenic properties of prolactin through a MAP kinase pathway (Das & Vonderhaar 1995).

In seasonally breeding mammals such as red deer, prolactin secretion is under seasonal regulation via the photoperiodic melatonin signal (Lincoln &
Clarke 1995). In red deer prolactin has been implicated in the regulation of seasonal rhythms of reproduction, pelage growth and metabolism (Curlewis et al. 1988). The receptor for prolactin in red deer has recently been cloned and sequenced and is predicted to encode a protein of 557 aa which differs from the sequence in other species by a 3’ truncation of the cytoplasmic domain located 34 residues before the stop codon (Jabbour et al. 1996). This truncation results in the deletion of the most C-terminal tyrosine residue shown in the rat receptor to be essential for activating the transcription of the β-casein promoter (Lebrun et al. 1995). However, despite this deletion the cervine receptor retains the ability to activate β-casein transcription thus outlining possible species variation in the signalling pathway to prolactin responsive genes.

Northern blot analyses have demonstrated that the prolactin receptor in red deer is expressed in numerous tissues and is encoded by transcripts of approximately 3·5 and 2·5 kb (Clarke et al. 1995). However, in addition to the 3·5 and 2·5 kb transcripts encoding the full length prolactin receptor, a further transcript of 1·7 kb is prevalent in the testis (Jabbour et al. 1996). In this study we describe the cloning and characterisation of a novel cDNA differentially expressed in the testis of red deer. The cDNA arises by alternative splicing of the prolactin receptor gene and is encoded by the previously described 1·7 kb testicular prolactin receptor RNA transcript (Jabbour et al. 1996). However, the putative protein encoded by the truncated cDNA does not bind prolactin, suggesting that the deletion alters the prolactin binding domain of the resultant protein. These data suggest that alternative splicing and hence down-regulation of expression of the full length and membrane bound prolactin receptor may be one physiological mechanism of modulating the function of prolactin in the testis of seasonally breeding mammals such as red deer.

MATERIALS AND METHODS

Tissue collection and RNA preparation

Testis and liver tissues were collected from male red deer (n=3) and immediately snap frozen in liquid nitrogen and stored at −80 °C until used. Total RNA was extracted using the guanidinium thiocyanate method as previously described (Chomczynski & Sacchi 1987). Polyadenylated (polyA+) RNA was purified on oligo(deoxythymidine)-cellulose affinity columns (Pharmacia Biotech, St Albans, Herts, UK). RNA yield and purity were estimated by spectrophotometry.

RT-PCR, cloning and sequencing of the testicular prolactin receptor isoform

Single stranded cDNA was generated from 5 μg polyA+ RNA from both liver and testis by reverse transcription (RT) using 1·6 μg oligo (deoxythymidine)12-18 primer, Superscript RT (Gibco BRL, Paisley, Strathclyde, UK) and conditions described by the manufacturer. The RT product was then diluted 25 times in double distilled H2O and 1 μl (10 ng) cDNA amplified by PCR using primer pairs designed from the untranslated regions of the red deer prolactin receptor cDNA sequence at base pair positions −105 to −86 (H3: 5’-GGC TGG-3’, forward primer) and 1750 to 1770 (Hrev: 5’-GGC CAG GTC AGC CTC GGC TGG-3’, reverse primer). The reaction was carried out in PCR buffer (50 mM KCl, 2 mM MgCl2 and 20 mM Tris–HCl, pH 8·3), 200 μM deoxy-NTPs, 25 pmol forward and reverse primers and 1 U Taq polymerase (Perkin-Elmer, Warrington, Cheshire, UK) in a total volume of 50 μl. The experiment proceeded for 35 cycles of 94 °C for 40 s, 52 °C for 75 s and 72 °C for 2 min 30 s. After a 10 min final extension at 72 °C, the products were isolated on a 1% agarose gel. The PCR products were precipitated at −80 °C for 1 h following the addition of 1 μl glycerol and two volumes ethanol and the precipitate resuspended in 10 μl double distilled H2O. One microlitre was ligated overnight into pMos-blue vector according to the manufacturer’s instructions (Amersham International, Little Chalfont, Bucks, UK). White colonies were isolated and grown to saturation at 37 °C in 2·5 ml Lauria-Bertani broth. Insert size of plasmid preparations were determined by digestion with EcoRI and HindIII and subsequently Southern blotting (Southern 1975) with [α32P]dCTP-labelled cDNA probe (P1; see section below) coding for the extracellular domain of the receptor. One positive clone with an approximately 1·7 kb insert was amplified by large scale plasmid preparation and sequenced in both directions using the dideoxynucleotide chain termination method. Subsequently, the 1·7 kb prolactin receptor cDNA insert was subcloned unidirectionally according to the manufacturer’s directions into pCR 3-Uni (TA Cloning kit, Invitrogen, NV Leek, The Netherlands) for use in expression studies.

Generation of cDNA probes and Northern hybridisation

Two cDNA probes termed P1 and P3 were utilised in Northern hybridisation in order to determine whether the testicular cDNA is encoded by the
The 1.7 kb transcript previously reported in the testis (Jabbour et al. 1996). P1 was previously generated by PCR and coded for part of the extracellular domain which is common to both the full prolactin receptor and the 1.7 kb testicular cDNA (Clarke et al. 1995). In addition, a second cDNA probe termed P3 was generated by PCR as described above using primers that flanked the region of the full length prolactin receptor cDNA which was deleted from the 1.7 kb testicular cDNA. The forward and reverse primers were designed at bp positions 539-558 (5'-GG GAG ATT CAT TTT GCT GCG-3') and 766-785 (5'-CT ATA GCC TTT CAA AGC CAC-3') respectively. The amplified product was subcloned into pMos-blue and sequenced in both directions.

The Northern hybridisation was conducted as previously described (Jabbour et al. 1996). Briefly, samples of 10 µg polyA+ RNA from testis and liver were electrophoresed through a 1% agarose-2.2 M formaldehyde gel and transferred to Hybond-N+ membrane (Amersham) by capillary blotting. The RNA was fixed to the membrane by 3 min UV transillumination and prehybridised at 65 °C for 4 h in 1% BSA, 1 mM EDTA, 0.5 M NaHPO4, 7% SDS and 100 mg denatured salmon sperm DNA. The membranes were then hybridised (initially with probe P1) overnight at 65 °C in the prehybridisation buffer in the presence of 10^6 c.p.m./ml [α^32P]dCTP-labelled cDNA probe previously denatured for 5 min at 100 °C. Washes were performed at 65 °C once in 1 × SSC-0.1% SDS and then twice in 0.1 × SSC-0.1% SDS, for 30 min each. Autoradiographic exposure was for approximately 1 week at −80 °C with an intensifying screen. To determine whether the 1.7 kb testicular prolactin receptor RNA transcript visible on the autoradiograph corresponded to the testicular cDNA amplified by PCR, the blot was stripped by soaking in boiling 10% SDS and then rehybridised to the cDNA probe P3 labelled with [α^32P]dCTP as above. Thus two autoradiographs were obtained for the same blot using two different cDNA probes.

**In vitro expression of the 1.7 kb testicular cDNA**

The human fibroblast 293 cell line was grown in Dulbecco’s Modified Eagle’s Medium (DMEM)/nutrient mix F12 (Gibco BRL) containing 10% fetal calf serum (FCS). Six hours before transfection, cells were plated on 100 mm culture dishes and grown to 50% confluence in a rich medium (two parts DMEM/F12 and one part DMEM containing 4.5 g glucose/l) containing 10% FCS. Transient transfection using the calcium phosphate precipitation procedure were carried out with 10 µg of the 1.7 kb testicular cDNA in the expression vector pCR 3-Uni. The transfected cells were incubated overnight at 37 °C in a 3% CO2 atmosphere. The following morning, the cells were changed to a 5% CO2 atmosphere and incubated for a further overnight period in serum free medium at 37 °C. Medium from transfected cells was then stored at −80 °C and subsequently used for HPLC analysis. In addition, parallel positive control reactions were conducted using 10 µg of the full length red deer prolactin receptor cDNA (Jabbour et al. 1996). These cells were subjected to membrane binding experiments using 125I-hGH and these confirmed the expression of membrane bound prolactin receptor protein in this in vitro model, as has been demonstrated previously (Jabbour et al. 1996). In addition, transfection success with the 1.7 kb cDNA was confirmed by conducting RT-PCR on total RNA extracted from the transfected cells. The primers used in the PCR reaction were designed at bp position 73–93 (forward: 5'-CAG TCA CCT GCT GGA AAA CCC-3') and 1750–1770 (reverse: 5'-GGC CAG GTC AGC CTC GGC TGG-3') of the prolactin receptor cDNA and amplified as expected a product of 1450 corresponding to the 1.7 kb testicular RNA transcript. Double distilled
water was used as a negative control. The transfection efficiency using this experimental model is approximately 30–40% (Buteau et al. 1998).

HPLC analysis was conducted following the protocol of Postel-Vinay et al. (1991). $^{125}$I-hGH was used as a tracer as previous experiments have demonstrated that the prolactin receptor displays similar binding affinity for hGH and ovine prolactin (Jabbour et al. 1996). In this experiment, concentrated medium (100 µl) from transfected cells was incubated overnight at 4°C in the presence of $2 \times 10^5$ c.p.m. $^{125}$I-hGH with incubation buffer (0·1 M potassium phosphate, pH 7·0, 0·1% BSA) in a total volume of 200 µl. Parallel incubations were carried out in the presence of excess (5 µg) ovine prolactin. After filtration through a 0·45 µm Millipore minifilter, the entire incubation mixture was injected onto an HPLC Protein Pak 300 sw column. Elution was performed automatically using degassed buffer (0·1 M Na$_2$SO$_4$ and 0·1 M potassium phosphate, pH 7·0) pumped at a rate of 0·5 ml/minute. Radioactivity was recorded on-line.

**FIGURE 2.** Sequence of the red deer 1·7 kb testicular prolactin receptor cDNA. Predicted amino acid sequence is also shown. The first 24 aa (shown in italics) correspond to the cleaved signal peptide thus resulting in a non-membrane bound protein of 174 aa (every tenth amino acid is indicated by a number in brackets). The four conserved extracellular cysteines are shown (bold and underlined). Alternative splicing resulting in the deletion of exons 7 and 8 (boundary between exons 6 and 9 indicated by //) results in a 17 aa novel hydrophilic tail and a premature stop codon (underlined). The sequences of the 5' and 3' untranslated regions are shown in lower case letters.
RESULTS

RT-PCR of testicular messenger RNA using primers flanking the entire prolactin receptor cDNA amplified two products of approximately 1·9 kb (1875 bp) and 1·7 kb (1633 bp). This is in contrast to the 1·9 kb (1875 bp) product amplified in the liver (Fig. 1) which has been shown previously to encode the full length prolactin receptor cDNA (Jabbour et al. 1996). The sequence of the 1633 bp cDNA amplified from the testis is presented in Fig. 2. Sequence analysis revealed that the sequence is almost identical to the liver clone, except for the deletion of 242 bp nucleotides at position 544 to 785 of the prolactin receptor cDNA. The deleted region corresponds precisely to exons 7 and 8 of the murine prolactin receptor gene (Ormandy et al. 1997). These missing exons encode: (a) the final 53 aa of the extracellular domain including the fifth conserved cysteine residue and the WS × WS motif, (b) the entire 24 aa transmembrane domain, (c) the first three cytoplasmic amino acid residues, and (d) two nucleotides of the codon encoding the fourth cytoplasmic residue resulting in a 1 bp shift of the codon reading frame distal to the deletion (Fig. 3). The sequence following the deletion thus encodes 17 new amino acid residues followed by a premature stop codon. The resultant cDNA encodes a putative protein of 198 aa consisting of a 24 aa signal peptide and a mature protein of 174 aa.

Northern blots of polyA+ RNA from testis and liver tissues hybridised to the homologous P1 and P3 cDNA probes are presented in Fig. 4. Different patterns of prolactin receptor mRNA transcripts were observed in the liver and testis following hybridisation with the extracellular domain cDNA probe P1. The receptor in the liver was encoded by transcripts of approximately 3·5 kb and 2·5 kb. This pattern was observed in the testis; however, an additional transcript of 1·7 kb was also evident. By contrast the cDNA probe P3 hybridised to the 3·5 kb and 2·5 kb transcripts but did not recognise the 1·7 kb testicular transcript. This suggests that the 1·7 kb transcript must lack the 242 bp region encoded by P3 and that the testicular cDNA amplified by PCR is encoded by this transcript in the testis.

A typical elution profile of 125I-hGH incubated with culture medium from cells transfected with the

![Diagrammatic representation of the generation of the red deer long and truncated testicular forms of the prolactin receptor by alternative splicing of the prolactin receptor gene. Exons 7 and 8 are omitted from the mRNA transcript encoding the truncated testicular form, resulting in the translation of a soluble protein with a novel hydrophilic tail (shaded region) and a premature stop codon (Early TGA). Position of the 24 aa signal peptide (SP), start codon (ATG), extracellular cysteine residues (Cys), WS × WS motif (WS), transmembrane domain (TMD), proline rich region (box 1) and long form stop codon (TGA) are shown.](image-url)
1.7 kb testicular cDNA is presented in Fig. 5. All the radioactivity was contained within a single peak, the elution time of which corresponded to unbound 125I-hGH. This peak persisted in the presence of unlabelled excess ovine prolactin. These data suggest that the putative protein encoded by the 1.7 kb testicular cDNA does not bind prolactin.

DISCUSSION

A cDNA encoding a putative non-membrane bound form of the prolactin receptor was isolated by RT-PCR from red deer testis. This novel cDNA isoform arises by alternative exon splicing during RNA transcription resulting in a deletion of 242 bp. The deleted region corresponds exactly to exons 7 and 8 of the murine prolactin receptor and is inclusive of the WS×WS motif, the fifth extracellular cysteine residue and the cytoplasmic region commonly associated with prolactin signal transduction (Rozakis-Adcock & Kelly 1991). As a result of this deletion, the predicted amino acid sequence encoded by this cDNA is altered. A 1 bp shift in the reading frame caused by incomplete codon deletion would result in the translation of 17 new amino acid residues distal to the deletion and a premature stop codon. The putative protein encoded by this transcript is a non-membrane bound polypeptide of 174 aa (exclusive of the 24 aa signal peptide) with a novel hydrophilic tail.

Alternative exon splicing is a well documented method by which multiple RNA transcripts encoding prolactin receptor isoforms are produced in certain species. The clearest example of this is in the rat where the cytoplasmic domain of the long and short prolactin receptor are encoded by exons 10 and 11 respectively (Kelly et al. 1991). In the red deer, the prolactin receptor is encoded by a major transcript of 3.5 kb and a minor transcript of 2.5 kb. The additional 1.7 kb transcript observed in the testis is generated by alternative splicing of exons 7 and 8. This has been confirmed following the differential Northern blot analyses in which the 242 bp cDNA (P3) does not hybridise to the 1.7 kb transcript. The possible generation of soluble forms of the prolactin receptor by alternative splicing of the prolactin receptor gene has been suggested previously in murine species (Davis & Linzer 1989, Nagano & Kelly 1995). In addition, similar observations have been reported with the growth hormone receptor in the chicken. Tissue specific alternative splicing due to a mutation in the 5'-splice donor site and the utilisation of an avian specific alternative polyadenylation signal result in the transcription of an additional 0.8 kb mRNA transcript that codes for part of the extracellular domain of the growth hormone receptor (Huang et al. 1993, Oldham et al. 1993). This truncated growth hormone receptor transcript appears to be translated based on its association in vivo with polyribosomes; however, the potential role of this putative protein product is not yet established (Oldham et al. 1993). The significance and molecular mechanisms for the generation of the truncated 1.7 kb transcript in red deer testis is not clear. A similar molecular pathway to that reported for the growth hormone receptor can be envisaged. Such a genetic modification may have evolutionary physiological significance for the role of prolactin on testicular function in seasonal breeders within the family Cervidae or more widely amongst the Ruminantia.

A secreted protein encoding the extracellular domain of the prolactin receptor has been previously reported in rabbit (Postel-Vinay et al. 1991) and human milk (Mercado & Baumann 1994). These function as prolactin binding proteins and display a higher binding affinity to prolactin than does the membrane bound prolactin receptor (Postel-Vinay et al. 1991). This is in contrast to the

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protein encoded by the 1.7 kb testicular cDNA, which displays no detectable binding capacity to prolactin by HPLC analysis and as expected by the deletion of the WS × WS motif. The importance of the WS × WS motif in conferring high affinity binding was demonstrated in the rat prolactin receptor following site-directed mutagenesis. Alanine substitution of the WS × WS motif has been shown to result in >300-fold reduction in hormone/receptor binding affinity (Rozakis-Adcock & Kelly 1991). This implies that the protein encoded by the 1.7 kb testicular cDNA may be structurally and functionally different from the prolactin binding protein previously reported in body fluid such as milk. This may be a reflection of the mode of generation of the two proteins. The rabbit prolactin binding protein is thought to be produced by post-translational proteolytic processing of the full length prolactin receptor (Dusanter-Fourt et al. 1991). By contrast, data from this study provide evidence that a transcript encoding the extracellular domain of the prolactin receptor is generated following alternative splicing of the prolactin receptor gene.

Prolactin receptors are expressed in the testis of a number of species (Hondo et al. 1995, Guillaumot et al. 1996) including red deer (Jabbour et al. 1998). Expression of the prolactin receptor gene has been demonstrated both in the seminiferous tubule and interstitial compartments suggesting a role for prolactin in spermatogenesis and steroidogenesis. This function of prolactin would be mediated through the membrane bound prolactin receptors encoded by the 3.5 and 2.5 kb RNA transcripts. By contrast, the exact role of the alternatively spliced 1.7 kb prolactin receptor RNA transcript is not clear. The HPLC data suggest that the putative protein encoded by the RNA is inactive and as such its transcription may be a physiological mechanism to modulate the activity of prolactin by down-regulating the expression of full length and functional membrane bound prolactin receptors. This would be similar to what has been proposed for the 0.8 kb transcript, which codes for a soluble growth hormone receptor in avian species. This 0.8 kb transcript may encode a non-functional protein and as such be a physiological mechanism for down-regulating the expression of the full length and functional chicken growth hormone receptor RNA transcript (Huang et al. 1993, Oldham et al. 1993). Such a mechanism for the prolactin receptor gene may be of particular relevance in seasonal breeders such as red deer which experience huge seasonal fluctuations in endogenous prolactin secretion and circannual cycles of testicular growth and regression (Curlewis et al. 1988). The generation of non-functional prolactin receptor isoforms may be one mechanism of overcoming any adverse effects that hyperconcentrations of prolactin (such as in the summer) may have on male reproduction. Hyperprolactinaemia has been demonstrated previously to have adverse effects on testicular function and spermatogenesis (Carter et al. 1978) and successful reduction of prolactin concentrations can restore
spermatogenesis and fertility (Franks et al. 1978, Thorner & Besser 1978). However, basal concentrations of prolactin are essential for successful and efficient spermatogenesis to occur (Regisdorf & Katz 1993) and mice with a homozygous prolactin receptor knockout are subfertile (Ormandy et al. 1997). In red deer, during the summer months prolactin concentrations are still elevated when testicular growth is initiated in preparation for the onset of the breeding season in autumn. Thus, down-regulation of expression of the full length prolactin receptor transcripts in favour of the 1.7 kb truncated transcript in the testis may be one mechanism of ensuring sufficient stimulation by prolactin while restricting the negative effects of the prevailing ‘hyperprolactinaemic state’ during summer. Such a mechanism would effectively ensure a homeostatic balance between the high prolactin concentration and the required level of membrane bound prolactin receptors for successful ‘priming’ and initiation of spermatogenesis in summer. This alternative hypothesis is strengthened by the observation that the pattern of expression of the 1.7 kb transcript in the testis of the red deer may be seasonally regulated (Clarke et al. 1995).

In conclusion, this study describes the cloning of a cDNA that codes for a putative 174 aa non-membrane bound form of the prolactin receptor. The putative protein encoded by the cDNA does not display any binding capacity for prolactin suggesting that the 1.7 kb transcript acts as a negative regulator of expression of the fully functional membrane bound prolactin receptor. An increase in the rate of alternative splicing towards the production of the 1.7 kb transcript could be a means of regulating prolactin function in the testis and hence fertility of seasonal breeders such as red deer.

The sequence reported in this paper has been deposited in the Genbank/EMBL data base with accession number Y14753.

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