Multiple and Tissue-specific Promoter Control of Gonadal and Non-gonadal Prolactin Receptor Gene Expression*

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Prolactin receptors (PRLRs) are widely expressed, and multiple mRNA transcripts encoding PRLRs are present in prolactin target tissues. The molecular basis for the control of the PRLR gene expression is currently unknown. Analyses of the 5′-untranslated regions of PRLR mRNAs expressed in gonadal and non-gonadal tissues and their genomic organization revealed three alternative first exons designated as E11, E12, and E13. Each of these exons is alternatively spliced to a common noncoding exon (exon 2, nucleotides −115 to −56) that precedes the third exon containing the translation initiation codon. Alternative utilization of exons E11, E12, and E13, as well as alternative splicing of exon 2, generates multiple 5′-untranslated regions in PRLR transcripts. These alternative first exons (E11, E12, and E13) were found to be utilized in a tissue-specific manner in vivo. E11 is predominantly expressed in the ovary, E12 is specifically expressed in the liver, and E13 is expressed as a predominant form in the Leydig cell and as a minor form in the ovary and liver. Genomic 5′-flanking regions containing the three putative PRLR gene promoters (PI, PII, and PIII) that initiate the transcription of E11, E12, and E13, respectively, were identified. E11 was found to initiate from a single site at −549, E12 from multiple sites at −405, −461, and −506, and E13 from two major sites at −340 and −351. These findings indicate that multiple promoters control transcription of the PRLR gene and provide a molecular basis for the differential regulation of PRLR expression in diverse tissues.

Prolactin exerts diverse biological functions including lactation, reproduction, steroidogenesis, metabolism, behavior, immune regulation, growth, and water-salt balance (1) through mechanisms by which the expression of the PRLR is controlled, partly through the control of luteinizing hormone receptor expression (2). Conversely, ovarian PRLRs and their mRNA levels were acutely up- and downregulated by administration of gonadotropins at different stages of ovarian development (6). In the male rat, luteinizing hormone treatment caused rapid and transient loss of Leydig cell prolactin receptors (8). These studies suggested that gonadotropins exert heterologous control of PRLR expression in the gonads. Furthermore, hepatic prolactin receptors were markedly induced by estrogen and during late pregnancy (4).

MATERIALS AND METHODS
RNA Isolation, 5′-RACE PCR, and Northern Blot Analysis—Poly(A)*
RNA was prepared from the ovary tissue of pseudopregnant rats (9), the Leydig cells of adult male rats (10), and the liver of pregnant rats. 5′-RACE PCR was performed according to the manufacturer’s protocol (Life Technologies, Inc.). Primers used for the 5′-RACE PCR were 5′-GGATCGGAGAAGCTCTC-3′ (#6, +312 to +332); 5′-CTGTC-GAGGATCCAC-3′ (#6, +125 to +142); 5′-TTGACCACTCTTCAGTACC-3′ (#4, −37 to −14) (Fig. 1, left panel). PCR products were subcloned into pCRII vector (Invitrogen, San Diego, CA). Northern blot hybridization was performed as described previously (6).

Screening of Genomic Library and Subcloning—A DASH rat genomic library (Stratagene, La Jolla, CA) was screened using cDNA probes that contained 5′-end sequences from the various PRLR mRNA 5′-UTRs (designated as E1, E2, and E3, Fig. 1)), as well as an oligomer probe 5′-GGGTCATGTCGAAACTCTGC-3′ (#6, −61 to −84). Phage DNA was analyzed by restriction mapping and Southern hybridization. Genomic fragments were subcloned into pGEM-4Z vector (Promega, Madison, WI). Sequencing was performed using T7 Sequenase version 2.0 (U. S. Biochemical Corp.).
Results and Discussion

Heterogeneity and Tissue-Specific Expression of PRLR mRNA 5'-UTRs—Comparison of 5'-UTR sequences of reported PRLR cDNAs revealed sequence divergence 5' from nucleotide -60 (ATG +1). Sequencing of 5'-RACE PCR products of the PRLR mRNAs from the rat ovary, Leydig cells, and liver verified the divergence of 5'-UTRs upstream from -60 and extended the 5'-UTRs to the 5'-ends (Fig. 1, left panel). Three distinct 5'-end mRNA sequences were identified and designated as E11 (442 nt), E12 (233 nt), and E13 (236 nt) (Fig. 1, right panel). Each of these sequences is followed by a region of either 55 nt (-115 to -60) or 23 nt (-115 to -93) with a 33-nucleotide deletion at positions -92 to -60. In addition, an ovarian form with deletion at -115 to -60 was identified. All sequences downstream of -60 were identical and conform to the reported PRLR cDNA forms. Tissue-specific expression of the PRLR mRNA 5'-UTRs was revealed by Northern hybridization of PRLR mRNA and Southern hybridization of 5'-RACE PCR products using 5'-end sequence probes. Northern blots showed that E11 was expressed only in the ovary (Fig. 2A, E11). However, very low levels of E11 were detected in Leydig cells but not in the liver by 5'-RACE PCR analyses. E12 was exclusively expressed in the liver and is the major form in this tissue (Fig. 2A, E12). E13 is expressed in the three tissues as the predominant form in Leydig cells (Fig. 2A, E13 and C) and as a minor form in the ovary and liver (Fig. 2A, E12 and B).

In addition, it was observed in the ovary that the difference between the 2.1- and 1.8-kb mRNA species corresponding to the short form of the receptor (with brief cytoplasmic domain) could be accounted for by the presence in the 5'-UTR of E11 (442 nt) and E13 (236 nt), respectively (Fig. 2B). However, this 5'-UTR difference was not resolvable for the 9.7-kb species that encodes the long form of the receptor (with extended cytoplasmic domain). Since both E11 and E13 sequences were associated with PRLR mRNAs of both receptor forms, it is suggested that the generation of the long and short form of the receptor is independent of promoter specificity (see below) and may result from a posttranscriptionally regulated process. In the Leydig cell, only the E11 mRNA species was detected on Northern blots with major bands at 9.7 and 1.8 kb (Fig. 2C). In the liver, both E12- and E13-containing species were resolved as one broad 1.8-kb band due to the small size difference between E12 (290-
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**Fig. 3.** Genomic organization of PRLR 5'-UTR and exon-intron boundaries. Top, the diagram of the genomic region corresponding to the 5'-UTR of the PRLR mRNA. E1, E12, and E13 are the three alternative first exons transcribed from three putative promoter regions PI, PII, and PIII, respectively (not in scale). The alternative splicing patterns are illustrated by lines and arrowheads connecting different exons. Gap regions between exons are indicated by //. Middle, the genomic clones with sizes (kb) and partial restriction enzyme mapping are indicated (E, EcoRI; X, XbaI; B, BamHI). Bottom shows sequences of the exon-intron boundaries and exon 2. Underlined TGAAGs at 293 and 256 are the two alternate 5'-donor recognition sites, a, the position of E1 in relation to E11 and E12 is arbitrary since no overlapping clones were isolated. b, this exon was deduced by the identification of the second intron position at +35 and another intron at +54 (revealed from isolation of an exon of 133 base pairs located within the coding region at +55 to +187, not shown).

**Fig. 4.** Mapping of PRLR gene transcription initiation sites from the three putative promoter regions. Primer extension was performed for PI (ovary mRNA), PII (liver mRNA), and PIII (ovary, Leydig cell, and liver mRNA). S1 nuclease protection assay was performed for PII (liver mRNA, lane 5). Yeast RNA was used for negative controls (lanes 2, 4, 6, 9, and 12). Sequence ladders were run along with the samples. Free probe at 362 base pairs was indicated (lanes 5 and 6). The specific extended or protected bands were determined within bandwidth error of ±2 base pairs (arrows). The sizes are in base pairs from primer locations 166 (lane 1); 162, 218, 263 (lanes 3 and 5); and 107 and 118 (lanes 7, 8, 10, and 11) corresponding to transcription initiation sites (initiation codon ATG as +1) at −549 (PI); −405, −461, −506 (PII); and −340 and −351 (PIII), respectively. The small arrow indicates a minor start site at −337 revealed only in the ovary.

390 nt, see below) and E12 (236 nt) (Fig. 2A, CRS, E12, E13).

Heterogeneity of PRLR mRNA species can arise from differential transcription initiation, alternative splicing of the coding region, and 3' alternative polyadenylation. Although the coding region of the long and short forms of the receptor partially accounted for the size difference between the mRNA transcripts encoding the two receptor forms (6, 7), it is evident from present data that differential transcriptional initiation also contributes to the mRNA heterogeneity (2.1 and 1.8 kb). In addition, the large size of the 9.7-kb species, which was more highly expressed in the ovary than in the testis (Fig. 2A), can be accounted for by a long stretch (−7 kb) of 3'-UTR (based on the sizes of the coding region, 1.8 kb, and the 5'-UTR, 0.35 and 0.55 kb).

Genomic Organization and Alternative Splicing of the PRLR mRNA 5'-UTR—The identification of three distinct 5'-end sequences was indicative of utilization of different promoters for transcription initiation of the PRLR gene. By employing the three 5'-UTR sequences (E1, E12, and E13) as probes, corresponding genomic clones were isolated from the rat genomic library (Fig. 3, middle). The gene region corresponding to the 5'-UTR spanned at least 60 kb (Fig. 3, top). Sequencing of gene fragments corresponding to E1, E12, and E13 confirmed the existence in the gene of the three 5'-end sequences identified by 5'-RACE PCR analyses. These sequences were designated as the alternative exons 1 of the PRLR gene. The first intron position was identified between the exons 1 (E1, E12, and E13) and nucleotide −115. Overlapping genomic clones containing both E1 and E12 were isolated, and E12 was localized −10 kb upstream of E12 in the PRLR gene (Fig. 3, top). Since E13 genomic clones did not overlap with any other clones, their position in relation to E1 and E12 remains to be determined. The doing of a genomic fragment corresponding to the oligomer probe 2 (−61 to −84) revealed the second exon at −115 to −56 and the second intron position at −55 (Fig. 3, bottom). An alternate splicing donor site was identified at −93 (TGAAG) of exon 2, which conforms to the consensus 5'-splicing site sequence (11) and accounts for the alternative splicing found in the PRLR mRNA 5'-UTRs (Fig. 1, left and Fig. 3, top). Since the alternate donor sequence TGAAG at −93 is identical to the one at −56, the divergence of the PRLR mRNA 5' UTR occurs upstream from −60. Thus, the 5'-UTR of the PRLR gene is composed of at least three alternative first exons (E1, E12, and E13) and one common exon 2. The three
alternative first exons are transcribed from three putative promoter regions, and each of these is spliced to the second exon before joining to the third exon containing the translation initiation codon. The organization of the 5'9-UTR in the gene has defined the alternate splicing variants of the PRLR mRNA 5'9-UTR. However, the presence of additional alternative first exons and promoters of the PRLR gene in other expressing tissues not investigated in this study (i.e. mammary glands, prostate, adrenal, kidney, pancreas, and thymocytes) cannot be excluded. We conclude that multiple forms of PRLR mRNA 5'-UTRs resulted from the alternative utilization of three first exons and alternative splicing of the second exon.

Mapping of Transcriptional Initiation Sites and Sequences of the Three Putative Promoter Regionsof the PRLRGene—A major transcription initiation site for E11 at −2549 was found in the ovary by primer extension analysis, conforming to the 5'9-end derived from the 5'-RACE PCR product (Fig. 4, lane 1). Multiple transcription initiation sites for E12 were identified at −2405, −2461, and −506 in the liver by both primer extension (Fig. 4, lane 3) and S1 nuclease protection analyses (Fig. 4, lane 5). Two major transcription initiation sites for E13 were identified at −340 and −351 in the ovary, Leydig cells, and the liver by primer extension analyses, consistent with the 5'-ends derived from 5'-RACE PCR analyses (Fig. 4, lanes 7, 8, and 10). In addition, a minor transcriptional initiation site for E12 at −337 was observed in the ovary (Fig. 4, lane 11) only after prolonged exposure of the gel.

Analyses of the three putative promoter region sequences (P1, P11, and P111) have revealed consensus sequences for several transcription factors, which may be important for the basal as well as hormonally regulated promoter activities. Although no canonical TATAA element was observed within an expected distance from the transcription initiation sites, TATA-like sequences are boxed, and consensus elements for transcription factor binding sites are underlined.

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expression may require being differentially regulated. The finding of three unique 5'-end exons in the 5'-UTR, which do not appear individually to associate with specific receptor forms, raises an intriguing question of whether the difference present in the PRLR mRNA 5'-UTR may play a role in regulation of the PRLR gene expression posttranscriptionally. It has been shown that the 5'-UTR was associated with the mRNA stability as well as the translatability in other genes (18, 19). Furthermore, the deletion of partial or entire exon 2 in some of the mRNA forms further diversifies the PRLR 5'-UTR. Interestingly, the sequence deleted at -93 to -60 can potentially form a stem loop structure, and therefore its presence or absence may be significant in regulating the PRLR mRNA stability and/or translatability.

In summary, three alternative first exons and corresponding putative promoter regions, PI, PII, and PIII, of the PRLR gene that are utilized in a tissue-specific manner in vivo were identified in gonadal and non-gonadal tissues. PI and PII function as major promoters in the ovary and in the liver, respectively, while PIII is the dominant promoter in Leydig cells and minor promoter in the ovary and liver. The differential control of these multiple promoters may provide the molecular basis of tissue-specific regulation of the PRLR expression in diverse prolactin target cells.

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