Differential effects of sex steroid hormones on the expression of multiple first exons including a novel first exon of prolactin receptor gene in the rat liver

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Abstract

In addition to the known four alternative first exons E1₁, E1₂, E1₃ and E1₄ of the rat prolactin receptor (PRL-R) gene, a novel first exon, E₁₅, was identified by cDNA cloning of the 5’-end region of PRL-R mRNA in the rat liver. Genomic fragments containing E₁₅ and its 5’- or 3’-flanking regions were also cloned from rat kidney genomic DNA. A sequence search for E₁₅ revealed that E₁₅ is located 49 kb upstream of exon 2 of the PRL-R gene in rat chromosome 2q16. RT-PCR analysis revealed that E₁₅ was preferentially expressed in the liver, brain and kidney. Expression profiles of E₁₂, E₁₃ and E₁₅-PRL-R mRNAs in the liver of male and female rats at 5 days of age and those at 8 weeks of age were examined by RT-PCR. The levels of E₁₂-PRL-R mRNA in the female rat increased remarkably in rats at 8 weeks of age compared with those at 5 days of age, and the levels of E₁₅-PRL-R mRNA in the male rat decreased markedly at 8 weeks of age compared with those at 5 days of age. In the female rat, the levels of E₁₂-PRL-R mRNA at 8 weeks of age decreased with ovariectomy performed at 4 weeks of age and recovered with the administration of β-oestradiol. On the contrary, the levels of E₁₅-PRL-R mRNA increased with ovariectomy and decreased with the oestrogen treatment. In the male rat liver, the levels of E₁₂-PRL-R mRNA at 8 weeks of age increased strikingly with castration performed at 4 weeks of age and became undetectable with the administration of testosterone. The levels of E₁₅-PRL-R mRNA increased slightly with castration and were restored by testosterone treatment. Removal of gonadal tissues and sex steroid hormone treatment had no effect on the expression levels of E₁₅-PRL-R mRNA in both female and male rat livers. These results indicated that the expression of the PRL-R gene in the liver is regulated by the differential effects of sex steroid hormones on the transcription of the multiple first exons including the novel one.

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Introduction

Prolactin (PRL) has many physiological functions in vertebrates (Doppler 1994, Freeman et al. 2000). The actions of PRL are mediated by the PRL receptor (PRL-R), which belongs to the type 1 cytokine receptor superfamily (Kelly et al. 1991). In mammals, the long and short forms of PRL-R differing in the length and sequences of their cytoplasmic domains are generated by alternative splicing of a primary transcript from a single PRL-R gene (Boutin et al. 1988, Davis & Linzer 1989, Shirota et al. 1990, Bignon et al. 1997, Schuler et al. 1997). The expression of the PRL-R gene is regulated by complex mechanisms. Multiple alternative first exons encoding 5’-untranslated regions have been identified in rat and human PRL-R genes, and the distinct expression patterns of these first exons are controlled by promoters located in the upstream regions of each first exon. In the rat PRL-R gene, three distinct first exons E₁₁, E₁₂ and E₁₃, together with their promoter regions P₁, P₁I and P₁II, have been identified (Hu et al. 1996, Moldrup et al. 1996). These three exons are located in the order of E₁₃, E₁₁, E₁₂ from the distal side of exon 2 of the PRL-R gene (Hu et al. 1998a). E₁₁ and E₁₂ are predominantly expressed in gonadal tissues and liver respectively, while E₁₃ is expressed in a wide range of tissues (Hu et al. 1996). The gonad-specific expression of E₁₁ is dependent on the activation of its promoter by steroidogenic factor 1 (Hu et al. 1997), and hepatocyte nuclear factor 4 (HNF4) participates in the expression of E₁₂ in the liver (Moldrup et al. 1996). The activation of the E₁₃ promoter by the transcription factors C/EBPβ and SP1 accounts for the generic expression of E₁₃ (Hu et al. 1998b). We have recently found another first exon, E₁₄, which is preferentially expressed in the brain, though its location in the rat PRL-R gene is not known (Tanaka et al. 2002). In the human PRL-R gene, six alternative first exons, hE₁₃, hE₁₇ₐ, hE₁₇₂, hE₁₇₃, hE₁₇₄ and
hE\textsubscript{N3}, have been identified (Hu et al. 1999, 2002). hE\textsubscript{13} is considered to be the counterpart of rat E\textsubscript{13} because its promoter shows high sequence similarity to that of rat E\textsubscript{13} and shows high activity in various human tissues. The other five first exons show unique expression profiles in human tissues such as liver, ovary, testsis and in a breast cancer cell line, T47D cells.

The presence of the multiple first exons allows the PRL-R gene to be regulated in different ways in individual tissues. We have previously demonstrated that the higher levels of PRL-R mRNA in the rat brain in late pregnancy and the following lactating period are dependent on the expression of E\textsubscript{14} (Sugiyama et al. 1994, Tanaka et al. 2002). It has also been shown that the expression levels of PRL-R mRNA in the different brain regions are regulated by oestrogen, which increases the utilization of E\textsubscript{12} and E\textsubscript{13} first exons (Pi et al. 2003). In the liver, the expression of the PRL-R gene is known to be regulated by sex steroid hormones; up-regulation by oestrogen and down-regulation by testosterone (Jolicoeur et al. 1989, Sakaguchi et al. 2005). It has also been shown that the molecular mechanisms of the effects of the sex steroid hormones on the expression of the PRL-R gene in the liver are not yet known.

In the present study, we have identified a novel first exon, E\textsubscript{15}, of the rat PRL-R gene, and have examined the effects of sex steroid hormones on the expression of E\textsubscript{12}, E\textsubscript{13} and E\textsubscript{15} first exons in the liver.

Materials and methods

Animals and hormone treatment

Sprague–Dawley rats were purchased from SLC (Sizuoka, Japan) and housed in a temperature-controlled room with 12 h light and 12 h darkness. Food and water were available ad libitum.

Castration or ovariectomy was performed on 4-week-old rats, and hormone treatment was started 2 weeks after surgery. Five micrograms of 17\beta-oestradiol (Nacalai Tesque, Kyoto, Japan) in 0·1 ml olive oil, 1 mg testosterone propionate (Nakalai Tesque) in 0·1 ml olive oil, or 0·1 ml olive oil alone were injected subcutaneously into the back of the neck. The hormone injection was carried out daily for 2 weeks. The animals were killed by decapitation 24 h after the last injection and the liver was rapidly removed and frozen in liquid nitrogen until used. All procedures were performed in accordance with the NIH guidelines regarding the principles of animal care.

cDNA cloning for 5′-regions of PRL-R mRNAs in the rat brain

Total RNA was extracted from the liver with the ISOGEN kit (Nippon Gene, Toyama, Japan), and poly (A)\textsuperscript{+} RNA was isolated with oligotex-dT30 (TAKARA, Tokyo, Japan) according to the manufacturer’s instructions. The 5′-end of the PRL-R cDNA was synthesized by the oligocapping method (Maruyama & Sugano 1994) as previously described (Tanaka et al. 2002), cloned into pGEM-T Easy vector (Promega, Palo Alto, CA, USA) and sequenced.

Cloning of the E\textsubscript{15} exon-containing region of the rat PRL-R gene

Genomic DNA was prepared from a rat testis by the SDS-protease K method, and 5′-flanking regions of E\textsubscript{15} were cloned with an LA-PCR in vitro cloning kit (TAKARA) according to the manufacturer’s instruction. Briefly, the genomic DNA was digested with EcoRI restriction enzyme, and ligated with EcoRI cassette. The 5′-flanking region of E\textsubscript{15} was then amplified by PCR with primer cassette C1 supplied by the manufacturer and primer 1 (5′-CTGGTTGTTGTTGTCAGTCACAA-3′) corresponding to the antisense sequence of nucleotides 21–42 of E\textsubscript{15} cDNA (Fig. 1). The second PCR amplification was performed with cassette primer C2 supplied by the manufacturer and primer 2 (5′-TCAGTCACAACTGCGTCCTGG-3′) corresponding to the antisense sequence of nucleotides 1091 of the 5′-flanking region of E\textsubscript{15}, by the PCR amplification was performed with cassette primer C2 supplied by the manufacturer and primer 2 (5′-TCAGTCACAACTGCGTCCTGG-3′) corresponding to the antisense sequence of nucleotides 8–30 of E\textsubscript{15} cDNA. For the cloning of the 3′-flanking region of E\textsubscript{15}, the inverse PCR method was employed (Ochman et al. 1990). The genomic DNA was digested with SacI restriction enzyme and self-ligated. The circularized DNA was subjected to PCR with primer 3 (5′-ATGCGTGGCTCCAGCTGCTTTTTGCTACAGCGACTGCTTTTTGGCCTG-3′) corresponding to nucleotides –146 to –122 and primer 4 (5′-ATGCGTGGCTCCAGCTGCTTTTTGCTACAGCGACTGCTTTTTGGCCTG-3′) corresponding to the antisense sequence of nucleotides –1115 to –1091 of the 5′-flanking region of E\textsubscript{15} (Fig. 2). The amplified DNA fragments were cloned into pGEM-T Easy vector and sequenced.

RT-PCR analysis

Total RNA (10 µg) from rat tissues was reverse transcribed using oligo-dT primer (TAKARA) and the
resulting cDNA was subjected to 25 cycles (E12-, E13- and E15-PRL-R mRNAs) and 17 cycles (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) of PCR (94°C for 1 min, 60°C for 1 min and 72°C for 1 min).

Primer 5 (5'-AAGAACACTTGCCTGGGAAAGAAGG-3', nucleotide positions –289 to –266 of E12 (Hu et al. 1996) and primer 6 (5'-TTCTGCTCTGTCTCACTCGCTCC-3', nucleotide positions –261 to –238 of E13 (Hu et al. 1996)) and primer 7 (5'-GCAGGCTGTACTACATGTGTTG-3', nucleotide positions 1–23 of E15 cDNA in Fig. 1) were used as sense primers for the respective PRL-R mRNAs. Primer 8 (5'-GAAGAAGGGCCAACCTGAAGAAAAAC-3', nucleotide positions 131–157 of E15 cDNA in Fig. 1) was used as the sense primer for the analysis of total PRL-R mRNA. Primer 9 (5'-AACTGCTTCCCATTTGGTTCGTGG-3', nucleotide positions 456–479 of E15 cDNA in Fig. 1) was used as the antisense primer for all of the PRL-R mRNAs. A sense primer (5'-TGAAGGTCGGTGTGACGGATTT-3', nucleotide positions 131–157 of E15 cDNA in Fig. 1) and an antisense primer (5'-CACAATCTTGCTTGAAATAGCTTGGGAAAGG-3') were used for the analysis of rat GAPDH mRNA. The PCR products were separated by electrophoresis on a 1·5% agarose gel and transferred to a nylon membrane. The membrane was subjected to Southern blot analysis with the AlkPhos direct labelling and detection system (Amersham Bioscience, Tokyo, Japan) according to the manufacturer’s instruction. A cDNA fragment derived from a common region of E15-PRL-R cDNA (nucleotides 131–479 in Fig. 1) was used as a probe. The fluorescent signal from the probe was detected on an X-ray film and the density of bands was measured by using the NIH image analysis system.

Statistical analysis

The data were analyzed for statistical significance using the Macintosh super ANOVA program and are expressed as the means ± s.d. The significance of the differences between the values was analyzed using Scheffe’s post-hoc test, and P<0·05 was considered significant.

Results

Identification of a novel first exon, E15, in the rat PRL-R gene by cDNA and genomic DNA cloning

Sequence analysis of the oligo-capping PRL-R cDNA clones obtained from the rat liver revealed the presence of a cDNA clone containing a unique sequence of 42 bp at its 5’-end. The unique sequence in the cDNA is followed by the full-length exon 2 sequence, hence the unique sequence is considered to be derived from a novel first exon and is referred to as E15 (Fig. 1). The 5'- and 3'-flanking regions of E15 were cloned by PCR-based methods and sequenced (Fig. 2). A computer-assisted search revealed that E15 was localized between E12 and exon 2 in the draft sequence of the PRL-R gene located in chromosome 2q16 (Fig. 3). No canonical TATA box or GC box was observed but consensus sequences for transcription factors such as CCAAT, GATA and GAS were present in the 5'-flanking region. The splicing donor site sequence, gt, was observed immediately downstream of E15.

Tissue distribution of E15-PRL-R mRNA

Tissue distribution of E15-PRL-R mRNA was examined by RT-PCR. As shown in Fig. 4, a 349 bp fragment derived from E15-PRL-R mRNA was detected in the liver, brain and kidney, but not in the other tissues examined.
Expression profiles of E12-, E13- and E15-PRL-R mRNAs in the liver during sexual maturation

Expression levels of E12-, E13- and E15-PRL-R mRNAs in the liver of male and female rats at 5 days of age and those at 8 weeks of age were examined by RT-PCR (Fig. 5). Levels of E12-PRL-R mRNA were faintly detected in the male rat liver at 5 days of age but were not detected at 8 weeks of age. In the female rat liver, the levels of E12-PRL-R mRNA were slightly higher than those in the male rat liver at 5 days of age, and increased remarkably at 8 weeks of age. The levels of E15-PRL-R mRNA did not differ with sex at 5 days of age, and decreased at 8 weeks of age with the male liver showing the higher extent. The levels of E13-PRL-R mRNA did not change with sex and age.

Effects of sex steroid hormone treatments on the levels of E12-, E13- and E15-PRL-R mRNAs in the liver

The effects of sex steroid hormone treatments on the levels of E12-, E13- and E15-PRL-R mRNAs in the liver were investigated (Figs 6 and 7). Two weeks after castration or ovariectomy of 4-week-old rats, 17β-oestradiol or testosterone propionate was subcutaneously administered daily for 2 weeks, and the levels of the PRL-R mRNAs were examined by RT-PCR 1 day after the final administration. In the female rat liver, the levels...
of E12-PRL-R mRNA decreased significantly with ovariectomy and recovered with oestrogen treatment. On the other hand, the levels of E15-PRL-R mRNA increased slightly with surgery, and decreased to the levels of sham-operated rats with oestrogen administration. The levels of E13- and total PRL-R mRNAs were not affected by ovariectomy and hormone treatment. In the male rat liver, the levels of E12-PRL-R mRNA increased strikingly with castration and decreased to the levels of sham-operated animals with the administration of testosterone. The levels of E13-PRL-R mRNA slightly increased with castration and were restored by testosterone treatment, while castration and testosterone treatment had no effect on the levels of E15-PRL-R mRNAs. The profile of the total PRL-R mRNA was similar to that of E12-PRL-R mRNA.

Discussion

In addition to the known four first exons, E1, E12, E13 and E14, a novel first exon E15 was identified in the rat PRL-R gene by cDNA and genomic DNA clonings, and found to be localized in the most proximal position to exon 2. E15 was preferentially expressed in the liver, brain and kidney. Such an expression profile differs from those of any other known first exons of rat and human PRL-R genes. Mouse counterparts of rat E11, E12, E13 and E15 are found in the 5′-flanking region of exon 2 of mouse PRL-R gene contained in a bacterial artificial chromosome clone derived from mouse chromosome 15. Although mouse E11 and E13 have been shown to be expressed in a mouse Leydig tumour cell line (Hu et al. 1997), expression profiles of each first exon in mouse tissues are not known. In the rat liver, E12 and E13 are also expressed with E12 as the major form (Hu et al. 1996). It has been shown that the expression of E12 in the liver depends on the action of HNF4 on the
promoter (Hu et al. 1998b) and that the expression of E1\textsubscript{3} in a wide range of tissues including the liver is operated by the activation of the promoter with the generic transcription factors, C/EBP\textbeta{} and SP1 (Moldrup et al. 1996). Within the 1·5 kb 5'-flanking region of E1\textsubscript{5}, there is no consensus element for these transcription factors participating in the activation of E1\textsubscript{2} or E1\textsubscript{3}. CCAAT, GATA and GAS elements are present, but no canonical TATA box or GC box exists in the 5'-region. The promoter regions directing the preferential expression in the liver, brain and kidney remain to be investigated.

It is well known that PRL-R is abundantly present in the female rat liver and that sex steroid hormones strongly affect the expression levels of PRL-R in the liver (Norstedt & Mode 1982, Yasui et al. 1999). We have previously shown that the PRL-R gene expression increases and decreases during sexual maturation in female and male rat liver respectively (Sakaguchi et al. 1996). The mRNA levels in the female liver decreased with ovariectomy and were restored by the administration of oestrogen. On the other hand, castration of male rats resulted in the induction of the PRL-R gene expression, and testosterone completely blocked the induction of the gene expression. Our present study revealed that expression of the three PRL-R mRNA species containing E1\textsubscript{2}, E1\textsubscript{3} or E1\textsubscript{5} first exons were distinctly regulated by sex steroid hormones in the liver. In the female, the levels of E1\textsubscript{2}-PRL-R mRNA markedly increased during sexual maturation while, in the male, the mRNA levels were very low irrespective of sexual maturation. E1\textsubscript{5}-PRL-R mRNA levels in both sexes decreased during sexual maturation with male rats showing the higher extent. No notable change was observed in the levels of E1\textsubscript{3}-PRL-R mRNA with sex or age. In female rat liver, oestrogen treatment after ovariectomy resulted in the up-regulation of E1\textsubscript{2}-PRL-R mRNA expression and the down-regulation of E1\textsubscript{5}-PRL-R mRNA expression. In male rat liver, testosterone treatment following castration showed a strong and moderate suppression of the expression of E1\textsubscript{2} and E1\textsubscript{5}-PRL-R mRNAs respectively. The expression of E1\textsubscript{5}-PRL-R mRNA was not affected by treatment with sex steroid hormones in either sex. The expression profiles of the total PRL-R mRNA in both sexes were represented by the profile of E1\textsubscript{2}-PRL-R mRNA, consistent with the reported finding that E1\textsubscript{2}-PRL-R mRNA is the most abundant PRL-R mRNA species in the liver (Hu et al. 1996). The presence of two potential half sites of the oestrogen-responsive element in the promoter region of E1\textsubscript{2} (Hu et al. 1996) suggested the possibility of a direct effect of oestrogen on E1\textsubscript{2} expression. The molecular mechanisms of the inductive effect of oestrogen together with the suppressive effect of androgen on E1\textsubscript{2} expression in the liver remain to be elucidated.

In addition to hepatocytes, the liver contains several cell types such as Kupffer cells, endothelial cells and hepatic stellate cells with minor populations. It has been shown that PRL-R is largely expressed in hepatocytes in the rat liver with higher levels in the female than in the male and that the PRL-R levels increase and decrease in both sexes with oestrogen and androgen treatments respectively (Smirnova et al. 1994). More recently, PRL-R gene expression has been observed in Kupffer cells as well as in hepatocytes in the male rat liver (Yokoyama et al. 2003). Although the profiles of the first exon usage in each cell types in the liver are not known, our present findings suggest that the E1\textsubscript{2} first exon is largely responsible for the stimulative and depressive effects of sex steroid hormones on the levels of PRL-R mRNA in hepatocytes. Moreover, it can be speculated that E1\textsubscript{3} and E1\textsubscript{5} are used in the minor hepatic cells to show the differential effects on their expression by sex steroid hormones.

In conclusion, the expression of the PRL-R gene in the liver is regulated by sex steroid hormones with different effects on the transcription of E1\textsubscript{2}, E1\textsubscript{3} and the newly identified E1\textsubscript{5} first exons. Oestrogen moderately increases the overall PRL-R mRNA level in the female liver by stimulating the expression of E1\textsubscript{2} mRNA, while the overall PRL-R mRNA level in the male liver is strongly depressed by testosterone due to the suppression of E1\textsubscript{2} and E1\textsubscript{5} mRNA expression. These findings contribute to the understanding of the regulatory mechanisms of PRL-R gene expression by sex steroid hormones in the liver.

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