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Modification of Prolactin Receptor (PRL-R) Expression by PRL in the Mouse Liver: Estimation of the Ratio of Two Forms of PRL-R mRNAs by "One-Sided Competitive PCR"

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ABSTRACT—We developed new means of measuring the ratio of the short to the long form (S/L ratio) of the mouse prolactin receptor (mPRL-R) cDNA by PCR using a primer common to the two forms and two specific primers. A means of estimating the amount of mPRL-R cDNA by competitive PCR was also established. We confirmed that these procedures were valid, since the S/L ratio of standard DNA was unaltered by one-sided cPCR amplification under the following conditions: the ratio was between 0.1 and 4, and the amount of cDNA was between 10^3 and 10^7 molecules/tube.

The result of one-sided cPCR showed that the short form was dominant in the mouse liver, while the long form was dominant in other tissues. In addition, pituitary grafting increased the S/L ratio in the liver, implying that prolactin down-regulated the functional long form of PRL-R and lowered tissue sensitivity to prolactin itself by modifying the post-transcriptional regulation of PRL-R.

INTRODUCTION

Since Boutin *et al.* (1988) first reported the primary structure of rat prolactin receptor (PRL-R) mRNA, the human (Boutin *et al.*, 1989), rabbit (Edery *et al.*, 1989), mouse (Davis and Linzer, 1989; Moore and Oka, 1993), rat (Shirota *et al.*, 1990), cattle (Scott *et al.*, 1992), chick (Tanaka *et al.*, 1992), pigeon (Chen and Horseman, 1994), and tilapia (Sandra *et al.*, 1995) PRL-R has been cloned. In rats and mice, there are at least two receptor isoforms (short and long) of which the cytoplasmic domains differ as the result of alternative splicing of a single gene (Kelly *et al.*, 1992). One long and three short forms of PRL-R mRNAs have been identified in several mouse organs, and they may correspond to high and low molecular weight PRL-R, respectively (Haldosen and Gustafsson, 1990). Although there are no differences in the binding activity between the long and short forms, only the long form functions in signaling to the milk protein gene in mammary gland cells (Lesueur *et al.*, 1991) or to interferon regulatory factor-1 in Nb2 T-lymphoma cells (O'Neal and Yu-Lee, 1994). Hence, the fact that PRL up-regulates the PRL binding activity of cells (Posner *et al.*, 1975; Amit *et al.*, 1985; Savoie *et al.*, 1985; Barash *et al.*, 1988) does not mean that PRL up-regulates the functional response of cells to the ligand. Tissue sensitivity to PRL may be regulated by modifying the composition of PRL-R molecules in post-transcriptional regulation, probably at the splicing step. In fact, the expression of multiple forms of mouse PRL-R (mPRL-R) mRNA is differentially regulated depending on the physiological or hormonal situation (Hu and Dufau, 1991; Buch *et al.*, 1992; Clarke and Linzer, 1993). Here, we investigated whether or not PRL regulates tissue sensitivity to PRL itself by modifying the composition of PRL-R.

A combination of reverse transcription (RT) followed by the polymerase chain reaction (PCR) is useful for analyzing low levels of mRNAs (Chelly *et al.*, 1988), but it sometimes does not yield quantitative information especially when the amount of the target mRNA is very small. Becker-Andre and Hahlbrock (1989) and Gilliand *et al.* (1990) have described competitive PCR (cPCR) in which DNA fragments containing the same primer template sequences as the target compete for primer binding and amplification (Siebert and Larrick, 1992). Competitive PCR allows low levels of mRNAs to be quantified and has been applied to studying the differential expression of gene isoforms such as the glucose transporter, GLUT1 and GLUT4 (Sivitz and Lee, 1991). Here we developed a means of examining the ratio of the short to the long form of mPRL-R (S/L ratio) cDNAs, by means of "one-sided cPCR". The procedure consisted of PCR using a primer common to both forms and primers specific to each of them. We then studied the effect of hyperprolactinemia induced by pituitary grafting on the S/L ratio. In addition, a means of measuring the level of cDNA encoding the extracellular domain of PRL-R was developed using cPCR, to estimate amount of PRL-R cDNA in cDNA samples.

MATERIALS AND METHODS

Animals

BALB/c mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) and housed in plastic cages with wood shavings under controlled temperature (25 ± 0.5°C) and lighting (12 hr from 6:00 to 18:00). They were given a commercial diet (CE-7; CLEA Japan) and tap water *ad libitum*. All procedures used on the mice were described in detail in a protocol that was approved by the Animal Care and Use Committee of the Graduate School of Science, University of Tokyo, and all experiments conformed to the regulations described in the NIH Guide to the Care and Use of Laboratory Animals.

The mice were killed at 2 months of age without further treatment. In addition, hyperprolactinemic mice were prepared as described (Matsuda *et al.*, 1995). Two-month-old female mice were transplanted with a single anterior pituitary gland obtained from male litter-mates under the left kidney capsule (PG mice), or were sham-operated as the control group. These mice were sacrificed at 15 days after the operation, when the circulating level of prolactin increased to more than 10-fold the control value (Matsuda *et al.*, 1994).

Preparation of cDNA samples

Tissues were dissected from decapitated mice and stored in liquid nitrogen until use. Total RNA was isolated from the frozen tissues using acid guanidinium-phenol-chloroform method (Chomczynski and Sacchi, 1987). The concentration of total RNA was estimated by the absorbance at 260 nm (1 OD unit was estimated at 40 μg/ml) using a spectrophotometer (Ubest-30; Japan Spectroscopic Co., Ltd., Tokyo). After denaturation at 90°C for 5 min, the RNA samples were ice-chilled, then template cDNAs for PCR were generated from the samples by RT according to standard procedures. RT of 2 μg total RNA was performed in a 20-μl reaction mixture containing 1x RT buffer (72 mM KCl, 3 mM $MgCl_2$, 50 mM Tris-Cl, pH 8.3), 10 mM dithiothreitol (DTT), 1 mM each of the four deoxyribonucleotide triphosphates (dNTP; Pharmacia LKB, Sweden), 20 U of ribonuclease inhibitor (RNasin; Promega, Madison, WI), 200 U of Superscript™ reverse transcriptase (GIBCO BRL, Gaithersburg, MA) and primers. The primers were 5 μM random hexamers (Takara Inc., Tokyo) or 100 nM oligo deoxythymidine (oligo $d(T)_{12\text{-}18}$; Pharmacia). The reaction mixture was incubated at 23°C for 10 min (annealing), at 42°C for 1 hr (elongation), at 95°C for 10 min (deactivation), then ice-chilled and divided into 2.5 μl of aliquots.

PCR primers and conditions

Eight oligonucleotides were synthesized (Sawaddy Technology, Inc., Tokyo) as PCR primers, designed to amplify the extracellular or cytoplasmic regions of mPRL-R cDNAs and to generate mPRL-R cDNA bearing a point mutation in the extracellular regions. The priming regions of cDNA synthesis using a truncated model of the two forms of mPRL-R mRNA, PRL-R_S3 and PRL-R_L1 (Clarke and Linzer, 1993) are shown in Fig. 1.

The sequences of primers were as follows: mPRLR-1; 5'-CTG AAGGGAGCCTCTGATCTATTGC-3', mPRLR-2; 5'-GGATTTGATA CTCATCTGCTAGAG-3', mPRLR-3K; 5'-CCTCGGTACCACTTTAT GTG-3', mPRLR-4; 5'-ATGCCATCTGCACTTGCTTACATG-3', mLTHR-1; 5'-GATTTCTCCTGGCCCCATCTACTCC-3', mLTHR-L1A; 5'-CATAGCTTCCATGACCAGAGTCAC-3', mLTHR-S3A; 5'-GCAT CCTTGAGACTAGATTATTGG-3', mLTHR-3K; 5'-ACATAAAGT GGTACCGAGGT-3' (*Kpn*I sites are underlined).

The cDNA sample was amplified by PCR in a 40-μl reaction mixture containing 1x PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-Cl, pH 8.0), 200 μM each of dNTP, and primers as indicated below in each reaction. Sometimes, 148 kBq of deoxycytidine 5'-[α-^{32}P]triphosphate ([α-^{32}P]dCTP) (Amersham, England) was added to

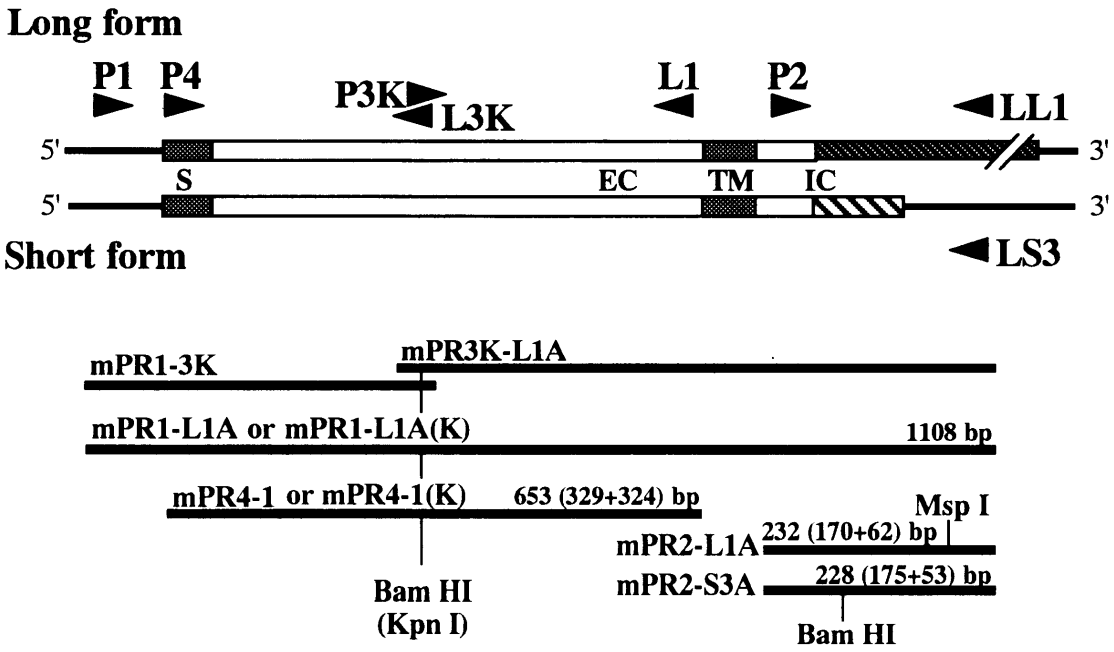


Fig. 1. A truncated model of two forms of PRL-R mRNAs (PRL-R_L1 and PRL-R_S3), regions of eight PCR primers, and DNA fragments amplified by PCR. The two forms have a common 5' flanking region and sequences corresponding to the signal peptide (S), extracellular domain (EC), transmembrane domain (TM) and part of the intracellular domain (IC). They differ in the 3' region corresponding to IC (indicated with striped box) and in the 3' flanking region. EC has a *Bam*HI site at which double-strand cDNA is digested by the RE. IC of the long or short forms contains *Msp*I site or *Bam*HI site, respectively. Arrowheads indicate regions of PCR primers. P1, mPRLR-1; P2, mPRLR-2; P3K, mPRLR-3K; P4, mPRLR-4; L1, mLTHR-1; LL1, mLTHR-L1A; LS3, mLTHR-S3A; L3K, mLTHR-3K. Bold lines below indicate DNA fragments amplified by PCR, and the names and sizes are indicated above or on the left of them. Vertical thin lines indicate RE sites, and the sizes of cleaved fragments are indicated in parentheses.

radioisotopically label the PCR products. The reaction mixture was overlaid with mineral oil (Sigma, St Louis, MO), and hot start PCR amplification proceeded using a thermal cycler (PTC-100; MJ Research, Inc., USA). After denaturation at 90°C for 5 min, 1 U of Taq DNA Polymerase (Takara) was added to the reaction mixture, then 40 cycles of PCR amplification were performed. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and elongation at 72°C for 1 min.

Preparation of competitor and standard DNA for cPCR and standard DNA for one-sided cPCR

Fragments of mPRL-R cDNA were amplified from mouse liver cDNA by PCR using three sets of primers; 500 μM each of mPRLR-1 and mLTHR-L1A, mPRLR-2 and mLTHR-L1A, and mPRLR-2 and mLTHR-S3A. The PCR reaction mixture was resolved by electrophoresis on 1 or 2% agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The gels were stained with ethidium bromide (EtBr), and the PCR products were visualized with a UV transilluminator. Gel strips containing a single PCR product of the appropriate size (1108, 232 or 228 bp, respectively) with a low background were excised. DNA was extracted from the gel using glass powder (Easytrap™; Takara, Inc.) and subcloned into the pT7Blue(R)-T vector (Novagen, Madison, WI) (mPR1-L1A(+)pT7B, mPR2-L1A(+)pT7B or mPR2-S3A(+)pT7B, respectively). XL1-Blue (Stratagene, La Jolla, CA) was the host bacterium. The insert DNA was isolated from the plasmid by digestion with restriction enzymes (REs), *Eco*RI and *Pst*I, subcloned into pUC118/119 vectors, then sequenced as described (Akazome *et al.*, 1994). We confirmed that they were derived from the target fragment of mPRL-R cDNAs. All REs used here were obtained from Takara.

PCR was also used to generate a mutant of mPR1-1LA(+)pT7B bearing a *Kpn*I site (GG**TA**CC) instead of a *Bam*HI site (GG**AT**CC) in the region corresponding to the extracellular domain of mPRL-R. MPR1-1LA(+)pT7B was linearized with *Hin*dIII, and PCR amplified using mPRLR-4 and mLTHR-3K or using mPRLR-3K and mLTHR-L1A as primers. Each PCR product was subcloned into pT7Blue(R) vector (mPR4-3K(+)pT7B and mPR3K-L1A(+)pT7B). Both plasmids were digested with *Kpn*I, and the mPR4-3K(+)pT7B digest was dephosphorylated using calf intestine alkaline phosphatase (Takara) and ligated with the smaller fragment of mPR3K-L1A(+)pT7B digest to generate the mutant mPR1-1LA(+)pT7B bearing a *Kpn*I site (mPR1-1LA(K)(+)pT7B).

Both plasmids, mPR1-1LA(+)pT7B and mPR1-1LA(K)(+)pT7B, were digested with *Pst*I and *Eco*RI, extracted with phenol-chloroform and precipitated with ethanol. They were then quantified by absorption at 260 nm (1 OD unit was estimated at 50 μg/ml), and used as the standard and the competitor DNA for cPCR, respectively.

Competitive PCR for the determination of rough amount of PRL-R cDNAs

PRL-R cDNA content was estimated by cPCR of cDNA samples (or standard DNA) with 10^3 or 10^7 molecules of competitor DNA using 500 μM each of mPRLR-4 and mLTHR-1 as primers. The PCR product derived from the competitor bearing *Kpn*I site was distinguished from that from the tissue-derived native cDNA or the standard DNA bearing *Bam*HI site by REs digestion. After digestion with either or both enzymes, the PCR products were resolved by electrophoresis on a 4% polyacrylamide gel in 1x TBE buffer (50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 8.0) and stained with EtBr.

One-sided cPCR for the determination of S/L ratio

PCR amplification of cDNAs proceeded in the presence of [α-^{32}P]dCTP using 100 μM of mPRLR-2 and 500 μM each of mLTHR-L1A and mLTHR-S3A as primers. Smaller amount of the common primer (mPRLR-2) than the identical primers (mLTHR-L1A and mLTHR-S3A) reduced potential alteration of the S/L ratio caused by exhaustion of the identical primers. The PCR product derived from the long form was distinguishable from that from the short form by having a restriction site for *Bam*HI or *Msp*I. The PCR mixture was digested with the enzymes, resolved by electrophoresis on 10% polyacrylamide gel in 1x TBE buffer, and stained with EtBr. The band corresponding to the larger fragment of digested PCR product was excised under UV light, transferred into a glass vial, then Cerenkov radiation was measured using a scintillation counter (LS6000IC; Beckman Instruments, Inc., Fullerton, CA). The PCR product without RE digestion was also resolved by electrophoresis, and a gel strip equivalent to the larger fragment of digested PCR product at the same position and size served as the background. The value of radiation intensity of gel corresponding to the larger fragment of *Msp*I or *Bam*HI digest was divided by the number of deoxycytidine residues in the alignment (83 or 77, respectively). The quotient was proportional to the number of long or short form mPRL-R cDNA molecules. Sometimes, the gels were exposed to a Kodak X-OMAT film (Eastman Kodak, USA) after electrophoresis.

Statistical analysis

All results are expressed as the mean ± S.E. Statistical significance of differences between means were assessed with Student's *t*-test. The number of samples is expressed as 'n' in parenthesis.

RESULTS

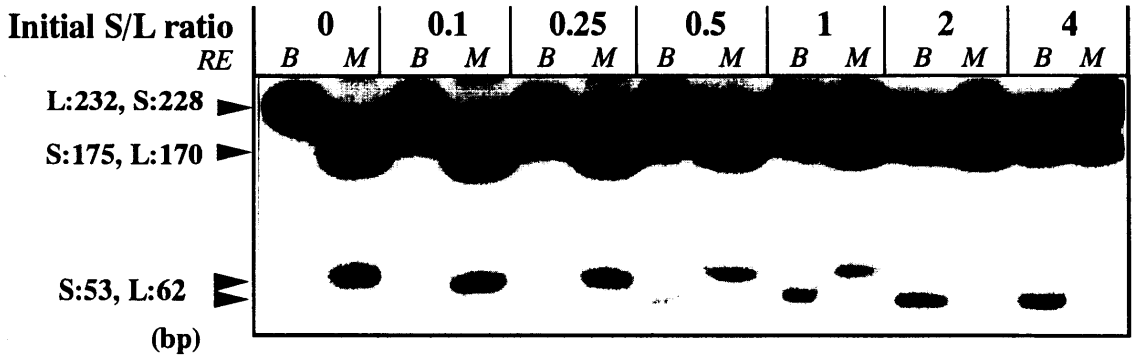
One-sided cPCR with standard DNA samples

To understand the relationship between the S/L ratio of one-sided cPCR products and that of the cDNA before amplification, one-sided cPCR was performed with samples containing fixed amounts of standard plasmid DNAs, mPR2-L1A(+)pT7B and mPR2-S3A(+)pT7B linearized by *Eco*RI digestion. After preparing standard samples containing 10^3 or 10^7 molecules in each tube and an S/L ratio of 0.1, 0.25, 0.5, 1, 2, or 4, one-sided cPCR amplification of mPRL-R cDNA was performed and the S/L ratio of the one-sided cPCR products was measured (Fig. 2). The results showed that S/L ratio was not altered by one-sided cPCR amplification. Hence, one-sided cPCR was considered to be reliable for measuring the S/L ratio, at least when it was between 0.1 and 4 and the amount of mPRL-R cDNA molecules was between 10^3 and 10^7.

Competitive PCR for determination of rough amount of mouse PRL-R cDNA

To reconfirm that the number of PRL-R cDNA molecules was between 10^3 and 10^7 in the cDNA sample, we developed cPCR with which to estimate the amount of mPRL-R cDNA. Competitive PCR amplification proceeded with samples containing 10^3 or 10^7 molecules of standard DNA (mPR1-L1A(+)pT7B) and competitor (mPR1-L1A(K)(+)pT7B) ($10^{2\text{-}4}$ or $10^{6\text{-}8}$ molecules, respectively) (Fig. 3A). The amount of PCR product cleaved by *Bam*HI was more than by *Kpn*I when there was more standard than competitor, and vice versa. We then performed cPCR of each cDNA sample using 10^3 or 10^7 molecules of standard (cf. Fig. 3B). All cDNA samples used here contained more than 10^3 and less than 10^7 molecules of mPRL-R cDNA.

a



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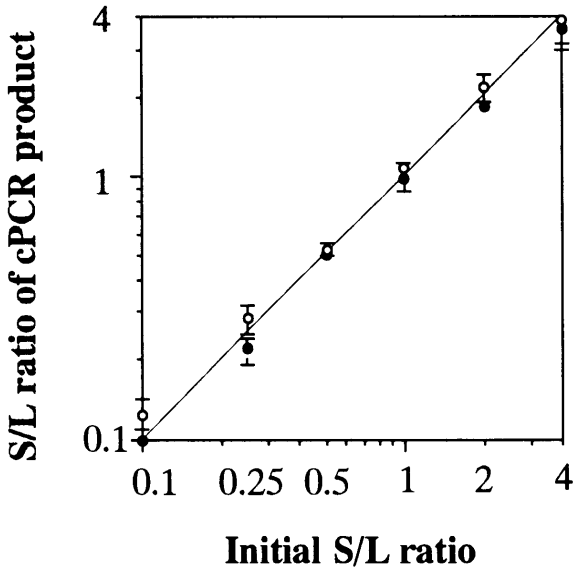

Fig. 2. One-sided cPCR products and its S/L ratio obtained from amplification of standard DNA for long and short form mPRL-R (mPR1-L1A/pT7B and mPR1-S3A/pT7B, respectively) combined in various initial proportions. a: After digestion with *Bam*HI (B) or *Msp*I (M), amplification products were resolved on a 10% polyacrylamide gel and autoradiographed. The initial S/L ratio and RE are indicated on the top of the lanes. The amplification generated 232 and 228 bp products that corresponded to the long and short form mPRL-R, respectively. The product from the long form was digested by *Msp*I into 170 and 62 bp fragments, and that from the short form was digested by *Bam*HI into 175 and 53 bp fragments. b: Relationship between initial S/L ratio and S/L ratio determined by one-sided cPCR after PCR amplification. The initial amount of standard DNA molecules was 10^3 (○) or 10^7 (●). Vertical bars indicate S.E. (n = 3-4).

S/L ratio in several tissues

The S/L ratio of cDNA in the liver, mammary gland, ovary, uterus, testis, small intestine, skeletal muscle and blood was determined by one-sided cPCR (Fig. 4). Oligo dT primer was used for the RT reaction. The S/L ratio was above 1 in the liver, but less than 1 in the mammary gland, ovary, uterus, testis, small intestine and skeletal muscle. No PCR product was observed in the blood. However, sufficient amount of one-sided cPCR product was obtained from cDNA of the buffy coat which was separated by 1% dexistran from heparinized blood. We found that the long form was also dominant in the blood (data not shown). Thus, long form PRL-R mRNA is dominant in almost all mouse tissues except the liver.

Effect of PG on S/L ratio in liver

Hepatic cDNA samples were prepared in PG and control mice by the RT reaction using oligo dT primer or random primer. The S/L ratios then determined by one-sided cPCR (Fig. 5), were above 1 even when the random primer was used, suggesting that the short form was dominant in the liver. The S/L ratio was consistently higher in PG mice than in controls. Therefore, PG increased the amount of the short, rather than the long form of PRL-R mRNA in the mouse liver.

DISCUSSION

We established one-sided cPCR to determine the ratio of two types of mPRL-R cDNAs. The procedure was proved useful when the ratio was in the appropriate range. This protocol will be able to determine the ratio of two cDNAs derived from alternative splicing as long as the appropriate PCR primers are available. Those used here were designed to generate PCR products of a similar length and GC content, so as to amplify two cDNAs with the same efficiency.

A cPCR was also established to estimate the amount of mPRL-R cDNAs. A competitor DNA bearing a single mutation is hardly different in amplification efficiency from the target cDNA or other competitor DNAs bearing a small fragment insertion or heterologous competitor fragments. Thus, cPCR with competitor DNA bearing single mutation will provide a reliable means of quantifying a target DNA. However, the generation of target and competitor DNA heterodimers makes it difficult to quantify more precise changes in the amount of the target DNA. Control of the heterodimerization must be

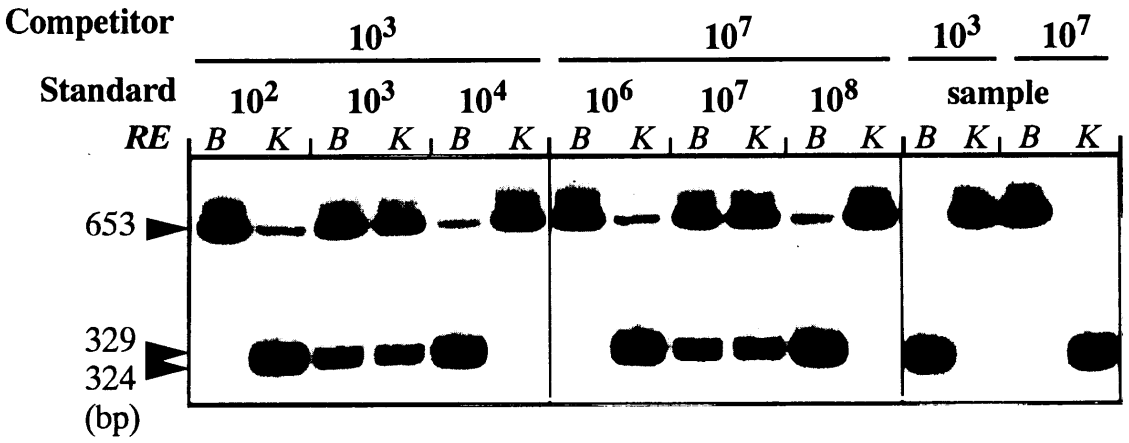

Fig. 3. Estimation of mPRL-R cDNAs in cDNA by cPCR. Left panel: cPCR of standard DNA ($10^{2\text{-}8}$ molecules/tube) with the competitor (10^3 or 10^7 molecules/tube). Right panel: cPCR of a hepatic cDNA sample with competitor cDNA (10^3 or 10^7 molecules). PCR products were digested with *Bam*HI (B) or *Kpn*I (K) and resolved on a 4% polyacrylamide gel. The amplification generated a 653 bp product, some of which was cut into two fragments (329 and 324 bp) by the REs. The amount of PCR products digested by *Bam*HI was more than that by *Kpn*I when the amount of standard DNA (or native mPRL-R cDNA in cDNA sample) was more than that of the competitor, and vice versa. The amount of native mPRL-R cDNA in the cDNA sample is more than 10^3 and less than 10^7 molecules.

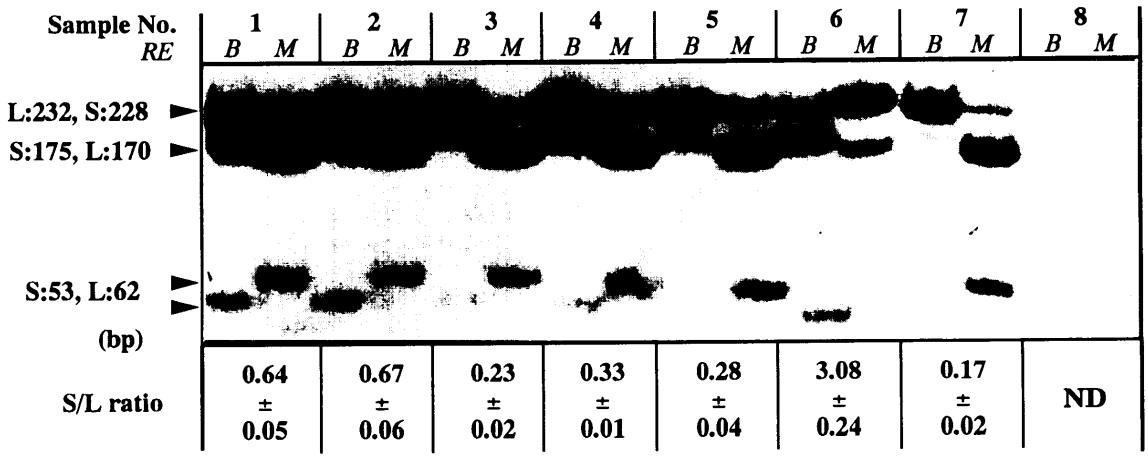

Fig. 4. One-sided cPCR products and the S/L ratio obtained from amplification of cDNA from tissues. Amplification products of cDNA from the small intestine, uterus, ovary, mammary gland, liver, skeletal muscle and blood of female mice and testis of males were digested by *Msp*I (M) or *Bam*HI (B), resolved on 10% polyacrylamide gels and autoradiographed. Means ± S.E. of their S/L ratios are indicated on the bottom of the lane (n = 3-4). ND, not detected. The long form PRL-R was dominant only in the liver.

established.

The S/L ratio of oligo dT-primed cDNA was considered to be larger than that of the original mRNA, since the efficiency of the RT reaction of the target fragment may be higher in the short, than in the long form of PRL-R mRNA, because of the short distance from the poly A site to the target sequence. In contrast, the S/L ratio of random-primed cDNA sample is considered to be smaller than that of the original mRNA sample, because the efficiency of the RT reaction of target fragment may be lower in the short, than in the long form. An RT product with the full length of target fragment will not be obtained when RT reaction starts from inside the target fragment. In fact, the S/L ratio of oligo dT-primed cDNA was larger than that of random-primed cDNA in the liver, and the S/L ratio of mRNA sample should be between the two values.

The biological meaning of PRL-R heterogeneity is not fully understood. The PRL-induced expression of milk protein gene is mediated by the long, but not the short form receptor. However, there is no difference in the binding with the ligand or Jak 2 kinase between the two forms. In this study, hyperprolactinemia increased the S/L ratio in the liver , suggesting that PRL affects the splicing or stability of PRL-R gene transcripts and up-regulates short form PRL-R expression. An increase in the S/L ratio may down-regulate the function of the long form by removing the ligand or Jak 2 kinase. Moreover, a small increase in S/L ratio will bring about a large reduction in the function of the long form, since homodimers of the long form receptor molecules only are functional in mediating the PRL-signal to the nuclei. Furthermore, not only the homodimer of the short form but

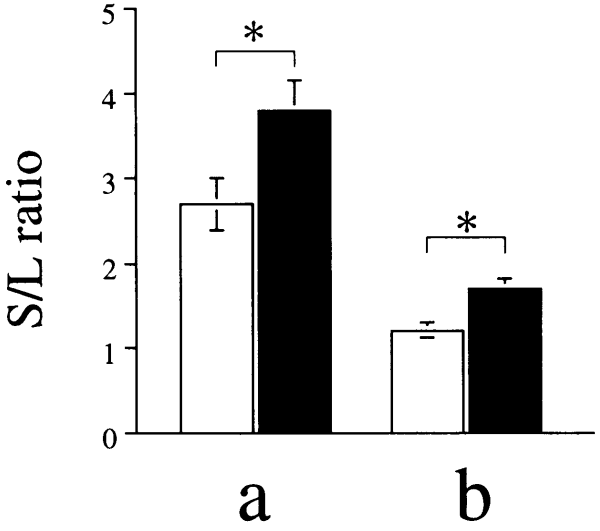

Fig. 5. Effect of PG on the S/L ratio in female mouse liver. Hepatic cDNA samples were prepared from female PG (black column) and control (clear column) mice by RT using oligo dT (a) or random hexamer (b) as the primer, and the S/L ratios were measured by one-sided cPCR. Vertical bars indicate S.E. (n = 4). Statistical significance; *p<0.05.

also heterodimers of the long and short forms are non-functional. Thus, PRL is considered to modify tissue sensitivity to the hormone itself by altering PRL-R gene expression during post-transcription, although information concerning other short form PRL-Rs is needed to understand the precise effect of PRL on PRL-R expression. Short form PRL-R in the liver may contribute to positive clearance of the ligand.

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