Expression of New Members of the Prolactin Growth Hormone Gene Family in Bovine Placenta

ISOLATION AND CHARACTERIZATION OF TWO PROLACTIN-LIKE cDNA CLONES*

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Two prolactin-like proteins (bPLP-I and bPLP-II) were deduced from the nucleotide sequence analyses of the cDNA clones derived from a bovine (Bos taurus) term placenta. These proteins resembled bovine prolactin but were different from the reported bovine placental lactogens or prolactin-related proteins. The predicted amino acid sequences of these clones showed 45–51% identity with bovine prolactin and 23–24% with bovine growth hormone. The two new clones show 62 and 39% overall homology with each other at the levels of nucleotide and amino acid sequences, respectively. bPLP-I, bPLP-II, placental lactogens, prolactins (PRLs), and other prolactin-like proteins isolated from cow, mouse, and rat share 7 common amino acid residues. Five of the 7 residues are conserved by other members of the family such as growth hormones, suggesting that they may be essential for the common structural features of the gene family. The other 2 residues are uniquely conserved in bovine, mouse, and rat placental lactogens, PRLs, and PRL-like proteins, predicting their indispensable roles in binding to the specific receptors. bPLP-I and bPLP-II, as well as bPLP-III, are shown to be expressed stage specifically and predominantly in full-term bovine placentas.

Several peptide hormones originating from the placenta are thought to play important roles in fetal development during pregnancy (1, 2). One of the dominant hormones of the placenta is placental lactogen (PL),1 which is a member of the prolactin (PRL)-growth hormone (GH) gene family (1, 3–8). In addition to the PLs, other members of this family have been identified in the placentas from mouse (9–12) and rat (13, 14). The expression of the genes of these new members during gestation has also been investigated, and the differentlly programmed appearances have suggested their important roles in the growth and development of the fetus and/or the placenta (9, 14–17). Recently, Schuler and Hurley (18) have reported the isolation of a new PRL-related clone, bPRLI, expressed in bovine fetal placenta from 6 months of gestation. They have suggested that multiple PRL-related genes expressed in the placenta may be a general phenomenon in nonprimates.

During the course of our study on the characterization and regulation of the PRL-GH gene family, we have analyzed the structure-function relationship of the PRLs and related proteins from different vertebrate species (19). Elucidation of the primary structures of avian, mammalian, and teleost PRLs enabled the extended analysis of the specific and conserved amino acid residues and domains on the PRL molecules. In order to extend our study further in this respect, we have investigated a bovine placental cDNA library and isolated multiple clones hybridizing to bovine PRL cDNA probes.

In this paper, we describe the isolation and characterization of two cDNA clones from bovine placenta, bPLP-I and bPLP-II, which have not been reported previously. The nucleotide and amino acid sequences of these clones indicate that they belong to the PRL-GH gene family and are related more closely to PRL (20) than to GH (21) or PLs (22, 23).

EXPERIMENTAL PROCEDURES

Construction of cDNA Library—A total RNA preparation was extracted and fractionated by the guanidinium thiocyanate-cesium

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J06456.

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The abbreviations used are: PL, placental lactogen; PRL, prolactin; GH, growth hormone; PLF, prolactin-like protein; PRC, prolactin-related cDNA; PD, prolactin domain; GD, growth hormone domain; LD, lactogen domain; SDS, sodium dodecyl sulfate.

FIG. 1. Restriction maps and sequencing strategy for bPLP-I and bPLP-II cDNA clones. The sequences of bPLP-I and bPLP-II were determined by the dideoxy chain termination method as described under "Experimental Procedures." The open and dotted areas indicate putative signal peptide and mature protein regions, respectively. The solid areas represent the 5'- and 3'-noncoding regions. Upper and lower panels are for bPLP-I and bPLP-II, respectively. kb, kilobases.

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was isolated by an oligo(dT)-cellulose column (25). A cDNA library pSI4001 (26) by a modification of the method of Okayama and Berg was constructed from 6.5 pg of poly(A+) RNA using plasmid vector chloride method (24) from frozen fetal cotyledons (0.83 g) of a bovine (Holstein-Friesian, Bos taurus) full-term placenta. Poly(A+) RNA (27). Recombinant plasmids obtained were used to transform Es&e-

Fig. 2. Nucleotide and deduced amino acid sequences of bPLP-I and bPLP-II. The termination codons are marked with asterisks. Potential glycosylation sites are underlined with dashed lines. The polyadenylation signals are shown by solid underlines.
blotting analysis was performed using the cDNA probes obtained from the cleavage sites. The arrows indicate the positions of putative signal peptide cleavage sites.

GeneScreen Plus membranes (Du Pont-New England Nuclear). The membranes were prehybridized with 200 µg/ml salmon sperm DNA at 42 °C for 6 h and then hybridized with radiolabeled DNA probes in 1.25 × SSPE, 5 × Denhardt’s solution, 0.2% SDS, 50% formamide, and 200 µg/ml salmon sperm DNA at 42 °C for 16 h. The hybridized membranes were washed once with 0.1 × SSC containing 0.2% SDS and with 0.1 × SSC containing 0.2% SDS before they were exposed to x-ray films.

DNA probes were prepared from the cDNA clones for bPRL-I, bPRL-II, and bPRL-III, cutting with AvaI/XbaI, AvaI/SspI, and SstII/HindIII, respectively. The cDNA fragments were radiolabeled by a multiprimer labeling method (38) using [α-32P]dCTP (ICN Radiochemicals, 375 Ci/mmol) and a DNA labeling kit (Nippon Gene).

RESULTS

Isolation and Nucleotide Sequence of bPRL-I and bPRL-II Clones—The bovine placental cDNA library was first screened with the bGH cDNA probe. However, under the conditions employed, no signal-positive clones were obtained with that cDNA probe. Then, the library was hybridized with the bPRL cDNA probes. From about 5000 colonies, 66 clones were detected to give positive signals. The positive clones were subjected further to the secondary screening, and 24 clones were confirmed to show strong positive signals. Restriction mapping analysis revealed that these positive clones could be classified into three major groups. These three groups of cDNA clones were found to be distinct from those of bPRL (20) or bGH (21). We designated these three clones as bPRL-I, bPRL-II, and bPRL-III. In this study, bPRL-I and bPRL-II have been sequenced and analyzed. The restriction maps and sequencing strategies are shown in Fig. 1. The sequencing analyses were performed as described under “Experimental Procedures.” The complete nucleotide sequences of bPRL-I and bPRL-II are presented in Fig. 2. Each cDNA clone possesses a single large open reading frame, and their deduced amino acid sequences are also shown in Fig. 2. These sequence data indicate that bPRL-I and bPRL-II are similar to bPRL but not identical with bovine PLs (bPLs), whose amino acid compositions (22) and other properties (23) have been reported previously. The proteins encoded by bPRL-I and bPRL-II are composed of 236 and 238 amino acid residues, respectively. The amino-terminal regions of both proteins are rich in hydrophobic amino acid residues, which are characteristic of signal peptides (39, 40). Schuler and her group have previously isolated a bovine placental PRL-related clone, bPRC-I (18), and recently a bPRL (41) and two other PRL-related clones, bPRC-II and bPRC-III (42). bPRL-I of this study was almost identical with the bPL clone except that the amino-terminal residue of bPL was alanine instead of valine found for bPRL-I. bPRL-II was not identical with any protein or clone ever isolated. bPRL-I had a consensus sequence for N-glycosylation, Asn-X-Ser/Thr (43) at the position of 89-91. On the other hand, bPRL-II contained four N-glycosylation sites at positions 70-72, 92-94, 146-148, and 160-162.

Codon Usage in bPRL-I and bPRL-II—As is the case for other eukaryotes (44-47), the codon usage in bPRL-I and bPRL-II cDNAs is apparently nonrandom. G or C in the third position of the codon is preferentially used (55% in bPRL-I and 58% in bPRL-II) over A or T, as has been observed in other members of the PRL-GH gene family (44, 48, 49).

Hydropathy Profile of bPRL-I and bPRL-II—Fig. 3 shows the hydrophathy profiles of bPRL-I and bPRL-II. Highly hydrophobic regions were found in the predicted signal peptide sequences of bPRL-I and bPRL-II. The profiles of bPRL-I and bPRL-II resemble those of bPRL and bPRC-I, indicating the structural similarity of these proteins.

Stage-dependent Expression of bPRL-I, bPRL-II, and...
The primary structures of bPLP-I and bPLP-II are compared with those of the preforms of bPLP-III (50), bovine prolactin-related cDNA I, II, and III (bPRC-I, Ref. 18; bPRC-II and bPRC-III, Ref. 49); bovine, mouse, and rat placental lactogens (bPL, Ref. 41); mPL, Ref. 52; and rPL-III, Ref. 53; and rPL-II, Ref. 54, respectively); mouse prolactin-related protein (mPRP, Ref. 9); mouse prolactin (mPRL, Ref. 11); and rat prolactin-like proteins A and B (rPLP-A, Ref. 10; rPLP-B, Ref. 14, respectively). Numbers correspond to the amino acid residues of bPLP-I. Residues identical to those of bPLP-I are shown in the black boxes. The amino acid residues conserved uniquely by bovine, mouse, and rat PRLs, PLs, and PRL-like proteins are indicated by the black arrows, and the residues conserved thoroughly by all the members of the PRL-GH gene family (19, 51) are shown by the white arrows. The putative signal peptidase cleaving site is indicated by a triangle. LD1 to LD4 indicate the conserved four domains of the lactogenic hormones.

**FIG. 5. Conserved amino acid residues in bovine, mouse, and rat PRL-like proteins, PLs, and PRLs.**

**TABLE I**

Homology among members of bovine PRL-GH gene family

Percent homology for identical amino acids (and for nucleotides in parentheses) is shown. The amino acid and nucleotide sequences of the preforms of bPLP-I, bPLP-II, or bPLP-III (50) are compared with those of bPRL, (41), bPRC-I (18), bPRC-II (42), bPRC-III (49), bPRL (20), and bGH (21).
bPLP-III mRNAs in Bovine Placenta—Another novel PRL-like cDNA clone, bPLP-III, has also been isolated from bovine placenta in this laboratory (50). Stage-specific expressions of bPLP-I, bPLP-II, and bPLP-III mRNAs were investigated in bovine placenta. As shown in Fig. 4, bPLP-I and bPLP-II were expressed dominantly in full-term placenta and slightly in placentas of 2-month or 3-month gestation. bPLP-III was also expressed in full-term placentas, but this species was not expressed in the early stages of gestation such as 2 or 3 months. Each of bPLP-I, bPLP-II, and bPLP-III showed a mRNA size around 1 kilobase, consistent with that of those cDNAs (Fig. 2 and Ref. 50). Another larger species of RNA of approximately 4 kilobases was also detected clearly in full-term placenta for bPLP-III and, to a lesser extent, for bPLP-I and bPLP-II. On the other hand, no mRNA expression was observed for bPLP-I, bPLP-II, and bPLP-III in the pituitary of nonpregnant cow (Fig. 4). Using cDNA of bPRL or bGH as the probe, no RNA was detected in the placenta of those gestation stages.

**DISCUSSION**

We report here the isolation and characterization of two novel prolactin-like cDNA clones, bPLP-I and bPLP-II, from bovine (*B. taurus*) term placenta. The third clone we have obtained recently, bPLP-III, is also shown to be a member of the gene family (50). Schuler and her group have recently isolated bovine placental PRL-related clones, bPRC-I (18), bPRC-II and bPRC-III (42), and a bPL (41) clone. The three clones we obtained were different from any of these four clones, but they were closely related to each other. In Fig. 5, the deduced amino acid sequences of bPLP-I and bPLP-II are compared with the preforms of bPLP-III, PLs, PRLs, and other PRL-like proteins from cow, mouse, and rat.

bPLP-I of the present study is very similar to bPRL reported previously (41), except for the predicted amino-terminal residues of the two mature forms. The nucleotide sequences for these residues are GTG and GCG, respectively, showing a single nucleotide difference. We analyzed seven individual clones isolated as bPLP-I for the corresponding sequences, finding that four of the seven clones had GTG, and the other three clones had GCG. Schuler and her group (41) have reported previously the presence of 2 amino-terminal residues, valine and alanine, in equal molar amounts for the isolated mature bPRL hormones, consistent with our results. We consider that these two clones (or proteins) are derived from the different genes. We propose to designate bPLP-I and bPRL as bPLP(Vai) and bPRL(Via), respectively.

bPLP-II, on the other hand, shows a close relationship to bPRL reported by Kessler et al. (42). Only 1 residue in the predicted mature portion of bPLP-II, Asp-189, is replaced by asparagine in mature bPRL-II. However, most of the predicted signal peptide of bPRL-II is quite different from those of bPLP-I, bPLP-II, bPRL-III, bPL, or bPRC-I, which are highly conserved (Fig. 5). The nucleotide sequence for the mature portion of bPLP-II is identical to that for bPRL-II (42), except that GAC (Asp-189) in bPLP-II is replaced by AAC (asparagine) in bPRL-II. However, the 5'-noncoding and signal-coding sequences of these two clones are quite different, and several nucleotide replacements are observed in the 3'-noncoding regions.

In Table I, percent identities in amino acid and nucleotide sequences among members of bovine GH-PRL gene family are shown. bPLP-I, bPLP-II, and bPLP-III are more homologous to PRL than to GH. The order of homology to PRL among the preforms of members is PRL > bPLP-1 > bPL > bPLP-II > bPRC-III > bPRC-I > bPLP-III > bPRC-II > bGH (Table I and Ref. 42). bPLP-I, bPLP-II, and bPLP-III are expressed in a stage-specific manner being predominant in the term placenta. Similar gestation stage-specific expressions of PLs and PRL-like proteins have been observed in the developing rodent placenta (9, 13-17). Protein of bPLP-I, or bPRL(Vai), has been identified by Schuler and her group (41); however, those for bPLP-II and bPLP-III remain to be isolated. We have also obtained three clones for bPRL(Via), but no clone of bPRC-I, bPRC-II, or bPRC-III was detected in our cDNA library constructed from full-term placenta. These clones have been obtained with the cDNA library constructed from fetal cotyledons of 6 months of gestation (18, 42). This difference of the source may have affected the species of isolated clones. Together with the stage-specific expressions and the structures of the mature hormones for these clones, the physiological roles of bovine PLs and other placental PRL-like proteins should be clarified hereafter.

When the primary structures of bovine PLs, PRL-like proteins, and PRL are compared with those for mouse and rat (Fig. 5), 7 amino acid residues are completely conserved in the 16 hormones. These 7 residues are distributed to four domains (LD1–LD4) of relatively high conservation. Recently, we have shown that the PRL molecules from various vertebrate species contain four highly conserved domains (PD1–PD4) (19), whereas the GH molecules are shown to possess five conserved domains (GD1–GD5) (51). LD1 falls on the PD2 or GD2 region, and LD2 fits PD3 or GD3. LD3 overlaps PD4, and LD4 falls within the PD4 or GD6 region. The four domains for PLs and PRL-like proteins from LdI to LD4 are considered to have important roles in the biological activities of those lactogenic proteins. Five of the 7 conserved amino acid residues, Cys-98 in LD1 and Cys-214, Asp-218, Cys-231, and Cys-236 in LD4, are shared not only by these 16 lactogenic hormone but also by all known members of the gene family including the GHs. This may suggest that the LD1 and LD4 domains are essential for the common structural features of the PRL-GH gene family and that LD2 and LD3 are important for the specific activities of the PI and PRL-like proteins.

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