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A Subfamily of Bovine Prolactin-Related Transcripts Distinct from Placental Lactogen in the Fetal Placenta†

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Received October 11, 1988; Revised Manuscript Received February 28, 1989

ABSTRACT: The placentae of many species express genes homologous to the pituitary hormones. In the bovine, two transcripts distinct from the pituitary hormones have been previously described: bovine placental lactogen (bPL) and bovine prolactin-related cDNA I (bPRCI). Here we provide evidence for a subfamily of prolactin-related transcripts quite different from bPL and the rodent placental homologues, which include proliferin and rat prolactin-related proteins. Bovine prolactin-related cDNAs II and III (bPRCII and bPRCIII) are about 75% similar in nucleotide sequence to one another and bPRCI, but only 56% similar to bPL, and about 45% to the rodent placental transcripts. The deduced amino acid sequences follow a similar pattern: they are about 60% similar to one another, but only about 35% similar to bPL as well as the predicted rodent placental proteins. mRNA levels corresponding to bPL, bPRCI, and bPRCIII in the fetal placenta show distinct patterns. The role of these predicted hormones during pregnancy remains to be determined.

Little is understood of the complex processes which control the growth and development of the fetus and placenta, as well as the modifications in maternal physiology that result in a successful pregnancy. While many of the activities observed during this period, including growth of the fetus and placenta, modulations in maternal energy metabolism, development of maternal mammary tissue, and maintenance of steroidogenesis, are related to functions regulated by prolactin (Prl)[1] and growth hormone (GH) postnatally, the pituitary hormones do not seem to be involved in these activities during gestation. Circulating levels of Prl are relatively low during most of pregnancy in nonprimates, including the cow (Kelly et al., 1976), and despite the presence of circulating GH, no receptors have been observed for this hormone in the ovine fetus (Gluckman et al., 1983; Parkes & Hill, 1985).

The placenta, which synthesizes homologues of a number of pituitary hormones, has been found to express members of the prolactin-growth hormone gene family. These placental products may play roles in fetal development analogous to those performed by GH and Prl in the adult. In several species, including ruminants, rodents, and primates, binding assays using lactogenic and somatogenic receptors have permitted the isolation of some of the placental hormones, referred to as placental lactogens (PLs). A single PL more closely related to Prl than to GH has been isolated in ruminant species (Beckers et al., 1980; Eakle et al., 1982; Murthy et al., 1982; Chan et al., 1976). However, additional hormones related to prolactin have been predicted in the cow, as well as the rat and mouse, by isolation of cDNAs from placental libraries. We have described bovine prolactin-related cDNA I (bPRCI), which predicts a secreted product quite different (35% amino acid homology) from bovine placental lactogen (bPL) and the pituitary hormones (Schuler & Hurley, 1987; Schuler et al., 1988). It is also quite unlike all proteins deduced from the

† This work was supported by NSF Grant DCB8608739, USDA Grant 85-CRCR-1-1837, and University of Wisconsin Graduate School Project 880525.
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[1] Abbreviations: Prl, prolactin; GH, growth hormone; PL, placental lactogen; bPRCI, bovine prolactin-related cDNA I; bPRCII, bovine prolactin-related cDNA II; bPRCIII, bovine prolactin-related cDNA III; SDS, sodium dodecyl sulfate.

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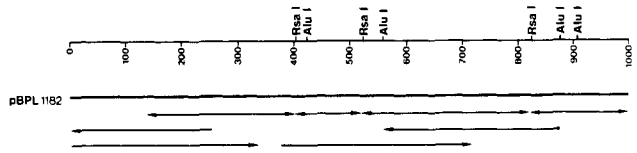

FIGURE 1: Partial restriction endonuclease map and sequencing strategy for pb1182 (bPRCII). The sequence obtained from a particular reaction is shown by the length and direction of the arrow.

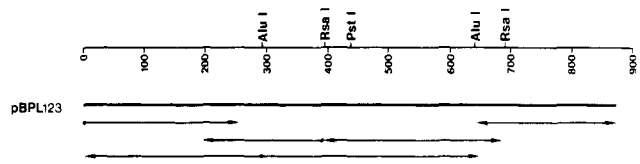

FIGURE 2: Partial restriction endonuclease map and sequencing strategy for pb123 (bPRCIII). The sequence obtained from a particular reaction is shown by the length and direction of the arrow.

described rodent placental transcripts related to this gene family, including the rat and mouse placental lactogens, mouse proliferin and proliferin-related protein, and rat prolactin-like protein A (about 30% amino acid homology; Linzer & Nathans, 1985; Linzer et al., 1985; Jackson et al., 1986; Duckworth et al., 1986a,b).

Here we report two additional distinct bovine prolactin-related placental transcripts, which together with the previously reported bPRCI comprise a subfamily of related predicted proteins quite different from bPL. All three members of this subfamily, bPRCI, -II, and -III, predict proteins which are more similar in amino acid sequence to each other than to any other members of the growth hormone–prolactin gene family. However, sequence differences within this subfamily as well as evidence presented below for differential regulation of expression during the course of gestation suggest that they may serve distinct functions in pregnancy.

Experimental Procedures

cDNA Library Construction and Screening. Two cDNA libraries prepared from bovine placental poly(A+) RNA from 6 months gestation were screened in these studies. The construction of the libraries in the λ phage vectors gt10 and ZAP (Stratagene) has been previously described (Schuler et al., 1988). Both libraries were screened by transferring plaque DNA to Biodyne A (ICN) using the conditions recommended by the manufacturer modified from Benton and Davis (1977). Placental prolactin-related cDNAs were identified by hybridization to nick-translated bovine prolactin cDNA (Rigby et al., 1977; Sasavage et al., 1982), or other placental prolactin-related cDNAs. A human β cytoplasmic actin cDNA (Gunning et al., 1983) was used to isolate the corresponding bovine cDNA (bp922) for use as a homologous bovine standard indicated as 100% control in Figure 7. Hybridization conditions to achieve varying stringency were as indicated below. Inserts of interest from the cDNA in the gt10 library were subcloned into Bluescript phagemid vectors (Stratagene). ZAP library cDNA inserts were recovered in Bluescript SK phagemid vectors by use of helper phage R408 (Stratagene).

Sequence Analysis. Nucleotide sequences of the entire length of both strands of the cDNAs were determined by the dideoxynucleotide chain-termination method (Sanger et al., 1977), either after subcloning into M13mp8, -mp18, or -mp19 (Yanisch-Perron et al., 1985) or following successive deletions with *Escherichia coli* exonuclease III modified from Henikoff (1984). Sequences were analyzed by using the University of Wisconsin Genetics Computer Group programs (Devereux et al., 1984). Sequencing strategies for bPRCII (clone pb1182) and bPRCIII (clone pb123) are shown in Figures 1 and 2.

Nucleic Acid Isolation. Bovine placental and pituitary tissue for RNA and genomic DNA isolation was obtained from an abattoir. Placental tissue was manually separated into fetal and maternal components (cotyledon and caruncle, respectively). Both placental and pituitary tissues were frozen immediately in liquid nitrogen and stored at −80 °C. Gestational age was estimated from the crown–rump length of the fetus (Rexroad et al., 1974). After the frozen placental tissue was pulverized and lyophilized, RNA was isolated as described (Kessler et al., 1985) and enriched for poly(A+) RNA by passage over an oligo(dT)–cellulose affinity column (Aviv & Lederer, 1972). Genomic DNA was purified essentially according to Maniatis et al. (1982). For Southern hybridizations, DNA was digested with restriction endonucleases (New England Biolabs), fractionated in 1% agarose with Tris/EDTA/acetic acid, and transferred to a Hybond nylon membrane (Amersham) using conditions recommended by the manufacturer modified from Southern (1975).

Southern, "Slot Blot", and Plaque Hybridization Conditions. For high stringency, filters were hybridized in 1.25× SSPE (1× SSPE contains 0.18 M NaCl, 5 mM sodium phosphate, pH 7.4, and 0.5 mM EDTA), 5× Denhardt's (1× contains 0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 0.2% SDS, 50% formamide, and 100 μg/mL herring sperm DNA at 42 °C for 24 h and washed in 2× SSC (1× contains 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) and 0.2% SDS at 50 °C for 1 h followed by 0.1× SSC at 65 °C for 1 h. For moderate- or low-stringency screening, filters were hybridized either in 5× Denhardt's, 0.2% SDS, and 100 μg/mL herring sperm DNA at 62 °C for 14–18 h (library screening) or in 5× SSPE, 5× Denhardt's, 0.2% SDS, 100 μg/mL herring sperm DNA, and 50% formamide at 42 °C for 24–36 h (genomic Southerns). Filters were washed in 2× SSC/0.2% SDS, 65 °C for 30 min, followed by a wash in 0.1× SSC/0.1% SDS, 65 °C for 30 min, to allow hybridization with moderately homologous species. For screening at low stringency, filters were washed in 2× SSC/0.2% SDS at room temperature for 30 min and twice in 2× SSC/0.2% SDS at 65 °C for 30 min. Antisense oligonucleotides corresponding to nucleotides 208–224 and 528–544 of bPRCII were used to specifically detect this cDNA and corresponding transcripts (below). Filters were prehybridized in 6× SSC, 1× Denhardt's, 0.5% SDS, 100 μg/mL herring sperm DNA, and 0.05% sodium pyrophosphate at 44 or 38 °C, respectively, for 2 h, and hybridized 12–14 h in the same buffer containing 20 μg/mL tRNA instead of herring sperm DNA and oligonucleotide end-labeled with [γ-^{32}P]ATP. Filters were washed in 6× SSC/0.05% sodium pyrophosphate at 44 or 38 °C, respectively, for 1 h, followed by a wash in fresh buffer at 54 or 48 °C, respectively, for 10 min.

Analysis of Relative mRNA Levels. The amount of mRNA corresponding to each of these cDNAs during gestation was assessed by a modification of the method of Kafatos and colleagues (Kafatos et al., 1979). Bovine prolactin cDNA, the placental prolactin-related cDNAs, and bovine cDNA corresponding to human actin in plasmid vectors were linearized with restriction enzymes, and 5-fold serial dilutions beginning with the equivalent of 770 fmol were applied to a Hybond nylon membrane (Amersham) through a "slot blot" apparatus (Schleicher & Schuell). Filters were prepared in duplicate, and with the use of the high-stringency conditions described above which were found to discriminate among bPrl, bPL, bPRCI, and bPRCIII, each was hybridized to ^{32}P-labeled cDNA prepared with reverse transcriptase (Life Sciences) from cotyledonary poly(A+) RNA from each of five different

times during gestation. This method gives a signal proportional to the relative abundance of the transcripts. Several exposures were made of the filters to XAR-5 film (Kodak) for 2–5 days using a Dupont Cronex Hi-Plus intensifying screen at −80 °C. Autoradiographic signal intensity was determined by using a LKB Model 2202 Ultrascan laser densitometer with a Model 2220 recording integrator. Signals from 6 to 154 fmol were found to be linear with concentration for all placental cDNAs; the endogenous actin signal was linear from 6 to 72 fmol. The slope over this range was compared and normalized to that for the endogenous actin control. The results from the two filters were averaged and expressed as percent of control. Replicates varied by less than 15%. In separate experiments, levels of endogenous actin transcript corresponding to pb922 were found to vary only 8.3% in the poly(A+) RNA preparations (data not shown). bPRCII cross-hybridized with bPRCI using these hybridization conditions. Therefore, an oligonucleotide complementary to bases 528–544 of bPRCII was used to distinguish bPRCI and -II in separate experiments (see hybridization conditions above).

Hybrid Selection, in Vitro Translation, and NEPHGE. Cotyledonary poly(A+) RNA was selected by hybridization to bPRCI and translated in the presence of [^{35}S]methionine (Amersham) in vitro using a rabbit reticulocyte lysate system as described (Schuler et al., 1988). The translation products were resolved by nonequilibrium pH gradient electrophoresis (NEPHGE, Figure 3) performed by the Kendrick Laboratory according to the method of O'Farrell (1977). Electrophoresis was carried out at 250 V for 12 h, using 1.25% pH 3.5–10 and 0.25% pH 9–11 ampholines (LKB). To confirm the pH gradient, six colored acetylated cytochrome p*I* markers, (p*I* 10.6, 9.7, 8.3, 6.4, 4.9, and 4.1; Calbiochem-Behring) were run in an adjacent tube. Purified calcium binding protein (M_r 27 000, p*I* 4.1; Bredderman & Wasserman, 1964) was added to the sample as an internal standard, although the nonequilibrium technique does not permit measurement of p*I*. The following proteins (Sigma) were added as molecular weight standards to the agarose which sealed the tube gel to the slab gel: myosin (220 000), phosphorylase A (94 000), catalase (60 000), actin (43 000), and lysozyme (14 000). These standards appear as fine horizontal lines on the Coomassie Blue stained 15% acrylamide slab gel. The bromophenol blue dye front was run off the bottom of the slab gels for 30 min. The gels were stained with Coomassie Blue, treated with Enhance (New England Nuclear) for 1 h, rehydrated in water for 30 min, and dried for fluorography.

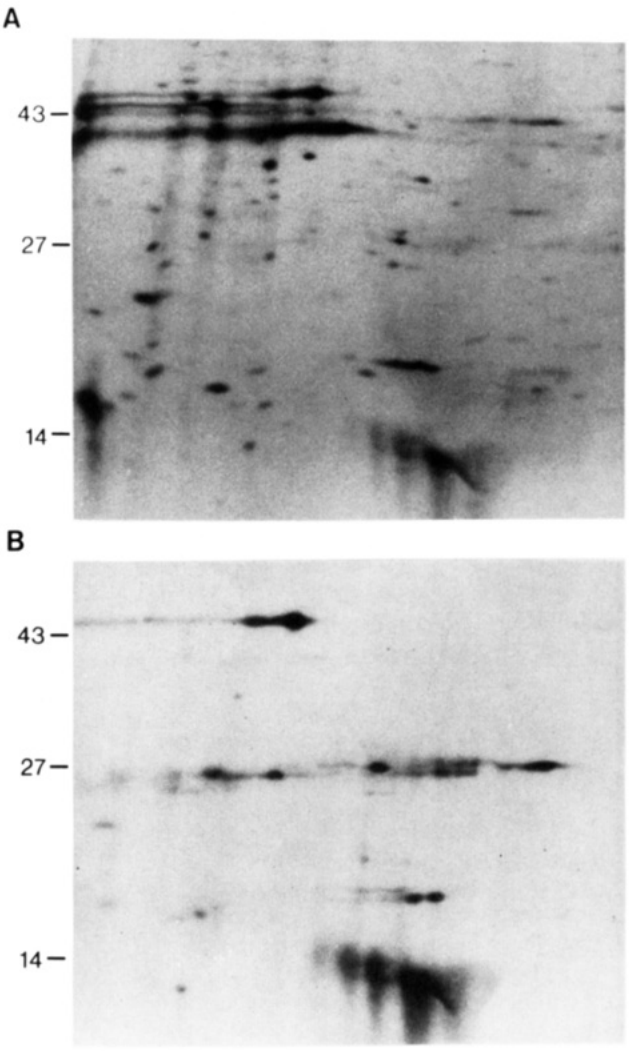

FIGURE 3: In vitro translation of placental mRNA. Total RNA and hybrid-selected poly(A+) RNA (prepared as described under Experimental Procedures) were translated in vitro using rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The products were resolved by NEPHGE, with the acid end on the left. The positions of molecular weight markers ($\times 10^{-3}$) are indicated at the left margin. A major spot at M_r 47K represents a nonenzymatically labeled endogenous reticulocyte protein. Hemoglobin is apparent as a smear right of center at the bottom of the gel. (A) Translation of total cotyledonary poly(A+) RNA from 180-days gestation. (B) Translation of poly(A+) RNA hybrid-selected with bPRCI.

Results

bPRCI-Related Subfamily. We have reported two members of the prolactin–growth hormone gene family, bPL and bPRCI, which are transcribed in the fetal placenta at 180-days gestation (term is about 280 days). bPRCI is quite distinct from bPL (35% amino acid homology) and was not detected by binding to classical somatogenic or lactogenic receptors (Schuler & Hurley, 1987; Schuler et al., 1988).

In order to determine if additional members of this gene family are transcribed in the placenta, and have the ability to be translated, we selected cotyledonary poly(A+) RNA prepared from 180-days gestation by hybridization to bPRCI under conditions of moderate stringency. The selected RNA was translated in vitro using a reticulocyte lysate system (Figure 3). Because preliminary results indicated that some of the translation products were too basic to be resolved using isoelectric focusing (O'Farrell, 1975), the products were separated by NEPHGE (O'Farrell et al., 1977). At least six products can be identified; four major signals are visible on the autoradiogram at about 27–30 kDa, ranging in p*I* from 5.8 to greater than 8.1 as estimated from equilibrium 2-D gel electrophoresis (O'Farrell, 1975). This is in the range of the predicted preprohormones corresponding to bPL and bPRCI, which are both about 27 kDa. Two additional smaller proteins are apparent at about 18 kDa (p*I* greater than 8.1). These results suggest a family of related genes transcribed at 180-days gestation.

To identify additional transcripts suggested by the multiple translation products observed, we screened both cDNA libraries from 180-days gestation with bPRCI under conditions of moderate stringency (see Experimental Procedures). Several additional cDNAs were isolated, of which two are described below. The nucleotide and predicted amino acid sequences of these clones are shown in Figure 4.

bPRCII. This cDNA (bp1182) isolated from the λ ZAP library delineates an open reading frame of 840 nucleotides from the beginning of the cDNA to the stop codon at nucleotide positions 841–843, followed by 140 bp 3′ untranslated

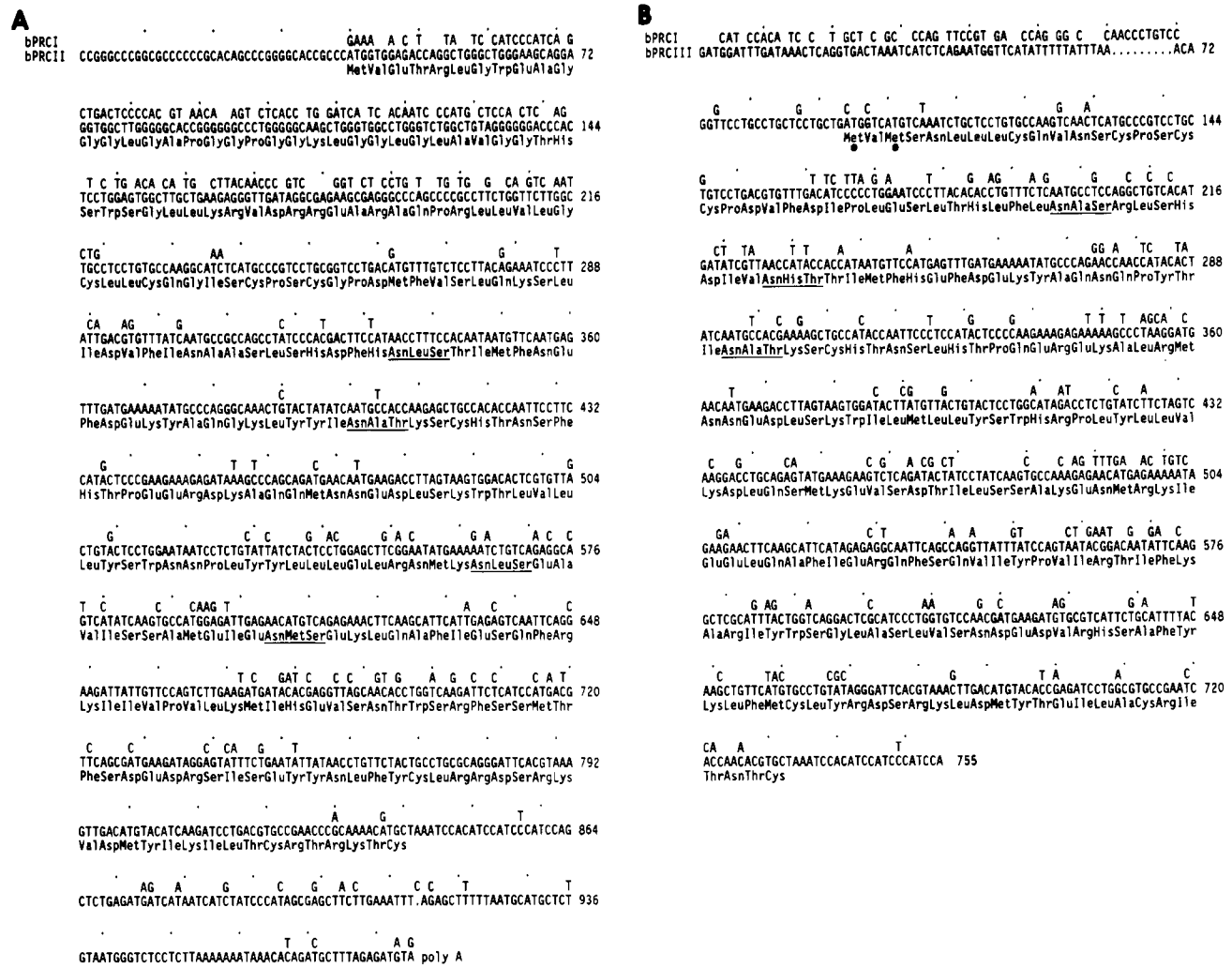

FIGURE 4: Nucleotide sequences of bPRCII (A) and bPRCIII (B). The nucleotide sequence for bPRCI (Schuler & Hurley, 1987) has been aligned with these sequences and is shown only where it differs. No sequence is available for bPRCI 5′ to the noted GAAA.... Gaps, marked by dots, have been introduced to maximize homologies (Devereux et al., 1984). The predicted amino acid sequences are shown below the nucleotide sequence. Consensus sequences for N-glycosylation are underscored (Bahl & Shah, 1977). Large dots indicate candidates for the initiation methionine in bPRCIII. The polyadenylation signal AATAAA (Proudfoot & Brownlee, 1976) in bPRCII is underlined with a dashed line.

Table I: Percent Homology among Members of the Bovine Prl–GH Gene Family [Nucleic Acid/Identical Amino Acid (Similar Amino Acid[a])]

	bPRCI	bPRCII	bPRCIII	bPrl	bGH
bPL	62/36 (56)	56/30 (51)	57/34 (57)	70/51 (65)	46/20 (53)
bPRCI		78/65 (78)	77/64 (70)	63/43 (60)	45/21 (49)
bPRCII			70/52 (67)	61/40 (58)	45/24 (46)
bPRCIII				65/44 (57)	40/21 (49)

[a] Similar amino acids were identified by the point acceptance mutation matrix of Dayhoff (1972), modified by Gribskov and Burgess (1986).

region containing a polyadenylation signal at nucleotides 959–964 (Proudfoot & Brownlee, 1976). The poly(A) tail begins 21 nucleotides further downstream. Although highly homologous to bPRCI throughout most of its length (Table I), the 5′ region is quite dissimilar beginning in the middle of the second exon of bPRCI (Ebbitt et al., 1989). To confirm that this unusual region of the cDNA was not an artifact of the cloning procedure, an oligonucleotide complementary to nucleotides 208–224, spanning the sequence where bPRCII diverges from other members of this gene family, was hybridized to placental RNA on a Northern blot (data not shown). It hybridized to RNA of about 1 kb, similar in size to the other related transcripts, consistent with the existence, in vivo, of an mRNA species corresponding to the bPRCII sequence.

Translation beginning with the AUG at nucleotide positions 40–42 of this clone predicts a protein of 267 amino acids with a calculated molecular weight of 30 321, approximately the size of larger products shown in the analysis of the in vitro translated hybrid-selected poly(A+) RNA. The 5′ untranslated region is markedly GC rich (36 of 39 nucleotides are G/C), possibly creating secondary structure that could affect translational efficiency (Kozak, 1988). bPRCII lacks an apparent signal peptide due to the loss of homology with other characterized members of this gene family near the amino terminus where the hydrophobic residues are located. Like all other bovine placental prolactin-related cDNA clones examined to date, bPRCII possesses consensus sequences for N-glycosylation (Asn-X-Thr/Ser) as indicated in Figure 4A (Bahl & Shah, 1977).

bPRCIII. A related but distinct clone, bPRCIII (pb123), was identified in the λ gt10 library. This cDNA contains an open reading frame beginning with nucleotide position 70 up to the stop codon TAA at nucleotide positions 733–735, followed by 20 bases of 3′ untranslated sequence. Candidates for the initiation codon are present at nucleotides 94–96 and

```
bPrl    .......... .......... .......... .........m dskgssqKGS rLLLLLVVSN  60
bPL     .......... .......... .......... MAPAsShRGH QWicdlvRGS CLLLLLVVSN
bPRCI   .......... .......... .......... MAPAPSFRGH QWTYNPPRGS CLLLLLLMSN
bPRCII  mvetrlgwea ggglgapggp ggklgglgla vggthSwsGl lkrvdrrear aqpr..LLvl
bPRCIII .......... .......... .......... .......... .......... .....MVMSN
bGH     .......... .......... .........m MAagPr.... .........t sLLLafaLlc
                                                              +

bPrl    LLLCQGVvS. tpvcPngpgn cqvSlrDlFd rAvmVSHyih dLSSeMFNEF ...DkRYAQG 120
bPL     VLLCQGaedy apycknqpgn cRiplqslFe rAtlVasnnY rLareMFNEF ...nkqFgeG
bPRCI   LLLCQG.KSC PSCGPDVFVS LRKSFTDRFM NAASLSHDFY NLSTIMFNEF ...DEKYAQG
bPRCII  gcLlcqgiSC PSCGPDMFVS LqKSliDvFI NAASLSHDFh NLSTIMFNEF ...DEKYAQG
bPRCIII LLLCQvn.SC PSCcPDVFdi pleSlThlFL NAsrLSHDiv NhTTIMFhEF ...DEKYAQn
bGH     LpwtQvVgaf Pa........ ..mSlSglFa NAvlraqhlh QLaadtFkEF ertyipegQr
         + +     +   ++       +

bPrl    KgFitmalnS CHTsSlptPE DKEqaQQThh EvLmsliLgL LrSWNdPLyH LVTEVrgMKg 180
bPL     KnFtskVinS CHTefmttPn nKEaaaNTed EaLlRlvIsL LhSWdePLHq aVTELlHrng
bPRCI   KLYYINVTKS CHTNSFHAPE ERDlVQQTNI EDLSKWRLVL LYSWNNPLHH LVTELQHMKE
bPRCII  KLYYINaTKS CHTNSFHtPE ERDkaQQmNn EDLSKWtLVL LYSWNNPLyy LLlELrnMKn
bPRCIII qpYtINaTKS CHTNSlHtPq EREkalrmNn EDLSKWiLML LYSWhrPLyl LVkDLqsMKE
bGH     ysiqntqvaf CfSeTipAPt gKneaQQksd lELlRisLLL IqSWlgPLqf Ls.rVftnsl
                  •                       ^       ^

bPrl    apdAiLSrAi eiEeenkRLl egMEmiFgqV IpgakeT..e pypvWSGLpS LqTkdEDaRy 240
bPL     aSpdiLarAk eiEdkTkvLl egVEmiqkRV hpgekkn..e pypvWSeksS LtaddEDvRq
bPRCI   LSNAFLSSAT RFENMSEKLQ AFIERQFSKI IVPVLNTMIQ ARSSWTGLPS LMSSAEDRRH
bPRCII  LSeAvISSAm eiENMSEKLQ AFIEsQFrKI IVPVLkmIhe vsnTWSrfsS MtfSdEDRsi
bPRCIII VSdtiLSSAk enmrkiEeLQ AFIERQFSqV IyPVIrTIfk ARiyWSGLaS LVSndEDvRH
bGH     Vfgtsdrvye K.lkdlEegi laLmReledg tpragQiLkQ t.....ydkf dtnmrsDdal
                                                    ^

bPrl    SaFYNLlhCL RRDSsKIDtY LKLLnCRiiy nnnC..
bPL     raFYrMFhCL hRDSsKIstY InLLkCRftp c.....
bPRCI   SEFYNLFYCL RRDSRKVDMY IKILTCRTHK TC....
bPRCII  SEYYNLFYCL RRDSRKVDMY IKILTCRTrK TC....
bPRCIII SaFYkLFmCL yRDSRKLDMY teILaCRitn TC....
bGH     lknYgLlsCf RKDlhKtEtY LRVMkCRrfg easCaf
           •       •       ++ +
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FIGURE 5: Comparison of the predicted amino acid sequences of cDNAs for bovine prolactin (bPrl; Sasavage et al., 1982), bovine placental lactogen (bPL; Schuler et al., 1988), bovine prolactin-related cDNA I (bPRCI; Schuler & Hurley, 1987), bovine prolactin-related cDNA II (bPRCII), bovine prolactin-related cDNA III (bPRCIII), and bovine growth hormone (bGH; Woychik et al., 1982). Gaps, marked by dots, have been introduced to maximize homologies (Devereux et al., 1984). Numbers correspond to the amino acid position of the aligned sequences. Residues identical with or similar (Dayhoff, 1972) to the protein predicted by bPRCI are in upper case and those differing are in lower case. Asterisks mark the positions of the conserved cysteine residues among all members of this family, and crosses denote positions of cysteines present in some members of these predicted proteins. Open arrowheads indicate tryptophan residues. Underlined residues are the first amino acids of the secreted proteins as determined by the sequence of the protein (bPRL, bPL, bGH) or the method of von Heinje (1986; bPRCI, bPRCIII).

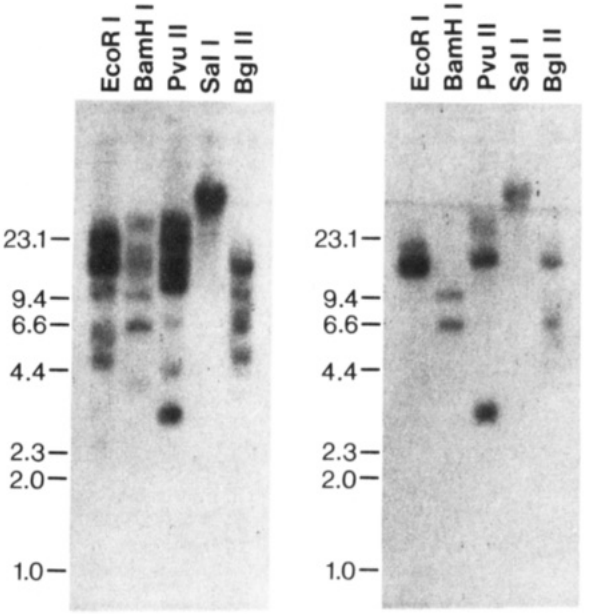

FIGURE 6: Southern blot analysis. Genomic DNA isolated from bovine pituitary tissue was digested with restriction endonucleases as noted, fractionated on a 1.0% agarose gel, transferred to a hybridization filter, and hybridized to bPRCIII under conditions of moderate stringency (left panel) or high stringency (right panel). Molecular size markers are indicated.

100–102. Translation of an in vitro generated transcript results in a product of about 25 kDa, consistent with the predicted size (25 or 24.7 kDa) of a protein initiating at one of these two AUG's (unpublished observations). An additional AUG at nucleotides 50–53 is followed six codons later by the termination codon TAA. The deduced amino acid sequence of bPRCIII also contains consensus sequences for N-glycosylation at several positions (underscored in this figure). Sequence homology with bPRCI begins with position 70 (Figure 4B); the 5′ untranslated region and first exon of bPRCIII are quite unlike the corresponding region of bPRCI (36% similarity).

The relative homology among members of this gene family in the bovine is shown in Table I. Like the other described bovine placental prolactin-related transcripts, and similar to those described in rodents, the deduced proteins are more similar to prolactin than to growth hormone. bPRCI, -II, and -III are more closely related to one another than to either bPL or bPrl. All of the bovine placental transcripts have approximately 45% homology to the rodent placental transcripts at the level of nucleotide sequence. Deduced amino acid sequence homology is only 30%, or 50% after correction for conservative amino acid replacements (Dayhoff, 1972; Gribosov & Burgess, 1986).

The deduced amino acid sequences of the placental members of this gene family in the bovine are compared to the pituitary hormones in Figure 5. Like prolactin, all of the predicted placental proteins have at least six cysteines as shown, suggesting the potential to form disulfide bonds. However, the relative proximity of the two cysteine residues near the amino termini of bPRCI, bPRCII, and bPRCIII (in contrast to bPL and bPrl) suggests that these predicted proteins may be unable to form the third loop characteristic of prolactin (Leszczynski & Rose, 1986). Positions of tryptophan residues are conserved among many members of this gene family (Miller & Eberhardt, 1983). All reported members of this gene family across species have a tryptophan at position 161 in Figure 5 (equivalent of amino acid 91 of bovine prolactin). Growth hormones have this single residue; prolactins of most species have an additional tryptophan at position 222 in Figure 5 (equivalent of position 150 of bovine prolactin). bPL, consistent with its closer relationship to prolactin, has both of these residues. The other placental cDNAs (bPRCI, -II, and -III) are consistent in predicting a third tryptophan in a common position (position 153 in Figure 5) in addition to those in Prl and bPL.

Chromosomal Genes. In order to examine the structure of the chromosomal genes corresponding to these transcripts, we hybridized bovine genomic DNA cut with various restriction enzymes to one of these cDNAs (bPRCIII; Figure 6). When conditions of moderate stringency are used, the resulting pattern is complex (see Experimental Procedures; Figure 6, left panel). In contrast, hybridization of the same blot to bPRCIII under conditions of higher stringency results in a much simpler pattern (Figure 6, right panel).

Pattern of Expression. The diverse amino acid sequences of bPL and the proteins predicted by bPRCI, -II, and -III raise the potential for different functions of these gene products. If this were true, the corresponding genes might be regulated differently, resulting in different levels of mRNA present at different times during gestation. The unusual amino terminus of the predicted product of bPRCII raises additional questions about its function. If the G/C-rich 5′ untranslated region serves to tightly regulate expression of this protein at the translational level, further control may be achieved by maintenance of a low level of mRNA. In order to investigate these issues, we examined relative steady-state levels of bPL, bPRCI,

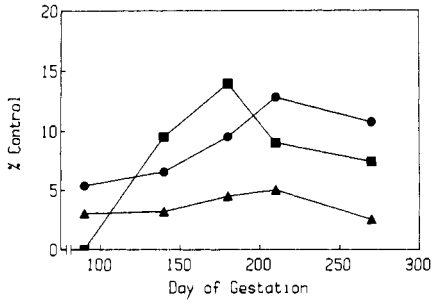

FIGURE 7: Relative levels of bovine prolactin-related cDNAs present in fetal placenta throughout gestation. Serial dilutions of linearized plasmids containing bPrl, bPL, bPRCI, bPRCIII, and bp922 were applied to a nylon membrane through a slot blot apparatus. Filters were hybridized under conditions of high stringency (see Experimental Procedures) to cDNA prepared from poly(A+) RNA from 90-, 140-, 180-, 210-, and 270-days gestation and exposed to XAR-5 film for 2–5 days. Relative levels were calculated from the slopes of the densities compared to that for an endogenous actin control sequence (100%). Points represent the average of two determinations. bPrl gave no signal under these conditions. bPL (●); bPRCI (■); bPRCIII (▲).

and bPRCIII from 90-days gestation through term. To maximize detection of differences in the relative levels of these transcripts, filter-bound cloned prolactin and prolactin-related cDNAs as well as a bovine actin standard were hybridized to a radioactive probe prepared from cotyledonary poly(A+) RNA prepared from the various times during gestation under conditions which are known to distinguish these related sequences. This approach had the additional advantage of minimizing differences in absolute levels of transcripts corresponding to these cDNAs between individuals. The results of these studies are shown in Figure 7 as a time course throughout gestation (term is 280 days). While transcripts corresponding to bPL and bPRCIII were detected at 90-days gestation, transcripts corresponding to bPRCI were not apparent at this early time. bPL-specific transcripts rose steadily through 210-days gestation, and remained elevated near term. bPRCI levels followed a slightly different pattern. Not detectable at 90 days, levels of transcripts hybridizing to bPRCI rose to a peak at day 180, when they were higher than bPL. Levels were slightly lower through the remainder of gestation. bPRCIII-hybridizing species varied only slightly over the gestational times examined. Levels of transcript detected at peak times (180- and 210-days gestation) were about one-third the level of bPRCI and bPL. No signal was observed with bPrl cDNA under these conditions. A portion of the signal designated as bPRCI may derive from the highly homologous bPRCII. To estimate the percentage of signal which may be due to this other transcript, an antisense oligonucleotide complementary to nucleotides 528–544 of bPRCII was employed to ascertain relative levels of bPRCI and -II in the cDNA libraries. In both libraries, about one-fourth of the cDNA clones identified with bPRCI under stringent conditions also hybridized to the bPRCII-specific oligonucleotide.

Discussion

The data presented here are consistent with transcription of multiple genes related to prolactin in the bovine fetal placenta during the second and third trimester of pregnancy. As demonstrated by NEPHGE analysis of the translation products of hybrid-selected RNA, multiple homologous transcripts also translate in vitro, although no one-to-one relationship has been established between the signals in Figure 3 and the isolated cDNAs. The identification of distinct placental transcripts by low-stringency hybridization has allowed us to predict members of this family which apparently do not interact with rat or rabbit prolactin or growth hormone receptors, the basis of early experiments to detect these hormones. The proteins deduced from the sequences of four of these transcripts, including bPL and bPRCI (Schuler & Hurley, 1987; Schuler et al., 1988), and the two additional transcripts described here are quite different from prolactin, as well as one another.

The distant relationship of these transcripts to prolactin suggests that duplication of the parent gene occurred relatively early, compared to the apparent recent origin of human placental lactogen from growth hormone (Shine et al., 1977; Miller & Eberhardt, 1983). Further, the extent of the divergence between bPL and the other predicted placental prolactin-related proteins suggests that these bovine placental transcripts fall into two subfamilies. The subfamily of transcripts represented by bPRCI, -II, and -III apparently arose subsequent to an additional early gene duplication event. The sequence similarity between the three predicted proteins, as well as the third tryptophan in a common position, which is not found in bovine growth hormone, prolactin, or placental lactogen, is consistent with a close evolutionary relationship.

In contrast to the homology over most of their length, the 5′ regions of both bPRCII and bPRCIII are quite different from bPRCI, ending within the second exon (bPRCII) or at the putative splice site between the first and second exons (bPRCIII). This may be due to distinct evolutionary events resulting in different 5′ regions for these genes, possibly conferring different regulatory sequences. Alternatively, homologous regulatory regions may be present further upstream, with the sequence disparity due to different transcription start sites and differential splicing between the first and second exons. Further analysis of the chromosomal genes will be necessary to elucidate the evolutionary history of this complex gene family.

While homologous, the predicted protein products of these bovine placental transcripts are quite distinct from one another, the pituitary hormones, and the described placental products in rodents. The significance of the differences in amino acid sequence will require experimental evaluation, since despite extensive study, the molecular elements responsible for interaction of the hormones of this gene family with their receptors are not understood. Current work on the lactogenic receptor suggests a complex relationship between specific moieties and steric factors [reviewed by Nicoll et al. (1986)]. Furthermore, the products of bPRCI, bPRCII, bPRCIII and several of the prolactin-like cDNAs described in rodents have not been detected by the classical prolactin or growth hormone receptor assays (utilizing liver or mammary gland receptors of the pregnant rabbit or rat). Although bPL does interact in vitro with these classically described receptors, in vivo it may have a distinct receptor, by analogy to ovine placental lactogen (oPL; Emane et al., 1986; Freemark & Handwerger, 1986). The marked differences in the primary structures of the predicted products of these bovine placental cDNAs, both from one another and from bovine prolactin (35–69% amino acid sequence similarity), offer many reasons for this variability in receptor binding and are consistent with the possibility of distinct functions mediated by specific receptors.

bPRCIII, like other described members of this gene family in all species, appears to code for a secreted protein. Although rather few in number, the amino-terminal 13 amino acids meet the criteria for a signal peptide proposed by two methods (McGeogh, 1985; von Heijne, 1986), with a predicted site of cleavage following the serine residue at position 13 (von Heijne, 1986). bPRCII is unusual in that the amino terminus of the deduced protein does not contain the stretch of hydrophobic

residues, raising the possibility that it acts intracellularly in the placental cells where it is synthesized. However, other factors including acidic and basic fibroblast growth factors and interleukin-1, share the lack of an obvious signal peptide (Abraham et al., 1986; March et al., 1985), although they act on membrane receptors of their target cells, arguing that a similar unorthodox mechanism of secretion cannot be ruled out. Several growth factors, receptors, and regulatory proteins, including basic fibroblast growth factor, also share the markedly GC-rich 5′ untranslated region, which may regulate the translation of this transcript (Abraham et al., 1986; Kozak, 1988).

The smaller proteins (18 kDa) observed by hybrid-select translation appear to result from differential splicing of these or related genes, resulting in either the omission of exons or the premature termination of translation (unpublished observations). Splicing variants for human growth hormone have been extensively studied for this family and are relatively common (Lecomte et al., 1987). Further, a 16-kDa metabolite of rat prolactin, believed to represent the amino portion of the intact molecule, has been isolated, and there is preliminary evidence for a specific renal receptor (Mittra, 1980; Clapp et al., 1988).

It has been hypothesized that placentally expressed members of the GH–Prl family may substitute for the pituitary hormones in processes specific to pregnancy. The distinct patterns of expression and sequence heterogeneity raise the possibility that the protein products predicted by bPL, bPRCI, bPRCII, and bPRCIII have unique roles during gestation. Investigations of the binding properties of some of the prolactin-like placental proteins in other species suggest the breadth of possible activities of these hormones. In the rat, Gibori and colleagues have identified a decidual prolactin-like protein which binds to lactogenic receptors in the corpus luteum and uterine decidua (Jayatilak & Gibori, 1986). Recombinant murine proliferin has an affinity for the IGFII/mannose 6-phosphate receptor (Lee & Nathans, 1988). Further, as noted above, a unique receptor for oPL has been identified in the fetal sheep liver, which correlates with stimulation of glycogen synthesis of oPL (Freemark & Handwerger, 1986). Because of protein sequence differences and species variations in reproductive physiology, however, further study of these related prolactin-related placental hormones must be performed in homologous systems. Such investigations may provide insight into the determinants of receptor binding and tissue specificity for this family of hormones, as well as the controlled proliferation and maternal adaptations which occur during pregnancy.

Acknowledgments

We are grateful to Dr. Fritz Rottman and his colleagues at Case Western Reserve Medical School for their gift of bPrl cDNA and to Dr. Larry Kedes at Stanford University School of Medicine for the human β-actin cDNA. We thank Dr. Jack Gorski for many helpful discussions.

Registry No. Prl, 9002-62-4; PL, 9035-54-5; DNA (ox clone pb1182 prolactin-related glycoprotein II mRNA complementary), 120636-66-0; glycoprotein (ox clone pb1182 prolactin-related II reduced), 120636-68-2; DNA (ox clone pb123 prolactin-related glycoprotein III mRNA complementary), 120636-67-1; glycoprotein (ox clone pb123 prolactin-related III reduced), 120636-70-6; glycoprotein (ox clone pb123 prolactin-related III precursor reduced), 120636-69-3.

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6-Fluorocholesterol as a Growth Factor for the Yeast Mutant GL7†

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Received October 18, 1988; Revised Manuscript Received February 9, 1989

ABSTRACT: 6-Fluorocholesterol supports the growth of the sterol-requiring yeast mutant GL7 albeit less efficiently than cholesterol or ergosterol. When the fluoro analogue is combined with very much smaller amounts of cholesterol, the growth response to the sterol pair is synergistic, i.e., greater than additive. On further addition of trace amounts of ergosterol to the 6-fluorocholesterol–cholesterol pair, an additional synergistic growth response is observed. On 6-fluorocholesterol alone, the growth rate of the yeast mutant is slow initially, but after several transfers of such cells to the same media containing the fluoro analogue, growth improves substantially. When incorporated into artificial membranes, cholesterol and its 6-fluoro analogue have essentially identical effects on membrane fluidity as judged from microviscosity measurements. The contrasting responses of artificial membranes and whole cells to the 6-fluoro analogue of cholesterol might be due to sterol–protein interactions in natural membranes.

There is growing evidence for multiple roles of sterols associated with eukaryotic plasma membranes. The first indication for such functional diversity came from the discovery of sterol synergism in the course of studies on the sterol requirement of pupating insect larvae (Clark & Bloch, 1959). At the time, already a distinction was made between structural and metabolic sterol functions. More recently, several laboratories have sought to define the phenomenon of sterol synergism in terms of two categories, with emphasis on the regulatory roles that sterols per se may play apart from modulating the bulk physical state of the membrane lipid bilayer (Dahl et al., 1980, 1981; Ramgopal & Bloch, 1983; Rodriguez et al., 1985).

Earlier this laboratory has reported extensively on the synergistic phenomenon displayed by the sterol auxotroph *Mycoplasma capricolum* on media supplemented with 20:1 mixtures of lanosterol and cholesterol (Dahl et al., 1980, 1981). Lanosterol can serve as the bulk sterol for this organism while the smaller cholesterol supplement controls the utilization of oleic acid for bacterial phospholipid synthesis. For studying sterol synergism in a eukaryotic cell, the yeast mutant GL7 has proven the organism of choice. Lacking squalene epoxide–lanosterol cyclase, this sterol auxotroph (Gollub et al., 1977) grows best on ergosterol, the principal sterol wild-type yeasts and fungi produce. Cholesterol supports growth of the mutant about half as effectively (Buttke & Bloch, 1981). In specified proportions, ergosterol–cholesterol combinations display the synergistic phenomenon as well (Ramgopal & Bloch, 1983). Here we compare the growth-promoting property of 6-fluorocholesterol with that of cholesterol, a choice motivated by the superiority of certain fluorosteroids as therapeutic agents. Cholesterol and its 6-fluoro analogue were

† This work was supported by grants from the National Science Foundation and National Institutes of Health.
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0006-2960/89/0428-5161$01.50/0