Cloning of bovine parathyroid hormone-related protein (PTHrP) cDNA and expression of PTHrP mRNA in the bovine mammary gland

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ABSTRACT

Parathyroid hormone-related protein (PTHrP) produced by the mammary gland has been postulated to have multiple functions in both the mother and neonate. In humans, alternative 3'-mRNA splicing and endoproteolytic processing result in multiple bioactive PTHrP peptides. Multiple PTHrP peptides also have been reported in bovine milk. To investigate the source of molecular heterogeneity of PTHrP in bovine milk, bovine PTHrP was cloned from a bovine brain cDNA library, sequenced and used to characterize the mammary PTHrP transcript. A 1065 bp clone (bP1) for bovine PTHrP was isolated from a brain cDNA library. The bP1 clone contained the entire coding sequence of PTHrP and 61 and 473 nucleotides of the 5'- and 3'-untranslated regions (UTRs) respectively. The predicted amino acid sequence of bovine PTHrP was 72-92% homologous to the sequences of chicken, rat, mouse,

human, and canine PTHrP with the highest sequence divergence present in the C-terminal region of the peptide. The 5'- and 3'-UTRs of bovine brain PTHrP have a high degree of homology to exons 4 and 9 of human PTHrP respectively. PTHrP was expressed as a single 1200 nucleotide mRNA transcript in lactating bovine mammary tissue. RT-PCR using region-specific oligonucleotide primers derived from bP1 demonstrated that PTHrP mRNA transcripts in bovine brain and lactating mammary gland utilize the same 5'- and 3'-UTRs. Expression of PTHrP mRNA was localized to secretory and ductular epithelial cells within the lactating mammary gland, as detected using *in situ* hybridization. Expression of PTHrP mRNA was demonstrated in the mammary gland during late pregnancy and throughout lactation in cows.

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INTRODUCTION

Parathyroid hormone-related protein (PTHrP) was initially identified as a product of human and animal tumors associated with humoral hypercalcemia of malignancy (Suva *et al.* 1987, Rosol & Capen 1992). Subsequently, the expression of PTHrP mRNA and protein has been reported in other neoplasms as well as many normal fetal and adult tissues in both animals and humans, including the mammary gland (Ikeda *et al.* 1988, Thiede & Rodan 1988, Liapis *et al.* 1993).

PTHrP is produced in the mammary gland during late pregnancy and lactation and is postulated to have multiple effects in the mammary gland as well as the neonate, including regulation of calcium transport, relaxation of smooth muscle, and regulation of cell growth and differentiation (Ratcliffe 1992, Thiede 1994). In addition, over-expression of PTHrP in the mammary gland of transgenic mice impaired branching morphogenesis of ducts during prepubertal development as well as lobuloalveolar development during pregnancy, supporting a physiologic role for PTHrP in

mammary gland morphogenesis (Wysolmerski *et al.* 1995).

PTHrP has been demonstrated in the milk of multiple species including human, bovine, rat, and goat where concentrations of PTHrP in milk are 1000-10 000-fold higher than the concentrations observed in maternal blood (Thiede & Rodan 1988, Budayr *et al.* 1989, Khosla *et al.* 1990, Ratcliffe *et al.* 1992, Kocabagli *et al.* 1995). Multiple bioactive PTHrP peptides have been identified in both human and bovine milk. In humans, these peptides are thought to arise from endoproteolytic processing of PTHrP as well as alternative splicing of PTHrP mRNA, which results in three different isoforms of PTHrP that differ in their C terminus. Three different PTHrP mRNA transcripts have been demonstrated in human breast tumors and, more recently, in normal lactating human mammary tissue (Southby *et al.* 1995, Andersson *et al.* 1997). The biologic significance of the different PTHrP isoforms has not been established. Alternative splicing of PTHrP mRNA transcripts has not been demonstrated in the rat, mouse, or chicken. The expression of PTHrP mRNA in the mammary tissue of bovines has not been previously reported.

In the present study, we describe the cloning and sequencing of bovine PTHrP cDNA. The cDNA sequence and the predicted amino acid sequence for bovine PTHrP were compared with genomic DNA and deduced amino acid sequences of multiple species. The bovine mammary PTHrP mRNA transcript was characterized and the temporal expression of PTHrP mRNA within the bovine mammary gland was investigated. In addition, *in situ* hybridization was used to localize PTHrP mRNA expression within lactating bovine mammary tissue.

MATERIALS AND METHODS

Cloning and sequencing of bovine PTHrP cDNA

A 434 bp bovine PTHrP cDNA probe was generated by RT-PCR from total RNA isolated from bovine hypothalamus using primers derived from the coding and 3'-UTR of human PTHrP corresponding to amino acids 6-12 (5'-CAG CTC CTC CAT GAC AAG GGG-3') of the mature peptide and nucleotides 6-25 (5'-CCT TGG AAG GTC TCT GCT GA-3') in the 3'-UTR, for the sense and antisense primers respectively. The bovine PTHrP cDNA probe was labeled with α -[32 P]dATP using a random primer labeling kit (Gibco-BRL, Gaithersburg, MD, USA) and used to screen approximately 5×10^5 plaques of a bovine

brain cDNA library prepared in phage λ UNI-ZAP XR (Stratagene Cloning Systems, La Jolla, CA, USA). Hybridization was carried out overnight at 42 °C in $2 \times$ PIPES, 50% formamide, 0.5% SDS, and salmon sperm DNA at 100 μ g/ml. After washing at 60 °C in $0.1 \times$ SSC and 0.1% SDS for 1 h, the membranes were exposed to X-ray film overnight at -80 °C. Putative clones were selected and rescreened until individual plaques were isolated. Plasmid DNA (pBluescript) from positive clones was isolated and a positive clone with an insert of greater than 1 kb (designated bP1) was further characterized. Both strands were sequenced using an Applied Biosystems Model 373A DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Internal oligonucleotide primers for step-wise sequencing were synthesized commercially (Integrated Data Technologies, Coralville, IA, USA).

Tissue procurement

Normal mammary tissue from 17 heifers and cows was obtained within 15 min of killing, frozen in liquid nitrogen, and stored at -80 °C until RNA isolation. Samples included normal mammary tissue from non-pregnant heifers, heifers pregnant with their first calf, and adult cows in various parities and stages of lactation as listed in Table 1.

RNA isolation

Total RNA was extracted using RNAzol B (Tel-Test, Inc., Friendswood, TX, USA) or Trizol (Gibco-BRL), quantitated, and stored in Formazol (Molecular Research Center, Inc., Cincinnati, OH, USA) at -80 °C. Messenger RNA was isolated from total RNA by incubating with oligo-dT cellulose, and washing and eluting retained poly (A)+ RNA using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA, USA).

Northern blot hybridization

Total RNA or poly (A)+ mRNA (10-30 μ g) was separated by gel electrophoresis in a 1.2% agarose/2.2 M formaldehyde gel and transferred overnight to a nylon membrane (Duralon UV, Stratagene) in $20 \times$ SSC. The membrane was incubated in pre-hybridization solution (50% formamide, $5 \times$ SSC, 10 mM Tris-HCl (pH 7.5), 125 μ g/ml salmon sperm DNA, $1 \times$ Denhardt's solution, 0.2% SDS and 1% background quencher (Molecular Research Center, Inc.)) for 2 h at 42 °C. [32 P]dATP-labeled 434 bp bovine PTHrP cDNA probe was added and hybridization was continued overnight at 42 °C.

TABLE 1. Animals used in the study

	Breed	Age (years)	Stage of lactation	PTHrP mRNA expression
1	Holstein	1	Non-pregnant, non-lactating	Negative
2	Charolais	2	6 months pregnant, non-lactating	Positive
3	Holstein	3	7 months pregnant, non-lactating	Negative
4	Holstein	9	3 days postpartum, lactating	Positive
5	Holstein	3	2 weeks postpartum, lactating	Positive
6	Holstein	4	2 weeks, postpartum, lactating	Positive
7	Brown Swiss	3	3 weeks postpartum, lactating	Negative
8	Holstein	5	2–4 weeks postpartum, lactating	Positive
9	Holstein	2	1 month postpartum, lactating	Positive
10	Holstein	4	1 month postpartum, lactating	Positive
11	Holstein	7	1 month postpartum, lactating	Positive
12	Holstein	5	4 months postpartum, lactating	Positive
13	Holstein	3	6 months postpartum, lactating	Positive
14	Holstein	7	13 months postpartum, lactating	Positive
15	Holstein	4	18 months postpartum, lactating	Positive
16	Holstein	3	60 days non-lactating, 9 months pregnant	Positive
17	Holstein	4	365 days non-lactating, 4 months pregnant	Negative

The membrane was washed in $1 \times \text{SSC}$ and 0.1% SDS at 50 °C for 1 h and exposed to X-ray film overnight at – 80 °C. Total RNA (10 µg) isolated from a canine apocrine adenocarcinoma associated with humoral hypercalcemia of malignancy was used as a positive control for PTHrP (Rosol *et al.* 1990).

Oligonucleotide primers

Oligonucleotides used to detect PTHrP mRNA expression in bovine mammary tissue by RT-PCR were derived from bP1 and the published sequence of human PTHrP (Fig. 1). To amplify the coding region of PTHrP, one of two primer pairs was used: primers P1 (5'-Cag TGG AGC GTC GCg GTG TTC-3'; mismatches shown in lower case) and P11 (5'-GTA cGT CTC CAC CTT GTT-3'; mismatches shown in lower case) which amplify a fragment of 320 bp, or primers P1 and P57 (5'-GAG TTG AGC TCC AGC GAT-3') which amplify a fragment of 499 bp. Region-specific oligonucleotide primers derived from bP1 were used to investigate the 5'- and 3'-UTRs of PTHrP transcribed in lactating bovine mammary tissue. Primer P84 (5'-GGT TGG AGT AGC TGA TTC-3') corresponding to nucleotides within the exon 4-like region of bP1 and primer P82 (5'-GCA CCG AGT AGC TCA GCA GG-3') were used to amplify a 108 bp fragment containing part of the 5'-UTR. Primer P56 (5'-AGA GCA GCC ACT GAA GAC-3') and primer P54 (5'-CAG CAG CAC CAA GAT ACA-3') corresponding to nucleotides within the exon 9-like region of bP1 amplify a 456 bp fragment containing part of the 3'-UTR.

Sense (5'-GAG ACG TTC AAC ACT CCT GC-3') and antisense (5'-GAG CTT CTC CTT GAT GTC AC-3') primers for bovine β -actin were based upon the published sequence of bovine β -actin lymphocyte cDNA (GenBank # K00622) and amplify a fragment of 276 bp. All oligonucleotides were synthesized commercially (Integrated DNA Technologies).

Reverse-transcription/polymerase chain reaction (RT-PCR)

Total RNA (1 µg) was reverse transcribed with 100 U Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) at 37 °C for 30 min in buffer containing 0.5 mM of each dNTP and 2.5 µM random hexamers. Complementary DNA was amplified using 1 U Taq DNA polymerase (Gibco-BRL) in buffer containing 2 mM MgCl_2 and 50 pmol of each oligonucleotide primer. The PCR amplification was conducted for 40 cycles in a thermocycler (Perkin-Elmer-Cetus 9600 Gene Amp PCR System; Perkin-Elmer-Cetus, Norwalk, CT, USA) with a thermocycle profile of denaturation at 94 °C for 20 s, primer annealing for 20 s at 60 °C and primer extension at 72 °C for 1 min. PCR products were separated and visualized in a 2% agarose gel containing ethidium bromide. Total RNA isolated from lactating rat mammary tissue amplified with oligonucleotide primers P1 and P11 was used as a positive control for PTHrP. RT-PCR reactions omitting reverse transcriptase or RNA were used as negative controls.

FIGURE 1. Nucleotide sequence of bP1. The predicted coding region is shown in capital letters. The 5'- and 3'-UTRs are shown in lower-case letters. Deduced amino acids are shown below their corresponding codons. The sequence encoding the mature protein begins at nucleotide 170. The AUUUA instability sequences in the 5'-UTR are underlined. The polyadenylation signal is boxed. The locations of the sense and antisense oligonucleotide primers used for RT-PCR are indicated by arrows.

Southern blot analysis

DNA from agarose gels was transferred to a nylon membrane (Duralon UV, Stratagene) by capillary action, hybridized overnight at 42 °C in hybridization solution containing 500 000 c.p.m. of either α -[³²P]dATP-labeled 434 bp or 1066 bp bovine PTHrP cDNA probe, washed at 37 °C, and exposed to X-ray film overnight at -80 °C (Sambrook *et al.* 1989).

Synthesis of riboprobes

A PTHrP riboprobe corresponding to the common coding exon of human PTHrP (exon 6) was used to localize the PTHrP mRNA transcript in lactating bovine mammary tissue by *in situ* hybridization (Zhou *et al.* 1994). A 422 bp genomic PCR fragment corresponding to nucleotides +102 through +524 of the human PTHrP cDNA sequence (Suva *et al.* 1987) was subcloned into the EcoRI site of pAM-19

(Amersham International, Buckinghamshire, UK) in both orientations. The plasmid was linearized with BamHI and transcribed by T7 RNA polymerase into antisense and sense riboprobes. The riboprobes were labeled with digoxigenin during RNA transcription using an RNA labeling kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

In situ hybridization

In situ hybridization was performed on formalin-fixed, paraffin-embedded lactating bovine mammary tissue as described (Zhou *et al.* 1994). Non-hybridized RNA was removed by treatment with 25 µg/ml of RNase A (Sigma Chemical Co., St Louis, MO, USA) in 2 × SSC. To block non-specific binding of antibody used for detection, sections were incubated for 30 min in PBS containing 30% normal rabbit serum, 3% BSA, and 0.1% Triton X-100 (Sigma). Hybridized probe was detected with alkaline phosphatase-coupled antidigoxigenin antibody (Boehringer Mannheim). Sections were counterstained with nuclear fast red. Sections used as negative controls were incubated either with sense riboprobe or treated with 100 µg/ml RNase A in 2 × SSC (2 h, 37 °C) prior to prehybridization.

RESULTS

Bovine PTHrP cDNA sequence and interspecies comparison

The bovine brain PTHrP cDNA clone (bP1) was 1065 nucleotides (Fig. 1) and contained a 61 nucleotide 5'-UTR, an open reading frame of 531 nucleotides encoding a protein of 177 amino acids, and a 473 nucleotide 3'-UTR. The first 39 nucleotides of the 5'-UTR of bP1 were 80% homologous to the 3'-region of human PTHrP exon 4, suggesting that in the brain, bovine PTHrP is transcribed from a P3-like (downstream TATA promoter) promoter (Mangin *et al.* 1990b, Southby *et al.* 1996). Nucleotides 40-61 in the 5'-UTR of bP1 were 82% homologous to the untranslated sequences present in human PTHrP exon 5.

Comparison of the deduced bovine PTHrP amino acid sequence with sequences for human, mouse, rat, canine, and chicken PTHrP predicted a 36 amino prepro-region and a 141 amino acid mature bovine PTHrP peptide (Suva *et al.* 1987, Karaplis *et al.* 1990, Mangin *et al.* 1990a, Thiede & Rutledge 1990, Rosol *et al.* 1995). Bovine PTHrP retains all putative di-, tri-, and multibasic proteolytic processing sites and the putative amidation sequences present at positions 86 and 94 that have been reported for human PTHrP (Orloff *et al.*

1994). Bovine PTHrP was 92, 90, 87, 85 and 72% homologous to canine, human, mouse, rat, and chicken PTHrP respectively (Fig. 2). Interspecies comparison of amino acids 1-111, which comprise the N-terminal and mid-region of PTHrP, demonstrated few conservative substitutions. Most of the amino acid sequence divergence was in the C-terminal region of PTHrP (amino acids 112-141). The 3'-UTR of bP1 was 473 nucleotides and had 96% sequence homology with the 3'-UTR of exon 9 of the human PTHrP gene including corresponding positions for the polyadenylation signal and multiple AUUUA instability sequences (Mangin *et al.* 1990b, Southby *et al.* 1996).

Identification and characterization of the bovine mammary PTHrP transcript

A Northern blot (Fig. 3A) containing 10-30 µg poly (A)⁺ mRNA derived from lactating bovine mammary tissue was probed with α-[³²P]dATP-labeled bovine cDNA (434 bp) corresponding to the coding sequence of PTHrP. A single 1200 nucleotide mRNA transcript for PTHrP was detected in lactating bovine mammary tissue.

RT-PCRs using region-specific oligonucleotide primers derived from the 5'- and 3'-UTRs of bP1 were used to characterize the 5'- and 3'-regions of mammary gland PTHrP mRNA. RT-PCR of total RNA from lactating bovine mammary tissue, using each of the oligonucleotide primer pairs depicted in Fig. 3B, amplified cDNAs of the expected sizes. Primers P1/P11 (lane 2, Fig. 3B) and P1/P57 (lane 3, Fig. 3B) amplified the expected 320 bp and 499 bp coding region fragments. Fragments of 108 bp and 456 bp were observed with primers P82/P84 (lane 4, Fig. 3B) and P54/P56 (lane 5, Fig. 3B) respectively, indicating that the same 5'- and 3'-UTRs used in brain PTHrP mRNA are also used in mammary PTHrP mRNA. Southern blot analysis using α-[³²P]dATP-labeled bP1 (full-length bovine brain PTHrP cDNA) probe demonstrated that these PCR products were PTHrP-specific (data not shown). The origin of the additional PTHrP-specific cDNAs amplified in lanes 4 (300 bp) and 5 (400 bp) is uncertain, but may be due to PCR artifact or amplification of genomic DNA. Amplification of human PTHrP genomic DNA with primers P82/P84 would produce a fragment of 270 bp encoding portions of exons 4 and 5 as well as the intervening intron. The additional 300 bp PCR product observed in lane 4 using primers P82 and P84 is consistent with an interpretation of genomic contamination although the genomic sequence of bovine PTHrP has not been reported. PCR products were not observed

bovine	AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHAEIRATSEVSPNSKPAP	(50)
human	S.	(50)
rat		(50)
mouse		(50)
canine		(50)
chicken	I..QN..EGVN.....P...T	(50)
bovine	NTKNHPVRFSGSDDEGKYLTQETNKVETYKEQPLKTPGKKKSKPGKRKEQ	(100)
human	R.....G.....	(100)
rat	R.....G.....R..	(100)
mouse	R.....G.....R..	(100)
canine	R.....G.....	(100)
chicken	Y.....E...R.....SQ.....VS.....A.....	(100)
bovine	EKKKRRTSAWLTSYVAGTGLEEDYLSDISATS--LELNSRRH	(141)
human	D.G.T.SN..G.H...T.T.--...D....	(141)
rat	--PGTT.S..N..PQPHT.P..TS..PS..T.	(141)
mouse	--PST.AS..L..P.PHT.R.--...PSL.T.	(139)
canine	N.G..ES...G.HPY.....--....L...	(141)
chicken	A.....N.GMY.SNVT.SPVL.N.V.T--HNHIL.	(139)

FIGURE 2. Comparison of amino acid sequences of mature PTHrP from bovine, human, rat, mouse, canine, and chicken. The top line represents the deduced amino acid sequence of bovine PTHrP derived from a bovine brain cDNA library. Conserved residues in human, rat, mouse, canine, and chicken are designated by a dot.

in RT-PCR reactions lacking RNA or reverse transcriptase.

Expression of PTHrP mRNA in the bovine mammary gland

The temporal expression of PTHrP mRNA in the bovine mammary gland was examined in non-pregnant heifers, pregnant heifers and cows during various stages of lactation using RT-PCR and Southern blot analysis (Fig. 4). PTHrP mRNA was expressed in the bovine mammary gland during late pregnancy and throughout lactation. Amplification of either a 320 or 499 bp PTHrP-specific RT-PCR product was detected in one of two (50%) heifers in the latter third of their first pregnancy, seven of

eight (88%) cows during early lactation (<100 days), both of two (100%) cows during mid-lactation (100-200 days), and both of two (100%) cows during late lactation (>200 days). In the single cow examined during the immediate preparturient non lactating period, PTHrP-specific RT-PCR products were not observed on agarose gels, but a faint signal was detected on Southern blots. Cow #17 was 4.5 months pregnant with an embryo transfer calf and was not lactating for greater than 1 year. PTHrP mRNA was not detected in the mammary gland of this cow. In addition, PTHrP-specific RT-PCR products were not observed in the mammary gland of a non-pregnant, non-lactating heifer. Amplification of β -actin mRNA by RT-PCR confirmed the quality of the mRNA within the

FIGURE 3. Characterization of bovine mammary PTHrP mRNA transcript. (A) Northern blot analysis. Lane 1, positive control, containing 10 μ g total RNA isolated from a canine apocrine adenocarcinoma. Lanes 2, 3, and 4 contain 10, 20, and 30 μ g respectively of poly (A)⁺ mRNA isolated from lactating bovine mammary tissue (cow #9). Membrane was probed with α -[³²P]dATP-labeled 434 bp bovine PTHrP cDNA. (B) Region-specific RT-PCR. Lane 1, 123 bp DNA ladder. Lanes 2–5, RT-PCR of 1 μ g total mammary RNA from cow #9 using primers P1 and P11 (lane 2), P1 and P57 (lane 3), P82 and P84 (lane 4), and P54 and P56 (lane 5).

FIGURE 4. Expression of PTHrP mRNA in bovine mammary tissue *in vivo*. RT-PCR and Southern blot analysis for PTHrP. Total RNA was isolated from bovine mammary tissue at various stages of pregnancy and lactation, reverse-transcribed and amplified with primers for PTHrP or β -actin (lane numbers correspond to the cows' identification numbers in Table 1). PCR products were transferred to a nylon membrane by capillary action and probed with 500 000 c.p.m. α -[³²P]dATP-labeled 434 bp bovine PTHrP cDNA. RT-PCR products for β -actin were visualized in agarose gels stained with ethidium bromide. Expression of RT-PCR products for β -actin confirmed the quality of RNA within the sample. Total RNA isolated from lactating rat mammary tissue amplified with oligonucleotide primers P1 and P11 was used as a positive control for PTHrP. RT-PCR of total RNA from lactating rat mammary tissue without using reverse-transcriptase was used as negative control.

samples. The temporal expression of PTHrP mRNA in the bovine mammary gland detected in this study is summarized in Table 1.

In situ hybridization was used to localize the PTHrP mRNA transcript within the lactating bovine mammary gland. Positive staining for PTHrP mRNA was observed within most mammary alveolar and ductular epithelial cells (Fig. 5).

DISCUSSION

PTHrP produced by the mammary gland has been postulated to have important functions in both the mother and neonate (Thiede 1994). Multiple PTHrP peptides as well as intact PTHrP have been detected in both bovine and human milk (Budayr *et al.* 1989); however, the biologic significance of

FIGURE 5. Cellular localization of PTHrP mRNA within lactating bovine mammary tissue using *in situ* hybridization. Formalin-fixed, paraffin-embedded sections of mammary tissue from a lactating cow were hybridized with 1–4 ng/ μ l digoxigenin-labeled PTHrP riboprobe for 16–18 h at 42 °C. Non-hybridized RNA was removed by treatment with RNase A. Hybridized probe was detected with alkaline-phosphatase coupled antidigoxigenin antibody. Sections were counterstained with nuclear fast red. Top panel: positive staining for PTHrP was detected in most alveolar and ductular epithelial cells. Bottom panel: negative control. Section was treated with RNase A prior to prehybridization.

these different molecular forms is uncertain. In other organs such as bone, different portions of the PTHrP molecule have been reported to cause different or opposing biologic effects (Kaji *et al.* 1995, Murrills *et al.* 1995, Seitz *et al.* 1995). The PTHrP peptides observed in milk could arise from multiple mechanisms including posttranslational endoproteolytic processing, extracellular proteolytic degradation, and alternative mRNA splicing.

The identification of a single 1200 nucleotide PTHrP mRNA transcript in lactating bovine

mammary tissue which utilizes the same 5'- and 3'-UTRs as the PTHrP transcript in brain suggests that bovine PTHrP has a genomic organization similar to that of the rat and mouse (Fig. 6). The mouse PTHrP gene contains at least four exons that are homologous to human exon 4 (5'-UTR), exon 5 (5'-UTR and prepro-region of PTHrP), exon 6 (the coding sequence of mature PTHrP), and exon 9 (3'-UTR) (Karaplis *et al.* 1990, Mangin *et al.* 1990a, Southby *et al.* 1996). In the rat, PTHrP is transcribed from a P3-like promoter located upstream from exon 4 and contains a single 3' exon resulting in a 1500 nucleotide PTHrP transcript which has been detected in many tissues (Thiede & Rodan 1988, Karaplis *et al.* 1990, Mangin *et al.* 1990a). In contrast, the human PTHrP gene is more complex consisting of nine exons with three promoters and alternative splicing of mRNA which result in multiple PTHrP mRNA transcripts (Southby *et al.* 1996). Direct evidence for alternatively spliced mRNAs in tissues from non-human species has not been reported. Our data suggest that the molecular heterogeneity of PTHrP observed in bovine milk does not result from alternative mRNA splicing.

The predicted sequence of mature bovine PTHrP (1–111) differs from human, rat, and mouse, and canine PTHrP by three or four amino acid residues. These substitutions occur at amino acids 49, 66, 92, and 98 and involve changes in either serine or basic amino acids which could potentially affect glycosylation or proteolytic cleavage of PTHrP. An O-linked glycosylated form of PTHrP has been detected in human keratinocytes but localization and significance of the glycosylation have not been determined (Wu *et al.* 1991). Amino acids 1–111 of chicken PTHrP contain multiple amino acid differences compared with mammalian PTHrPs; the biologic significance of these changes is unknown.

The C-terminal portion of PTHrP (112–141) is not highly conserved among reported species. The C-terminal region of bovine PTHrP is 30, 43, 50, 67, and 70% homologous to chicken, rat, mouse, human, and canine PTHrP respectively. The biologic function of the C-terminal PTHrP peptide is not clearly understood and may have divergent functions in different species.

PTHrP mRNA expression was demonstrated in the bovine mammary gland during late pregnancy and throughout lactation. The regulation of PTHrP mRNA expression in bovine mammary tissue is unknown. Although prolactin is not required for the maintenance of lactation in cows, serum prolactin is increased during lactation after milking and prolactin has been demonstrated to

FIGURE 6. Comparison of the organization of the human and mouse PTHrP genes with the cDNA sequences of bovine and rat PTHrP. UTRs are shown in hatched boxes, the prepro-region is shown in white, and the coding sequence of the mature peptide is shown in black. Numbers correspond to exons of the human PTHrP gene. Arrows indicate observed and predicted mRNA splicing patterns.

increase production of PTHrP in bovine primary mammary cell cultures (Okada *et al.* 1996). These results are similar to *in vivo* findings in rats where transcription of PTHrP mRNA in the lactating rat mammary gland was stimulated by prolactin and was dependent upon suckling for continued expression (Thiede 1989). It is possible that milking regulates PTHrP expression by the mammary gland of ruminants since milking increased the concentration of PTHrP detected in the milk of goats (Thompson *et al.* 1994). Lack of milking for an extended period before tissue sampling may explain why PTHrP mRNA expression was not detected in 1 of 12 lactating cows in this study.

In summary, we have described the cloning and sequencing of bovine PTHrP and examined the expression of PTHrP mRNA within the bovine mammary gland. Analysis of the cDNA and amino acid sequences of bovine PTHrP and characterization of the mammary PTHrP mRNA transcript suggest that the multiple PTHrP fragments observed in bovine milk are the result of endoproteolytic processing or extracellular degradation of PTHrP, rather than alternative splicing of the PTHrP mRNA transcript. PTHrP synthesized within epithelial cells in the lactating mammary gland is the likely source of PTHrP observed within milk. The expression of PTHrP mRNA observed in the mammary gland of cows in this study is consistent with a physiologic role for PTHrP in both mammary development and lactation.

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