Cloning and Expression of Bovine Polyadenylate Binding Protein 1 cDNA in Mammary Tissues

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ABSTRACT : A pregnant-induced clone was identified by differential screening from a cDNA library of bovine mammary gland. The clone was identified as a cDNA encoding a polyadenylate binding protein 1 (PABP). The cDNA clone had a total length of 1,911 nucleotides coding for 636 amino acids. The nucleotide sequence of the bovine PABP was 95% and 94% identical to those of human and mouse species, respectively. Comparison of the deduced amino acid sequences of bovine PABP with those of human species showed 100% identity. Induction of the PABP mRNA was observed in bovine mammary tissues at pregnant 7 and 8 months compared to virgin, lactating and involuted states. Expression of the PABP gene was examined in mammary epithelial HC11 cells at proliferating, differentiated and apoptotic conditions. The mRNA levels of PABP gene were similar between proliferating and differentiated cells, but expression levels were very low in apoptotic cells compared to other conditions. Results demonstrate that the PABP gene is induced during pregnancy at which stage mammary epithelial cells are actively proliferating. *(Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 6 : 771-776)*

Key Words : Bovine, Polyadenylate Binding Protein 1 cDNA, Mammary Epithelial Cells, Lactation

INTRODUCTION

Mammary gland growth during pregnancy involves enormous increases both in branching of ducts and in the number of epithelial cells (Topper and Freeman, 1980). The mammary epithelial cell population influences milk yield considerably. Increasing the number of mammary alveolar epithelial cells has the potential to increase efficiency of milk production (Tucker, 1987). Thus, understanding of the mechanism that increases epithelial cell numbers will lead to new methods to increase milk yield in the cow. Efforts have been made to understand the role of hormones, growth factors, and unknown factors in the proliferation of mammary epithelial cells (Borellini and Oka, 1987; Imagawa et al., 1990), but many questionson the mechanism that regulates mammary epithelial cell proliferation remain unanswered. The present study was performed to identify pregnant-nduced cDNAs by differential screening of a cDNA library of bovine mammary gland, characterize the selected clone by nucleotide sequencing and northern analysis, and to understand the molecular events involved in proliferation of mammary epithelial cells.

MATERIALS AND METHODS

Tissue samples

Bovine mammary tissues were obtained by biopsy as described (Knight et al., 1992) from virgin, pregnant (5, 6, 7, and 8 months), lactating (4 months) Holstein cows, and at involution (1 and 5 weeks postpartum). For biopsy, cows were starved for 15-21h. Deep sedation was achieved by intravenous injection of xylazine (10 mg/50 kg body weight). All animals were unable to support their weight within 1-2 min and were tipped so as to be held recumbent on their right side. The feet were restrained for safety and allow clear access to the udder. The head was lowered to allow drainage from the respiratory track. The left side of udder was shaved and cleaned with surgical scrub. Biopsy sites were selected in the upper portion of the udder by palpation, avoiding fat and larger subcutaneous blood vessels. Local anesthesia was achieved by a subcutaneous injection of 5 ml of 2% lignocaine hydrochloride. The skin was swabbed with 70% methylated spirits. An about 5 cm incision was made through skin using a scalpel, and mammary tissues were exposed by blunt dissection through a capsule. A 5-10 g portion of mammary tissue was removed by scalpel. Hemostasis was achieved with hemostats and ligation using 3 metric catgut. The wound was closed with 4 metric catgut sutures in the deep tissues and skin closure achieved with interrupted blanket sutures using 6 metric nylon. Animals received 3,000,000 IU procaine penicillin and 3.75 g dihydro-steptomycin sulphate intramuscularly daily for 3 days following surgery. Other tissues were obtained at slaughter house.

Construction of cDNA library of bovine mammary gland

Total RNA was extracted by the acid/guanidinium thiocyanate/phenol chloroform method (Chomzynski and

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Sacchi, 1987). For construction of pregnant-specific cDNA library, poly A RNA was extracted from total using Poly (A) Quick mRNA isolation Kit (Stratagene, USA). The directional cDNA library was constructed in the λ TriplEx vector using pooled poly A RNA extracted from mammary gland at pregnant 5, 6, 7, and 8 months according to the manufacturers protocol (Clontech, USA). Briefly, the first strand cDNA was synthesized using the poly A RNA, Xba 1-(dT)15 primer and AMV reverse transcriptase. The second strand cDNA was synthesized using RNase H, DNA polymerase and DNA ligase. The EcoR I adapted cDNAs were ligated to the λ TriplEx vector, and the recombinant vectors were subjected to *in vitro* packaging and transfected into the XL1-Blue cells. The unamplified titers were 3.4×10^6 plaque-forming units per milliliter (pfu/ml). The insert size distribution of the library determined by PCR was 0.3~1.8 kb.

Differential screening and sequencing

Differential screening method was used to isolate pregnant-induced clones as described earlier (Choi et al., 1996; Lee et al., 1996). For the primary screening, the library was plated with XL1-Blue cells in a low density (about 2,500 pfu/150 mm plate). Following an 8h incubation at 37℃, the plates were cooled at 4℃ for 2 h. Phage DNA from each plate was transferred onto the nylon membranes in duplicate. The differential hybridization was carried out at 68℃ overnight with ^{32}P labeled cDNA probe prepared from mRNAs of virgin tissues and of pregnant tissues, respectively. After comparing signals from the two films, the pregnant-induced positive plaques were identified.

Partial sequencing of the clone was done by the dideoxy nucleotide chain- termination method using the Sequenase DNA Sequencing Kit (USB, USA) and pTriplEx 5' and pTriplEx 3' primer. The sequences of the clone were compared to the sequence data of GenBank in NIH, USA.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Partial sequencing revealed that the clone was a homologue of human polyadenylate binding protein 1 cDNA. Since the clone had a missing portion in the 5' coding region, RT-PCR method was used to clone the polyadenylate binding protein 1 cDNA containing the full-coding region. Total RNA was extracted from pregnant 8 months mammary tissues. Total RNA was reverse-transcribed with *Moloney murine leukemia virus* reverse transcriptase (Promega, USA) and oligo d(T) primers at 42℃ for 1h. This cDNA template was amplified by PCR using a 5' degenerate primer designed by human and mouse amino acid sequences and a 3' primer based on sequencing results in this study. The amplification was performed with AmpliTaq DNA polymerase (Perkin Elmer, USA) for 40 cycles (preheating at 94℃ for 5 min; cycling at 94℃ for 30 sec, 63℃ for 1 min, and 68℃ for 2 min 30 sec; a final elongation at 72℃ for 10 min). PCR products were separated on an agarose electrophoresis and the fragments were cloned into the pGEM-T easy vector (Promega, USA). For the full-sequencing, the Pst I- and Hind III-digested fragments were subcloned into pGEM vector and the fragments was sequenced by the dideoxy chain termination method using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer).

Northern analysis

For northern analysis, twenty micrograms of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde, and blotted onto a membrane. The lambda DNA containing the cDNA insert was converted into the pTriplEx clone by in vivo excision. The plasmid was digested with EcoR I and Xba I, and the insert was obtained after low melting agarose gel electrophoresis. The insert of cDNA clone was labeled using a Prime-It Random Primer Labeling Kit (Stratagene, USA). The membrane was hybridized with the ^{32}P-labeled insert of the cDNA clone.

Mammary epithelial HC11 cell culture

The HC11 cell is a clonal mammary epithelial cell that is derived from spontaneously immortalized COMMA-D epithelial cells, isolated from the mammary gland of midpregnant BALB/c mice (Ball et al., 1988). HC11 cell were grown in RPMI1640 medium (GIBCO BRL, USA) with 10% fetal bovine

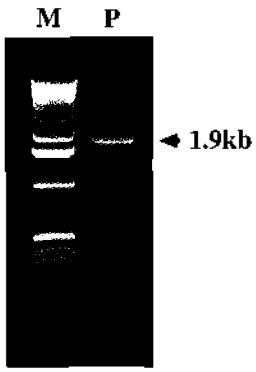

Figure 1. The RT-PCR of bovine polyadenylate binding protein 1 cDNA. The cDNA of bovine PABP gene were amplified by RT-PCR using total RNA of 8 months pregnant mammary tissues (P) and a 5' degenerate primer designed by human and mouse amino acid sequences and a 3' primer based on sequencing results in this study. The PCR products were electrophoresed on a 1% agarose gel. M; 1 kb ladder.

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atgaaccccagcgcccccagctaccccatggcctcgctctacgtgggagacctacaccccgacgtgaccgaggcgatgctctatgagaag   90
 M  N  P  S  A  P  S  Y  P  M  A  S  L  Y  V  G  D  L  H  P  D  V  T  E  A  M  L  Y  E  K    30
ttcagcccggccgggcccatcctctccatccgggtctgcagggacatgatcacccgccgctccttgggctacgcgtatgtgaacttccag  180
 F  S  P  A  G  P  I  L  S  I  R  V  C  R  D  M  I  T  R  R  S  L  G  Y  A  Y  V  N  F  Q    60
cagccggcggacgcggagcgtgctttggacaccatgaattttgatgtgataaagggcaagccagtacgcatcatgtggtctcagcgcgat  270
 Q  P  A  D  A  E  R  A  L  D  T  M  N  F  D  V  I  K  G  K  P  V  R  I  M  W  S  Q  R  D    90
ccatcacttcgcaaaagtggagtgggcaacatattcattaaaaatttggataaatccattgataataaagcactgtatgatacatttttct  360
 P  S  L  R  K  S  G  V  G  N  I  F  I  K  N  L  D  K  S  I  D  N  K  A  L  Y  D  T  F  S   120
gcttttggtaacatcctttcatgtaaggtggtttgtgatgaaaatggttccaagggttatgggtttgtgcattttgagacacaagaagca  450
 A  F  G  N  I  L  S  C  K  V  V  C  D  E  N  G  S  K  G  Y  G  F  V  H  F  E  T  Q  E  A   150
gctgaaagagctattgaaaaaatgaatgggatgcttctaaatgatcgcaaagtatttgttggaagatttaagtctcgtaaagaacgagaa  540
 A  E  R  A  I  E  K  M  N  G  M  L  L  N  D  R  K  V  F  V  G  R  F  K  S  R  K  E  R  E   180
gcagaacttggagctagggcaaaagagttcaccaatgtttacatcaagaattttggagaagacatggatgatgagcgccttaaggatctc  630
 A  E  L  G  A  R  A  K  E  F  T  N  V  Y  I  K  N  F  G  E  D  M  D  D  E  R  L  K  D  L   210
tttggcaagtttggacctgccttaagtgtgaaagtaatgactgatgaaagtggaaaatccaaaggctttggatttgtaagctttgaaagg  720
 F  G  K  F  G  P  A  L  S  V  K  V  M  T  D  E  S  G  K  S  K  G  F  G  F  V  S  F  E  R   240
catgaagatgcacaaaaagctgtggatgagatgaatggaaaagagctcaatggaaaacaaatttatgttggtcgagcccagaaaaaagtg  810
 H  E  D  A  Q  K  A  V  D  E  M  N  G  K  E  L  N  G  K  Q  I  Y  V  G  R  A  Q  K  K  V   270
gaacgacagacagaacttaagcgcaaatttgaacagatgaagcaagacaggatcaccagataccagggtgttaacctttatgtgaaaaat  900
 E  R  Q  T  E  L  K  R  K  F  E  Q  M  K  Q  D  R  I  T  R  Y  Q  G  V  N  L  Y  V  K  N   300
cttgacgatggtattgatgatgaacgtctccggaaggagttttctccatttggcacaatcaccagtgcaaaggttatgatggagggtggt  990
 L  D  D  G  I  D  D  E  R  L  R  K  E  F  S  P  F  G  T  I  T  S  A  K  V  M  M  E  G  G   330
cgcagcaaaggttttggttttgtatgtttctcctccccagaagaagccactaaagcagttacggaaatgaacggtagaattgtggccacc 1080
 R  S  K  G  F  G  F  V  C  F  S  S  P  E  E  A  T  K  A  V  T  E  M  N  G  R  I  V  A  T   360
aagccattgtatgtagctttagctcagcgcaaagaagagcgccaggctcacctcactaaccagtatatgcagaggatggcaagtgtaaga 1170
 K  P  L  Y  V  A  L  A  Q  R  K  E  E  R  Q  A  H  L  T  N  Q  Y  M  Q  R  M  A  S  V  R   390
gctgtgcccaaccccgtaatcaacccctaccagccagcacctccttcaggttacttcatggcagctatcccacagactcagaaccgtgct 1260
 A  V  P  N  P  V  I  N  P  Y  Q  P  A  P  P  S  G  Y  F  M  A  A  I  P  Q  T  Q  N  R  A   420
gcatactatcctcctagtcaaattgctcaactaagaccaagtcctcgctggactgctcagggtgccagacctcatccattccaaaatatg 1350
 A  Y  Y  P  P  S  Q  I  A  Q  L  R  P  S  P  R  W  T  A  Q  G  A  R  P  H  P  F  Q  N  M   450
cccggtgctattcgcccagccgctcctagaccaccatttagtactatgagaccagcttcttcacaggttccacgagtcatgtcaacacag 1440
 P  G  A  I  R  P  A  A  P  R  P  P  F  S  T  M  R  P  A  S  S  Q  V  P  R  V  M  S  T  Q   480
cgtgttgctaacacatcaacacaaacgatgggtccacgtcccgcagctgctgcagctgcagctactcctgctgttcgcaccgttccacag 1530
 R  V  A  N  T  S  T  Q  T  M  G  P  R  P  A  A  A  A  A  A  A  T  P  A  V  R  T  V  P  Q   510
tacaaatacgctgcgggagttcgcaatcctcaacagcatctgaatgcacagccgcaggtcaccatgcagcagcccgctgttcatgtacaa 1620
 Y  K  Y  A  A  G  V  R  N  P  Q  Q  H  L  N  A  Q  P  Q  V  T  M  Q  Q  P  A  V  H  V  Q   540
ggtcaggagcctctgactgcttccatgttggcatctgcccctcctcaagagcagaagcaaatgttgggtgaacggctctttcctctaatt 1710
 G  Q  E  P  L  T  A  S  M  L  A  S  A  P  P  Q  E  Q  K  Q  M  L  G  E  R  L  F  P  L  I   570
caagccatgcaccctactcttgctggtaaaatcactggcatgttgttggagattgataattcagaacttcttcatatgcttgagtctcca 1800
 Q  A  M  H  P  T  L  A  G  K  I  T  G  M  L  L  E  I  D  N  S  E  L  L  H  M  L  E  S  P   600
gagtctctccgttctaaagttgatgaagctgtagctgtactccaagcccaccaagcaaaagaggctgcccagaaagcagttaacagtgcc 1890
 E  S  L  R  S  K  V  D  E  A  V  A  V  L  Q  A  H  Q  A  K  E  A  A  Q  K  A  V  N  S  A   620
actggtgttccaactgtttaa 1911
 T  G  V  P  T  V  *   636
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Figure 2. The nucleotide and the deduced amino acid sequences of bovine polyadenylate binding protein 1 cDNA. The nucleotide sequence data are in the EMBL, GenBank, and DDBJ nucleotide sequence databases with the accession number AJ401269.

serum (GIBCO BRL), 5 μg/ml insulin, 10 ηg/ml epidermal growth factor (EGF), and 50 μg/ml gentamycin (SIGMA, USA) in a 5% CO_2 at 37℃. To induce proliferation of the cells, cells were cultured until 70% confluent stage in growth medium containing insulin, EGF and 10% FBS. To induce differentiation of the cells, confluent cells were cultured in differentiation medium containing 10% FBS, insulin, 0.1 μM dexamethasone and 5 μg/ml prolactin for 2 days without EGF. For apoptotic cells, confluent cells were cultured in medium containing 2% FBS and insulin for 2 days and incubated in medium containing insulin but neither FBS nor EGF for 3 days. Total RNA was prepared as described above.

RESULTS AND DISCUSSION

Differential screening and cloning of polyadenylate binding protein 1 cDNA

To isolate pregnant-induced genes, the directional cDNA library was constructed in the λTriplEx vector using pooled poly A RNA extracted from mammary gland at pregnant 5, 6, 7 and 8 months. Differential screening method was used to isolate pregnant-induced clones. One clone was selected for nucleotide sequencing and partial sequencing revealed that the clone was a homologue of human polyadenylate binding protein 1 (PABP) cDNA. Since the clone had a missing portion in the 5' coding region, RT-PCR was performed to clone the PABP cDNA containing the full-coding region, and approximately 1.9 kb fragments were amplified by RT-PCR (figure 1). The cDNA clone had a total length of 1,911 nucleotides coding for 636 amino acids (figure 2). The nucleotide sequence of the bovine PABP was 95% and 94% identical to those of human and mouse species (Grange et al., 1987; Wang et al., 1992), respectively. Comparison of the deduced amino acid sequences of bovine PABP with those of human and mouse species showed 100% and 99% identity, respectively (figure 3). Results demonstrate evolutionary conservation of PABP gene.

Expression of polyadenylate binding protein 1 gene

Northern analysis was used to examine expression of the PABP gene in bovine mammary tissues at several physiological states. Northern analysis gave a band of the PABP mRNA at 2.9 kb (figure 4). It was highly expressed at pregnant 7 and 8 months. Expression of the PABP was very low or not detected at virgin, lactating, and involuted mammary tissues. In tissue-specific northern, the expression was detected only in mammary tissues. The PABP mRNA was not detected in other tissues examined such as bile, heart, kidney, liver, lung, spleen, stomach, and uterus tissues. We have also examined expression of the PABP gene in mammary epithelial HC11 cells at proliferating,

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BOVINE: MNPSAPSYPMASLYVGDLHPDVTEAMLYEKFSPAGPILSIRVCRDMITRRSLGYAYVNFQQPADAERALDTMNFDVIKGK  80
HUMAN : ................................................................................
MOUSE : ................................................................................

BOVINE: PVRIMWSQRDPSLRKSGVGNIFIKNLDKSIDNKALYDTFSAFGNILSCKVVCDENGSKGYGFVHFETQEAAERAIEKMNG 160
HUMAN : ................................................................................
MOUSE : ................................................................................

BOVINE: MLLNDRKVFVGRFKSRKEREAELGARAKEFTNVYIKNFGEDMDDERLKDLFGKFGPALSVKVMTDESGKSKGFGFVSFER 240
HUMAN : ................................................................................
MOUSE : ..............Q.............................E.............................

BOVINE: HEDAQKAVDEMNGKELNGKQIYVGRAQKKVERQTELKRKFEQMKQDRITRYQGVNLYVKNLDDGIDDERLRKEFSPFGTI 320
HUMAN : ................................................................................
MOUSE : ................................................................................

BOVINE: TSAKVMMEGGRSKGFGFVCFSSPEEATKAVTEMNGRIVATKPLYVALAQRKEERQAHLTNQYMQRMASVRAVPNPVINPY 400
HUMAN : ................................................................................
MOUSE : ................................................................................

BOVINE: QPAPPSGYFMAAIPQTQNRAAYYPPSQIAQLRPSPRWTAQGARPHPFQNMPGAIRPAAPRPPFSTMRPASSQVPRVMSTQ 480
HUMAN : ................................................................................
MOUSE : ................................................................................

BOVINE: RVANTSTQTMGPRPAAAAAAATPAVRTVPQYKYAAGVRNPQQHLNAQPQVTMQQPAVHVQGQEPLTASMLASAPPQEQKQ 560
HUMAN : ................................................................................
MOUSE : ................................................................................

BOVINE: MLGERLFPLIQAMHPTLAGKITGMLLEIDNSELLHMLESPESLRSKVDEAVAVLQAHQAKEAAQKAVNSATGVPTV 636
HUMAN : ..............................................................................
MOUSE : ..............S...............................................................
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Figure 3. Comparison of deduced amino acid sequences of bovine polyadenylate binding protein 1 to those of other species. Dot "." represents same amino acid residues in different species.

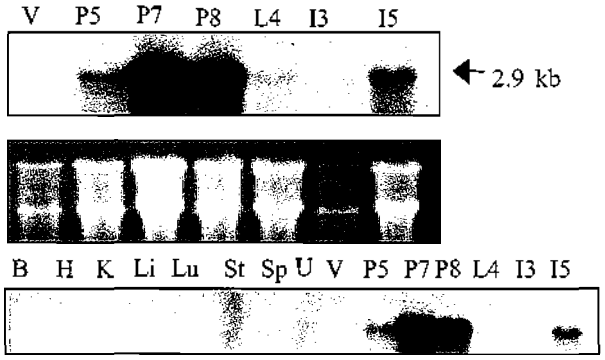

Figure 4. Northern analysis of bovine polyadenylate binding protein 1 gene. Twenty micrograms of total RNA isolated from bile (B), heart (H), kidney (K), liver (Li), lung (Lu), stomach (St), spleen (Sp), and uterus (U) and virgin (V), pregnant 5 months (P5), pregnant 7 months (P7), pregnant 8 months (P8), lactating 4 month (L4), involution 3 weeks (I3), and involution 5 weeks (I5) of bovine mammary gland were separated on a 1% formaldehyde/agarose gel. Total RNA on the gel was transferred onto the membrane by capillary reaction. The blot was hybridized with the ^{32}P-labeled cDNA probe. That amounts of RNA were present in each lane was checked by the intensities of 28S and 18S bands as shown.

differentiated and apoptotic conditions. The mRNA levels of PABP gene were similar between proliferating and differentiated cells (figure 5), but expression levels were very low in apoptotic cells compared to other conditions.

Our study demonstrates that the PABP gene is induced during pregnancy at which stage the proliferation of mammary epithelial cells is extensively observed. Tissues such as heart, kidney, liver, lung, spleen, and uterus that did not show expression of PABP in this study may be fully differentiated. And HC11 cells that showed expression of PABP in differentiated condition may still contain portions of dividing cells. Therefore, induction of PABP may not be specific in pregnant 7 and 8 months mammary tissues, merely reflecting proliferating state of mammary gland.

It has been known that PABP plays important roles in the control of translation and message stability in a complex with the 3' poly (A) tail of eukaryotic mRNAs (Bernstein and Ross, 1989; Gallie, 1998). The PABP binds to the 3' tails of eukaryotic mRNAs that contain multiple adenine nucleotides (Blobel, 1973). At the same time, PABP is able to bind translation initiation factors located at the 5' end of the mRNA, stimulating translation initiation (Gingras et al., 1999). The resulting circularization of mRNA is thought to

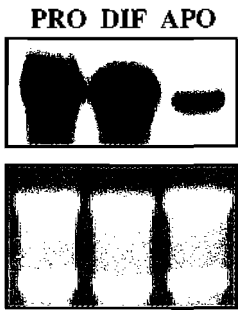

Figure 5. Northern analysis of the PABP gene in proliferating (PRO)-, differentiated (DIF)- and apoptotic (APO)- mammary epithelial HC11 cells. Cells were cultured as described in Materials and Methods. The mRNA levels were analyzed by northern method using ^{32}P-labeled bovine PABP cDNA probe.

increase the efficiency of translation by delivering terminating ribosomes to the translation start site. The mammary gland growth during pregnancy involves enormous increases both in branching of the ducts and in the number of epithelial cells. Results suggest that induction of the PABP gene expression during late pregnancy may contribute to an enhancement of translation efficiency and an increase in mRNA stability for the genes involved in proliferation of mammary cells.

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