Induction of Lysozyme Gene Expression During Involution of Mouse Mammary Gland

M. J. Lee, O. Han, K. Back, Y. J. Choi[1] and M. G. Baik*
Department of Genetic Engineering, Biotechnology Research Institute, College of Agriculture
Chonnam National University, Kwangju 500-757, Korea

ABSTRACT : To understand molecular mechanisms of mouse mammary gland involution, clones were isolated by differential screening of a cDNA library. Partial sequences of a clone showed 100% identity to cDNA sequences of mouse lysozyme P gene. Northern analysis was performed to examine expression levels of lysozyme mRNA in mammary gland at several physiological states. Expression of lysozyme gene was induced at involution day 5 compared with lactating stage. High levels of lysozyme mRNA were also detected at virgin tissues. Two types of separate genes, P and M lysozyme, have been known in mouse, and we found that both lysozyme P and M genes were expressed in mammary tissues by reverse transcriptase-polymerase chain reaction. The lysozyme enzyme activity determined by lysoplate assay was also higher in involuted mammary tissues compared with lactating tissues, showing a similar trend to its mRNA levels. Lysozyme is an antimicrobial protein and involved in host defense mechanism. The increase in lysozyme gene expression may help to prevent microbial infection during mammary gland involution at which stage the residual milk in the mammary gland provides good nutritional sources for microbial growth. *(Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 4 : 462-466)*

Key Words : Mouse, Lysozyme, Gene Expression, Mammary Gland, Involution

INTRODUCTION

Mammary gland is a unique tissue with a developmental potential after birth since, following the onset of pregnancy, epithelial cells in the mammary gland proliferate and differentiate into milk-secreting cells during lactation. Involution of the lactating mammary gland following weaning is accompanied by a reduction in the number of epithelial cells. Mammary glands can repeat this cycle periodically between the pregnant and the non-pregnant stage. Therefore, mammary gland is an excellent model organ for researching involution events.

After completion of lactation, the mammary gland undergoes involution, regressing to a state resembling that of a virgin animal. This phase of mammary gland development is characterized by dramatic epithelial cell death and tissue remodeling. The involution of mammary gland requires active gene expression. The expression of stromelysin, sulfated glycoprotein-2, Fas antigen, and interleukin-1 β converting enzyme has been induced during involution of the mammary gland (Baik et al., 1995; Boudreau et al., 1995; Strange et al., 1992). Previously, we have isolated involution-induced genes by differential screening of a cDNA library and reported that expression of sulfated glycoprotein-2, WDNM1, ferritin heavy chain, and lactoferrin genes is induced during involution of mammary gland (Choi et al., 1996; Lee et al., 1996). In those papers, we reported that the lysozyme gene was induced at involution stage of the mammary gland.

The purpose of this study was to examine expression levels of lysozyme mRNA and its enzyme activity levels from mouse mammary gland at several physiological states. Two types of separate genes, mouse P and M lysozyme, have been reported (Hammer et al., 1987), but which type of lysozyme gene is expressed in mammary tissues is not known. In this study, we have performed reverse transcriptase-polymerase chain reaction to determine which type of lysozyme gene (P and M) is expressed in mammary tissues.

MATERIALS AND METHODS

Differential screening and sequencing

To find involution-induced genes in mouse mammary gland, we used an involution-specific cDNA library constructed earlier (Lee et al., 1996), identifying by differential screening as described there. Briefly, the library was plated with XL1-Blue cells, and phage DNA was transferred onto the membrane in duplicate. The differential hybridization of membrane was done with ^{32}P-labeled cDNA prepared from mRNAs of lactating tissues and of involuted tissues, respectively. After comparing signals from two films, the involution-induced plaques were identified and characterized by northern and sequencing analyses.

Partial sequencing of the clone was done by the dideoxy nucleotide chain-termination method using the Sequenase DNA Sequencing Kit (USB, USA) and T3

* Address reprint request to M. G. Baik. Tel: +82-62-530-2164, Fax: +82-62-530-2169, E-mail: mgbaik@chonnam.chonnam.ac.kr.
[1] School of Agricultural Biotechnology, College of Agriculture, Seoul National University, Suwon 441-744, Korea.
Received October 9, 2000; Accepted December 11, 2000

primer. The sequences of the clone were compared to the sequence data of GenBank in NIH, USA.

Tissue sampling and northern analysis

Tissues obtained from ICR mice were used in all experiments. Total 72 female mice were used to isolate mammary tissues; eighteen animals were used for an experiment, and four separate experiments were performed for northern analysis and enzyme activity assay. Since mice have relatively small amounts of tissues at virgin and involution 3 and 5 day stages, mammary tissues were collected and pooled from four animals at each stage and divided into two portions, used for a northern analysis and enzyme activity assay. Mammary tissues were pooled from two animals at pregnant day 10, lactating day 10, and involution day 1 for one experiment since mice have relatively large amounts of mammary tissues at these stages. For the induction of involution, the young were removed 10 days after parturition, and the mammary tissues were obtained at the indicated time after weaning.

Total RNA was extracted by the acid guanidinium thiocyanate phenol/chloroform method (Chomczynski and Sacchi, 1987). 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 phagemid, pBluescript, by lambda ZAP II Automatic Excision Process (Stratagene, USA). The plasmid was digested with EcoR I and Xho 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). The membrane was hybridized with the ^{32}P-labeled insert of the cDNA clone. The equal amount of RNA loading was confirmed by the intensities of 28S and 18S bands, and the efficiency of transfer was monitored by ethidium bromide staining.

RT-PCR of lysozyme gene expression

To identify whether P and M lysozyme genes are expressed in mouse mammary gland, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as described below. Total RNA was extracted from mammary tissues at virgin, pregnant, lactating and involuted states and from small intestine. Total RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, USA) and oligo d (T) primers at 42℃ for 1 h. This cDNA template was amplified by PCR using primers specific to lysozyme P cDNA (Cortopassi and Wilson, 1990). The primers were 5'-TGCCCAGGCCAAGGTCT ACA-3' (5' primer; 45-64th sequence of lysozyme P cDNA) and 5'-TTGATCCCACAGGCATTCTT-3' (3' primer; 274-293th sequence of lysozyme P cDNA), which generated 248 bp products. The primers specific to lysozyme M cDNA were 5'-TGCTCAGGCCAAGG TCTATG-3' (5' primer; 45-64th sequence of lysozyme M cDNA) and 5'-TTGATCCCACAGGCATTCAC-3' (3' primer; 274-293th sequence of lysozyme M cDNA). The amplification was performed with AmpliTaq DNA polymerase (Perkin Elmer, USA) for 43 cycles (preheating at 94℃ for 5 min; cycling at 94℃ for 30 sec, 60℃ for 1 min, and 72℃ for 1 min; a final elongation at 72℃ for 10 min). PCR products were electrophoresed on 1% agarose gel containing ethidium bromide.

Lysozyme enzyme assay

Dissected tissues were frozen in liquid nitrogen and ground. Determination of lysozyme activity was done by the lysoplate assay as described below (Lie et al., 1986; Osserman and Lawlor, 1966). *Micrococcus lysodeikticus* known to be particularly susceptible to lytic action of lysozyme was prepared by incubation with tryptic soy broth at 30℃ for 24 h. The cells were harvested and suspended in 0.07 M sodium phosphate buffer. Suspended cells were freeze-dried, and dried cells were inactivated by heating at 80℃ for 30 min. The lysoplate was prepared using 40 ml of a 1% agarose gel in 0.07 M sodium phosphate buffer containing 100 μg/ml of *M. lysodeikticus*.

The lysozyme standards were prepared by dilution of a chicken egg white lysozyme stock (48,000 units/mg; Sigma) to 500 μg/ml, 250 μg/ml, 100 μg/ml, 50 μg/ml, 25 μg/ml, 10 μg/ml, 5 μg/ml, and 0.5 μg/ml. Sample wells of 3 mm in diameter were punched and the lysozyme sources were applied in 9 μl volumes. Plates were incubated in a humidity chamber. Diameters of the cleared zones were measured, and a semilogarithmic plot was made of the diameters given by the standard solutions. The diameters of the cleared zones of standard lysozyme were proportional to the log amount of the lysozyme (μg/ml). The amount of lysozyme in tissue samples was calculated using the diameters of the cleared zones and the standard curve for lysozyme stock. Protein concentrations were determined by using the Micro BCA protein assay (Pierce, USA). The lysozyme activity in samples was calculated with the value of activity of lysozyme stock (48,000 units/mg) ×(mg of tissue lysozyme/mg of tissue protein).

RESULTS

Differential screening and mRNA levels of lysozyme gene

To find genes induced during involution of the mammary gland, we used an involution-specific cDNA library of mammary gland constructed earlier (Lee et al., 1996), identifying clones by differential screening

as described there. Partial sequences of a clone showed 100% identity to cDNA sequences of mouse lysozyme P gene (Cortopassi and Wilson, 1990).

Northern analysis was used to examine expression of lysozyme gene in mammary tissues. Lysozyme gene was not detected in the lactating mammary gland and expressed at only low levels in involution day 1. Expression of lysozyme gene was dramatically induced at involution day 5 (figure 1A). In addition, high levels of expression were observed in virgin tissues. The lysozyme gene was highly expressed in lung tissues (figure 1B). The relative levels of lysozyme mRNA in mammary gland were low compared with lung tissues.

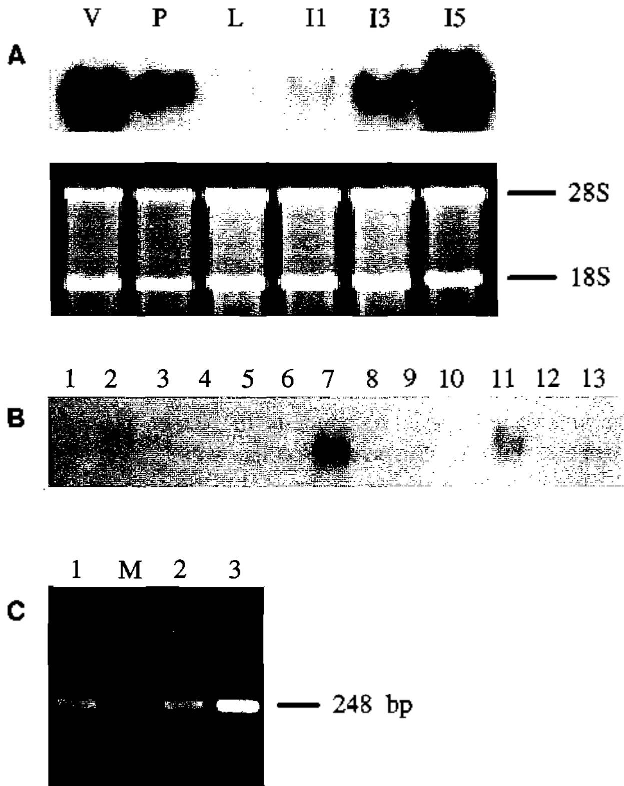

Figure 1. Expression of lysozyme gene in mammary gland. A. The 20 μg of total RNA isolated at virgin (V), pregnant day 10 (P), lactating day 10 (L), and involution 1 (I1), 3 (I3), and 5 (I5) days of mouse mammary gland were separated on a 1% formaldehyde/agarose gel and transferred onto the membrane. The blot was hybridized with the [^{32}P] labeled cDNA probe. B. The 20μg of total RNA isolated from thymus (1), spleen (2), uterus (3), ovary (4), liver (5), brain (6), lung (7), heart (8), kidney (9), mammary gland at involution days 2 and 4 (10, 11) of the female mouse, and testis (12) and mammary gland (13) of male mouse, were analyzed by northern method. Representative data are shown here for panels A and B from four separate experiments. C. Total RNA was reverse-transcribed and the cDNA template was amplified by PCR using primers specific to either lysozyme M (lane 1) or lysozyme P cDNA (lanes 2 and 3). PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide. M, USB PCR marker; lanes 1 and 2, mammary tissues of involution day 5; lane 2, small intestine.

RT-PCR of lysozyme gene expression

It has been reported that two types of separate genes, P and M lysozyme, are present in mouse (Hammer et al., 1987). The predominant form of lysozyme mRNA in the small intestine is encoded by the lysozyme P gene, while the lysozyme M gene accounts for the vast majority of lysozyme mRNA detectable in other tissues examined such as lung, spleen, and thymus (Cross et al., 1988). The lysozyme P coding sequence differs from that of M lysozyme by 21 base substitutions out of 447 base, showing 95% identity to each other (Hammer et al., 1987). The mRNAs of lysozyme P gene have been indistinguishable by hybridization to probes from either translated or untranslated regions of the M gene (Cross et al., 1988).

To determine which type of lysozyme gene is expressed in mouse mammary tissues, the first-stranded cDNA template generated by reverse transcriptase was amplified by PCR with primers specific to either lysozyme P or lysozyme M cDNA. The expected RT-PCR products (248 bp) for lysozyme P gene were detected in involuted mammary tissues as well as in small intestine (figure 1C). The PCR products were also detected in virgin, pregnant, and lactating mammary tissues (data not shown). The RT-PCR products for lysozyme M gene were also detected in the involuted mammary tissues. The result of RT-PCR demonstrates that both P and M lysozyme genes are expressed in mammary tissues.

Lysozyme enzyme levels

Comparisons of mRNA and enzyme levels of lysozyme reveal an accumulation of disproportionately high concentrations of lysozyme protein in some tissues (Cross et al., 1988), while its mRNA levels are close to background. Enzyme levels of lysozyme were examined in mammary tissues as well as other tissues by lysoplate assay to compare with mRNA levels. High levels of lysozyme activity were observed in lung (11,400 units/mg protein), kidney (34,814 units/mg protein), and spleen (16,983 units/mg protein); similar values were reported by Cross et al. (1988). The lysozyme activity in mammary glands was relatively low compared with other tissues such as kidney, lung, and spleen. Highest levels of lysozyme activity in mammary gland were detected at involution day 5 and lowest levels at lactating stage (figure 2);

these values were generally correlated with mRNA levels at involution and lactation stages, but there were differences between enzyme levels of lysozyme and its mRNA levels in virgin mammary tissues. Relatively high mRNA levels were detected, but enzyme levels of lysozyme were low in virgin tissues.

DISCUSSION

We found that expression of lysozyme gene in mRNA levels was induced at the involution stage of the mammary gland compared with the lactating stage. High levels of lysozyme mRNA were also detected in virgin tissues. The lysozyme enzyme activity was also higher in the involution compared with lactation stage, showing a similar trend to mRNA levels in mammary gland. Considering that lysozyme is an antimicrobial protein and involved in host defense mechanism, the increase in lysozyme enzyme levels may contribute to prevention of microbial infection during mammary involution, at which stage the residual milk in the mammary gland provides good nutritional sources for microbial growth.

We found that there were differences between enzyme levels of lysozyme and its mRNA levels in virgin mammary tissues. Relatively high mRNA levels were detected, but enzyme levels of lysozyme were low in virgin tissues. Reasons for this difference between reproductive stages of mammary tissues are not clear. Differences in the mRNA/enzyme ratios between reproductive stages may reflect variable degrees of posttranscriptional control. These ratios may also be affected by differences in the rates of secretion and accumulation of enzyme in different stages: relatively high amounts of blood lysozyme may be accumulated at pregnant- and involution- stages in the mammary secretion, while low amounts of blood lysozyme are accumulated at the virgin stage. Steinhoff et al. (1994) have isolated bovine lysozyme cDNA clones from a mammary gland derived cDNA library, but they have suggested that their isolates originate from blood cells that are known to invade the lactating mammary gland (Doubravsky and Trappmann, 1992). Cross et al. (1988) have observed extremely high levels of lysozyme in kidney, while it contains only background levels of the mRNA. They have suggested that high levels of enzyme are due to an accumulation of blood lysozyme in kidney. We confirmed this suggestion in that the lysozyme was found in the kidney, but mRNA for lysozyme was not detected in this organ. Differences in the mRNA/enzyme ratios between reproductive stages may also reflect differential expression of P and M lysozyme genes in virgin mammary tissues. Cross et al. (1988) have reported that there was a noticeable difference between the pattern of lysis mediated by small intestine extracts (clear plaques) and those from other tissues (turbid plaques). They have suggested that extracts from small intestine have a characteristically low activity in lysoplate assays and that the values of lysozyme activity from small intestine are therefore underestimated and could not be compared to the data of other tissues. These observations are consistent with reports that most of the lysozyme enzyme activity in the small intestine derives from the lysozyme P gene and that the lysozyme proteins differ between P and M type in amino acid sequences at 6 of 49 positions near the amino terminus (Hammer et al., 1987). In this study, we have found that both P and M lysozyme genes are expressed in mammary tissues. In our study, lysoplate assay showed turbid plaques in all tissue samples as well as in standard, suggesting that some of the lysozyme enzyme activity is derived from M gene. But, the present study does not show how many portions of mRNA levels as well as enzyme levels detected in mammary tissues come from the expression of either lysozyme P or M type. A more sensitive method is required to differentiate which type of lysozyme gene (P or M) expression is dominant in mammary tissues.

ACKNOWLEDGMENT

This study was supported by Korea Research

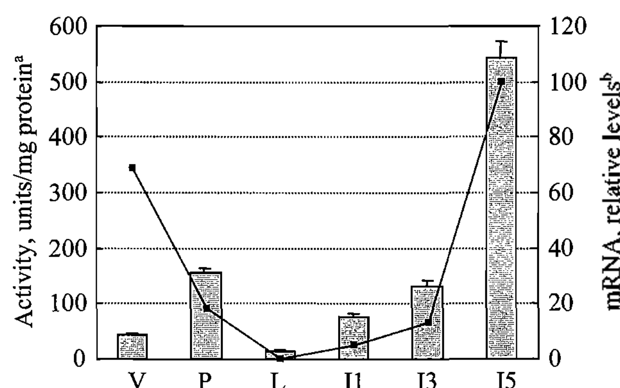

Figure 2. Quantitation of lysozyme enzyme activity and its mRNA levels in mouse mammary gland. Bar graph: Lysozyme enzyme activity of mouse mammary tissues at virgin (V), pregnant day 10 (P), lactating day 10 (L), and involution 1 (I1), 3 (I3), and 5 (I5) days was calculated with the value of activity of a chicken egg white lysozyme stock (48,000 unit/mg)$\times$(mg of tissue lysozyme/mg of tissue protein). Values are mean$\pm$S.E.M (n=4). Line graph: Relative levels of lysozyme mRNA were determined by scanning densitometry of northern autoradiograms presented in figure 1A. Values were normalized to 100 for the highest levels. Band was not detected in lactating mammary tissues.

Foundation Grant (KRF-99-005-G00008).

REFERENCES

Baik, M. G., J. K. Seol and Y. J. Choi. 1995. The induction of Fas mRNA and DNA fragmentation in the involution stage of mouse mammary glands. Mol. Cells. 5:81-85.

Boudreau, N., C. J. Sympson, Z. Werb and M. J. Bissell. 1995. Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science. 267:891-893.

Choi, B., K. Myung, M. Lee, H. Kim, D. Jeon, I. Hwang, Y. Choi, S. Paik and M. Baik. 1996. Cloning of involution-induced cDNAs from mouse mammary gland. Mol. Cells. 6:311-315.

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. Anal. Biochem. 162:156-159.

Cortopassi, G. A. and A. C. Wilson. 1990. Recent origin of the P lysozyme gene in mice. Nucleic Acids Research. 18:1911.

Cross, M., I. Mangelsdorf, A. Wdel and R. Renkawitz. 1988. Mouse lysozyme M gene: isolation, characterization, and expression studies. Proc. Natl. Acad. Sci. USA. 85: 6232-6236.

Doubravsky, P. and W. Trappmann. 1992. Use of somatic cell counts for genetic improvement of udder health. Zuchtungskunde. 64:323-333.

Hammer, M. F., J. W. Schilling, E. M. Prager and A. C. Wilson. 1987. Recruitment of lysozyme as a major enzyme in the mouse gut: duplication, divergence, and regulatory evolution. J. Mol. Evol. 24:272-279.

Lee, M., H. Kim, D. Jeon, I. Hwang, B. Choi, K. Myung, H. Kim, Y. Choi, S. Paik and M. Baik. 1996. Iron metabolism-related genes and mitochondrial genes are induced during involution of mouse mammary gland. Biochem. Biophys. Res. Commun. 224:164-168.

Lie, O., M. Syed and S. Solbu. 1986. Improved agar plate assays of bovine lysozyme and haemolytic complement activity. Acta Vet. Scand. 27:23-32.

Osserman, E. F. and D. P. Lawlor. 1966. Serum and urinary lysozyme (muramidase) in monocytic and mono-myelocytic leukemia. J. Exp. Med. 124:921-951.

Steinhoff, U. M., B. Senft and H. M. Seyfert. 1994. Lysozyme-encoding bovine cDNAs from neutrophile granulocytes and mammary gland are derived from a different genes than stomach lysozymes. Gene. 143: 271-276.

Strange, R., F. Li, S. Saurer, A. Burkhardt and R. R. Friis. 1992. Apoptotic cell death and tissue remodeling during mouse mammary gland involution. Development. s115:49-58.