유생산증진 및 신선유 생산을 위한
젖소유선내 유용유전자원의 개발과 이용
에 관한 연구

Development and Utilization of Useful Genes for Increase of Milk Production and Fresh Milk in Dairy Cow

연구기관

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농　림　부

190 × 268

2000

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1.   10

2.   1

2000   12   29

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( )

2000   12   29

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.

.

multiple linkage marker   . Australia,   ,

   bovine genome

                                        .   bovine genime

                                .

,

                                        .

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•

1.   ,

   (   )

                                .

1)   cDNA clone

2) Northern   cDNA

3)   cDNA

4) E. Coli

                                                        .

   ,

                                                .

                              RNA

   cDNA clone   .   clone

Northern   .

               ,   .   ,
                         ,
            .

   E. Coli   E. coli
                .

 2.   ,
   (   )

                                cDNA library   cDNA
library   .   cDNA
clone   northern

         .   stat5a
                      .   stat5a
         .

 3.   (   )

                   Staphylococcus aureus   ,
    -interferon   .
          antigen presenting cell (APC)   phagolysosome
               Nramp   ,   major histo-
compatibility complex (MHC)
    BoLA DRB3.2 exon   allele typing
                                        .

·

1.

1)   ,

(   )

total RNA   , differential hybridization, differential display RT-PCR subtractive hybridization

                      .

         clone   ,
lactoferrin, glycoprotein III   , glycoprotein III   clusterin similar sequence

                 . WDNM1

         ,

            . , serum amyloid A3

           metalloprotenase   ,
inflammation
cytokine TNF   .

      clone glycoprotein III, serum amyloid A3, WDNM1

. serum amyloid A3 WDNM1 full-coding
cDNA clone   , glycoprotein III full-coding
1.3kb RT-PCR   .

              Northern

. Total RNA   , 1/2 , 1 , 1 1/2 , 2 ,
2 1/2 , 9 total RNA   , glycoprotein III
1 , 1 1/2   , serum amyloid A3
glycoprotein III   5

.

      glycoprotein III, serum amyloid A3 WDNM1 cDNA
, glycoprotein III, serum amyloid A3 WDNM1

. hormone ,
, cytokine , glycoprotein III, serum amyloid A3
WDNM1   .

in vivo test   in vitro test

E. coli   .

Glycoprotein III, serum amyloid A3   WDNM1

,   DNA

.   glycoprotein

III, serum amyloid A3   WDNM1   ,

.

2)   ,

(   )

cDNA library   , cDNA library

. 4

clone   -casein,   -casein,

S1-casein   -lactalbumin   . Bovine

elongation factor alpha   stat5a   . 4   clone

human secretory carrier membrane protein, human

polyadenylate binding protein 1, hamster ribosomal protein S14   human

ribosomal protein L21   . Polyadenylate binding

protein 1 cDNA   full-coding

cDNA clone   .

,   1,911   636

. Bovine polyadenylate binding protein 1 cDNA   human

mouse   cDNA   95%   94%   identity   .

northern   ,

-casein,   -casein,   -S1-casein   -lactalbumin

. Bovine

stat5a   5

7   8   .

Stat5a   .

Polyadenylate binding protein 1

7   8   . Polyadenylate binding

protein 1   mRNA   ,   (translational

efficiency)   . Stat5a

stat5a   , stat5a

.

stat5a cDNA   stat5a

.   , stat5a cDNA   control neo

24, 48   72   26%,

95%   85%   .   , bovine stat5a

.

Stat5a   polyadenylate binding protein 1

,   DNA

.   stat5a

polyadenylate binding proteinp 1   ,

.

3)   (   )

,   *Nramp*   BoLA

,

.

2.

5   9 , SCI   6   ,

13   ,   7

3   .

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SUMMARY

( )

I. Subject

Development and utilization of useful genes for increase of milk production and fresh milk in dairy cow

II. Objectives and importance of the project

Mammary gland growth during pregnancy involves enormous increases both in branching of ducts and in the number of epithelial cells. The mammary epithelial cell population influences milk yield considerably. Numbers of mammary epithelial cells are a major determinant of milk yield. Thus, understanding of the mechanism that increases epithelial cell numbers will lead to develop new methods to enhance milk yield in the dairy cow. Efforts have been made to understand the roles of hormones, growth factors, and unknown factors in the proliferation of mammary epithelial cells. But many questions on the mechanisms that how these factos regulates mammary epithelial cell proliferation remain unanswered. This study was conducted to identify candidate genes involved in the proliferation of mammary epithelial cells, characterize the identified cDNA clones, to examine expression patterns of the these genes, and finally to characterize the roles of these identified genes on the proliferation of mammary epithelial cells.

Cessation of milk removal leads to rapid changes in the mammary tissue and initiation of the process of mammary involution. Changes in the compositoin of mammary secretions during the early phases of involution indicate rapid changes in the normal mechanisms involved in milk synthesis and secretion. These compositional changes in the mammary gland secretion include a rapid decline in lactose concentration in the mammary gland secretions, indicating that lactose synthesis, and the associated water transport mechanism, decline soon after cessation of milk removal. However, total protein concentrations increase in early involuiton,

partially because of water resorption from the secretion and partly due to increased concentrations of lactoferrin, serum albumin and immunoglobulins. Lactoferrin is a major protein found in mammary gland secretions during involution. Its synthesis is increased during involution in contrast to milk-specific proteins such as casein whose synthesis is decreased. Lactoferrin has a number of potential functions in the mammary epithelial gland, particularly as a nonspecific disease resistance factor.

Involution-associated ultrastructural changes in bovine mammary cells begin within 48 hours after cessation of milk removal. The most apparent change is the formation of large stasis vacuoles in the epithelial cell, formed largely as a result of intracellular accumulation of milk fat droplets and secretory vesicles. These vacuoles persist to at least 14 days of involution and are usually gone by day 28. Alveolar lumenal area declines during this period, while interalveolar stromal area increases. A substantial reduction in fluid volume in the gland occurs between day 3 and 7 of involution, probably accounting for the reduction in lumenal volume. The collapsed alveolar structures by day 28 are considerably smaller than those during lactation, with a very small lumen. General alveolar structure in the mammary gland is maintained thoughout involution period.

Mastitis continues to have a major economic impact on the dairy industry. Incidence of mastitis in the dairy cow is increased by invading various pathogenic bacteria due to poor hygienic milking management. More importantly, host defense system can influence the severity of disease in the bovine mastitis. Host immune response are individually different, therefore, the host immunogenetic properties may provide the selection and establishment of disease-resistant group against certain pathogens.

This study was carried out to determine immunogenetic chracteristics of mastitis-resistant dairy cows and to, apply to select individuals, genetically resistant dairy cow to certain specific pathogens.

III. Contents and ranges of the project

1. Development and utilization of useful genes in involution stage of

mammary gland in dairy cow

The contents are below.

1) Selection of involution-specific cDNA clone by subtractive hybridization

2) Confirmation of the selected cDNAs by Northern blot and analysis and identification of full-coding region of selected cDNA

3) Transfection of the selected cDNAs into mouse mammary epithelial cell line, HC11, and characterization of the roles of these cDNA in in vitro cell culture system

4) Production of recombinant protein in E. coli system

The purpose of this research was to obtain the selectively genes at the involution stage of bovine mammary gland, and to know their function whether these genes induce cell death or not. The bovine involution-induced cDNAs were isolated by subtractive hybridization method. We selected involution specific clones compared with lactation-specific clones using dot blot analysis. From this method we cloned H. sapiens polyadenylate binding protein, serum amyloid A3 protein, lactoferrin, bovine glycoprotein III(clusterin sequence similarity), WNDM1 protein, human homology of mRNA for ornithine decarboxylase antizyme, 38kDa heparin binding glycoprotein and unknown gene. Three clones among these selectively genes were further confirmed to be specifically expressed in involution stage by Northern blot analysis. And we cloned full sequence these three cDNAs.

Expression vector for bovine glycoprotein III(clusterin), serum amyloid A3 and WDNM1 cDNA containing full-coding region were constructed into pIRES, expression vector, in which glycoprotin III, serum amyloid A3 and WDNM1 cDNAs expression is driven by cytomegalovirus(CMV) promoter(they were called pIRGPIII, pIRSAA3 and pIRWDNM1, respectively). pIRES- EGFP vector containing GFP reporter gene was used as a control. The glycoprotin III, serum amyloid A3, and WDNM1 expression vector was transfected into mouse mammary epithelial cell line, HCll cells. The transfectants were further confirmed by RT-PCR.

To further characterize regulated expression of these genes in in vitro system, we treated death inducing agents in HC11. In the course of HC11

differentiation by lactogenic hormones, the steady-state mRNA levels of clusterin, serum amyloid A3 and WDNM1 were induced with maximal induction at day 5 of differentiation. The effects of individual hormones on GPIII(clusterin is same word), serum amyloid A3 and WDNM1 gene expression were further characterized and found that prolactin(PRL), dexamethasone (DEX), PRL+EGF and PRL+DEX treatment induced clusterin mRNA expression. Similarly, the steady-state serum amyloid A3 mRNA level was increased by PRL, DEX+PRL and EGF+PRL treatment, but was not changed by DEX and EGF treatment alone. In other hand, the steady-state WDNM1 mRNA level was not changed by any above treatments. The steady-state levels of clusterin and WDNM1 mRNA were down-regulated by serum. Also, the regulated expression of clusterin, serum amyloid A3, and WDNM1 mRNA were further characterized by cytokines(TGF and TNF). Finally, GPIII, serum amyloid A3 and WDNM1 recombinant proteins were produced in E. coli using pET-expression vectors system

2. Development and utilization of useful genes in mammary gland for increase of milk production in dairy cow

 Pregnant-specific cDNA library of bovine mammary tissues was constructed in the TriplEx vector using pooled poly A RNA extracted from mammary gland at pregnant 5, 6, 7, and 8 months to identify genes involved in mammary gland growth. Differential screening method was used to isolate pregnant-induced clones. Partial sequencing and northern analysis was performed to characterize the clones and to examine expression pattern of the genes. The cDNA clone that was not reported in bovine species was subjected to sequencing of the full-coding region. To examine function of bovine stat5a in mammary epithelial cell proliferation, expression vector for bovine stat5a cDNA containing full-coding region was constructed by using pBK-CMV expression vector, in which stat5a cDNA expression is driven by cytomegalovirus (CMV) immediate early promoter. pBK-CMV vector containing neo gene was used as a control. The stat5a expression vector was transfected in mammary epithelial HC11 cells. Effect of stat5a transfection on mammary epithelial cell proliferation was examined under various hormonal treatments. Stat5a protein was produced in *E. coli* cells.

3. Development of Immunogenetical Analysis for the Selection of Mastitis Resistant Cows

   This study was carried out to determine immunogenetic characteristics of mastitis-resistant dairy cattle. Host immune cell activity was determined by analyzing the proportion of lymphocyte subpopulation of mastitis-resistant cows. Functional activity of host immune cell was determined by cytokine production and phgocytic activity. *Staphylococcus aureus* vaccine using enterotoxin C was prepared to examine the possibility of enhancement of host defensive immune system through this study. Immunogenetic characteristics of bovine mastitis resistant cows could be defined by *BoLA class II DRB3* genotyping and *Nramp* expression in the involvement of host adaptive immunity.

Ⅲ. Research results and suggestions for application of results

1. Research results

1) Development and utilization of useful genes in involution stage of mammary gland in dairy cow

   To identify genes involved in mammary gland involution, we isolated total RNA from bovine mammary gland during lactation and involution stage. Differential hybridization, differential display RT-PCR and subtractive hybridization method were used to isolate involution-induced clones. Partial sequencing and Northern analysis revealed that expression of unknown and known genes(H. sapiens polyadenylate binding protein, serum amyloid A protein, lactoferrin, bovine glycoprotein 3-clusterin sequence similarity, WNDM1 protein, human mRNA for ornithine decarboxylase antizyme, 38kDa heparin binding glycoprotein) in bovine mammary gland involution stage. Three clones among these selected genes revealed that the clones were homologue of mouse WDNM1 protein, rabbit serum amyloid A3 and bovine glycoprotein III(clusterin). Serum amyloid A3 and WDNM1 were

contained full-coding region, and the glycoprotein III contained partial-coding region and further cloned full-coding region by RT-PCR method. Functional role of glycoprotein III, serum amyloid A3 and WDNM1 have not been well studied in bovine mammary gland involution. To examine function of bovine glycoprotein III, serum amyloid A3 and WDNM1 in mammary epithelial cell death, expression vector for bovine glycoprotein III, serum amyloid A3, and WDNM1 cDNA containing full-coding region were constructed into pIRES expression vector. pIRES vector containing pEGFP-IRESneo was used as a control. The glycoprotein III, serum amyloid A3, and WDNM1 expression vectors were transfected into mammary epithelial HC11 cells and the transfectants were further confirmed by RT-PCR.

From this project, we found that bovine glycoprotein III, serum amyloid A3, and WDNM1 were responsible for mammary gland death in in vivo and in vitro.

Numbers of mammary epithelial cells are a major determinant of milk yield. Further studies regarding the polymorphisms of glycoprotein III, serum amyloid A3, and WDNM1 genes will lead to find DNA marker for efficient breeding of dairy cow. Further understanding for role of glycoprotein III, serum amyloid A3 and WDNM1 in death of mammary epithelial cells may lead to develop new methods to enhance milk yield in the dairy cow and to inhibit new methods to reduce mastitis.

2) Development and utilization of useful genes in mammary gland for increase of milk production of dairy cow

To identify genes involved in mammary gland growth, pregnant-specific cDNA library of bovine mammary tissues was successfully constructed in the TriplEx vector. Differential screening method was used to isolate pregnant-induced clones. Partial sequencing and northern analysis revealed that expression of known genes (-casein, -casein, S1-casein, -lactalbumin, stat5a)in bovine species was induced in mammary tissues of holstein cow during pregnancy. One clone revealed that the clone was a homologue of human polyadenylate binding protein 1. The clone containing full-coding region of bovine polyadenylate binding protein 1 was cloned by RT-PCR method. The cDNA clone of bovine polyadenylate binding protein 1

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 and mouse species showed 100% and 99% identity, respectively. In this study, we have found that expression of stat5a gene is highly induced at late pregnant stage at which proliferation of mammary epithelial cells is extensively occurred. Functional role of stat5a has not been well studied in bovine mammary gland development. To examine function of bovine stat5a in mammary epithelial cell proliferation, expression vector for bovine stat5a cDNA containing full-coding region was constructed by using pBK-CMV expression vector. pBK-CMV vector containing neo gene was used as a control. The stat5a expression vector was transfected in mammary epithelial HC11 cells. Stat5a transfection increased mammary epithelial cell proliferation. Overall mean values of cell proliferation at 24h, 48h and 72h after seeding were 26%, 95%, and 85% higher in stat5a-transfected cells than in control vector-transfected cells. The results demonstrate that bovine stat5a is responsible for mammary gland development.

From this project, we found that bovine stat5a is responsible for mammary gland development. We also found that bovine polyadenylate binding protein 1 gene was highly induced in bovine mammary tissues during pregnancy, suggesting 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. The mammary epithelial cell population influences milk yield considerably. Numbers of mammary epithelial cells are a major determinant of milk yield. Further studies regarding polymorphism of stat5a and polyadenylate binding protein 1 and genes will lead to find DNA marker for efficient breeding of dairy cow . Further understanding for role of stat5a in proliferation of mammary epithelial cells may lead to develop new methods to enhance milk yield in the cow.

3) Development of Immunogenetical Analysis for the Selection of Mastitis Resistant Cows

The study has indicated the immunogenetic characterization determined by innate and adaptive host immune responses in bovine mastitis-resistant cows may be useful for early selection and further, can be applied to define the individuals genetically resistant to certain specific pathogens causing bovine mastitis.

2. Suggestions for application of results

From the results of this project, total 15 papers (9 Korean journals and 6 SCI journals) were published. Also, total 13 abstracts were presented in academic society(e.g. Korean Society for Molecular Biology) and 3 patents were achieved.

From this achievment, further investment is necessary for the industrial application of these results.

Contents

(   )

Chapter 3. Development and utilization of useful genes in
           mammary gland for increase of milk production of
           dairy cow

Chapter 4. Development of Immunogenetical Analysis for the Selection of Mastitis Resistant Cows ················· 122

1

1

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,   ,

.   1960

2000

.   5,800kg

6,800kg   1,000kg

.

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2000

25%   (, 1993).

,   ,

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,   ,   ,

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1)   ,

2)   ,

3)   .

.

.   somatic cell count (SSC)

.

1   6   .

lactoferrin   . Lactoferrin

                  2   . Lactoferrin

anti-bacterial   .

            lactoferrine   (mastitis-resistance

gene)   .   lactoferrin

                                                                  .

                                                            .

        ,   .

                                                                  .

                              ,

                .

                                                  .

                                                      .

                              ,

                                                                  .

2

1 (1996)		(2, 3, 4, 5, 6, 7, 8) Total RNA mRNA cDNA library (0.5, 1, 1 1/2, 2, 2 1/2, 9) Total RNA mRNA cDNA library BoLA typing Typing BoLA

2 (1997)		cDNA clone PCR/Southern cDNA clone clone insert Lambda DNA insert plasmid Northern clone cDNA clone PCR/Southern cDNA clone clone insert Lambda DNA insert plasmid Northern clone MHC clone

3
(1998)
		cDNA clone
		    cDNA clone
		GenBank clone
		    cDNA clone
		
		cDNA clone
		GenBank clone
		    cDNA clone
		
		                    clone
		
		Gene mutation
		
		        ( )

4 (1999)		cDNA 　　　　　　cDNA Northern cDNA 　　　　　cDNA Northern

5 (2000)		E. coli E. Coli E. coli E. Coli ,

2

1

(UR)   (WTO)

,

.   ,

,

(CODEX)   (HACCP)   .

HACCP   ,   HACCP

.

,

,   ,

,

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,   ,   ,   ,

.

,

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,

.

.   6   8   ,

.   3

.   .

1/3   .

2/3

.

.

.

,

.

(Seyfert , 1994).

. ,

DNA .

.

.

2

1.

Fig . 1   .

Xylazine(100mg, 1M, Haver-Lockhard, Shawness)   .

.

.

.

ephedrine/procaine hydrochloride (100mg procaine, 400mcg ephedrine)   .

.

4cm   .

.

10g   .

.
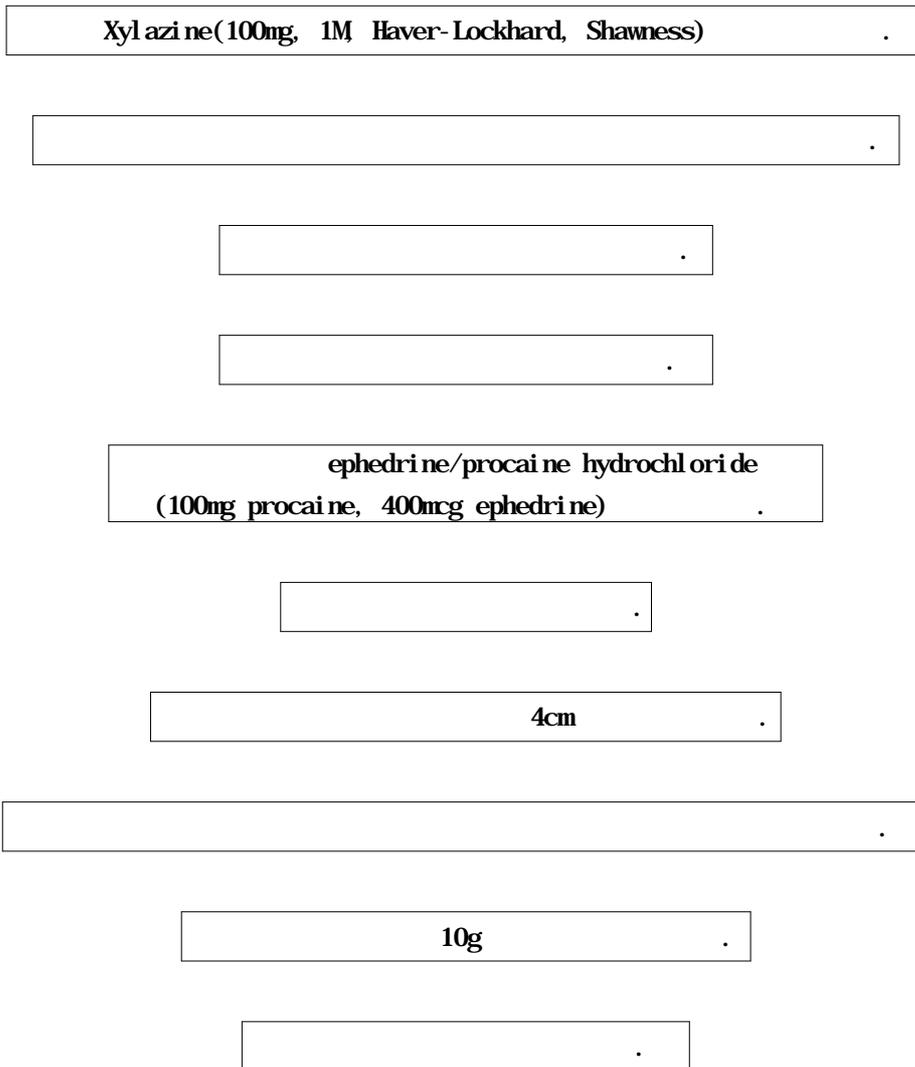
| 5   penicillin   ,   . |

Fig 1.   (biopsy)

. Biopsy

                             .

                                                                    .

                                    penicillin

              .

2.   ,   (1/2, 1, 1 1/2, 2, 2 1/2, 9   )

1)

                                    .   xylazine   (
)   (Fig. 2).   ephedrine/
procaine hydrochloride(100mg procaine, 400mcg ephedrine)   , 4cm
                        .   2g-5g   (Fig. 3).
                            4   penicillin   .
                                                RNA   -70
deep freezer   .
            (1/2, 1, 1 1/2, 2, 2 1/2, 9   )
                                    .

Fig 2. (   )
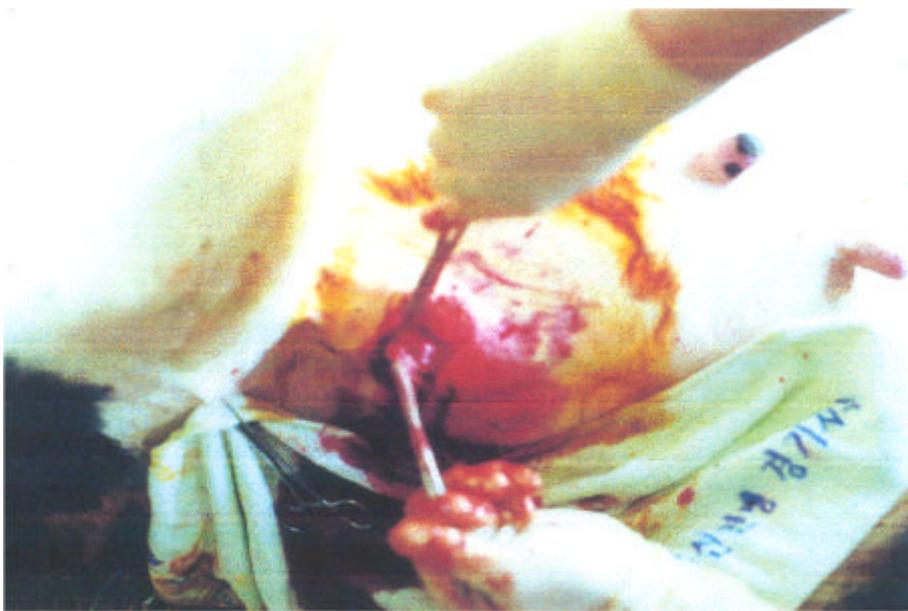
Fig 3. 2g-5g

3. Total RNA

total RNA   acid   guanidinium   thiocyanate-
phenol-chloroform   (Chomozenski and Sacchi, 1987).
RNA   (denaturing solution)   homogenization   2M Na
citrate, acidified phenol   chloroform   .
Aqueous phase   phenol extraction   2-propanol   RNA
          .

4.   cDNA library

              total RNA   Clonetech   cDNA library
        .   cDNA library   diffrential hybridization

. Table 1   cDNA library   .

Table 1.   cDNA library construction

Unamplifed cDNA library titer	4.0 × 10⁵pfu/ml
Amplified cDNA library titer	6.7 × 10⁵pfu/ml
Clear plaques/blue plaques	80%
Number of independent clone	1.0 × 106
Average insert size	1.0 kb
Insert range	0.4 2.5 kb

5.   cDNA clone

1) Differential hybridization
1   cDNA library   amplification   10⁶pfu/μl
      20,000 plaques/plate   12   master plate   .

nitrocellulose filter   transfer   32P-dATP
                        cDNA probe   labeling   .   cloned
                        probe   cDNA   hybridization
                        screening   signal   .

   2) Differential Display RT-PCR
   3   total   RNA   oligo   dT   primer,   reverse
transcriptase   cDNA   .   cDNA 2μ , oligo   dT
primer, random primer, *Taq* polymerase   32P-dATP   labeling
     annealing temperature 39   RT-PCR   .   PCR
6% polyacrylamide gel   cDNA   .   cDNA
         band
      cloning   .

   3) Subtractive hybridization
                        Fig. 4   .

| cDNA_   _ |
| Tester(   ), Driver(   ) ds cDNA |

| Rsa I_digestion |
| Tester(   ), Driver(   ) ds cDNA   , blunt-ending |
| molecule   digestion |

| Adaptor_ligation |
| Tester   adpator(adaptor 1, adaptor 2R)   , |
| driver   adaptor   . |

| First_hybridization |
| Rsa I digested driver   ligated adaptor 1 or 2R tester   hybridization |

| Second_hybridization |
| Fresh driver   ligated adaptor 1   adpator 2R   hybridization |

First_PCR_amplification
adaptor(A1, A2R)   ds cDNA

Second_PCR_amplification
Background   first

TA_cloning
Subtracted   ,   pGEM Teasy vector   ligation

Probe_____Dot_blotting
,   probe   white colony   direct PCR   PCR   product   dot blotting   .

Colony_selection
Dot blotting   product   clone   sequencing

Fig 4. Subtraction

6. cDNA clone

1)

clone   BigDye Terminator
ABI PRISM™BigDye™Terminator Cycle Sequencing Ready Reaction Kit
                    . BigDye terminator   sequencing kit
                    plasmid   kit
200 500ng   plasmid   (25cycle   PCR   ),
terminator   ddATP, ddTTP, ddCTP, ddGTP
,   A-, C-, G-, T-dye terminator   mixture
                    .

                              .
PCR   denaturation   96   30 , annealing   50
    15 , extension   62   4   25cycle   .
        PCR   ethanol precipitation   sequencing
        .

7. Northern blotting

cDNA clone　　　　　　　　　　　　　　　　　　　　　Northern
hybridization　　　　　　　　　.　　　　　clone　　insert
　hybridization　　　　　32P-labeled probe　random priming kit (Takara)
　　　　　　　　. Membrane　　　　probe　hybridization

　.

1) Total RNA
　　　　　denaturing　　　　　homogenization　　total RNA
(Chomozenski　　Sacchi, 1987). 50　　　　　　6　　RNA　　　(denaturing
solution　　　), 600 ㎕ 2 M Na acetate (pH 4), 6　　phenol (acidify
saturated phenol : Bioner), 3　　chloroform　　　,　　　　vortexing
(RNA　　　phenol　　　　　　　　　　　　　)　　　10 -15
　　. 4 , 15,000 rpm, 20　　　　　　　　　　　　　50
　　　. 　　(6) 6　acidify saturated phenol, 3　chloroform :
isoamylalcohol (49:1),　600 ㎕ 2 M Na acetate
　　vortexing　　. 4 , 15,000 rpm, 10
　50　　　　　　. RNA　　　　　　　　2 - 3　　　　.
RNA　(6)　　　volume　2-propanol　　　-20　　1
　　　. 4 , 10,000 rpm, 30　　　RNA　　　　　RNA
pellet　70% ethanol　　. RNA pellet　　　　　　DEPC
treated ddH2O　　　　　　　RNA　(OD
1=40 ug/ml RNA)　　.　　-70　deep freezer　RNA
.

2) Northern
Total RNA　　　　　membrane　　　　　　RNA
　　　　　(Sambrook , 1989). Formaldehyde-1.0% agarose gel
　. RNA loading buffer　RNA sample 10　　　　total volume
20ul　65 , 15　heating　gel　well　loading　.　(100
V, 1 - 2) UV　28S 18S band
mRNA band　　　　　　.
gel　　total RNA　capillary

nenbrane   . 20 x SSC solution   agarose gel   total RNA
nenbrane   .   nenbrane   UV crosslink   5
baking   nenbrane   RNA   .

3) Hybridization
cDNA insert   hybridization   32P-labeled probe   random
prining kit (Takara)   . Nenbrane   probe
hybridization   clone   . 0.5% SDS, 6X
SSC, 5× Denhard's reagent, 50% deionized fornamide, 100 ug/nl sperm DNA
          hybridization   . 42   2   prehybridi-
zatoin   32P-labeled probe   hybridization   16
   (Thonas, 1980). Hybridization   nenbrane   2X SSC/0.1 % SDS (
   15   2), 1X SSC/0.1 % SDS (15), 1X SSC/0.1 % SDS (42
15), 0.5X SSC/0.1 % SDS (42   15)   washing   .
Washing   nenbrane   rap   X-ray filn   2-4
      .

8. GenBank   clone

   clone   ,   Internet
      GenBank database   clone
   .

9.   cDNA clone

                              bovine
                                    clone
                        screening   RT- PCR cloning
      .
Screening   bovine nannary gland cDNA   2   5× 104
   plating   clone   probe   hybridization   .
            RT-PCR   full-length cDNA
cloning   .
   RT-PCR   template   total   RNA   acid   guanidinium
thiocyanate-phenol-chloroform   (Chonozenski

Sacchi, 1987). Total RNA   1.0% fornaldehyde agarose gel
                                    total RNA   template   oligo(dT)
primer   MMLV (Promega) reverse transcriptase   first-strand cDNA
              . PCR   1.2% low meting gel
         PCR band   DNA purification kit   .
PCR product   pGEM-T easy vector (Promega)   ligation   E. coli Top
10F' cell   transfornation   .

    10.   cDNA   vector

                    expression vector   pIRES vector   vector
eukaryotic cell   cytomegalovirus (CMV) immediate
early pronoter,   translation site   biocistronic mRNA
                 .   neo   (neomycin resistance gene) system
        G418   selection   eukaryotic   cell   line
transfection   .   Fig. 5   .
  Glycoprotein III, serum amyloid A3, VDNM1 gene   expression vector
pIRES   Not I   QIAEX II
Gel Extraction kit(QIAGEN)   .   glycoprotein
III(1.3kb), serum amyloid A3(0.7kb), VDNM1(0.5kb)   pIRES vector(5.3kb)
T4 ligase   ligation   .   ligation products   E. coli Top10F'
cell   transfornation   LB/anpicillin/tetracyclin agar plate   plating
    . Colony   vector   primer   insert   primer
   colony PCR   insert   vector   insertion
                  .   colony   LB/anpicillin/tetracyclin
broth   plasmid   Aetna spin Midiprep kit(Aetna genetech)
          .   .   .   pIRES-GPIII, pIRES-SAA3,
pIRES-VDNM1 plasmid   pIRGPIII, pIRSAA3, pIRVDNM1   .
       vector   Not I   vector(5.3kb)
insert   .

Nru I

PCMV ie

MCS

Not I

EcoR I

Amp

IVS

pIRESneo
5300 bp

IRES

poly A

Neo

Sma I
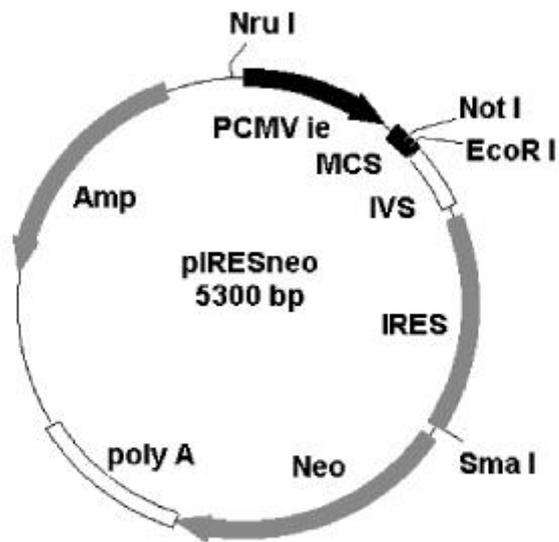
Fig 5. Restriction Map and Multiple Cloning Site(MSC) of pIRESneo vector

11.   (HC11)   cDNA

  1) Transfection   HC11 cell culture

                 mouse normal mammary epithelial cell line   HC11 cell
bovine glycoprotein III, serum amyloid A3, WDNM1

                   . Cell   ampule   liquid nitrogen tank
                37   water bath   . Ampule   70% ethanol
ampule   cell suspension   label   flask   . 9 ml
                       . RPMI 1640 media, 10% heat-inactivated fetal
bovine serum, 5 ug/ml insulin, 10 ng/ml epidermal growth factor(EGF)
                          5% CO₂, 37   .   2
                   .   1 , 2   cell growth, cell attachment
      .   monolayer   confluency   subculture
          2ml   0.25% trypsin   monolayer
trypsin   monolayer   flask   1   trypsin

incubator   cell round up/dissociation
(2-3). Cell dissociation   8ml   monolayer
      pipetting   cell dispersion   cell suspension
15ml tube   pipetting   homogenous single cell
suspension   .   cell number   counting
                          .   subculture   36   60%
confluency   , 48   confluency

2) HC11 cell
HC11 cell   liposome   Effectene
Transfection Reagent kit(QIGEN   )   .
1 X 105 HC11 cell   60mm dish   2ml media   seeding   24
5% CO₂, 37   subcloning   40   80% confluent
                  . TE buffer   dissolve   DNA 1   total
volume 150   buffer EC   Enhancer   8   vortexing
    1   . 2 5   incubation   .   DNA-
Enhancer mixture   Effectene Transfection Reagent   10   .
      vortexing   5 10   . Mixture
incubation   cell   plate   medium   , PBS solution
      cell   washing   4ml   fresh   growth medium   .
fresh cell growth medium 1ml   transfection complexes   mixing
    cell   plate   .   plate   5% CO₂, 37
    24 48   incubation   cell   attachment
              .   ,
      DNA   RNA   .

12.

                        vector   control   vector
pEGFP-IRESneo Control Vector(6kb)   transfection
    . HC11 cell   control vector   pEGFP-IRESneo Control Vector
            liposome   Effectene Transfectin Reagent kit
(QIGEN   )   .
pEGFP-IRESneo Control Vector   GFP protein   vector

vector   gene   insert   .
Transfection   Effectene transfection Reagent kit   , Enhancer
(4, 8, 16), Effectene(5, 10, 12.5, 20, 25, 50, 100)   DNA(0.5, 1, 2)
                           transfection   transfection
                    . HC11 cell
    transfection   24   5% CO₂, 37   incubation
    9   sample
              pIRGPIII,   pIRSAA3,   pIRVDNM1   HC11   cell
transfection   .

13. RT-PCR

                                                    total RNA   RT-PCR
                      .   confluent
    PBS   washing   monolayer cell   denaturing solution overlay   .
                  lysis   scraper
Trisol (GibcoBRL   )   . Trisol   (bGPIII, bSAA3,
bVDNM1   )   total RNA
  .
                      pIRES vector   5' primer
              3' primer   RT-PCR
          .

14.

    HC11   hormone   (e.g. cytokine)
                      (bGPIII, bSAA3, bVDNM1)
  .

1) Differentiation induction medium
    HC11   .   bGPIII, bSAA3, bVDNM1

differentiation induction medium   .

2) Apoptosis induction medium

Apoptosis   HC11
                    .   , apoptosis   hormone
insulin, dexamethasone, prolactin
     .
     Table 2   .

Table 2. HC11 Medium

Components \ Medium	GM	DIM	AIM
RPMI 1640			
5 0 u g / m l Gentamycin			
10% FBS			
5ug/ml Insulin			
10ng/ml EGF			
5 u g / m l Prolactin			
1 u M Dexamethasone			

   3) Serum restimulation experiment
   Serum   factor   factor
              .   serum   serum
starvation   serum
              .

   4) TGF , TNF   experiment
   Cytokine   . TGF , TNF
             cytokine   .

   5) Antimycin, oligomycin experiment
   Antimycin   oligomycin   mitochodria synthesis inhibitor   ,

antimycin A   Fas mRNA   , antimycin A   5
caspase activity   .   , oligomycin   oligomycin
2   cell   2/3   .
        , antimycin oligomycin
                  ,   signal transduction pathway   mitochondria
              .

   6)   hormone   dose
   Lactogenic hormones   insulin, EGF, prolactin, dexamethasone   dose
                   hormone
   .

      hormone   3   , serum
       serum   apoptosis
                                    .

      dose   band   Gel Documentation system(Bio Rad   )
                   band   .

   15.

                                                  ,
   (bovine GPIII, SAA3, VDNM1)   E. coli

                                  .

                   bGPIII, bSAA3, bVDNM1 protein   E. coli
                              . Expression vector   cloning
   stability   IPTG inducible   N
       hisidine-tag   . IPTG induction vector   gene expression
       3   reading frame   integration system   ,
   fusion protein   affinity-purification   colorimetric
   immunologic detection   . T7 promoter   1-5mM IPTG
        induction   , 10 40 kDa   His-tag   fusion protein
          .   ATG, ribosome binding site, T7 promoter   .
   pET-22b(+) vector   his-bind resin
                          (Fig. 6A).

(A)
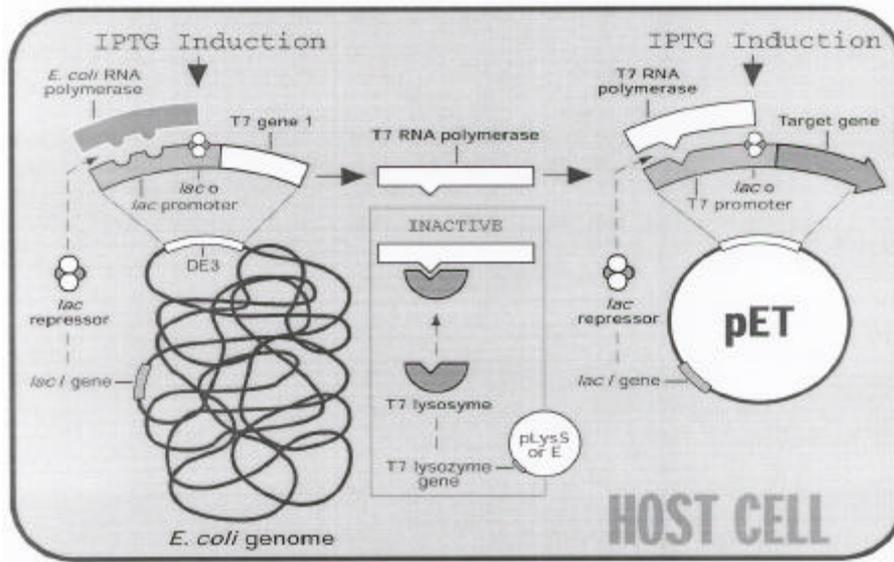
pET-22b(+) vector system

(B)
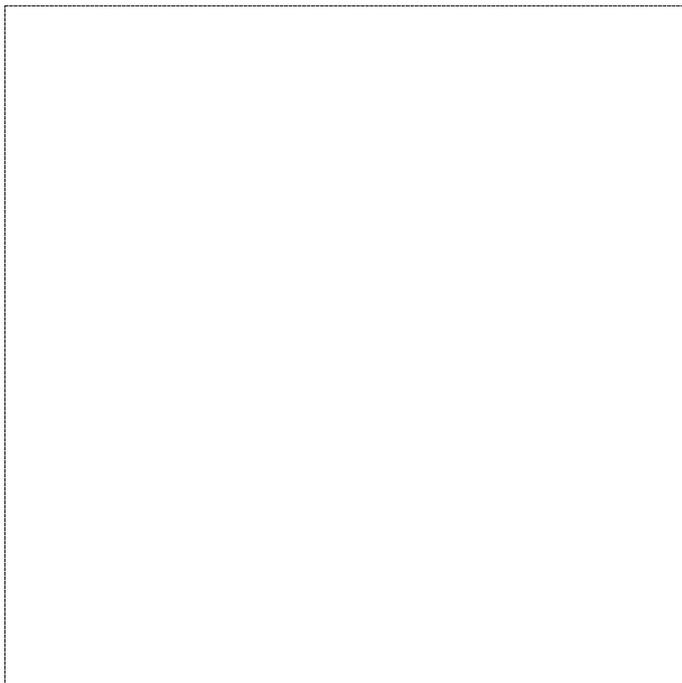
pET vector

Fig 6. pET-22b(+) vector   vector system

GPIII, SAA3 VDNM1 protein E.
coli   pET expression vector system
.

Fig. 6(B)   pET-22b(+) vector   . bGPIII, bSAA3, bVDNM1
insert vector   reading frame   Nde I   Xho I
site   insert   5'   3' primer design   . pIRES
eukaryotic experssion vector   cloning   bGPIII, bSAA3   bVDNM1
cDNA 5'   3'   linker primer   Taq polymerase (Takara
)   PCR   bGPIII, bSAA3   bVDNM1 band   .
bGPIII, bSAA3   bVDNM1 PCR product   1   low melting agarose gel
                band   gel elution kit(Bio101)
insert   . Vector   Nde I   Xho I
nucleotide removal kit(Qiagen)   .   pET vector   gel
elution   insert   Nde I   Xho I(Promega)
cohesive end   T4 ligase (Takara)   16   12h
ligation   . Ligation product   Top 10F' cell
transformation   ampicillin, tetracyclin resistant colony
plasmid   Nde I Xho I   , 5.5 kb pET-22b vector
GPIII, SAA3   VDNM1 gene insert   ligation
.   construction   E. coli   pETGPIII, pETSAA3,
pETVDNM1   .

16.   E. coli

Construction   pETGPIII, pETSAA3   pETVDNM1 expression vector
         host   BL21 E.coli cell   transformation   .
colonies   colony PCR
plasmid   .   Nde I   Xho I   bGPII, bSAA3
bVDNM1 cDNA   .

17. E.coli

pETGPIII, pETSAA3, pETVDNM1 expression vector   BL21 cell
LB/amp. plate   single colony   selection   .
0.1mM IPTG   inductino 0h, 1h, 2h   induction 0h   OD

1   protein loading buffer   SDS-PAGE
        fusion protein   .

3

1.

(1/2,
1, 1 1/2, 2, 2 1/2, 9)   .

2. Total RNA

acid guanidinium thiocyanate-phenol-chloroform
total RNA   . Fig. 7   RNA   .

1	2	3	4	5	6	7
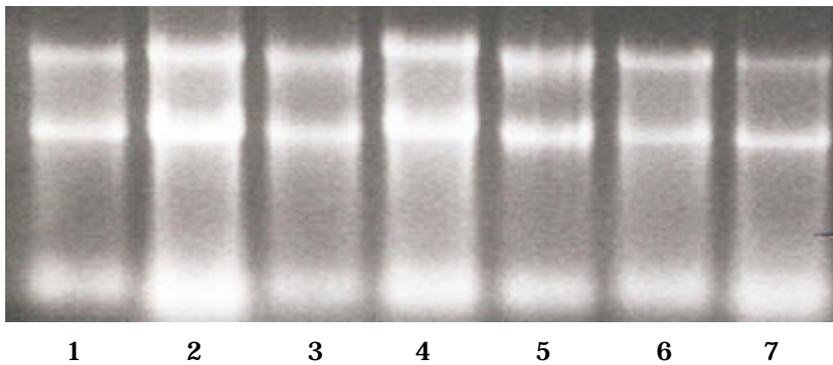
Fig 7.   total RNA
1:     , 2:   1/2, 3:   1, 4:   1 1/2, 5:   2, 6:   2 1/2, 7:   9

3.   cDNA clone

. cDNA library   differential
hybridization, oligo dT primer   differential display RT-PCR
adaptor   subtractive hybridization,
subtractive hybridization
cDNA clone   .
Fig. 7   total RNA   cDNA   tester(cDNA)
driver(cDNA)   adaptor   ligation   hybridization

.   primary, secondary PCR   cDNA   Fig. 8
.
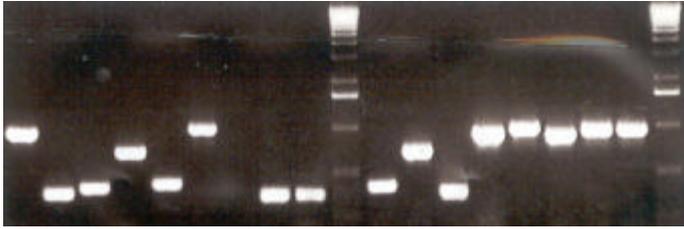
Fig. 8 PCR   cDNA clone

4. cDNA clone

                                    clone   BigDye
Terminator   ABI PRISM™BigDye™Terminator Cycle Sequencing Ready
Reaction Kit   .
 Fig. 9   clone   clone
      .   clone   B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11, B12
          .

     26   clone   clone
GenBank data-base   , 11   clone
                          .   clone B2-2,
6-2, 13-2, 1-3   bovine lactoferrin, clone B4-2, B9-2, B10-2, B2-3, B3-3,
B6-3, B8-3   bovine glycoprotein   homology   .
   12   clone   homology   .
Clone b1-1   homo sapines genomic DNA chromosome 21q2, b6-1   human P311
HUM(3.1) mRNA, clone 11-1   Homo sapiens polyadenylate binding protein,
clone 12-1   homo sapiens splicing factor   homology   . Clone 1-2,
3-2, 11-2, 1-3   rabbit serum amyloid A3 protein, clone 12-3   human
melanoma-associated antigen ME 491 mRNA, clone 12-2   mouse WDNM 1 protein
          .
      bovine   homology   3   . Clone b7-1, b9-1,
b5-2   bos taurus prostaglandin G/H synthase-2 gene,   bovine capillary

E-selectin mRNA, Bos taurus chitinase-like protein 1

.

1   clone 7-2   GenBank database   unknown
clone   .
Table 2   clone   table 3,
4, 5   clone   GenBank data
                    .   table 3, 4, 5   clone
subtractive hybridization   1, 2, 3   .

Table 2. Nucleotide partial sequences of clones from b1 to b12

Clone No.	Primer	Reading	Sequences
B1	T7 SP6	222	AAGCGACCCAAGGNCAATNTCATTTTCAACACTTCTCTTGGAGCCATTTTTGGGGTCAAGAAGTATGCAG ATGCCCTACAGAAGATTATNCAGGAGAGGGCCCTCACCGNTAACTACAAGCAAAATCTCATTGAAGTCCG AGCTGATAAGCAAGAGGCTGTTTTTGAGAATNTGGACAAACCTGGAGAGACCCAAGTGATTTNATATGAA ATGCTTNACGTT
B2	T7 SP6	166	CACTCTATGAACAGTCACTNGNCATCAAGNCTGCCATCCCTCATCCATTGATCATGGGAGTTATCAGAGA ATGTGGTGGNAAAATGCACTTGAGAGAAGGNGAATTTGAAAAGGCACACACTGATTTTTTTTGAAGCCTTT AAGAATTATGATGAATCAGGAAGTCC
B3	T7 SP6	165	TGGACACTTAGTGCAACTTTTTATAAAGAAAATAATGCTAAAATAAGACCNAAACTGATGNCATCACNGA AATTAACAGTTTTCAATATGTTCATATTTTAATTCACAATGGAAAAATGTGTTCCAAAACTGGAAATTCA TAATGCTTGNGTAAAGCGNGGAAGA
B4	T7 SP6	167	CAGAGGNGGAAAAACAGCATGNAGGGGNTTNTCANNTACTTAANCAAAACTCAGATGTGAGTGNCTCTAT CTTNTTACCTTTCATACACTAGCCNTGGCCTCTTTCCTCANNCNTAAGAACCANCTGCCAAAAAANTACN GAACCNCGCATGATGGCAGNCATAGNG
B5	T7 SP6	168	CAAAANCGGNGNCCAANANGCCACGGCNGANGCCCCACANAAANAAGACNCNNNCANAGGGGCCCCANCNC NAAANACNAGNNNCAACCCNAACGNACACCNNNCCGANAAAACAAGACGNNGCNNACGANAAAANNGGACC AANCCNNAGAGACCAAANGANCCGGNAN
B6	T7 SP6	167	ACAGNGGANATACCTACTCTAACAAAGGAGGTGGGGGGAAANGCACAGGAAACTCAGGCCAATGGGGAGC GAGAAGAGAACGAAGAAGTGCCAGCCGCATGCCGTCAGNCGATGNGTAACAGTCAGAAGCGGAACAGTTCANA ACAAAGCCTGNCCTGNCAAAGGAAGAG
B7	T7 SP6	168	AATATCTACCTACGTATNTANTTCTGGNCACACCACACGCCTTGNAGGATCTTAGTTCCCTGACCAGGGA CTGAACCCCGGGGCCCTGACAGTGAAAGNCCCAAGNCATAACCACTGAACCACCNGGGAANTTCCTCTAG ACTTTTANAATGGAANTATAAAGCANNT
B8	T7 SP6	166	AGTATNATAGCAAGAAGCATTTTTTAACAAAGATGAAAAAATAGTTCTTATAATATACATCCAGATTCTA GTAACTTNTCATTACCTNACCACTTAACAGATACTTTCTCTCTGACCCACTATGAATAATTTCCAACAAA GCACCTTTACTGGTTGGAATGGAAAC
B9	T7 SP6	167	CTGAACAACAACCCAANCCTCNCCCGCAAACCNGGAAACCCTNNNGGNANCNANNTAAAAAAAAAAAAAAAA AACGNANCCTCGANAAAACACCGGGGANGGGGGAACCANAAGCCCAAACNANAAANNGGCCCAAAACCGGN CATNNGGAACCCGGGGGAAAGGGGCCG
B10	T7 SP6	168	ACCTACTCTAACAAAGGAGGTGGGGGGAAATGCACAGGAAACTCAGGCCAATGGGGAGCGAGAAGAGAAC GAAGAAGTGCAGCGCATGCCGTCAGNCGATGNGTAACAGTCAGAAGCGGAACAGTTCAGAACAAAGCCTGC CCTGNCAAAGGAAGAGCTAAAAGACAGT
B11	T7 SP6	166	GGAGTTGCTGAATGCCCTCTTCTCTCTAAANCCTATGGATGACAACTTAANCTGNGCAGTAAAATTACTGAAG TTGACAGGGNCAGTTTTAGAAGATGCCTGGAAGGAAAAAGGAAAGACTGACATGGAAGAAATTATTCAGA GAATTGAAAATGTTGTCCTANATGCA
B12	T7 SP6	167	GCAATTGATGGGCACTTCACATACACATTGCCCTGAGCTGAATTTTTGNCAACATAAATATGAATAACTC CTCCATGNTTATTACATTCTTCAATCACATCATCTTTAATCTCTGTATCCCATCCAACTTCTTCTTCTGT TTGAGGATTAAACATGNTAGAGAGNTG

- 29 -

Table 3. Clone   GenBank data   (1   )

Clone No.	Primer	Reading	Homology with GenBank data	
			Clone or gene	Homo-logy (%)
b1-1	T7	165	Homo sapines genomic DNA chromosome 21q2	89
b6-1	T7	167	Human P311 HUM(3.1) mRNA	87
b7-1	T7	168	Bos taurus prostaglandin G/H synthase - 2 gene	96
b9-1	T7	335	Bovine capillary E-selectin mRNA	92
b11-1	T7	166	H. sapiens polyadenylate binding protein	88
b12-1	T7	167	Homo sapiens splicing factor	96

Table 4. Clone   GenBank data   (2   )

Clone No.	Primer	Reading	Homology with GenBank data	
			Clone or gene	Homo-logy (%)
b1-2	T7	165	Serum amyloid A3 protein	82
b2-2	T7	167	Lactoferrin	97
b3-2	T7	168	Serum amyloid A3 protein	81
b4-2	T7	335	Bovine glycoprotein	97
b5-2	T7	166	Bos taurus chitinase-like protein 1(CLP-1)	95
b6-2	T7	167	Lactoferrin	95
b7-2	T7	170	Unknown	
b8-2	T7	185	Human mRNA for ornithine decarboxylase antizyme	95
b9-2	T7	167	Bovine glycoprotein	92
b10-2	T7	198	Bovine glycoprotein	93
b11-2	T7	164	Serum amyloid A3 protein	80
b12-2	T7	175	WDNM 1 protein	82
b13-2	T7	188	Lactoferrin	92

Table 5. Clone   GenBank data   (3   )

Clone No.	Primer	Reading	Homology with GenBank data	
			Clone or gene	Homo-logy (%)
b1-3	T7	165	Lactoferrin	89
b2-3	T7	167	Bovine glycoprotein	99
b3-3	T7	168	Bovine glycoprotein	99
b6-3	T7	335	Bovine glycoprotein	99
b8-3	T7	166	Bovine glycoprotein	99
b10-3	T7	167	Rabbit serum amyloid A3 protein	83
b12-3	T7	167	Human melanoma-associated antigen ME 491 mRNA	79

5. Northern blotting

    Clone   GenBank data

               ,

          .   Northern
blot   .   clone
serum amyloid A3, ornithine decarboxylase antizyme, glycoprotein III,
VDNM1   .   clone   Northern
       .   (,   )
       .   total RNA   acid guanidinium thiocyanate-phenol-
chloroform   . Total RNA 10 ug   1.0% formaldehyde
agarose gel   size-fractionation   .   agarose gel

RNA   capillary   menbrane   .
clone   insert   hybridization   32P-labeled probe
random priming kit (Takara)   . Menbrane   probe
   hybridization   clone   .

1) Serum amyloid A3
Fig. 9   tissue remodeling   serum amyloid A3 protein
                                              .   1
    1 1/2   2   2 1/2
                         9   .

2) Ornithine decarboxylase antizyme
Clone 8-2   ornithine decarboxylase antizyme   ribosomal frameshift
  ORF   2   ornithine decarboxylase   cell growth   division
                     ornithine decarboxylase antizyme   remodeling
    . Fig. 10   .
    ,   2   .

3) Glycoprotein III
Clone 4-2   glycoprotein III
,   homology   clusterin   . Clusterin
                                         clusterin   sequence
         glycoprotein III(clusterin
glycoprotein III   )
              Northern blot   .
Glycoprotein III   1   1 1/2
       .
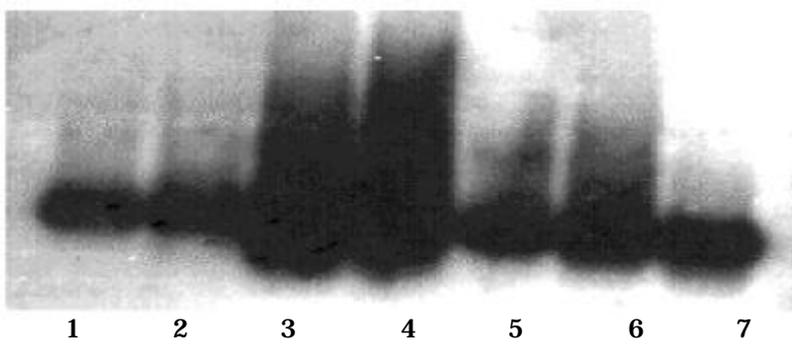
1:    , 2:   1/2, 3:   1, 4:   1 1/2, 5:   2, 6:   2 1/2, 7:   9

Fig 9. Serum amyloid A3   Northern blot

1:    , 2:   1/2, 3:   1, 4:   1 1/2, 5:   2, 6:   2 1/2, 7:   9

Fig 10. Ornithine decarboxylase antizyme   Northern blot

1:    , 2:   1/2, 3:   1, 4:   1 1/2, 5:   2, 6:   2 1/2

Fig 11. Glycprotein III   Northern blot

6.　　　cDNA clone

Clone　　　　　　　GenBank data　　　　　　　serum amyloid A3(Fig. 12)　VDNM1(Fig. 13)　　　　　　　　coding

.

, glycoprotein III　　　coding
cloning　　　　, GenBank　　　　data
.　　　total RNA　template　　　　oligo(dT) primer
first-strand cDNA　　　　. First-strand cDNA　template
PCR　　　　　　　1.3kb　　　band　　　.　　PCR product
pGEM-T easy vector (Promega　)　ligation　　　transformation
sequencing　　plasmid　.　　plamid　　sequencing
coding　　　　　.

```
GCAGTGGGTAACAACGCAGAGTACGCGGGGAGCACAGGCAGCTCAGCTTCACCAGGAGCC          60
TCAGCAGGAGGGCACGGCCACAGGATGAACCTTTCCACGGGCATCATTTTCTGCTTCCTG         120
                         M  N  L  S  T  G  I  I  F  C  F  L          12
ATCCTGGGCGTCAGCAGCCAGAGATGGGGGACATTCCTCAAGGAAGCTGGTCAAGGGGCT         180
  I  L  G  V  S  S  Q  R  W  G  T  F  L  K  E  A  G  Q  G  A          32
AAAGACATGTGGAGAGCTTACCAAGACATGAAAGAAGCCAACTACAGGGGTGCAGACAAA         240
  K  D  M  W  R  A  Y  Q  D  M  K  E  A  N  Y  R  G  A  D  K          52
TACTTCCACGCCCGTGGAAACTATGACGCTGCCCGAAGGGGACCTGGGGGTGCCTGGGCT         300
  Y  F  H  A  R  G  N  Y  D  A  A  R  R  G  P  G  G  A  W  A          72
GCTAAAGTGATCAGTAACGCCAGAGAGACTATTCAGGGAATCACAGACCCTCTGTTTAAG         360
  A  K  V  I  S  N  A  R  E  T  I  Q  G  I  T  D  P  L  F  K          92
GGTATGACCAGGGACCAGGTACGGGAGGATTCGAAGGCCGACCAGTTTGCCAACGAATGG         420
  G  M  T  R  D  Q  V  R  E  D  S  K  A  D  Q  F  A  N  E  W          112
GGCCGGAGCGGCAAAGACCCCAACCACTTCAGACCTGCTGGCCTGCCTGACAAAGTACTG         480
  G  R  S  G  K  D  P  N  H  F  R  P  A  G  L  P  D  K  V  L          132
AGCCTGCTCTCTCTCTGCTCAGGAGATGGGCTGTGAGTCCCTAAGGGCAGGGACACTGAC         540
  S  L  L  S  L  C  S  G  D  G  L  *                                 143
CTAGAGAGTTCTCTGTCCTCAGAAGGCAGCAGATCTAATAAATGCTCAAGAGATGGAATA         600
CTT                                                                 603
```

Figure 12. Nucleotide and deduced amino acid sequences of bovine serum amyloid A3 cDNA. A putative start codon and polyadenylation signal sequence are underlined, and the stop codon is indicated by an asterisk (*) - GenBank　　　　　AF160867

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```
  1 acgcggggag agccaac**atg** aagacagcca cagtctttgt tctggtagct ttgattttca
 61 tgacaatgac tactgcctgg gctctgtcta accccaaaga aaaacctggc gcttgtccta
121 agccgccccc acgcagtttt ggaacttgtg atgaacgatg cacaggagat ggatcgtgct
181 ctggcaacat gaagtgctgc agcaatggct gtggtcatgc ctgcaaacct cctgtctttt
241 **aa**ccatgtgg aagatggatc tttataagca ggactgatgg ctagccccag aagatttttt
301 tgaacctaca gaccccatgc ttggctcctc cttagaccta gaattgcatc cttggaagag
361 gaagttctat actgtggtga cagcttccta acgtgtttgt gtctaa**aata_a**actatcctt
421 agcatccgaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaa
```

              <Nucleotide sequences of bovine VDNM1 cDNA>
           A putative start, stop codon and polyadenylation signal sequence are underlined.

```
  1 nktatvfvlv alifntntta walsnpkekp gacpkpppprs fgtcderctg dgscsgnmkc
 61 csngcghack ppvf
```

             <Deduced amino acid sequences of bovine VDNM1 cDNA>

Figure 13. Nucleotide and deduced amino acid sequences of bovine VDNM1 cDNA- GenBank   AF159701

7.   cDNA   vector

                       Northern blot
            .   bovine serum amyloid A3(bSAA3), bovine VDNM1(bVDNM1)   bovine glycoprotein III(bGPIII)   .

               eukaryotic expression vector   pIRES vector
         expression vector   .
 bSAA3, bVDNM1, bGPIII gene   expression vector   pIRES   Not I
                             QIAEX II Gel Extraction kit(QIAGEN)   (Fig. 14).   bSAA3(0.7kb), bVDNM1 (0.5kb), bGPIII(1.3kb)   pIRES vector(5.3kb)   T4 ligase   ligation
   .

                expression vector   pIRGPIII, pIRSAA3, pIRVDNM1   (Fig. 15).

1: bGPIII(1.3kb), 2: bSAA3(0.7kb), 3: bVDNM1(0.5kb)

Fig 14. pIRES vector

(A)

1kb marker   bSAA3 bVDNM1

(B)

1kb bGPIII
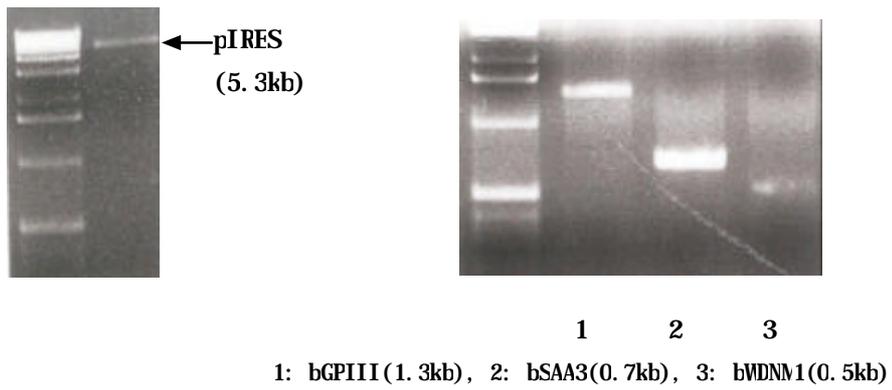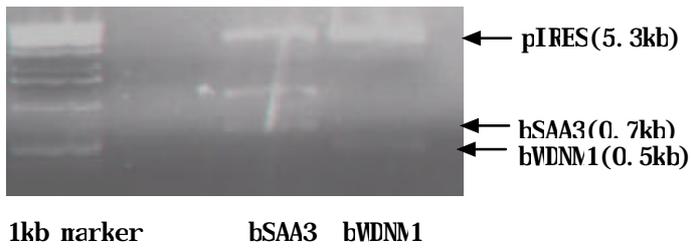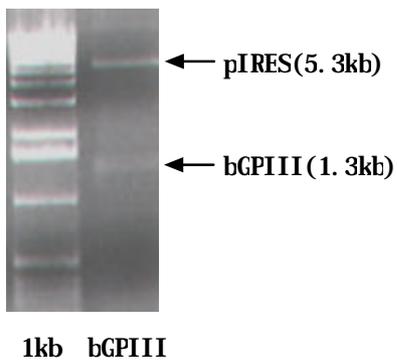
Fig 15. pIRGPIII, pIRSAA, pIRSVDNM1

8.　　　　　(HC11)　　　　cDNA

HC11 cell　　　　　　　　　　　　　　　liposome　　　　　　　Effectene
Transfection Reagent kit(QIGEN　)　　　　　　.
1 X 105 HC11 cell　　60mm dish　2ml media　　　seeding　　24
5% CO2, 37　　　　subcloning　　　　　40　80% confluent
　　　　　　　　　　　. TE buffer　　dissolve　　　　　DNA 1　　total
volume 150　　　　　buffer EC　　　　Enhancer　8　　　　vortexing
1　　　.　2 5　　　　　incubation　　.　　　DNA-
Enhancer mixture　Effectene Transfection Reagent 10　　　　　.
　vortexing　　　　　5 10　　　　　. Mixture
incubation　　　cell　　plate　　medium　　　　, PBS
solution　cell　washing　4ml　fresh　growth medium
　. fresh cell growth medium 1ml　transfection complexes
mixing　cell　　　　plate　　.　plate　5% CO2,
37　　24 48　　　incubation　　cell　attachment
　　　.
　　　,　　　　DNA　RNA　　　　　.

9.

　　　　　　　vector　　　　　control vector
pEGFP-IRESneo Control Vectot(6kb)　　　transfection
　.
pEGFP-IRESneo Control Vectot　GFP protein　　　　vector
　vector　gene　insert　　　　　　.
Transfection　　Effectene transfection Reagent kit　　　　,
Enhancer (4, 8, 16), Effectene(5, 10, 12.5, 20, 25, 50, 100)　DNA
(0.5, 1, 2)　　　　transfection
　transfection　　　.
HC11 cell　　　　　　　　　transfection
24　　5% CO2, 37　　incubation　　9　sample
　　　(Fig. 16).
　　Fig. 16C　　　pIRGPIII, pIRSAA3, pIRVDNM1
HC11 cell　transfection　　.

(A)

(B)

(C)

(D)
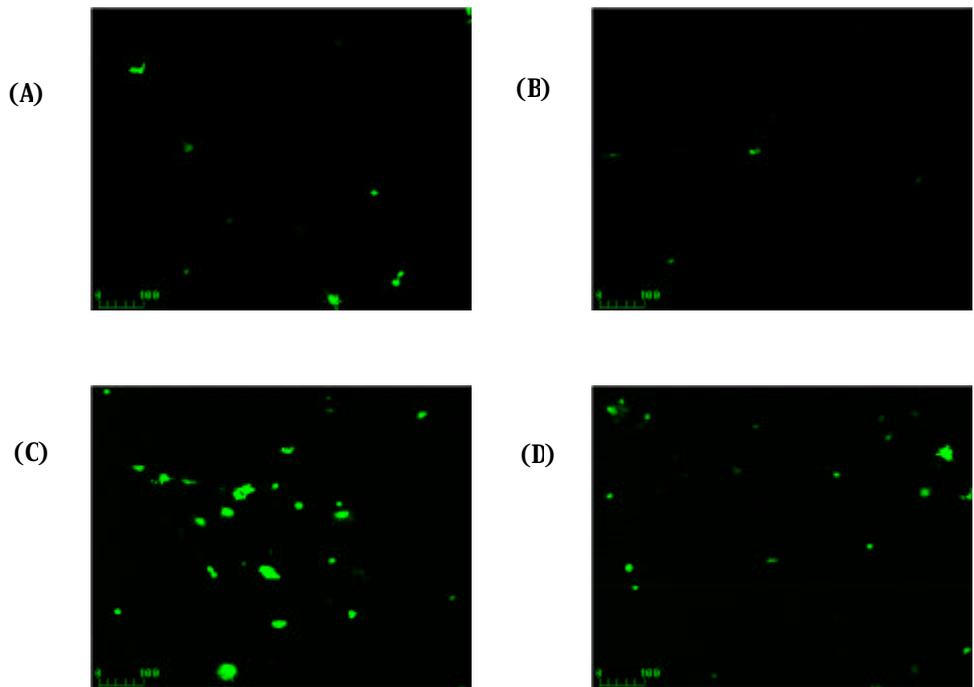
7Fig 16. Control vector　transfection　　　　　(　　　　　　　　)
                (A) DNA 0.5 ，Enhancer 4 ，Effectene 5
                (B) DNA 0.5 ，Enhancer 4 ，Effectene 12.5
                (C) DNA 1 ，　Enhancer 8 ，Effectene 10
                (D) DNA 2 ，　Enhancer 16 ，Effectene 20

10. RT-PCR

                        total RNA　　　　　　　RT-PCR
    ．
        pIRES vector　　　　　　　　　　5' priner
   3' priner　　　　　　RT-PCR
  (Fig. 17).

← 1.3kb   ← 500bp   ← 250bp

pIRGPIII   pIRSAA3   pIRVDNM1
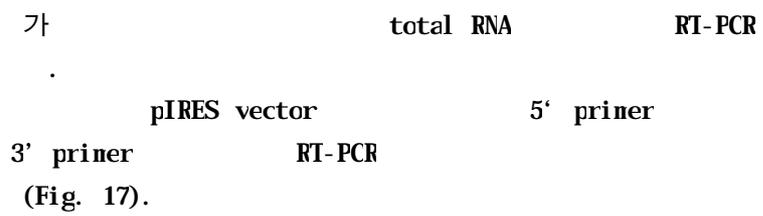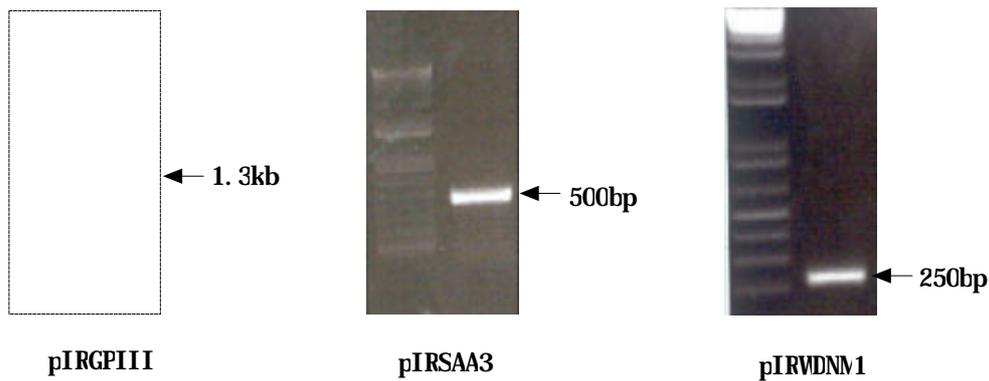
Fig 17. HC11   RT-PCR

11.

HC11   .   sequence
      bGPIII   mouse clusterin , bSAA3   mouse SAA3 , bVDNM1
mouse VDNM1   sequence   mouse   primer   RT-PCR

1) Differentiation induction medium
   Lactogenic hormones   HC11 cell   differentiation   ,
clusterin, SAA3   VDNM1 mRNA()   , differentiation
                        , differentiation 5
(Fig. 18).

2) Apoptosis induction medium
        hormones   clusterin, SAA3   VDNM1 mRNA
   , clusterin   ,   prolactin(PRL),   dexamethasone(DEX),   PRL+EGF
PRL+DEX   mRNA   , SAA3   PRL, DEX+PRL
EGF+PRL   mRNA   , DEX   EGF
                    .   , clusterin   SAA3   PRL
synergic   .   VDNM1 mRNA   hormone
                  (Fig. 19).

3) Serum restimulation experiment

Serum   restimulation   clusterin   VDNM1 mRNA

down-regulation   .   serum   factor
clusterin   VDNM1   (Fig. 20).

4) TGF , TNF   experiment

TGF   clusterin   ,   cell type
              , HC11   , TGF   clusterin mRNA
                    VDNM1 mRNA
(Fig. 21).

TNF   clusterin   VDNM1 mRNA   .
   , SAA3   TNF
(Fig. 22).

5) Antimycin, oligomycin experiment

Antimycin   mitochondrial synthase inhibitor   ,   morphology
36   48   cell
                    67   cell   2/3
           .   clusterin, VDNM1   mRNA
(Fig. 23).

Antimycin   mitochondirial synthase inhibitor   , oligomycin
     morphology 12   18
            cell   26   cell
2/3   (Fig. 24).

6)   hormone   dose

Lactogenic hormones   insulin, EGF, prolactin, dexamethasone   dose
                hormone
.

) Prolactin

Serum free medium(SFM), apoptosis induction medium(AIM)   mclusterin,
mVDNM1, mSAA3   .
prolactin   0.5 /ml   5 /ml
                    (Fig. 25).

) Dexamethasone

SFM, AIM

,   5μM

.   0.1μM   0.7μM   1,

2, 5μM   (Fig. 26).

) EGF

EGF   dexamethasone   . EGF   0.1ng

5ng   10, 15, 20ng

(Fig. 27).

) Insulin

Insulin   , Insulin

(Fig. 28).
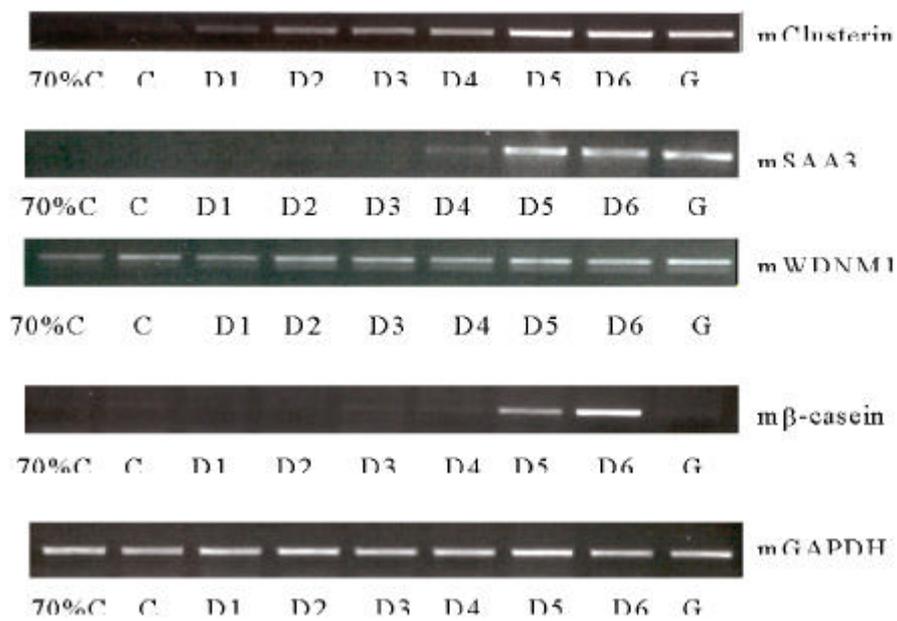
Fig 18. Effects of differentiation induction on clusterin, SAA3 and WDNM1 mRNA expression

HC11 cell was grown until confluent in GM. After the cells were reached to confluent(C), cultures were exposed to DIM and continued until day 6(lanes; D1 to D6). On day 7, cultures were refed to GM again(G). The relative abundance of clusterin, SAA3 and WDNM1 at the indicated times was determined by RT-PCR. mGAPDH and β-casein expression served as a control and a positive control, respectively.　C : confluence,　D : differentiation

•
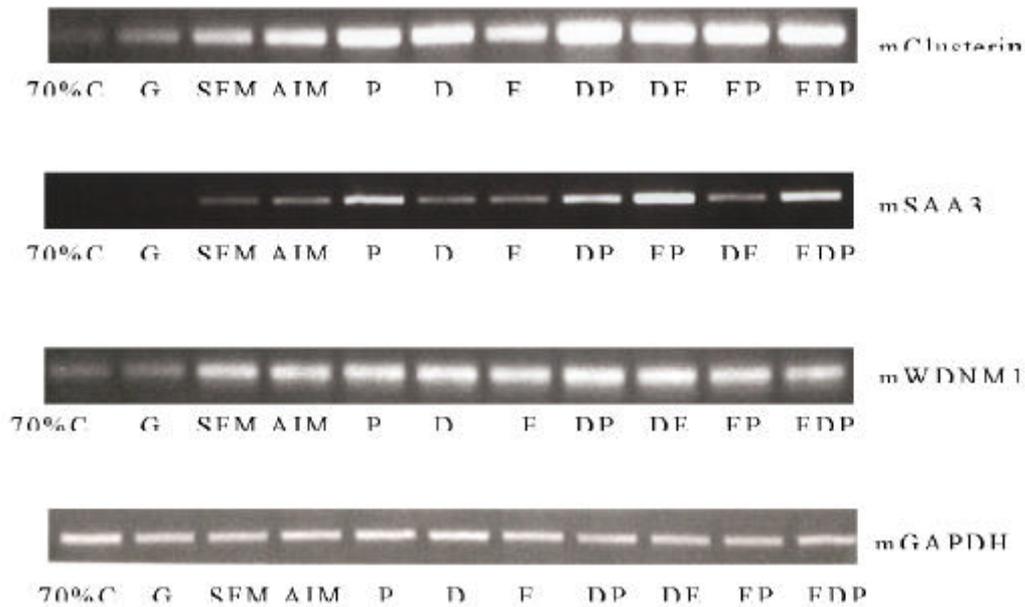
Fig 19. Effects of hormone treatments on clusterin, SAA3 and WDNM1 mRNA expression

Cells were grown until confluent, and exchanged to SFM, AIM and AIM + individual hormone. After 3 days, RNA was prepared from cultures of each treatment. mGAPDH expression served as a control, respectively.

C : Confluence, G : Growth medium, SFM : Serum free medium, AIM : Apoptosis induction medium, P : Prolactin, D : Dexamethasone, E : EGF
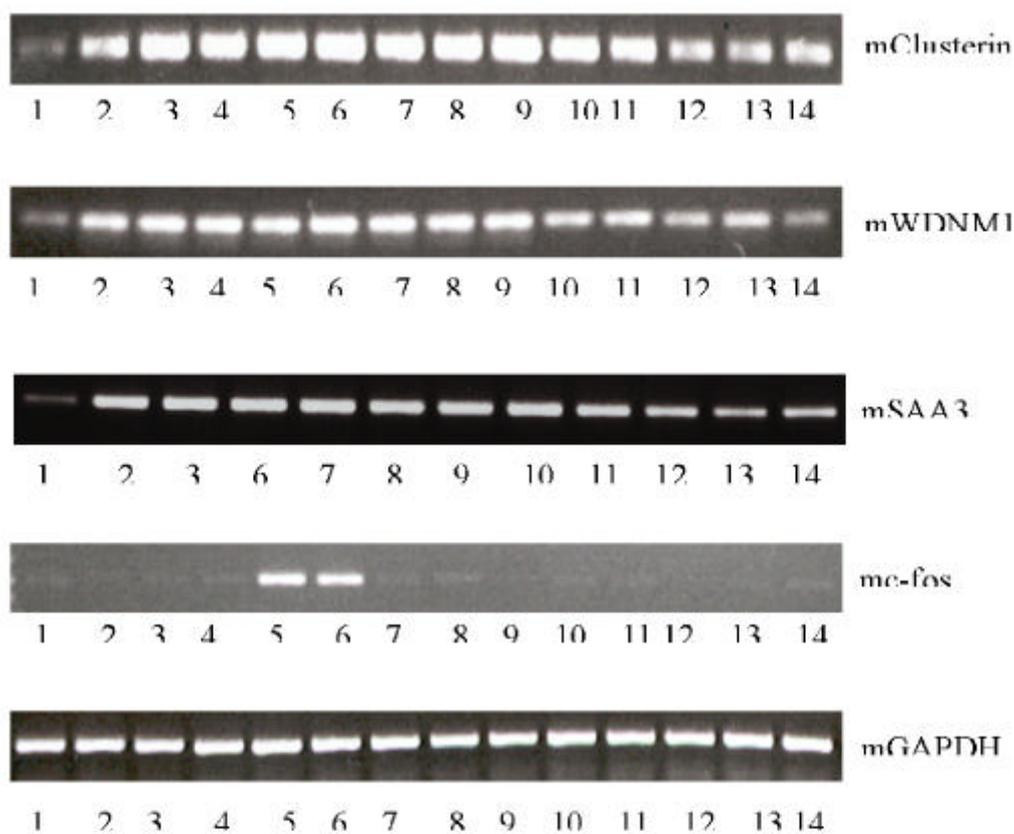
Fig 20. Time course for down regulation of clusterin and WDNM1 mRNA in serum stimulation cultures.

Cells were grown until confluent, exchanged to SFM for 2days, and then shifted to 10% serum. mGAPDH and c-fos mRNA expression served as a control and a positive control, respectively.

1: confluence, 2: SFM 1day, 3: SFM 2day, 4: 10% serum 0h, 5: 0.5h, 6: 1h, 7: 2h, 8: 4h, 9: 6h, 10: 12h, 11: 18h, 12: 24h, 13: 30h, 14: 36h
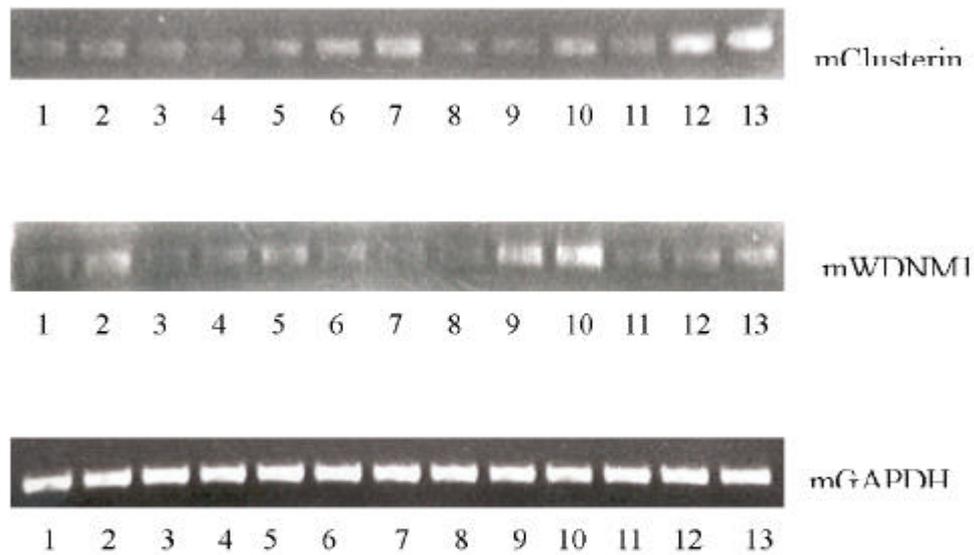
Fig 21. Experiment of TGFβ treatment

1: 70% confluence, 2: GM 1h, 3: GM 12h, 4: GM 24h, 5: GM+TGFβ 1h, 6: GM+TGFβ 12h, 7: GM+TGFβ 24h, 8: Serum 10% 1h, 9: Serum 10% 12h, 10: Serum 10% 24h , 11: Serum 10%+ TGFβ 1h , 12: Serum 10%+ TGFβ

1) Clusterin experssion
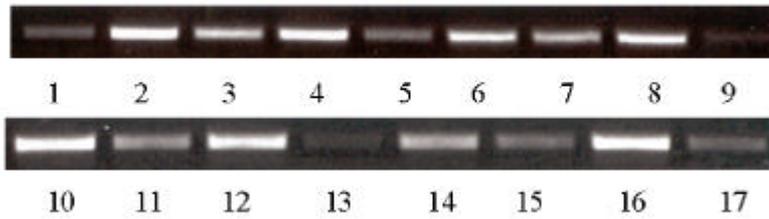
2) SAA3 experssion

2) WDNM1 experssion
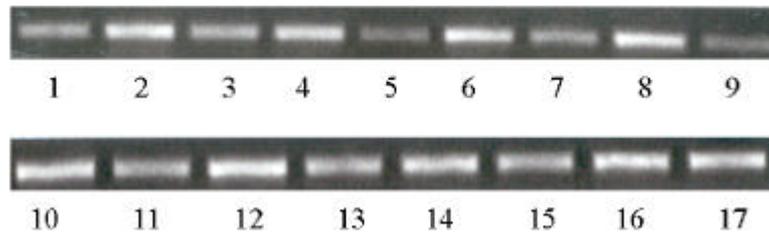
3) GAPDH
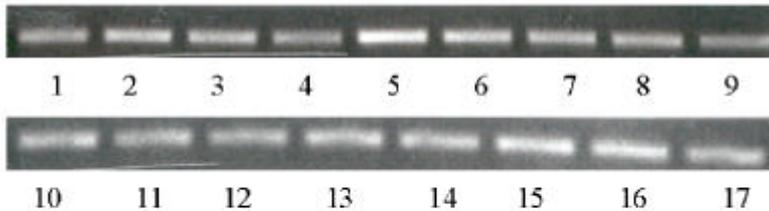
Fig 22. Experiment of TNF alpha treatment
1: 70% confluence, 2: GM 6h, 3: GM+TNF 6h, 4: Serum 10% 6h, 5:
Serum+TNF 6h, 6: GM 12h, 7: GM+TNF 12h, 8: Serum 10% 12h, 9: Serum
10% +TNF 12h, 10: GM 18h, 11: GM+TNF 18h, 12: Serum 10% 18h, 13:
Serum 10%+ TNF 18h . 14: GM24h. 15: GM+TNF 24h. 16: Serum 10% 24h.
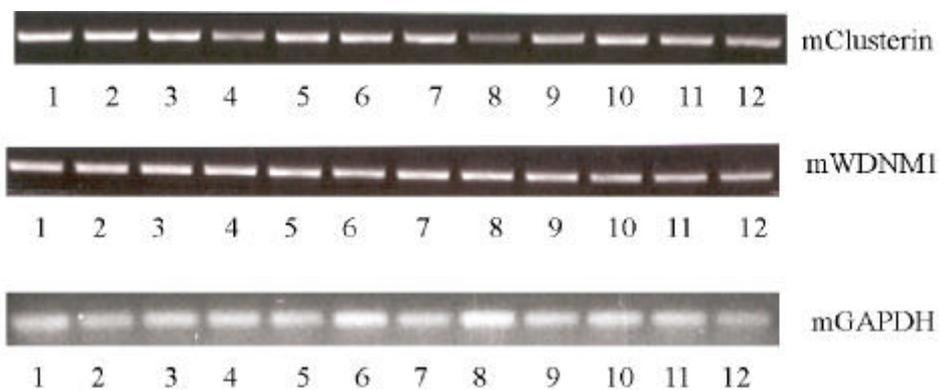
Fig 23. Experiment of antimycin treatment
1: Confluence, 2: 1h, 3: 6h, 4: 12h, 5: 16h, 6: 18h, 7: 20h, 8: 24h,
9: 36h, 10: 48h , 11: 67h attached cell, 12: 67h floating cell
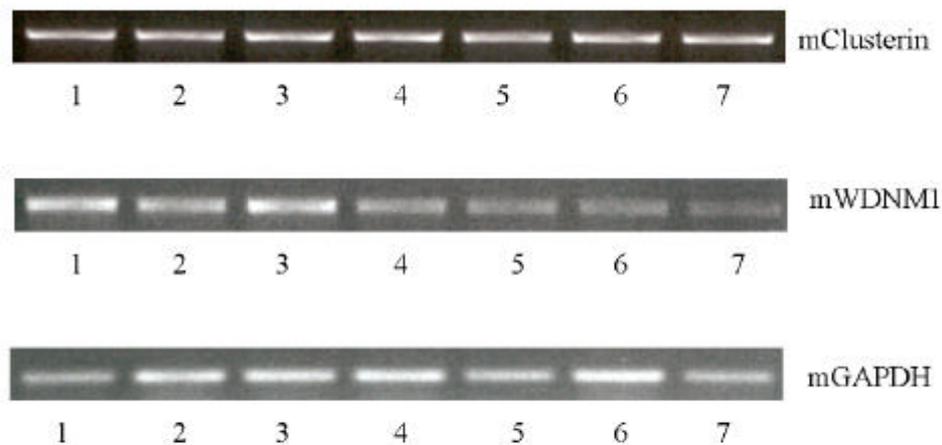
Fig 24. Experiment of oligomycin treatment
1: Confluence, 2: 3h, 3: 6h, 4: 12h, 5: 18h, 6: 24h, 7: 26h

1) Prolactin

SFM+PRL

GM	0	0.5	1	3	5	10	20μg/ml
1	2	3	4	5	6	7	8

mClusterin

mWDNM1

mSAA3

mGAPDH

SFM+PRL-mClusterin

SFM+PRL-mWDNM1

SFM+PRL-mSAA3

AIM+PRL

	GM	0	0.5	1	3	5	10	20μg/ml
	1	2	3	4	5	6	7	8

mClusterin
mWDNM1
mSAA3
mGAPDH

AIM+PRL-mClusterin

AIM+PRL-mWDNM1

AIM+PRL-mSAA3

Fig 25. Prolactin dose dependent

GM: growth medium, SFM: serum free medium

AIM: apoptosis induction medium

2) Dexamethasone

SFM+DEX

GM	0	0.1	0.3	0.7	1	2	5μM
1	2	3	4	5	6	7	8

mClusterin

mWDNM1

mSAA3

mGAPDH

SFM+DEX-mClusterin

SFM+DEX-mWDNM1

SFM+DEX-mSAA3
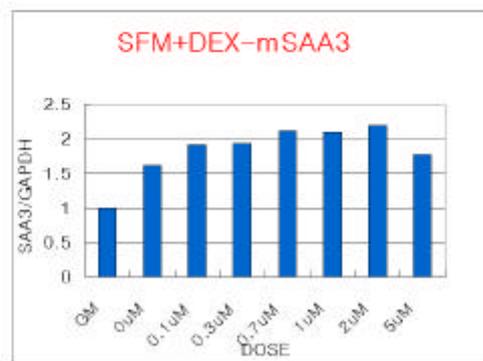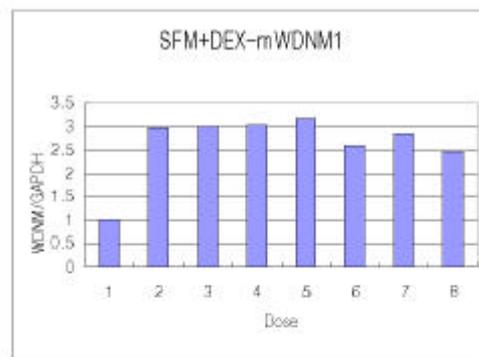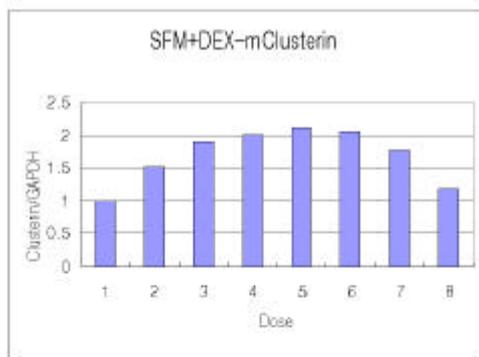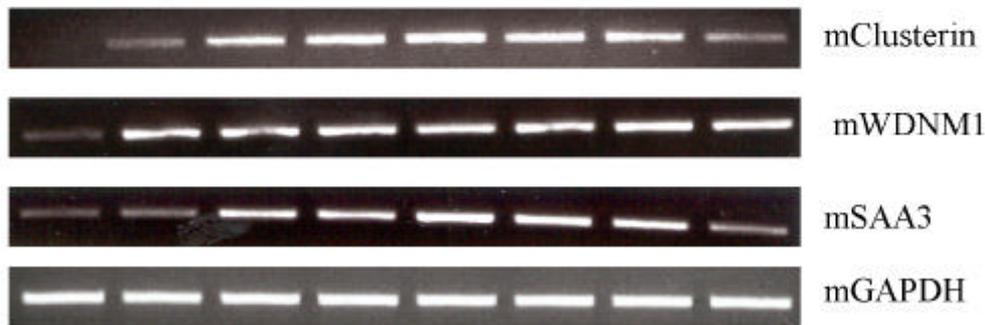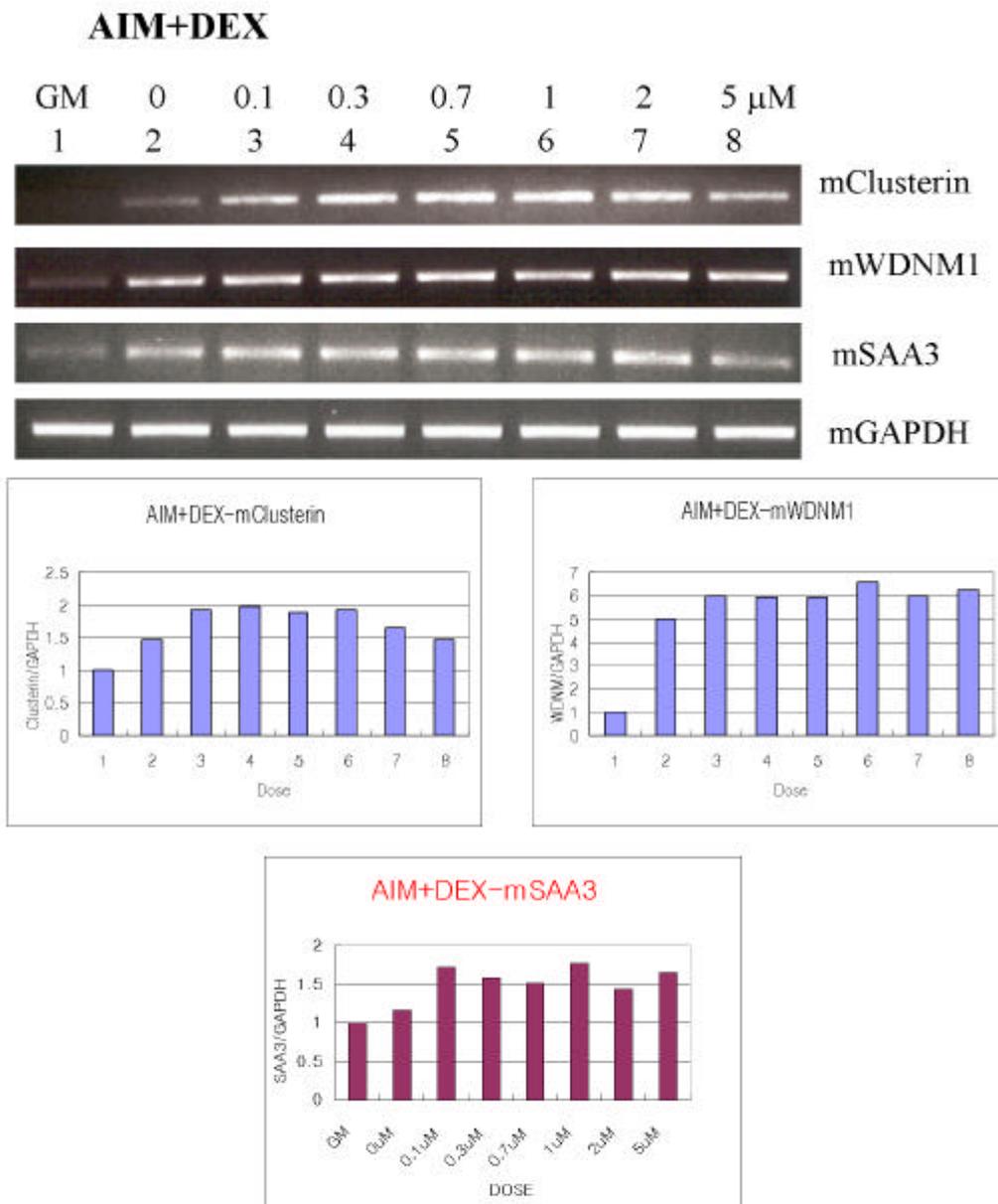
Fig 26. Dexamethasone dose dependent

GM: growth medium, SFM: serum free medium

AIM: apoptosis induction medium

3) EGF

SFM+EGF

GM	0	0.1	1	5	10	15	20 ng/ml
1	2	3	4	5	6	7	8

mClusterin

mWDNM1

mSAA3

mGAPDH

AIM+EGF

GM	0	0.1	1	5	10	15	20 ng/ml
1	2	3	4	5	6	7	8

mClusterin

mWDNM1

mSAA3

mGAPDH
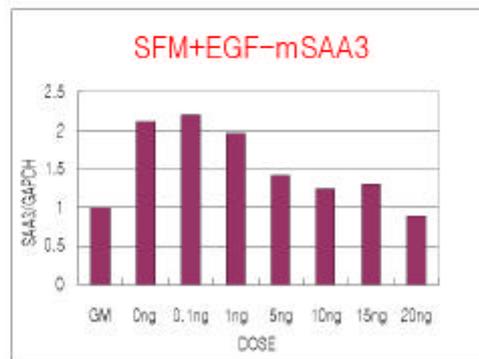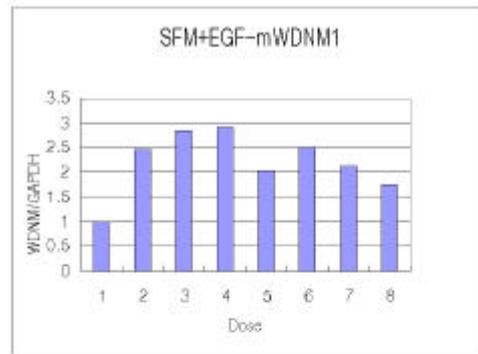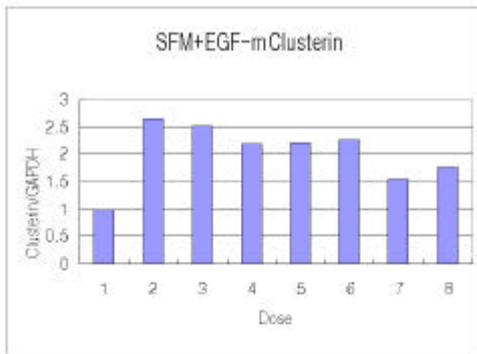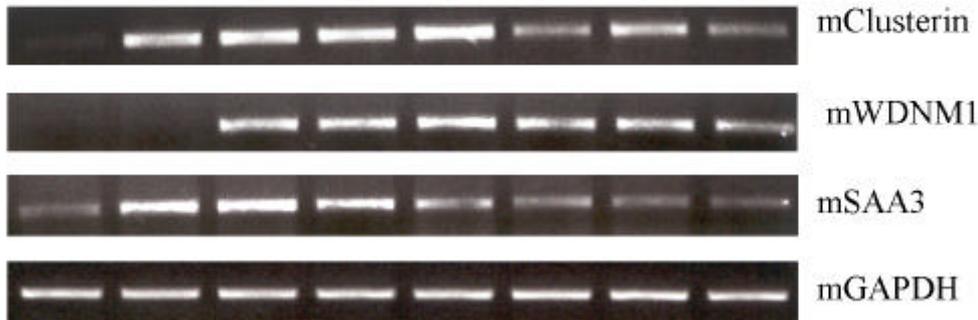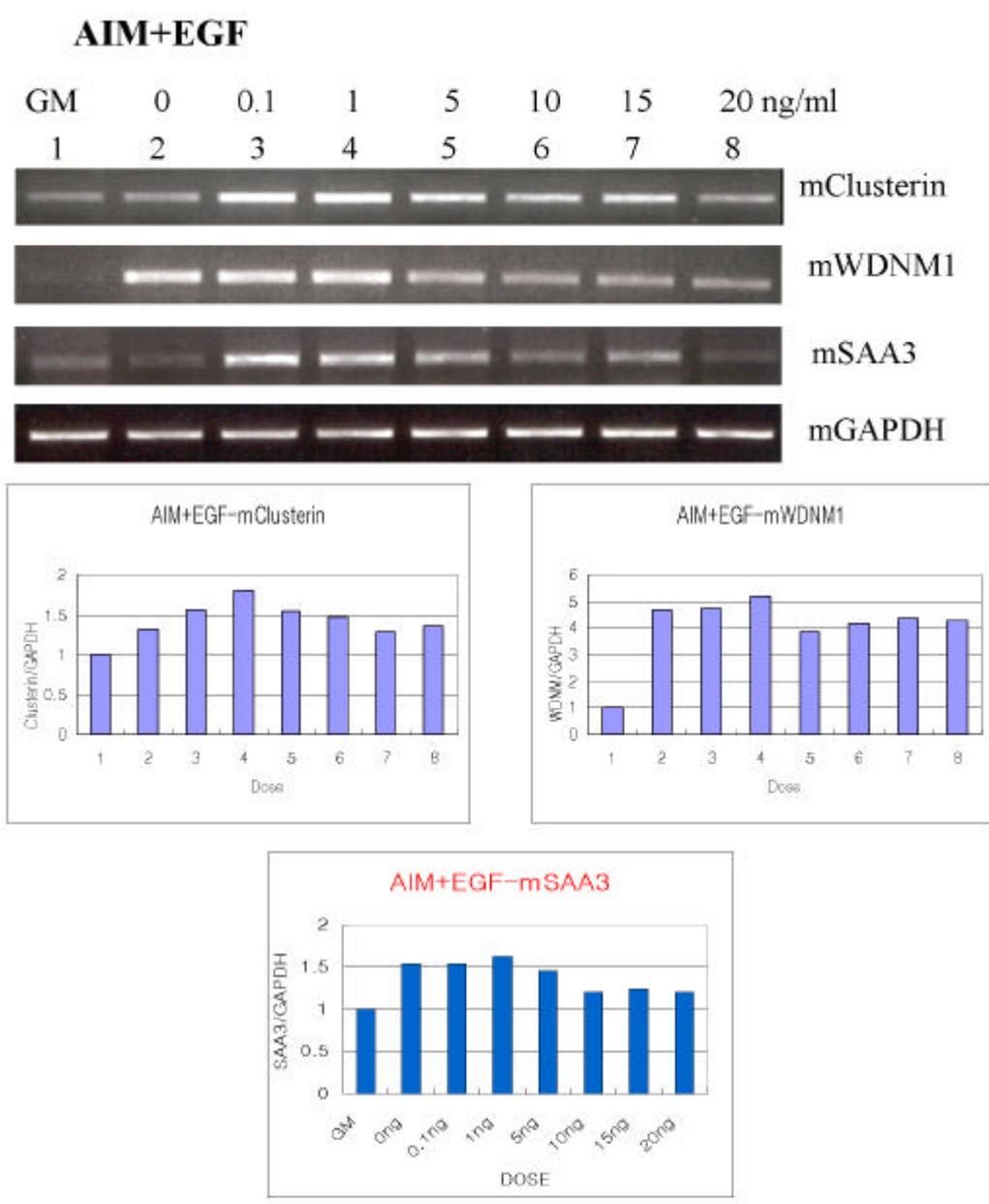
Fig 27. EGF dose dependent

GM: growth medium, SFM: serum free medium

AIM: apoptosis induction medium

4) Insulin

SFM+Ins

GM	0	0.5	1	3	5	10	20 $\mu g/ml$
1	2	3	4	5	6	7	8
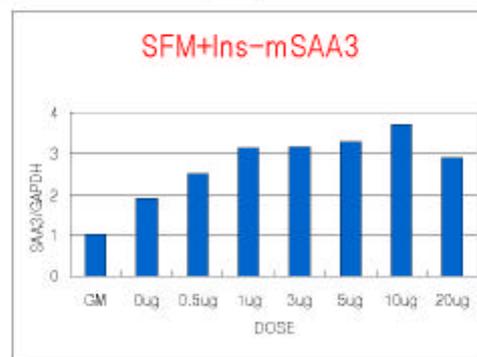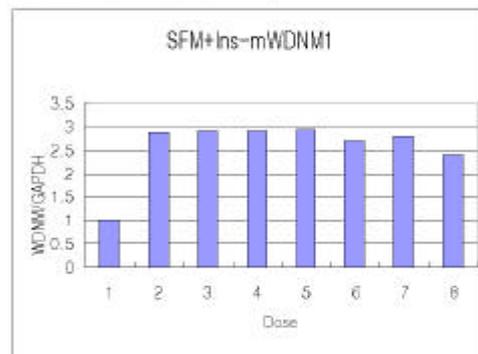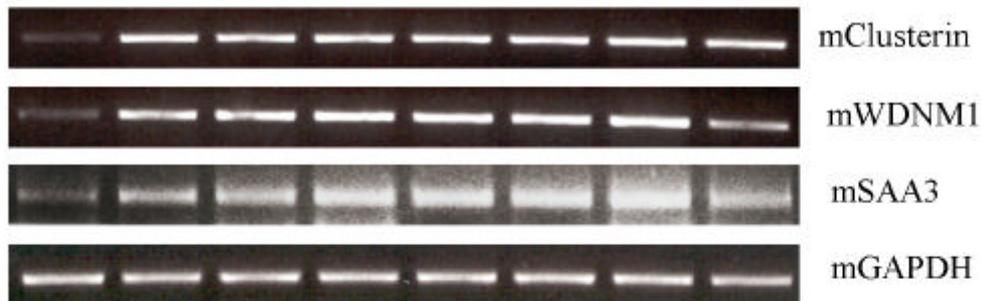
Fig 28. Insulin dose dependent

GM: growth medium, SFM: serum free medium

12.

(bovine
GPIII, SAA3, VDNM1)   E. coli

.

bGPIII, bSAA3, bVDNM1 protein   E. coli
pET expression vector system
bGPIII, bSAA3, bVDNM1 protein   E. coli

.

bGPIII, bSAA3, bVDNM1 insert   vector   reading frame
Nde I   Xho I   site   insert   5'   3' primer
design   PCR   .   bGPIII, bSAA3   bVDNM1 PCR product
1   low melting agarose gel   band   gel
elution kit(Bio101   )   insert   (Fig. 29). Vector
Nde I   Xho I   nucleotide removal kit(Qiagen   )
(Fig. 30).   pET vector   gel elution   insert
Nde I   Xho I(Promega   )   cohesive end   T4
ligase (Takara   )   16   12h   ligation
. Ligation product   Top 10F' cell   transformation
ampicillin, tetracyclin resistant colony   plasmid   Nde I
Xho I   , 5.5 kb   pET-22b vector   clusterin, SAA3   VDNM1
gene   insert   ligation   .
construction   E. coli   pETGPIII, pETSAA3, pETVDNM1
.
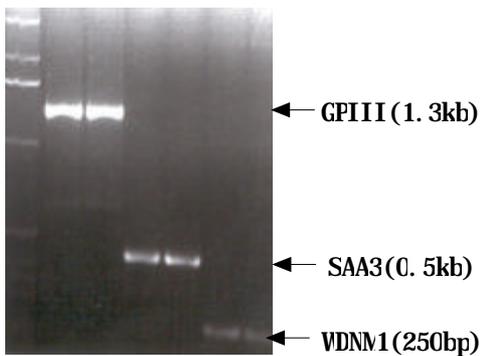
Fig 29. PCR-product
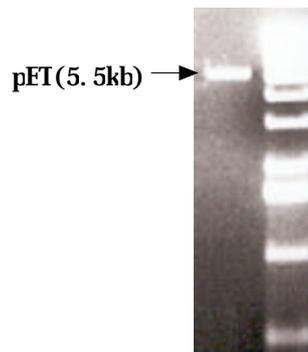
Fig 30. pET vector

13.　　　　　　E. coli

Construction　　pETGPIII, pETSAA3 expression vector
　　host　　BL21 E.coli　cell　　transformation　　　　.
colonies　colony PCR　　　　　　　　　　　　　　　plasmid
　　　　.　　　　Nde I　Xho I　　　　　　　　pETGPIII, pETSAA3 cDNA
　　　　　　　　　　　　　.

14. *E. coli*

pETGPIII, pETSAA3 expression vector　　　　　BL21 cell　　　LB/amp.
plate　　single colony　selection　　　　　　.　　　　　　0.1mM
IPTG　　　　　　　　inductino Oh, 1h, 2h　induction Oh　OD　1
　　　　　　　　　protein loading buffer　　　SDS-PAGE
　fusion protein　　　　　　　.
Fig 31. 32　　　SDS-PAGE　　.

pETGPIII_protein

IPTG induction   marker   0h   1h   2h

45kDa →   ← 47kDa

Figure 31. pETGPIII protein SDS-PAGE

pETSAA3_protein

IPTG induction   marker   0h   1h   2h

14.5kDa →   ← 15kDa
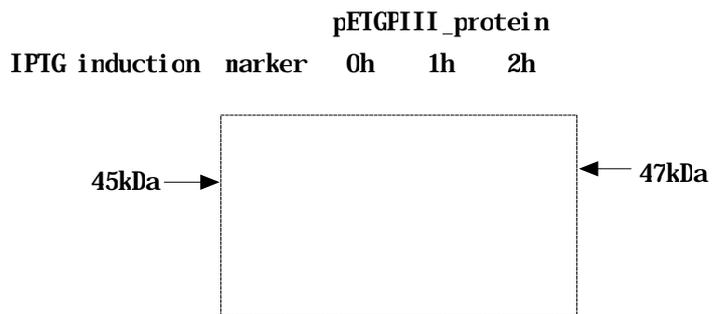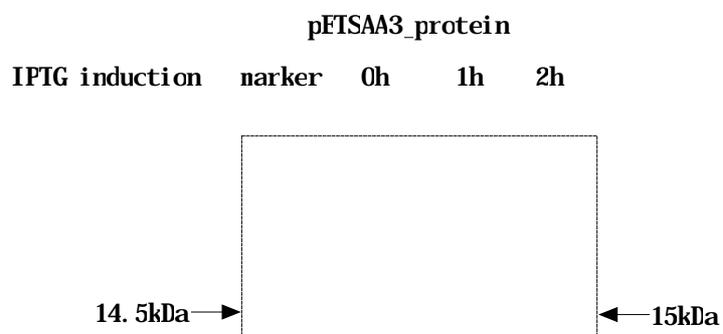
Figure 32. pETSAA3 protein SDS-PAGE

4

,
.

total RNA
subtractive hybridization
. glycoprotein III, serum amyloid
A3, VDMM1
Northern analysis .
Glycoprotein III, serum amyloid A3, VDMM1
in vivo ,
, glycoprotein III clusterin
, serum amyloid A3
metalloprotenase inflammation
. VDMM1
. . ,
glycoprotein III, serum amyloid A3 VDMM1 ,
,
.

Glycoprotein III, serum amyloid A3 VDMM1
, DNA
. glycoprotein
III, serum amyloid A3 VDMM1 ,

.

, .

1. : 1 , SCI 2
1. , , , , , , , ,
(1998) (Apoptosis, Programmed Cell Death) . Korean J. Dairy
Sci. 20(4), 289-310
2. Y.J. Kho, K.K. Cho, S.C. Kim, S.H. Kim, M.I. Chung, M.G. Baik and Y.J.
Choi (2000) Cloning of Bovine Serum Amyloid A3 cDNA. J. of Animal Science.

78, 2756-2757.
3. S.H. Ha, D. Y. Lee, Y. J. Kho, M. G. Baik and Yun-Jaie Choi (2000) inc-415 Gene Expression in the Prolliferation and Cell Death Phases of Mammary Epithelial Cells. Asian-Australasian J. Animal Sciences. 13(9), 1201-1204.

2.   :   5 ,   1
1. Hyung-Gee Kim, Young-Min Kim, Ju-Yong Lee, Yoon-Jung Kho, Kwang-Keun Cho, Jung-Hee Woo, Myung-Gi Baik and Yun-Jaie Choi. 1997. Cloning and characterization of Involution-Induced Gene from bovine mammary gland.   p90.
2. Hyung-Gee Kim, Young-Min Kim, Ju-Yong Lee, Yoon-Jung Kho, Kwang-Keun Cho, Myung-Gi Baik and Yun-Jaie Choi. 1998. Cloning and characterization of Involution-Induced Gene from bovine mammary gland. Proceedings of 8th World Conference on Animal Production p377.
3. Yoon-jung Kho, Dae-Yeon Lee, Myung-Gi Baik and Yun-Jaie Choi. 1999. Cloning of Involution-Induced Gene from bovine mammary gland.
        p119.
4. Seck-Ho Ha, Yun-Jung Kho, Dae-Yeon Lee, Myung-Gi Baik and Yun-Jaie Choi. 1999. inc-415 gene expression in the proliferation and cell death phase of mammary epithelial cells.   p130.
5. Yoon-Jung Kho, Sung-Chan Kim, Yan-Hua Yin, Sung-Wook Kwak, Seung-Kwon You, Myung-Gi Baik and Yun-Jaie Choi. 2000. Characterizatino of Up-regulated Genes During Involution Stage of Mammary Gland.
        p60.
6. Yoon-Jung Kho, Sung-Chan Kim, Yan-Hua Yin, Sung-Wook Kwak, Seung-Kwon You, Myung-Gi Baik and Yun-Jaie Choi. 2000. Regulated Expression of the Serum Amyloid A Gene Isoforms Mouse Mammary Gland Epithelial Cell, HC11.
                    p188.

3.
 1)   : 1997-0052590

 2)   : 1997-0052591

5

Adams, J. M., and S. Cory. 1998. The Bcl-2 family: arbiters of cell survival. Science 281:1322-1326.

Alison, J. M., and M. J. Betenbaugh. 1998. Overcoming apoptosis: new methods for improving protein-expression systems. Tibtech. 16:88-95.

Alnemri, E. S., D. J. Livingston, D. W. Nicholoson, G. Salvesen, N. A. Thornberry, W. W. Wong, and J. Yuan. 1996. Human ICE/CED-3 protease nomenclature. Cell 87:171.

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

Baik, M., M, Lee, and Y. Choi, 1998. Gene expression during involution of mammary gland (review). Int. J. Mol. Med. 2:39-44

Boldin, M. P., T. M. Goncharov, Y. V. Goltsev, and D. Wallach, 1996. involvment of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1 and TNF receptor-induced cell death. Cell 85:803-815

Bouderau, 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.

Chien C. T., P. L. Bartel, R. Sternglanz, and S. Fields. 1991. The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA 88:9578-82.

Chinnaiyan, A. M., K. O'Rourke, M. Tewari, and V. M. Dixit. 1995. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 81:505-512.

Chittenden, T., C. Flemington, A. B. Houghton, R. G. Ebb, G. J. Gallo, B. Elangovan, G. Chinnadurai, and R. J. Lutz. 1995. A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. EMBO J. 14:5589-5596.

Cohen, J. J. 1993. Apoptosis. Immunol. Today 14:126-131.

Cohen, G. M. 1997. Caspase:the executioners of apoptosis. Biochem. J. 326:1-16.

Danielson, K. G., C. J. Oborn, E. M. Durban, J. S. Butel, and D. Medina. 1984. Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proc. Natl. Acad. Sci. USA. 81:3756-3760.

Doppler, W., B. Groner, and R. K. Ball. 1989. Prolactin and glucocorticoid hormones synergistically induce expression of transfected rat -casein gene promoter constructs in a mammary epithelial cell line. Proc. Natl. Acad. Sci. USA. 86:104-108.

Faraldo, M. M., M. A. Deugnier, M. Lukashev, J. P. Thiery, and M. A. Glukhova. 1998. Perubation of beta1-integrin function alters the development of murine mammary gland. EMBO. J. 17:2139-2147.

Feng, Z., A., Marti, B. Jehn, H. J. Alternatt, G. Chicaiza, and R. Jaggi. 1995. glucocorticoid and progesterone inhibit involution and programmed cell death in the death in the mouse mammary gland. J. Cell Biol. 131:1095-1103.

Fernandes, A. T., A. Takahashi, and R. Armstrong. 1995. Mch3, a novel human apoptotic cystein protease highly related to CPP32. Cancer Res. 55:6045-6052.

French, L. E., A. Wohlwend, A. P. Sappino, J. Tschopp, and J. A. Scgifferli. 1994. Human clusterin gene expression is confined to surviving cells during in vitro programmed cell death. J. Clin. Invest. 93:877-884.

Frisch, S. M. and H. Francis. 1994. Disruption of epithelial cell-matrix interaction induces apoptosis. J. Cell Biol. 124:619-626.

Gervais, C., and M. Suh. 1990. Serum amyloid A protein-related mRNA expression in herpes simplex virus type 2-transformed hamster cells. Mol. Cell. Biol. 10: 4412-4414.

Girard, M. T., M. Matsubara, C. Kublin, M. J. Tessier, C. Cintron, and M. E. Fini. 1993. Stromal fibroblasts synthesize collagenase and stromelysin during long-term tissue remodeling. J. Cell. Sci. 104: 1001-1011.

Mitchell, T. I., C. I. Coon, and C. E. Brinckerhoff. 1991. Serum amyloid A (SAA3) produced by rabbit synovial fibroblasts treated with phorbol esters or interleukin 1 induces synthesis of collagenase and is neutralized with specific antiserum. J. Clin. Invest. 87: 1177-1185.

Rossevatin, K., P. K. Andresen, K. Sletten, A. Husebekk, G. Husby, K. Nordstoga, K. H. Johnson, G. T. Westermark, and P. Westermark. 1992. The complete amino acid sequence of bovine serum amyloid protein A (SAA) and of subspecies of the tissue-deposited amyloid fibril protein A. Scand. J. Immunol. 35: 217-224.

Sellar, G. C., M. C. DeBeer, J. N. Lelias, P. W. Snyder, L. T. Glickman, P. J. Felsburg, and A. S. Whitehead. 1991. Dog serum amyloid A protein. Identification of multiple isoforms defined by cDNA and protein analyses. J. Biol. Chem. 25: 3505-3510.

Stearnan, R. S., C. A. Lowell, C. G. Peltzman, and J. F. Morrow. 1986. The sequence and structure of a new serum amyloid A gene. Nucleic. Acids. Res. 24: 797-809.

Strachan, A. F., W. F. Brandt, P. Woo, D. R. van der Westhuyzen, G. A. Coetzee, M. C. de Beer, E. G. Shephard, and F. C. de Beer. 1989. Human serum amyloid A protein. The assignment of the six major isoforms to three published gene sequences and evidence for two genetic loci. J. Biol. Chem. 264: 18368-18373.

Strissel, K. J., N. T. Girard, J. A. West-Mays, W. B. Rinehart, J. R. Cook, C. E. Brinckerhoff, and N. E. Fini. 1997. Role of serum amyloid A as an intermediate in the IL-1 and PMA-stimulated signaling pathways regulating expression of rabbit fibroblast collagenase. Exp. Cell. Res. 237: 275-287.

Thompson, C. B. 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267: 1456-1461.

Thompson, C. B., R. Strnage, and P. J. Schedin. 1992. Apoptosis in the genesis and prevention of cancer. Cancer Epidemiol. Biomarkers. Prev. 1: 597-602.

Thornberry N. A., and Y. Lazebink. 1998. Caspase : enemies within. Science 281: 1312-1316.

Tsujinoto, Y., and S. Shimizu. 2000. Bcl-2 family: life-or-death switch. FEBS 466: 6-10.

Wyllie, A. H., 1987. Apoptosis: Cell death in tissue regulation. J. Pathol. 153: 313-316.

Xue, D., and H. R. Horvitz. 1995. Inhibition of the Canorhabditis elegans cell-death protease CED-3 by a CED-3 cleavage site in

baculovirus p35 protein. Nature 377:248-251.

Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B.. and Korsmeyer, S. J. 1995. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. 80:285-291.

Zervos, A. S., J. Gyuris, and R. Brent. 1993. Mxil, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell 72:223-232.

3   ,

1

                                                      ,

  , 1   .   ,
                                                                            (Knight   Wilde,
1987; Tucker, 1987).   0.85
                                    (Tucker, 1966).

                                                    .

                                                                                    ,

                          .   48-98%
                                    (Knight   Peaker, 1982; Borellini   Oka, 1987;
Inagawa   , 1990; Joshi   , 1986).
                        .
                              ,

              .

              .
                                                                  cDNA library
  , cDNA library   .
      cDNA clone   northern

                        .   stat5a
                                          .   stat5a
              .

2

1.   **cDNA library**

.

(Knight   ,
1992).

.   xylazine
( )   .
ephedrine/procaine hydrochloride(100mg procaine, 400mcg ephedrine)
, 4cm   .   2g - 5g
.   4   penicillin (3,630 units/kg   /)
.

RNA   ,   .
cDNA library   (
4, 5, 6, 7, 8   )   biopsy
.

. Total RNA   poly A RNA
total RNA   acid guanidinium thiocyanate- phenol-
chloroform   (Chomozenski and Sacchi, 1987).
denaturing   (guanidinium thiocyanate, Na citrate, 10% sarcosyl,
2-mecaptoethanol)   homogenization   RNA   denaturing
2M Na citrate, acidified phenol   chloroform
. Aqueous phase   phenol extraction   2-propanol   RNA
.

cDNA library   poly A RNA   total RNA
Stratagene poly A RNA purification column   .

. cDNA library
cDNA library   Clontech   TriplEx vector
. First strand cDNA   Poly A RNA   Xba I- d(T)
primer   42   40

. Second strand cDNA RNA/DNA ribonuclease H
DNA polymerase I 12 22 60
, 70 10 . T4 DNA
polymerase 37 10 cDNA double strand blunt-end
. 500 bp cDNA column . cDNA
phosphorylated EcoRI adaptor T4 DNA ligase 15
18 ligation 70 10
. linker spin column .
cDNA EcoRI-XbaI digested TriplEx vector T4 DNA ligase
ligation packaging mix in vitro packiging cDNA library
E. coli XL1-Blue cell transfection .

. cDNA library
cDNA library recombinant X-gal IPTG blue/white
plaque . cDNA library titer unamplified cDNA
library serial dilution plaque forming unit ,
independent clone . cDNA library
insert insert PCR agarose gel
. cDNA library , titer -70
.

2.

. cDNA clone
cDNA clones system
. Differential hybridization T-cell cDNA
clones cDNA clones
(Cochran , 1984). (growth factor
mRNA species)
clones (Hedrick , 1984)
mRNA species .
mRNA species
mRNA species .
single stranded cDNA , probes
cDNA library membrane duplication

differential hybridization   screening   ,
                clones   .
                        mRNA species   differential hybridization
                                 (1   ).
                stage-specific poly A RNA   , labeled first strand cDNA
probe   32P-CTP, oligo (dT) primer   reverse transcriptase
            .   cDNA library   recombinant bacteriophage
plaques   nylon membrane   duplicate   .   nylon membrane
                                        stage-specific labeled-first
strand cDNA probe   hybridization   probe   hybridization
      clone   signal   clone   . Primary screening
      low density (2,500 pfu/150 mm plate)   plating
single plaque   .
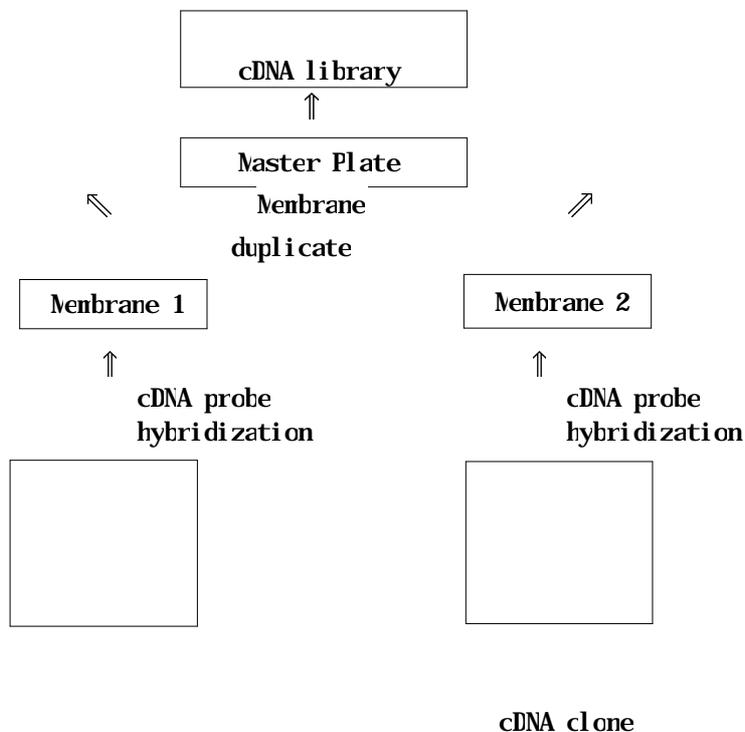
cDNA clone

Figure 1. Identification method of pregnant-induced clones by differential
          screening

. PCR/Southern   cDNA clone
               primary screening   single plaque
primary screening   false plaque
        .   clone   northern
    sequencing   .   PCR/Southern   differential
screening   positive clone   (Luo   ,
1994).   2   . Primary screening
positive plaques   freezing/thawing   2-3   lambda DNA
phage   . Template lambda DNA   5'/3' primer   30
cycle   PCR   . PCR product   duplicate   1.0% agarose gel
        . Southern blotting   gel   DNA   nenbrane
        nenbrane   cDNA probe   hybridization
                              nessage   clone
     .   20   clone
   .

   . Lambda DNA   plasmid   insert
         cDNA clone   λTriplEx vector   in vivo excision
      insert   pTriplEx plasmid   .
LB/kan/cam agar plate   BM25.8 stock cell   streaking   37
overnight   colony   . Working stock plate
single colony   LB/MgSO4/kan/cam agar plate   streaking
single colony   LB/MgSO4 broth 10ml   31   , 150rpm
overnight   , BM25.8 cell   pellet   MgCl2(10mM)   OD
       OD600 = 1.0   . Screening plate   single
plaque   1× lambda dilution buffer 200ul   vortexing   phage solution
        . In vivo excision   BM25.8 cell culture 100ul
eluted positive plaque 10ul   20ml tube   shaking   31
30   . LB broth 200ul   31   , 225rpm   1
   . Infected cell suspension 2ul   LB/carbenicillin plate   plating
overnight   , single colony   LB/carbenicillin broth
overnight   . Tube   glycerol stock
plasmid   .

Primary screening
plaque
lambda DNA

⇑

5'/3' primer ,
PCR

↙ Duplicate ↗
agarose gel ,
Southern blotting

Membrane 1   Membrane 2

⇑ cDNA probe   ⇑ cDNA probe
hybridization   hybridization

1 2 3 4   1 2 3 4
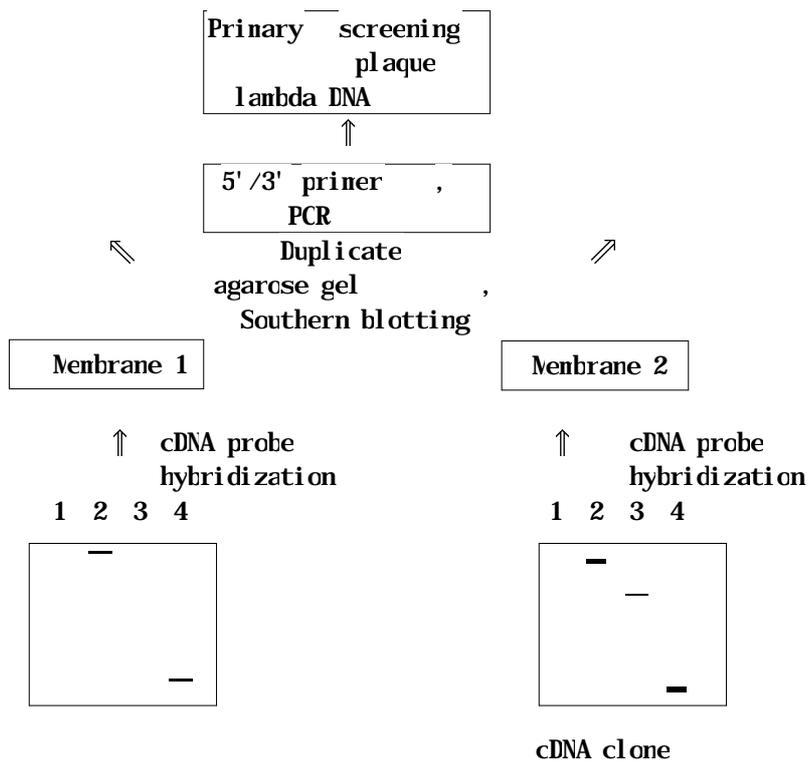
cDNA clone

Figure 2. Identification method of pregnant-induced cDNA clones by
PCR/Southern differential screening. Lane 1 clone

probe   message   false clone
. Lane 3 clone   message   , lanes 2 4
clone   message
clone   .

In vivo excision colony   miniprep.   plasmid
.   plasmid DNA Qiagen plasmid purification column
.   cDNA insert EcoRI XbaI
plasmid EcoRI XbaI   cDNA insert   .
Insert   plasmid   low melting agarose   ,
insert   gel   -20   , northern
³²P-labeled cDNA probe   template   .

3.

. cDNA clone database
clone
. ³⁵S-dATP double-stranded dideoxy
chain termination PCR sequencing kit [Stratagene Exo(-)
Pfu cyclist] . PCR
plasmid 10 fmol 500 fmol template DNA
.

PCR sequencing primer
strand primer , 5' primer
pTriplEx 5'(pTEx5) , 3' primer pTriplEx 3'(pTEx3) T7
primer sequencing . 500fmol template DNA 1pmol
primer, 10× sequencing buffer, 10μCi ³⁵S-dATP, 2.5U Exo(-) Pfu DNA
polymerase, 4 DMSO 30 mixture
ddNTP(ddATP, ddTTP, ddGTP, ddCTP) tube 7 mixture
PCR sequencing . PCR denaturing 94 , 30 ,
annealing 47 30 , extension 72 30 30
. PCR 5 stop dye mix 8% polyacryamide gel
.

clone GenBank database
clone .

. Northern
cDNA clone
northern . , ,
total RNA northern .
Total RNA membrane RNA
(Sambrook , 1989). Formaldehyde-1.0% agarose gel
. RNA 15,000 rpm, 4 , 30 , RNA pellet 70%
ethanol . RNA loading buffer 20ul pellet , 95 ,
2 heating gel well loading . (100 V, 1 - 2)
UV 28S 18S band mRNA
band .
gel total RNA capillary

membrane   . 10 x SSC solution   agarose gel   total RNA
membrane   .   membrane   microwave oven   2.5
baking   membrane   RNA   .
    cDNA insert   hybridization   32P-labeled probe   random
priming kit (Stratagene)   . Membrane   probe
hybridization   clone   . 0.5% SDS, 6X
SSC, 10% dextran sulfate, 100 ug/ml sperm DNA   hybridization
        . 65   2   prehybridizatoin   32P-labeled probe
    hybridization   12   (Thomas, 1980). Hybridization
    membrane   2X SSC/0.1 % SDS (10   2), 2X SSC/0.1 % SDS (4
2   30), 0.1X SSC/0.1 % SDS (42   30), 0.1X SSC/0.1 % SDS (5
5   30), 0.1X SSC/0.1 % SDS (65   30)   washing
  . Washing   membrane   wrap   X-ray film   2-4
            .
        cDNA clone
        northern hybridization   .

    .   cDNA clone
                                        bovine
      clone
      ,   subcloning
    , PCR cloning   .   database program
sequence   clone   site   .
    Clone   GenBank data   ,   clone bP51   human
polyadenylate binding protein (PABP)   85% homology   .
    ,   clone   insert   full-length coding region
  . RT-PCR   full-length PABP cDNA   cloning
  .
        human   mouse PABP cDNA   coding
5' primer   3' primer   . RT-PCR   template   8
            total RNA   . Total RNA   DNA
    DNase   . Total RNA   template   oligo(dT) primer
        MMLV (Clontech) reverse transcriptase   first-strand cDNA
      . PCR   1.2% low melting gel
    PCR band   DNA purification kit   . PCR

product   pGEM-T easy vector (Pronega)   ligation   E. coli JM109
cell   transformation   .

4.

                        bovine stat5a gene

                         . Stat5a   prolactin
                                      .   bovine stat5a gene
                  HC11   stat5a
                                          .   , bovine stat5a gene
eukaryotic expression vector   vector   stat5a insert
          control vector   HC11 cell line   transfection   , stat5a
gene transfection
        .   3   .

    . Stat5a cDNA   vector
    Bovine stat5a gene   (pstat5a)   neo
gene   control vector (pNeo)   .
                    bovine stat5a   cDNA   eukaryotic expression vector
                            4   .
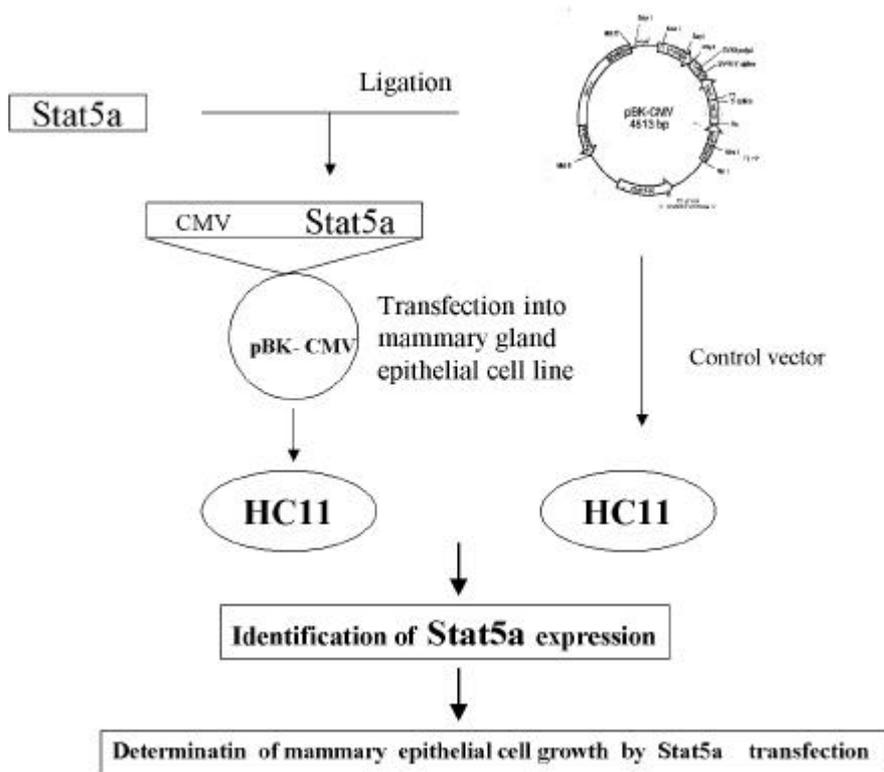
Figure 3. Strategy for the determination of mammary epithelial cell growth by bovine stat5a transfection in HC11 cells.
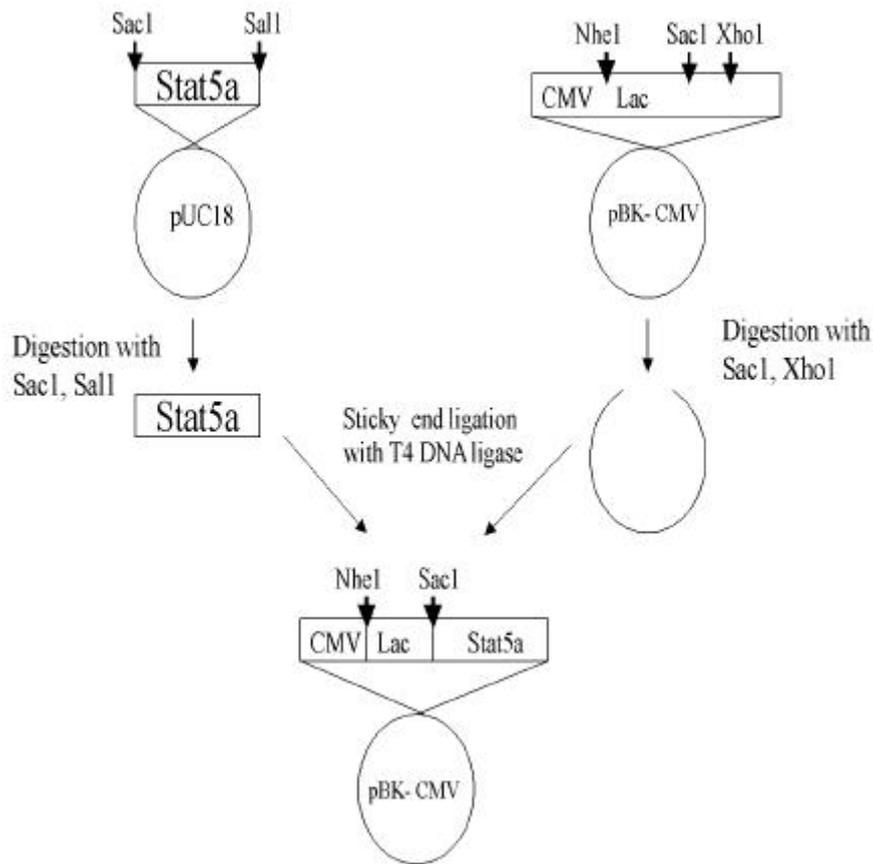
Figure 4. Strategy for the construction of bovine stat5a gene into eukaryotic expression vector(pBK-CMV)

expression vector   pBK-CMV vector (Stratagene)
vector   eukaryotic cell   cytomegalovirus (CMV)
immediate early promoter, SV40 transcription terminator   polyadenylation
signal   SV40 3' splicing site   .   SV40 early promoter   TK
transcription termination   polyadenylation signal
    neo   (neomycin/kanamycin resistance gene) system
G418 selection   eukaryote cell line   transfection
              .

    Bovine stat5a insert   pUC18-stat5a   Sac I(5')   Sal I(3')
                  , pBK-CMV vector (4.513kb)   multi-cloning site   Sac
I(5')   Xho I(3')   QIAEX
II Gel Extraction kit(QIAGEN)   .   stat5a insert
(2.6kb)   pBK-CMV vector   T4 ligase   16   12   cohesive
end ligation   . Ligation product (7.1kb)   100mM CaCl₂
    XLI-blue MRF'competent cell   transformation   LB/kanamycine (50
ug/ml) agar plate   plating   14   37   incubation   . Colony
        LB/Kanamycine (50ug/ml) broth   12   37
plasmid   QIAGEN Plasmid mini Kit   .   .   .
    pBK-CMV-stat5a plasmid   Sac I (5')   one cut   plasmid
size 7.1kb   .   Not I (5', 3'   )
    stat5a (2.6kb) insert   pBK-CMV (4.5kb) vector   ligation
              .

    Eukaryote cell HC11   Nhe I   Sac
I   200bp   ß-galactosidase   promoter   start codon
AUG   . lac promoter   AUG   plasmid
    QIAEX II Gel Extraction kit (QIAGEN)   .   pN-S
    DNA Polymerase Large klenow Fragment(Promega)   fill-in
    blunt end   T4 ligase (NEB)   16   12   ligation
      . Ligation product (7.1kb)   E.coli XLI-blue MRF'competent cell
transformation   LB/kanamycine agar plate   plating   14   3
7   . Colony   LB/Kanamycine broth   , 12
        37   plasmid   QIAGEN Plasmid mini Kit
    .   . Ligation junction   correct ligation
              .

    Plasmid   pBK-CMV vector   stat5a insert   Xba I

6.9kb   ,   Not I   4.3kb
pBK-CMV vector   2.6kb   stat5a insert   .

.   (HC11)   stat5a cDNA

1) Transfection   HC11 cell culture
                   mouse normal mammary epithelial cell line   HC11 cell
bovine stat5a   (Ball   ,
1988). Cell   ampule   liquid nitrogen tank
37   water bath   . Ampule   70% ethanol   ampule
      cell suspension   label   flask   . 9 ml
(dropwise   2   )   .   RPMI 1640   media,   10%
heat-inactivated fetal bovine serum, 5 ug/ml insulin, 10 ng/ml epidermal
growth factor (EGF)   5% CO2, 37
          .   2-3   .   1 , 2   cell growth,
cell attachment   .   monolayer   confluency
            subculture   3ml   0.25% trypsin   monolayer
             trypsin   monolayer   flask
1   trypsin   incubator   cell round up/dissociation
                 (5-30). Cell dissociation   3ml
         monolayer   pipetting   cell dispersion
      cell suspension   15ml tube   pipetting
homogenous single cell suspension   .   cell
number   counting   .   subculture
36   60%   confluency   , 48   confluency

2) HC11 cell
1 X 105 HC11 cell   35mm dish   2ml media   seeding   18   24
                50   80% confluent
. Lipofectamine   (GIBCO BRL) 2, 4,   6 ul   100 ul serum-free media
          , Quagen Endofree plasmid Maxi Kit   .
stat5a expression plasmid   control plasmid 2 ug   100 ul serum-free
media   .   30
   DNA-liposome complex   . DNA-liposome complex

cell   2ml   serum-free medium   rinse   . DNA-liposome
complex tube   0.8ml serum-free medium
       1.0ml   solution   overlay   . Transfection
antibacterial agent   media   . 5   incubation   20%
FBS   media 1.0 ml   , 24   10% FBS
              fresh medium   .
    Medium   24   200 ug/ml G418   G418
           transfected cell   selection   .   3
              10-14   selection   G418   colonies
  . Control vector   stat5a vector   lipofectamine
              4   ,   12   colonies   . Colony
  cell   PBS   2   plate   cloning
cylinder   colony   plate   . Cloning
cylinder   trypsin   1 drop   repeated pipetting   cell
   dissociation   24 well plate (1 ml media)   . Cell
    2-5   cell   attachment   fresh
medium   . Cell   confluency   (1) 6 well   plate
            ,   subculture
                  ,   DNA   RNA   .

    . Stat5a

                              genomic DNA   PCR   .
Confluency   TBS solution
      monolayer   cell
pellet   40 ul TE buffer   cell   dissociation   . DNA
extraction buffer 360 ul   40 ul proteinase K   55   30
                     3   .   400 ul chloroform
phenol isoamyl alcohol   genomic DNA
                       .   1 ml ethanol   genomic DNA
pellet   70% ethanol   TE buffer   DNA   .
                 genomic DNA   template
          primer   PCR   .
Control   vector   CMV5'   primer   CMV3'
primer   , stat5a vector   CMV5'   primer
stat5a3' primer   . PCR   94   preheating 5   94

denaturation 1 30 , 53 annealing 1 30 , 72 extension 1 30
40 cycle   72 10 final elongation   . PCR
product 1.0% agarose gel   .

. Northern   stat5a mRNA
    stat5a   total RNA
northern   .   confluent
   PBS   washing   monolayer cell   denaturing solution
overlay .   lysis   scraper
   homogenization . Lysed   total RNA
   labeled-stat5a insert probe   hybridization
   .

. Stat5a cDNA
  Stat5a cDNA   control
vector   stat5a vector   104 cells/well 24
well plate seeding 24 , 48   72
   .   trypsin   dissociation
hemocytometer   counting .   MTT assay
   (cell proliferation rate)   . MTT
(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
   mitochondrial dehydrogenase   soluble formazan
 , formazan dye solution
   .

   cytotoxicity   (Mosmann,
1984). MTT assay   96 well plate well 0.1× 104 cell seeding
  well   100ul   . 37 CO2 incubator
  24, 48 72   50ul MTT (PBS   5mg/ml MTT solution,
Sigma)   . 37 3-4   incubation   media
   well plate PBS media washing . Well 70ul
propanol-2-ol (50ml   isopropanol 50ml 100ul HCl
0.1-0.2% HCl   )   insoluble blue
formazan crystal   . Acidified propanol-2-ol 30   ELISA
reader 570nm optical density .   5
   .

5. stat5a

. Bovine stat5a

bovine stat5a overexpression

, stat5a

.

stat5a protein E.coli
expression vector cloning . pGEX expression vector
. pGEX GST gene fusion vector . GST fusion vector gene
expression fusion protein affinity-purification
. Tac promoter 1-5mM IPTG
induction , 26 kDa glutathione S-transferase (GST) fusion
protein , thrombin fusion protein GST carrier protein
.

Stat5a insert vector reading frame Not I
site 5' linker primer (5'-AGCGGCCGCATGGCGGGCTGGATC-3')
design . pBK-CMV vector cloning stat5a cDNA 5'
linker primer 3' T7 primer Taq polymerase PCR
2.6kb stat5a band . stat5a PCR product 1 low
melting agarose gel band phenol
extraction . PCR product stat5a Not I
cohesive end , phenol extraction
. pGEX-4T-3 vector Not I
cohesive end . Vector self ligation
CIAP 5' dephosphorlyation . Not I stat5a
cDNA (2.6kb) vector (4.9kb) T4 ligase (Takara) 16 12h
ligation . Ligation product DH5 cell
transformation ampicillin resistant colony plasmid
Not I ligation .

. Stat5a *E. coli*
Construction stat5a expression vector host
BL21 E.coli cell transformation . colonies
ampicillin plasmid Not I ,
stat5a cDNA .

. *E.coli*   stat5a

pGEX-4T-3-stat5a expression vector   BL21 cell   LB/amp.
plate   single colony   selection   .   0.1mM
IPTG   2h, 4h, 6h   1ml   protein loading
buffer   SDS-PAGE   fusion protein   .

3

1.   cDNA library

.

.

.

RNA   ,
.   cDNA library
4, 5, 6, 7, 8
.

. Total RNA   poly A RNA
total RNA   acid guanidinium thiocyanate- phenol-
chlorcform   .   total RNA   formaldehyde gel
28S   18S ribosomal RNA   28S
band   intensity 18S   2   ,   total RNA
intact   (5).
cDNA library   poly A RNA   total RNA
Stratagene poly A RNA purification column   .
poly A RNA   28S   18S RNA
mRNA   enrich   (6).
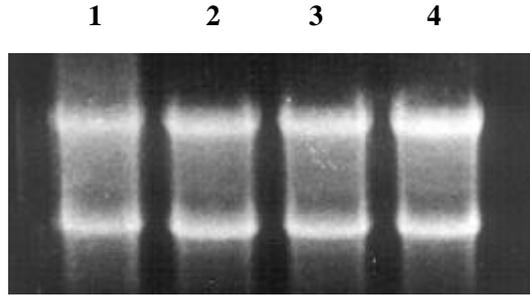
Figure 5. Electrophoresis of total RNA. The 20 ug of total RNA extracted from bovine mammary gland at pregnant 4 (lane 1), 6 (lane 2), 7 (lane 3), and 8 months (lane 4) were fractionated on 1.0% agarose gel containing formaldehde.
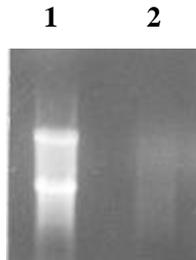
Figure 6. Electrophoresis of poly A RNA. The 20 ug of total RNA (lane 1) and poly A RNA (lane 2) purified from the pooled total RNAs of bovine mammary gland at pregnant 4, 5, 6, 7, and 8 months were fractionated on 1.0% agarose gel containing formaldehyde.

. cDNA library

　　　　　　　cDNA library　　　TriplEx vector
　　　　　. First strand cDNA　　poly A RNA　　Xba I- d(T) primer
　　　　　　　　　　　　　　　　　. Second strand cDNA
　　　　　　　　　　　　RNA/DNA　　　　　　ribonuclease H　　DNA
polymerase I　　　　　　．　　　　　　T4 DNA polymerase　　　　cDNA
double strand　　　　blunt-end　　　．　　　cDNA　　phosphorylated EcoRI
adaptor　　　　　T4 DNA ligase　　　　ligation　　　．
linker　　spin column　　　　　　　　　　．　　cDNA　　EcoRI-XbaI
digested　TriplEx　vector　　T4　DNA　ligase　　　　　ligation
packaging mix　　in vitro packaging　　cDNA library　　　　　E. coli
XL1-Blue cell　　transfection　　　．

. cDNA library

cDNA library　　reconbinant　　　X-gal　　IPTG　　　　　　blue/white
plaque　　　　　　　　　　　, 85%　　reconbinant plaques　　　　　. cDNA
library　titer　　unamplified cDNA library　serial dilution　　plaque
forming unit　　　　　　　　　　　, 　　　　　　　independent clone
　　　　. Unamplified library　7.0 x 106 pfu/ml　　　titer
　．

cDNA library　insert　　　　insert　PCR　　　　　agarose gel
　　　　. 15　　plaques　　　　　　0.3 kb - 1.8 kb
insert　　　　　　　，　　insert size　0.7 kb　　(　7).
cDNA library　　　　, titer　　　　　　-70　　　　　　．
library　titer　1.0 x 109 pfu/ml　　．
cDNA library　　　　　, 　　cDNA library
　　　, 　　　　　　．

Figure 7. Construction of pregnant specific cDNA library from bovine mammary gland. Pregnant-specific cDNA library was constructed using the pooled poly A RNA extracted from pregnant mammary gland. Fifteen clear plaques (lanes 2 - 16) were randomly picked and subjected to the PCR using insert screening primers, and amplified products were analysed on an 1.0% agarose gel.

2.

. cDNA clone

cDNA library
(differential hybridization) .

mRNA species
mRNA species
. single stranded cDNA
, probes cDNA library membrane
duplication differential hybridization screening ,
clones .
mRNA species differential
hybridization .
poly A RNA kit

. Labeled first strand cDNA probe   ₃₂P-dCTP, oligo (dT) primer
reverse transcriptase   .
cDNA library   recombinant bacteriophage plaques   nylon menbrane
duplicate   .   nylon menbrane
            labeled-first strand cDNA probe   hybridization
    probe   hybridization   clone   signal
clone   .   8   .   18   150 mm
petridish   ,   109   primary
positive candidate plaques   . cDNA library   plating   150 mm
petridish   2,000 - 2,500   plaque   ,   (80%,
87/109)   clone   single plaque   . 1   screening
    87   clone   single plaque   , 20   clone   2   plaque
        2   clone   3   plaque   .
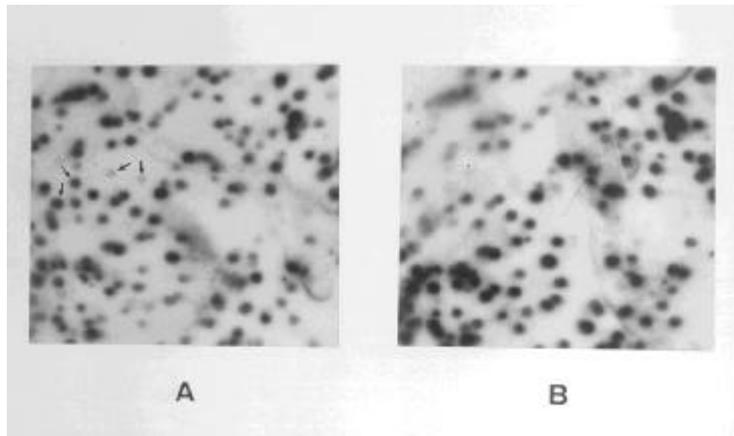
Figure 8. Differential screening of cDNA library constructed from pregnant
        bovine mammary gland. Approximately 2,500 pfu of phage solution
        were plated. Duplicate filters were prepared from the same plate
        and hybridized with cDNA probes prepared from virgin mRNA (B) or
        pregnant mRNA (A). The spots indicated by an arrow represent
        examples of pregnant-induced cDNA clones.

. PCR/Southern   cDNA clone

                    primary screening   single plaque

primary screening   false plaque

        . clone   northern

sequencing   .   PCR/Southern differential

screening   positive clone   . Primary screening

positive plaques   freezing/thawing   2-3   lambda DNA

phage   . Template lambda DNA   5'-   3'- vector primer

        30 cycle   PCR   . PCR product   duplicate   1.0%

agarose gel   . Southern blotting   gel   DNA

        membrane   membrane   cDNA probe

hybridization   message

clone   .   cDNA clone   PCR/Southern

differential screening   9   . 9   positive

plaque   template   PCR   9A   . PCR

agarose   Southern   2   membrane   mRNA

(9V)   mRNA probe (9P)   hybridization   , 4 , 6 , 7

,   9   lane   clone   probe   signal   .

A   B
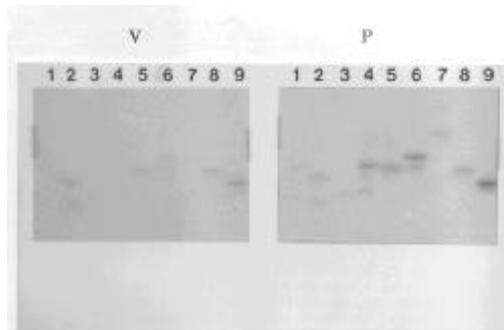
Figure 9. PCR/Southern differential screening of primary positive clones.
Panel A: Inserts of positive plaques (lanes 1-9) identified from primary
differential screening were amplified by PCR using 5'- and 3'-vector
primers and analyzed on a 1.0% agarose gel. Marker, γ-BstE II digest. The
lanes of panels V and P correspond to those in panel A. Panel V represents
an autoradiogram hybridized with the cDNA probe prepared from the virgin
mammary gland. Panel P represents an autoradiogram hybridized with the
cDNA probe from the pregnant mammary gland.

. Lambda DNA   plasmid   insert
       cDNA clone   γTriplEx vector   in vivo excision
       insert   pTriplEx plasmid   . In vivo
excision   positive plaque   γDNA   pTriplEx plasmid   .
   Plasmid   LB/carbenicillin broth   cell
miniprep.   plasmid   .   cDNA insert
EcoRI   XbaI   plasmid   EcoRI   XbaI
   cDNA insert   . Plasmid
       10   .   clone   insert size   0.5 - 1.0
kb   ,   0.3 kb,   1.9 kb   . Insert
plasmid   low melting agarose   , insert
northern   32P-labeled cDNA probe   template   .
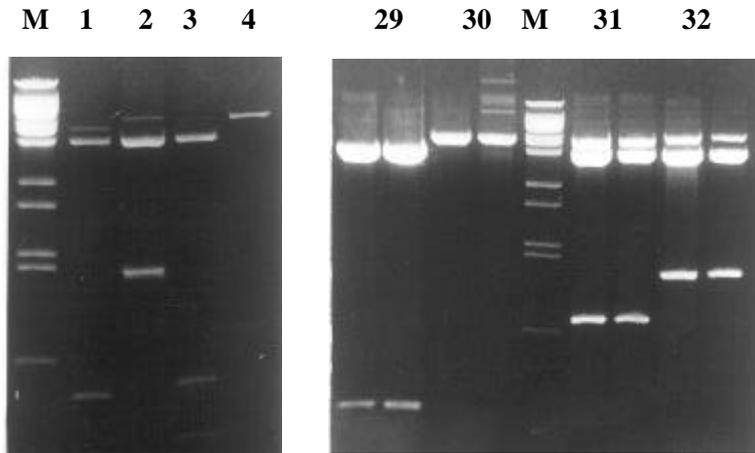
Figure 10. Electrophoresis of plasmid containing bovine mammary gland cDNA
          after digestion with EcoRI/XbaI. The γDNA containing the cDNA
          insert was converted into the phagemid by Automatic Excision
          Process. The plasmid was digested with EcoRI and XbaI, and the
          DNA fragments were analysed by a 1.0% agarose gel
          electrophoresis. M;   DNA-Bst E II digest. Numerical number on
          each lane indicates clone number.

3.

　　　　　　clone　　　　　　　　　　　　　　　database
　　　　　　　　　　　　　　　　clone　　　　　　[35]S-dATP
　　　double-stranded　dideoxy　chain　termination　　　　　　PCR
sequencing　　　　　　　　　　　　　　　　　.
　　　20　　clone　　　　　　　　　　　clone
GenBank database　　　　　　　　　, 10　　clone
　　　　　　　　　　（　　1）.　　　　bP1, bP4, bP31, bP32　bovine-
-casein cDNA ,　　　bP42　bP86　bovine- -lactalbumin cDNA ,
bP22, bP61　bP100　bovine- S1-casein cDNA , bP18　bovine- -casein
cDNA　　　　　　　　.　　bP2　bovine elongation factor alpha
　, 　bP95　bovine stat5a　　　　　　　　　(Schroder　, 1998;
Seyfert　, 2000).
　　4　　clone　　　　　　　　　　　　　　　　　　　（
1). Clone bP50, bP51, bP67　bP88　human secretory carrier membrane
protein, human polyadenylate binding protein 1, human ribosomal protein
L21　hamster ribosomal protein S14 gene　　　　　　　　　.
　　4　　clone　GenBank database　　　　　　　unknown clone
　.　3　clone　　　　　　　　　　　　　　　.

Table 1. Comparison of nucleotide sequences of the selected clones with GenBank database

1. Known genes in bovine species	
Clone Number	Gene Name
bP1, bP4, bP31, bP32	Bovine β-casein mRNA
bP18	Bovine κ-casein mRNA
bP22, bP61, bP100	Bovine αS1-casein mRNA
bP42, bP86	Bovine α-lactalbumin mRNA
bP2	Bovine elongation factor alpha mRNA
bP95	Bovine stat5a mRNA

2. Known genes in other species		
Clone number	Gene Name	% Identity
bP50	1) H. sapiens secretory carrier membrane protein	90
	2) Human thymosin β10 mRNA	80
bP51	Human polyadenylate binding protein gene	95
bP67	Human ribosomal protein L21	80
bP88	Chinese hamster ovary ribosomal protein S14 mRNA	90

Table 2. Partial nucleotide sequences of the clones.

Clone No.	Primer	Reading	Sequences
bP33	pTEx5	159	AAACCATGTGTTTAATATTTTCATTAAACTGTTGTCAATACAATTTC ANGCAAATTGCACCAACTGGAAGTACCAGGCCCAGAAATCATAACTA ATCTTCCAAAGGAATTGCCAAGGTNCTCTTCACTCCAGCTGCACCAT AGNGCAGAGGTGCAGCAG
	pTEx3	61	GGGCAAACCGGAGAAATTTGGCAGTCCGATGGGAAGGCCCCTCAGGA GACCCGCGGGCCGG
bP41	pTEx5	85	GAATTAAATTTAATTAACTTNNCAACTGGNAATTAAATTCAAAATNG GTTAAGGTTTAAGGAATTAATTAATGAATTAAAGGAAA
	T7	62	AAATTGGNNNGGNNNAAAGCGCAATTAATTGAGATTAAAAGGAANNN GGCCGGNNTTAACCC
bP43	pTEx5	218	GGCTTGCACTGTCCTGAAAATTGCTTCTGCAAGGAATGAGTCCAATTA ACAATTGGCAATTCTTTTTTTCCTAATCAGATACCAAATTTGCAGATT TCTGGCTCTGGTTTTATTTTACTATGTTCCATTCAGTTGACTCTTGGA CTGCACAGGCTAAGGAAACATGCATATAATGTCAGCATACTGTGAAGC ATGATAAGTAGTAATCATTGAGCGCG
	pTEx3	190	TTCCTAATCAGATACCAAATTTGCAGATTNCTGGCTCTGGTTTTATTT TACTATGTCCATTCAGTTGACTCTTTGTCATCTGCACAGGCTAAAGGA AACATGCATATAATGTAGCATACTGTGAATCATGATAAGTAGTAAATN TTGAGGCTGTCTGCTGTGGTGCCCTASTCACTCATCGCTAGGCGGC
bP52	pTEx5	156	CTTTACTGCTCTAATGCTGCTAGTTTTAGTCTTTAGCACACTAGGTG GTTTATGCCTTTTATAGCTAGAAAAAACCTAACAGCTATTACTATAAC TGGGGTGGGGGTGGTCAGGACTGGGTGGGCGGCTGCTGAGCCCTTTG TGGAGTTCTCTCGTGTTGGTGCTTCTCGTC
	T7	177	ACATTCAGGCTCTTATAATGTCAGGGGAGGTAGGAAGAAGTCAGTGC TGAGGTCTCTGGGAAATTCTGCAGACCTTGTAGGCTCTCTAAGCCCC TCTAGCAACATCTGGATATGGGCCTTGATATTCATGGAGTCCTTGGT AGGTTGTCGCTGAGCTCTGTGAGGAGACTGCTCGAC
bP88	pTEx5	218	GTCAGCGCTCAGAGCCCTCGCCCCGCTCAGGATGAAGATTGGGCGGATTG AGGATGTCACCCCCATCCCCTCCGACAGCACCCGCAGAAGGGGGGTCGC CGTGGTCGCCGTCTGTGAACAGGACTTCTCAAATTATTGCTTTCTGTTA ATAAATTRVTTTGATGTAAGCGCAAAAAAAAAAAAAAAAAAATCTAGACT CGAGCAAGCTTATGCATGCATGC
	pTEX3	129	GACGTTACATCAAAGSAATTTRTTAACAGAAAGCRRTAATTTGAGAAGT SCTGTTCASAGRCGGCGRCCACGGCGRSCCGCCTTGCTGCGGGTGSTGT CGGAGGGGATGGGGGTGACATCCTCAAKCCGCCCAATCT

. Northern   cDNA clone

                    clone
     ,   ,   total RNA
   northern   .
        11   mRNA
   .   -casein,   -casein,   -S1-casein,
-lactalbumin
        8
   .   ,   ,   ,   ,   ,
   ,   ,   .
           12   bovine elongation factor   bP2
northern   .
           13   bovine stat5a   . Stat5a
                                          5
          7   8
  . Stat5a   . Stat5a

       stat5a   , stat5a
                                                        .

           14   human secretory carrier membrane protein
clone bp50   . Clone bP50   5
           7   ,   ,
              . Clone bP50   (lung),
(spleen)   8   ,   (stomach)
    (uterus)   .
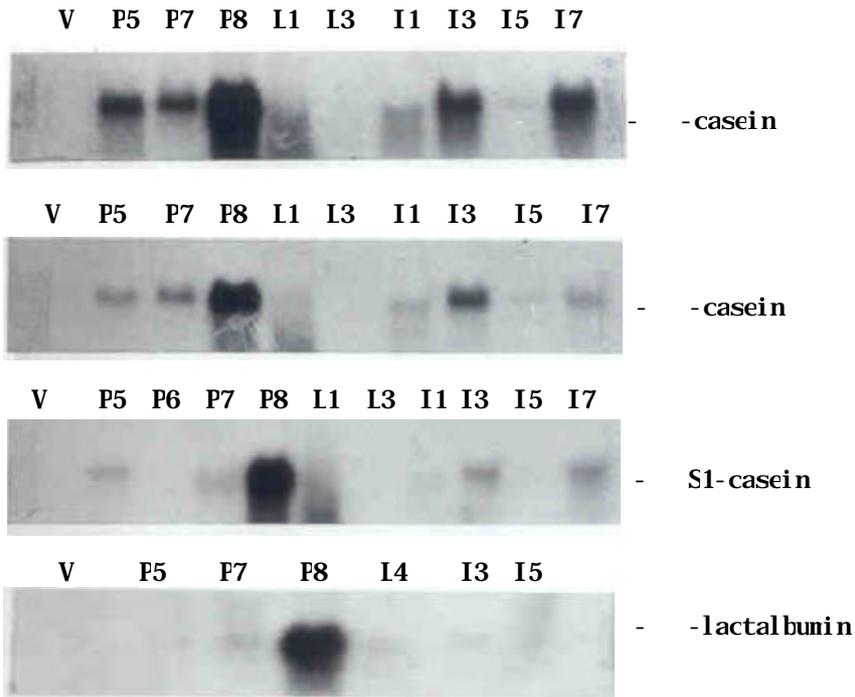
Figure 11. Northern analysis of known clones (milk protein genes) in various tissues of Holstein cow. The 20 ug of total RNA isolated at virgin (V), pregnant 5 months (P5), pregnant 6 months (P6), pregnant 7 months (P7), pregnant 8 months (P8), lactating 1 month (L1), lactating 3 months (L3), involution 1 week (I1), involution 3 weeks (I3), involution 5 weeks (I5), and involution 7 weeks of bovine mammary gland were analyzed by northern method.
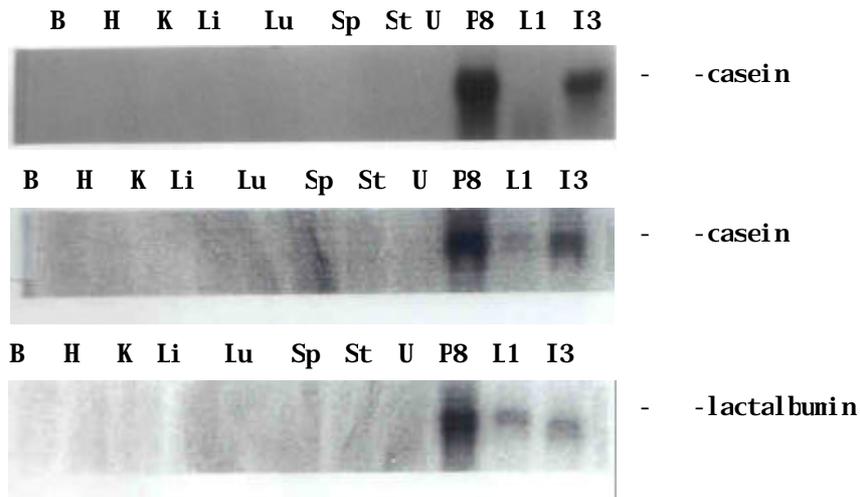
Figure 11. Northern analysis of known clones (milk protein genes) in various tissues of Holstein cow (continued). The total RNA isolated from bile(B), heart(H), kidney(K), liver(L), lung(Lu), spleen(Sp), stomach(St), uterus(U) tissues of holstein cow were analyzed by northern method.
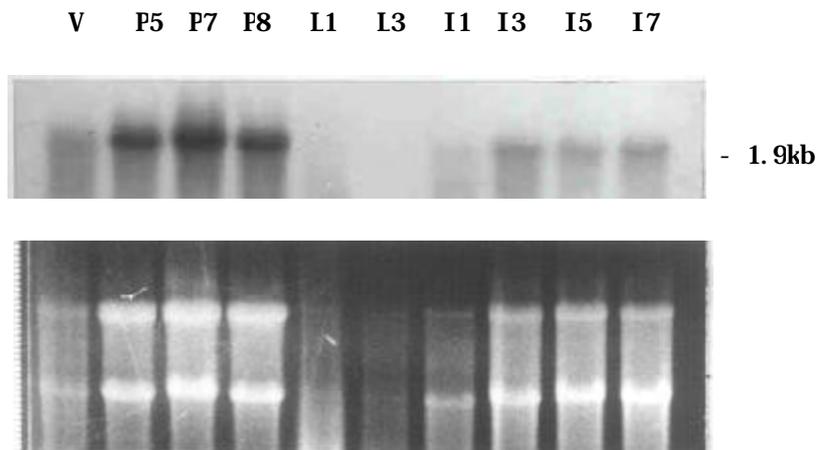
Figure 12. Northern analysis of clone bP2 (bovine elongation factor   ) in bovine mammary tissues.
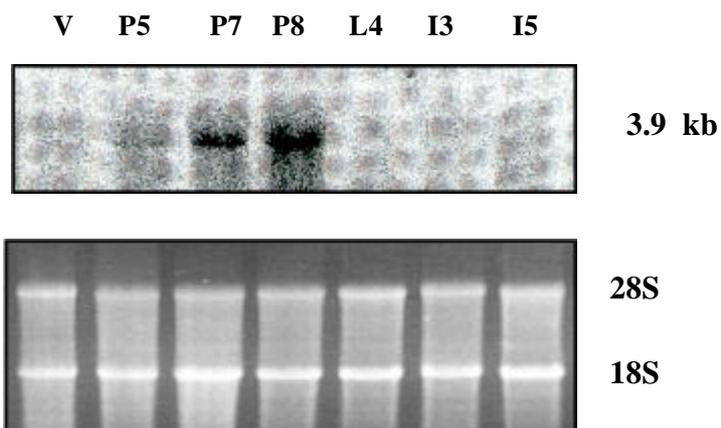
Figure 13. Northern analysis of the stat5a gene in bovine mammary gland at several physiological states. Twenty micrograms of total RNA isolated from mammary gland of Holstein cow at virgin (V), pregnant 5 months (P5), pregnant 7 months (P7), pregnant 8 months (P8), lactating 4 months (L4), involution 3 weeks (I3), and involution 5 weeks (I5) were separated on a 1% formaldehyde/agarose gel. Total RNA was analyzed by northern method. That equal amounts of RNA were present in each lane was checked by the intensities of 28S and 18S bands as shown.
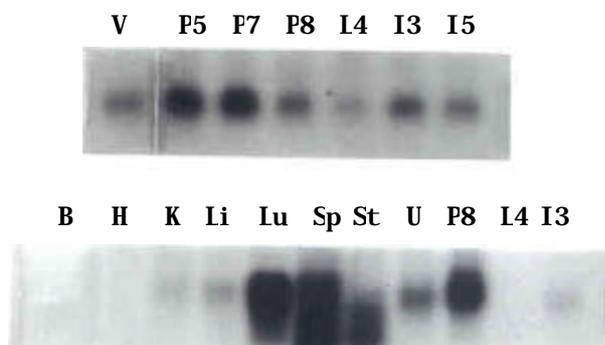
Figure 14. Northern analysis of the clone bP50 that showed high similarity with human secretory carrier membrane protein.

15 bovine polyadenylate binding protein 1 gene (PABP)

. PABP gene

7   8   ,

. PABP

,   ,   ,   ,   ,   ,   ,   ,

.

16 human ribosomal protein L21 gene   clone bP67
northern   ,

.   northern

,   ,   ,   .

17 hamster ribosomal protein S14   clone bP88

. Clone bP88   7   8

,   ,   .

,   ,   ,   ,

.   ,

3   total RNA loading

.
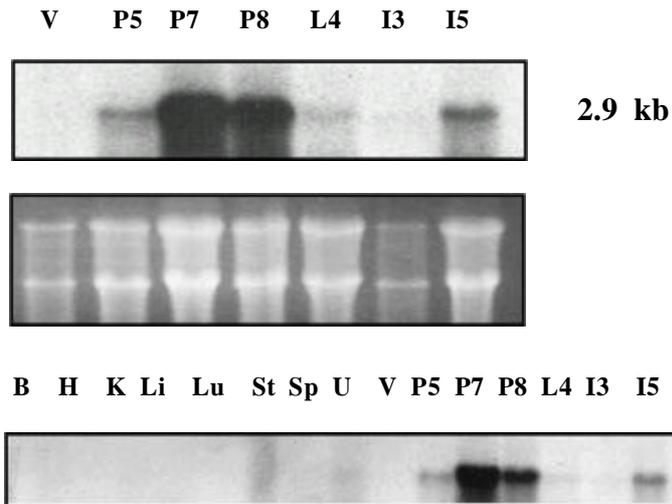
Figure 15. Northern analysis of bovine polyadenylate binding protein 1 gene. Twenty micrograms of total RNA isolated from bile (B), heart (H), kidney (K), liver (L), lung (Lu), stomach (St), spleen (Sp), and uterus (U) and virgin (V), pregnant 5 months (P5), pregnant 7 months (P7), pregnant 8 months (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 32P-labeled cDNA probe. That amounts of RNA were present in each lane was checked by the intensities of 28S and 18S bands as shown.
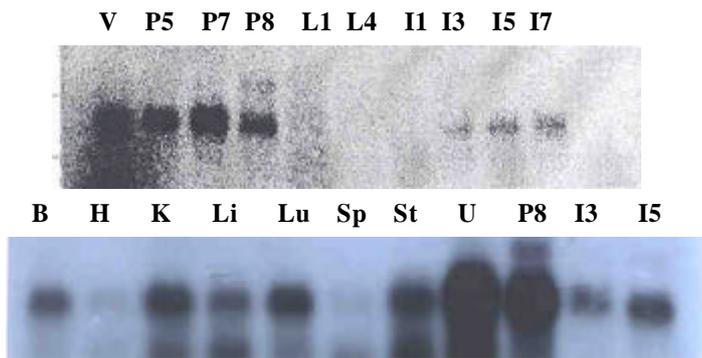
Figure 16. Northern analysis of the clone bP67 that showed high similarity with human ribosomal protein L21 gene.
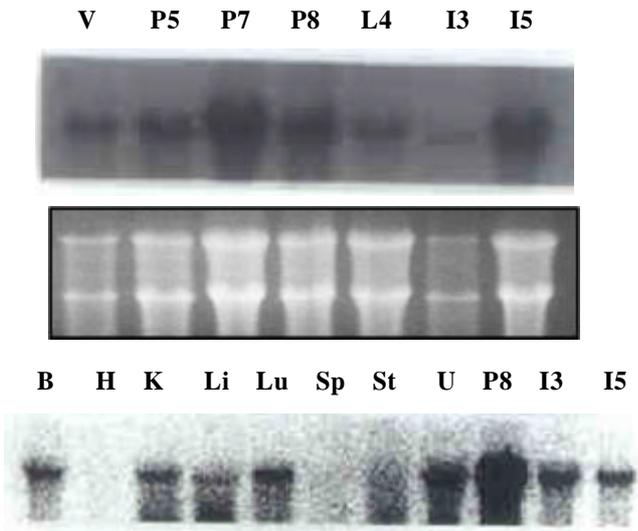
Figure 17. Northern analysis of the clone bP88 that showed high similarity with hamster ribosomal protein S14 gene.

. cDNA clone

GenBank database   bovine
                                    2   clone
                                . Clone bP51   human polyadenylate
binding protein gene   , clone bP88   hamster ribosonal
protein S14 gene   .
                            , clone bP51   3'
BanH I site   , clone bP88   5'   Ava I   Hind III site
            (18).

bP51
                                        BanH I(211bp)
5'   3'

bP88
                                Ava I(196bp)
5'   3'

                                Hind III (203bp)
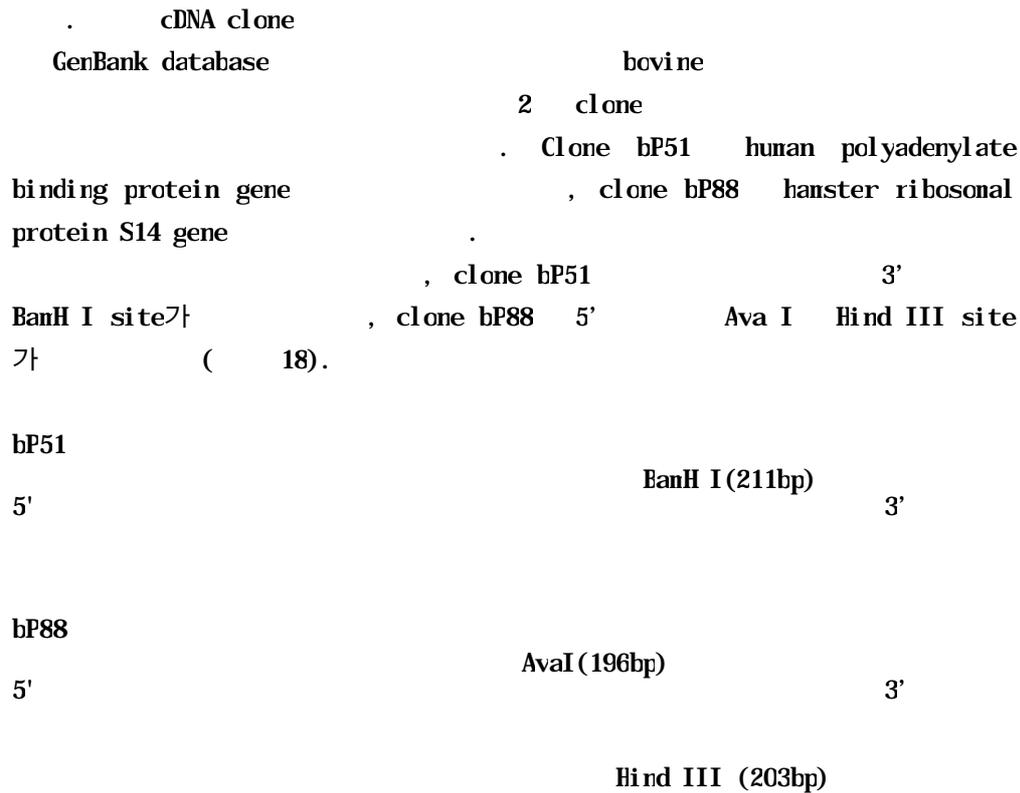
Figure 18. Restriction enzyne nap of clones bP51 and bP88.

    Clone   GenBank data   clone bP51   human
polyadenylate binding protein   honology   .   ,
                    coding   , bovine PABP   full
length cDNA   PCR   cloning   .
        8   total RNA   template
oligo(dT) priner   first-strand cDNA   . First-strand
cDNA   template   PCR   1.9kb   band
            (19). PCR product   pGEM-T easy vector (Pronega)   ligation
        transfornation   sequencing   plasnid   . EcoRI
        , PCR product   plasnid   (20).

M P

1.9 kb
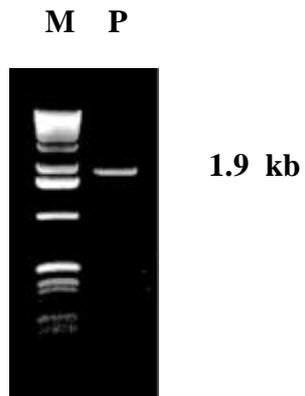
Figure 19. The RT-PCR of bovine polyadenylate binding protein 1 cDNA. The cDNA of bovine PABP gene were amplified by RT-PCR using total RNA of 8 months pregnant mammary tissues (P) and a 5' degenerate primer designed by human and mouse amino acid sequences and a 3' primer based on sequencing results in this study. The PCR products were electrophoresed on a 1% agarose gel. M; 1 kb ladder.

M 1 2 3 4
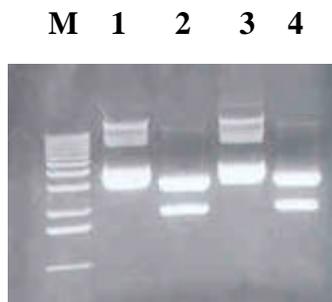
Figure 20. Cloning of PCR product into pGEM-T easy vector. M: 1 kb DNA ladder, Lanes 1 and 3: undigested plasmid. lanes 2 and 4: EcoRI digested plasmid after transformation.

RT-PCR   cloning   clone   ,
1,911   ,   636   coding   start
codon   stop codon   full-coding sequences   (
   21). GenBank data   ,   human polyadenylate binding
protein 1   95% identity, mouse polyadenylate binding protein 1   94%
identity   (Grange   , 1987; Wang   , 1992).
   , human   mouse polyadenylate binding protein 1   100%   99%
identity   (22).   ,   bovine polyadenylate
binding protein 1 (PABP)   full-coding   cDNA
cloning   .
   PABP   eukaryotic mRNA   3' poly (A) tail   mRNA
      translation   (Bernstein   Ross,
1989; Blobel, 1973; Galli, 1998).   eukaryotic mRNA   5' end
      translation initiation   (Gingras   ,
1999).
               PABP   .   ,
               PABP
                         mRNA   translation
         ,
         .

```
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
cagccggcggacgcggagcgtgctctttggacaccatgaattttgatgtgataaagggcaagccagtacgcatcatgtggtctcagcgcgat   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
ccatcacttcgcaaaagtggagtgggcaacatattcattaaaaatttggataaatccattgataataaagcactgtatgatacattttct   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  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
```

Figure 21. 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.

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: QPAPPSGYFMAAIPQTQNRAAYYPPSQIAQLRPSPRVTAQGARPHPFQNMPGAIRPAAPRPPFSTMRPASSQVPRVMSTQ 480
HUMAN : ....
MOUSE : ....

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

BOVINE: MLGERLFPLIQAMHPTLAGKITGMLLEIDNSELLHMLESPESLRSKVDEAVAVLQAHQAKEAAQKAVNSATGVPTV 636
HUMAN : ....
MOUSE : ....S..

Figure 22. 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.

4.

. Stat5a cDNA　　　vector

　　　　　　　　　stat5a

　　　　　　　　　　　　　　　　　　.

prolactin　　　　　　　　　　　　　　　stat5a

　　　　(Watson　Burdon, 1996), stat5a

　　　　　　　　　　.　　　　,　　　　　　　stat5a

.

　　　　　　　　　　　　　　bovine　stat5a　　cDNA　(2.6kb)

eukaryotic expression vector　pBK-CMV vector

　　　　. Bovine stat5a insert　pUC18-Stat5a　　Sac I(5')　Sal I(3')

　　　　　　　　　　　, pBK-CMV vector (4.5kb)　multi-cloning site

Sac I(5')　Xho I(3')　　　　　　　　　　　　　　QIAEX

II Gel　Extraction kit　　　　　　　　　(23).　　　stat5a

insert (2.6kb)　pBK-CMV vector (4.5kb)　T4 ligase　cohesive end

ligation　　　. Ligation product (7.1kb)　XLI-blue MRF'competent

cell　transformation　LB/kanamycine agar plate　plating　14　3

7　　incubation　. Colony　　　　　LB/Kanamycine broth

,　　plasmid　QIAGEN Plasmid mini Kit　　　　　　.　　.

·　　pBK-CMV-Stat5a plasmid(pC-S)　　Sac I (5')　one

cut　plasmid size 7.1kb　　　　　(24A).　　　Not I

(5', 3'　　　)　　　　stat5a (2.6kb) insert　pBK-CMV (4.5kb)

vector　　　ligation　　　　(24B).
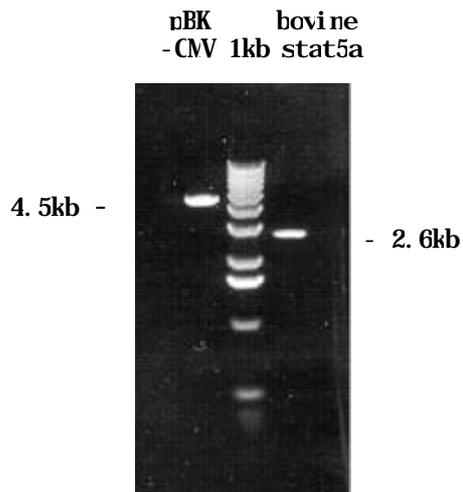
Figure 23. Preparation of pBK-CMV vector and bovine Stat5a insert. pBK-CMV vector was digested by SacI(5') and Xho I (3'). Bovine Stat5a was digested by Sac I(5') and Sal I (3'). 1kb; 1 kb ladder.
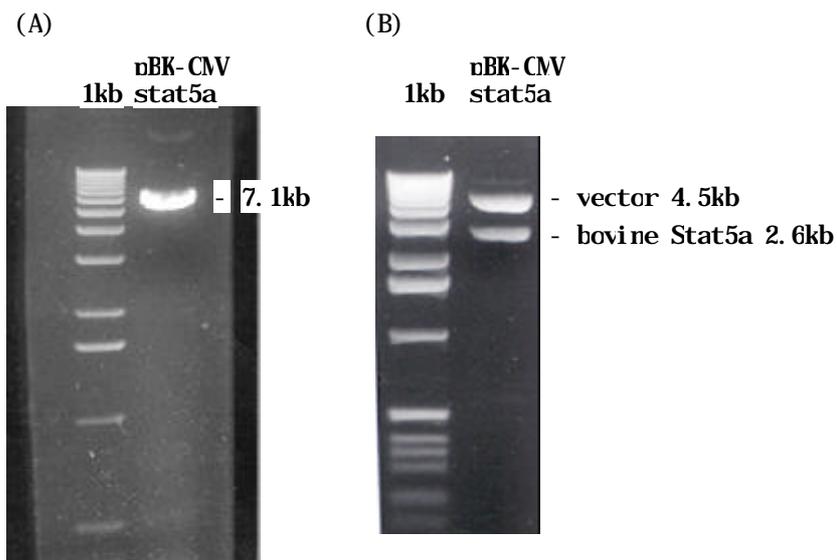
Figure 24. Identification of cloned pBK-CMV stat5a.(A) Size of pBK-CMV stat5a was checked by Sac I digestion. (B) Insertion of bovine stat5a into pBK-CMV vector was checked by Not I digestion. 1kb; 1 kb ladder.

Eukaryote cell HC11   Nhe I   Sac
I   200bp   ß-galactosidase   promoter   start codon
AUG   fill-in   blunt end   ligation   .
Ligation   product   (6.9kb)   transformation   colony
plasmid   ·   . Plasmid   pBK-CMV vector   stat5a
insert   Xba I   6.9 kb   (
25A),   Not I   4.3 kb   pBK-CMV vector   2.6kb   Stat5a
insert   (25B). Neo   control
vector (pNeo)   4.3kb band   (26).

(A)   (B)

1kb   pstat5a   pstat5a 1kb

- 6.9kb

4.3kb

2.6kb
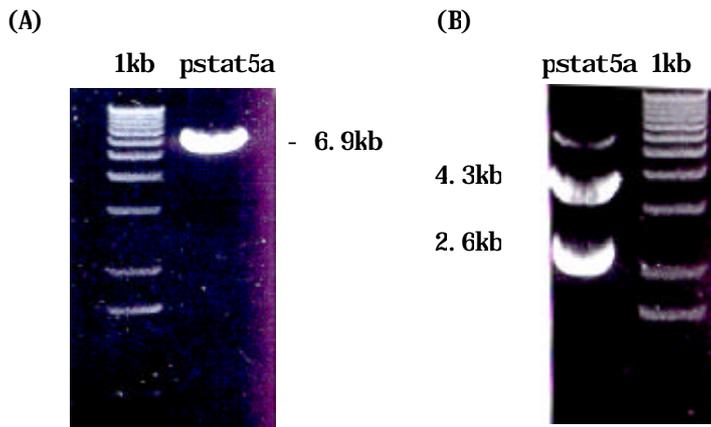
Figure 25. Construction of pstat5a expression vector. To increase
            efficiency of Stat5a expression in eukaryotic cells, about
            200 bp region containing bacterial lac promoter and AUG codon
            was removed from pBK-CMV vector by Nhe I and Sac I digestion.
            Linear fragments were blunt-ended by fill-in reaction and
            self-ligated, generating final pstat5a expression vector.
            After transformation, the recombinant plasmid was purified.
            (A) Size of pstat5a was checked by one-cut Xba I digestion,
            generating 6.9kb band containing pBK-CMV vector and bovine
            Stat5a insert. (B) Correct size of pstat5a vector containing
            pBK-CMV vector (4.3kb) and bovine Stat5a insert (2.6kb) was
            also confirmed by Not I digestion. 1kb; 1 kb ladder.

4.3kb
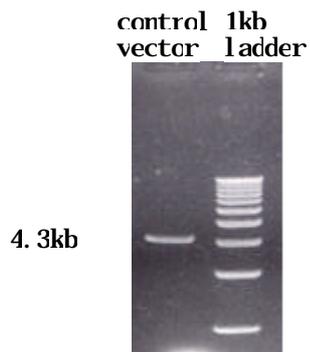
Figure 26. Construction of control vector.

. HC11   stat5a cDNA

mouse normal mammary epithelial cell line   HC11 cell
bovine stat5a   . Cell
         ampule   liquid nitrogen tank   seeding 48
         monolayer   confluency   (27A),
subculture   .
  HC11 cell   1 X 105 HC11 cell   35mm dish   2ml
media   seeding   18   24   50   80% confluent
                   . Stat5a recombinant DNA-liposome complex
transfection   . Transfection   200 ug/ml G418   G418
               transfected cell   selection   .   3
         10-14   selection   G418   colonies
. Control vector   stat5a vector   lipofectamine
          4   ,   12   colonies   (27B). Cloning
cylinder   trypsin   1 drop   repeated pipetting   cell
   dissociation   24 well plate, 6 well plate
          subculture
          ,   DNA   RNA   .

A   B
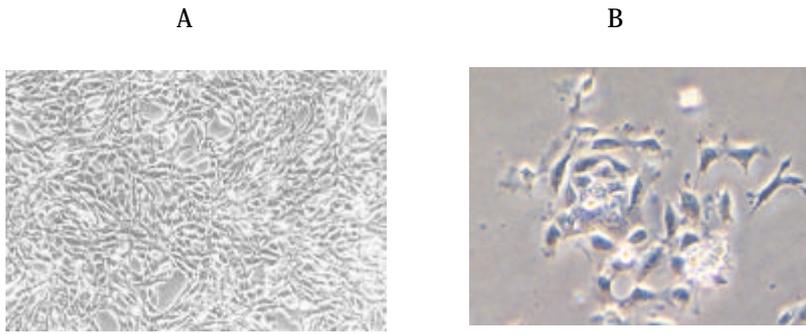
Figure 27. HC11 mammary epithelial cell culture and colony formation of HC11 cells containing bovine stat5a cDNA after 14 days of G418 selection. (A) Mammary epithelial cell line were grown in RPMI 1640 medium with 10% fetal bovine serum, 5 / insulin, 10ng/ epithelial growth factor, and 50 / gentamycin in a 5% CO2 at 37 . Confluent cells were formed at around 48 hours. (B) Colonies of HC11 cells containing bovine stat5a cDNA were formed after 14 days of G418 selection.

. Stat5a
Confluency   genomic DNA   40 cycle   PCR
. Control vector
       CMV5' primer   CMV3' primer   , Stat5a vector
       CMV5' primer stat5a3' primer   .
PCR   , control vector   0.42kb band
     (28A), stat5a cDNA   0.7kb
band   (28B).

(A) pNeo   pstat5a   (B) pNeo   pstat5a
   2 1 M 2   1 1kb   2 1 1kb 4 3 2

- 0.42kb   -0.70kb
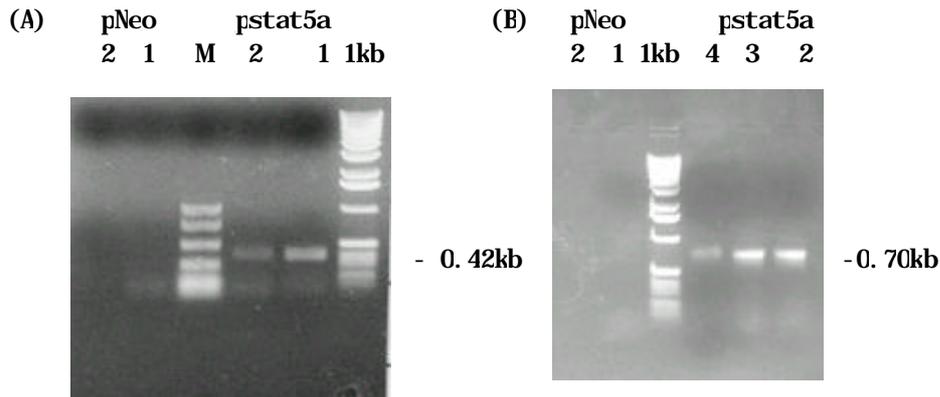
Figure 28. The PCR identification of stat5a recombinant DNA integration
          into genomic DNA of HC11 cells. (A) Genomic integration of
          control vector (pNeo) was confirmed by PCR using genomic DNA
          isolated from pNeo-transfected HC11 cells and CMV5' primer and
          CMV3' primer. M: PCR marker, 1 kb: 1 kb ladder. (B) Genomic
          integration of stat5a recombinant vector (pstat5a) was
          confirmed by PCR using genomic DNA isolated from
          pstat5a-transfected HC11 cells and CMV5' primer and Stat5a 3'
          primer. 1 kb: 1 kb ladder.

       . Northern   stat5a mRNA

                                    total RNA   northern
                    .   confluent
    PBS   washing   monolayer cell   denaturing solution overlay   .
                lysis   scraper
homogenization   . Lysed   total RNA   labeled-stat5a
insert   probe   hybridization   .   , control
vector   stat5a mRNA   detection   , stat5a
cDNA   mRNA message   (29).

pNeo   pstat5a

Figure 29. Northern analysis of stat5a gene expression in transfected
            mammary epithelial HC11 cells. The twenty micrograms of total
            RNA were isolated from pNeo- and pstat5a-transfected cells. The
            mRNA levels of bovine stat5a gene were analyzed by northern
            method.

     . Stat5a cDNA
    Stat5a cDNA   control
vector (pNeo)   stat5a expression vector (pstat5a)
                   24   , 48   72   MTT assay
   .

    Stat5a   control vector
         24, 48   72   26%, 95%   85
(30).   (insulin, prolactin   dexamethasone)
   , prolactin   dexamethasone   control   73%   71%
           insulin   58%   .
bovine stat5a   .
                      stat5a
   (Humphreys   Hennighausen, 1999), stat5a knock-out
                                                           (Liu   ,
1997;   Akira,   1999).   Stat5a   homodimer   stat5b
heterodimer   ,   HC11
lactogenic   stat5a homodimer
   (Cella   , 1998).   stat5a
                      bovine stat5a   homodimer
                        .
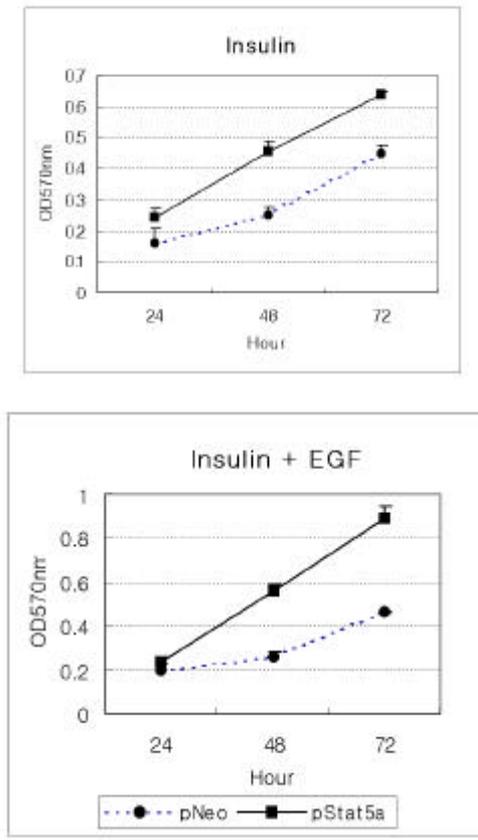
Figure 30. Proliferation of mammary epithelial HC11 cells expressing bovine stat5a gene under various hormonal treatments. pNeo- and pstat5a-transfected HC11 cells were cultured in RPMI1640 media containing 10% FBS with insulin, insulin plus EGF, insulin plus prolactin, and insulin plus dexamethasone. Cell proliferation was quantitated by MTT assay as described in Materials and Methods at 24h, 48h and 72h after seeding. The optical densities (OD570nm) are directly proportional to the number of viable cells. The values are the averages + the standard deviations (n=5). Overall mean are the averages of all hormonal treatments. All data except insulin plus prolactin and insulin plus dexamethasone treatments at 24h (no differences: p>0.05) showed statistical differences (p<0.01) between stat5a-transfected cells and control cells at each time.
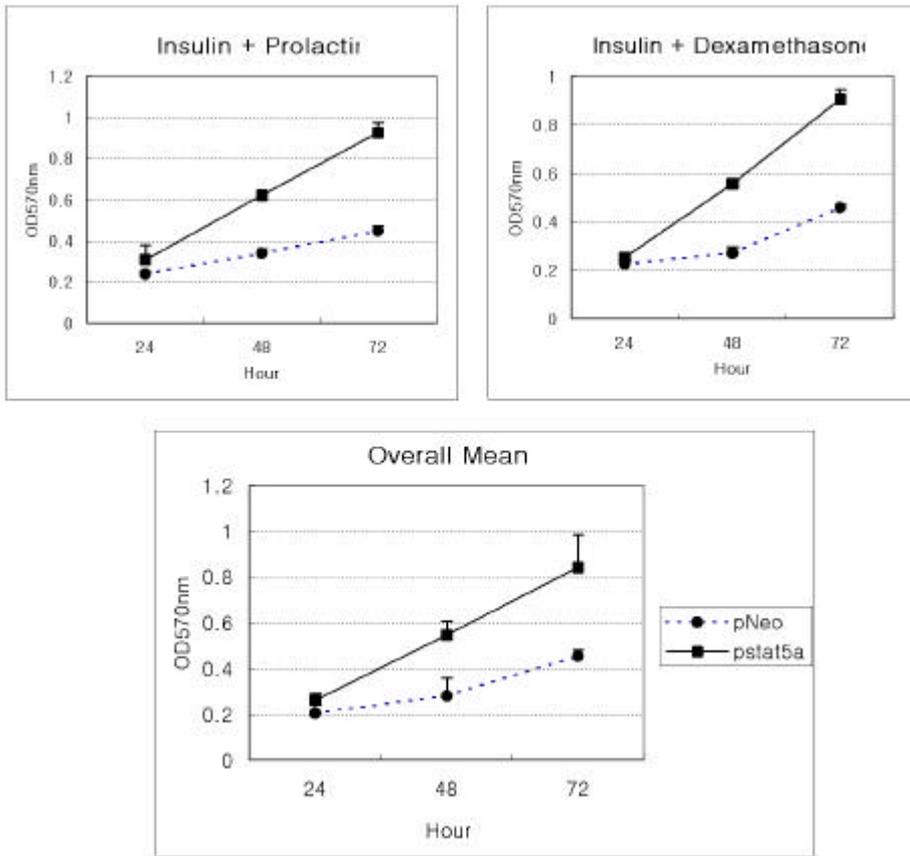
Figure 30. Proliferation of mammary epithelial HC11 cells expressing bovine stat5a gene under various hormonal treatments (continued).

5.

. Stat5a

stat5a

expression vector pGEX vector

. pGEX GST fusion vector fusion protein

affinity-purification . Tac promoter
1-5mM IPTG induction 26 kDa glutathione
S-transferase (GST) fusion protein , thrombin
fusion protein GST carrier protein .

Stat5a insert vector reading frame Not I
site 5' linker primer (5'-AGCGGCCGCATGGCGGGCTGGATC-3')
design . pBK-CMV vector cloning Stat5a cDNA 5'
linker primer 3' T7 primer Taq polymerase (Takara) PCR
2.6kb stat5a band (31). stat5a PCR
product 1 low melting agarose gel band
phenol extraction . PCR product stat5a
Not I (Takara) cohesive end ,
phenol extraction . pGEX-4T-3 vector Not I
cohesive end . Vector self
ligation CIAP (Takara) 5'
dephosphorlyation . Ligation stat5a cDNA (2.6kb) vector
(4.9kb) size (32). Not I stat5a cDNA
(2.6kb) vector (4.9kb) ligation ligation product DH5 cell
transformation . Ligation ampicillin resistant colony
plasmid Not I , 4.9 kb pGEX-4T-3 vector
2.6kb stat5a gene insert ligation (
33A).

Stat5a
1kb PCR product

2.6kb
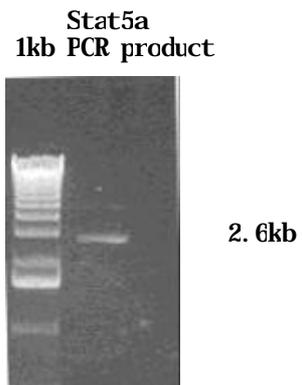
Figure 31. PCR amplification of stat5a cDNA. PCR was performed using linker primer and T7 primer.

stat5a 1kb pGEX-4T-3

2.6kb-

- 4.9kb
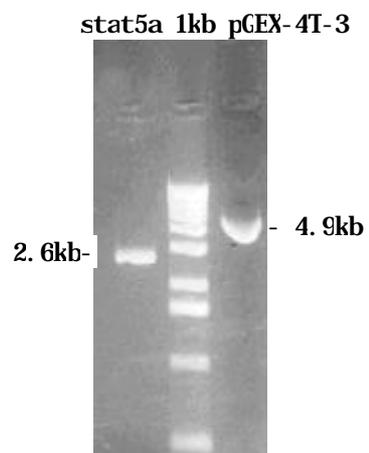
Figure 32. Preparation of pGEX-4T-3 vector and bovine stat5a cDNA insert. The pGEX-4T-3 vector and stat5a PCR product were digested by Not I, purified using phenol extraction method, and electrophoresed on 1% agarose gel. 1kb; 1 kb ladder.

.   E. coli

  Construction   stat5a expression vector   host
BL21 E.coli cell   transformation   .   colonies
ampicillin   plasmid   Not I   ,
stat5a cDNA   (33B).

A   **B**

pG3-S
1   2 1kb   pG3-S_1kb

pGEX-4T-3 (4.9kb)-   pGEX-4T-3 (4.9kb)-
Stat5a (2.6kb) -   Stat5a (2.6kb) -
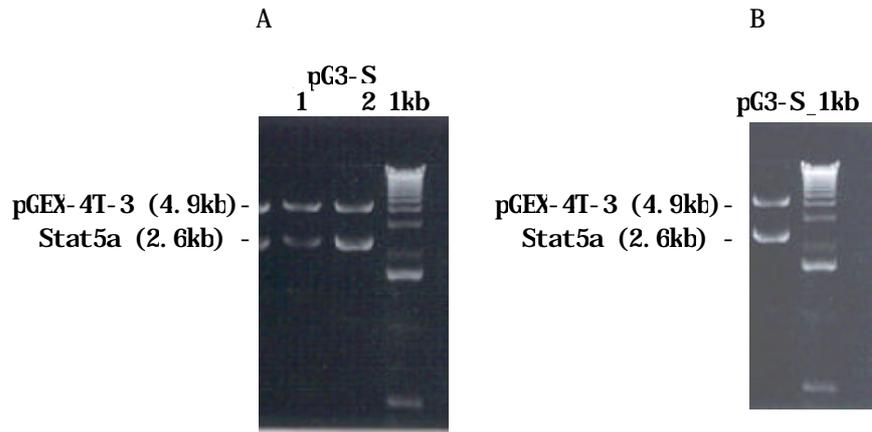
Figure 33. Identification of bovine Stat5a expression recombinant DNA
          after transformation into DH5   (A) and BL21 (B) cells.
          Plasmid DNA isolated from ampicillin-resistant colonies after
          transformation of ligation product into either DH5   or BL21
          cells was digested with Not I, and the digests were
          electrophoresed on 1% agarose gel. 1kb; 1 kb ladder.

. E. coli   stat5a

pGEX-4T-3-stat5a expression vector   BL21   GST-stat5a fusion
protein   LB/amp. plate   single colony   0.1mM
IPTG   .   0h, 2h, 4h, 6h   cell
1ml   protein loading buffer   100   crude
    lysis   10% SDS-PAGE gel   SDS-PAGE   .   0.1mM
IPTG induction   GST-stat5a fusion protein (113kD + 26kD =
139kD)   (34).

pGC3-stat5a_protein
IPTG induction   0h   2h   4h   6h

- 139kD
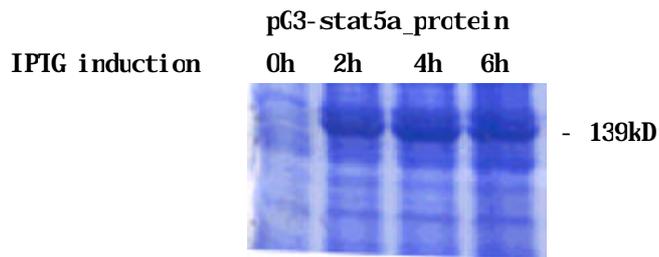
Figure 34. Expression of GST-stat5a fusion protein in BL21 cells. BL21
         cells containing pGC3-stat5a expression vector were collected at
         0, 2, 4, and 6h after 0.1 nM IPTG induction and lysed by boiling
         method containing loading buffer. Cell lysate was subjected to
         SDS-PAGE.

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cDNA library   cDNA library   .

stat5a

. Stat5a

stat5a   , stat5a

. Stat5a

,

.   polyadenylate binding protein 1 cDNA
cloning   , 1,911
,   636   . Bovine polyadenylate binding
protein 1   .
Polyadenylate binding protein 1   eukaryotic mRNA   ,
(translational efficiency)
.

Stat5a   polyadenylate binding protein 1
,   DNA
.   stat5a
polyadenylate binding protein 1   ,
.

,   .

1.   (SCI): 2   , 2

1) Lee, M., I. Hwang, Y. Choi and M. Baik (1998) Sequence of a cDNA encoding mouse ribosomal protein S14. Biosci. Biotechnol. Biochem. 62(3), 573-574.
2) Baik, M. G., M. J. Lee and Y. J. Choi (1998) Gene expression during involution of mammary gland (Review). Int. J. Molecular Medicine 2, 39-44.
3) Jeon, D. H., Y. J. Choi and M. G. Baik. (2000) Expression of stat5a

gene in bovine mammary gland and its effect on proliferation of mammary epithelial cells. Biosci. Biotechnol. Biochem. (submitted)

4) Kim, J. H., D. H. Jeon, Y. J. Choi and N. G. Baik. (2000) Cloning and expression of bovine polyadenylate binding protein 1 cDNA in mammary tissues. Asian-Australasian J. Animal Sciences. (submitted)

2.   :   5 ,   1

1) Kim, H. A., D. H. Jeon, J. H. Kim and N. G. Baik. (1996) Induction of genes during the development of mammary gland.   .
p177.

2)   ,   ,   . (1997) Cloning of pregnant-induced cDNAs from bovine mammary gland.   . p101.

3)   ,   ,   ,   . (1997) Differential expression of genes in mammary gland.   . p104.

4) Kim, J. H., I. T. Hwang, Y. J. Choi and N. G. Baik. (1998) Partial cloning of pregnant-induced cDNA from bovine mammary gland. Proceedings of 8th World Conference on Animal Production. Vol II, 642-643.

5) Kim, J. H., Y. J. Choi and N. G. Baik. (1999) Cloning of bovine polyadenylate binding protein cDNA from mammary gland.
        . p125.

6) Jeon, D. H., J. Y. Kim, Y. J. Choi and N. G. Baik. (2000) Expression of bovine stat5a gene and its effect on proliferation of mammary epithelial cells.   . p63.

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Akira, S. 1999. Functional roles of STAT family proteins: lessons from knockout mice. Stem Cells 17:138-146.

Ball, R. K., R. R. Friis, C.-A. Schonenberger, W. Doppler and B. Groner. 1988. Prolactin regulation of -casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. EMBO J. 7:2089-2095.

Bernstein, P. and J. Ross. 1989. Poly(A), poly(A) binding protein and regulation of mRNA stability. Trends in Biochem. Sci. 14:373-377.

Blobel, G. 1973. A protein of molecular weight 78,000 bound to the polyadenylate region of eukaryotic messenger RNAs. Proc. Natl. Acad. Sci. USA 70:924-928.

Borellini, F. and T. Oka. 1987. Growth control and differentiation in mammary epithelial cells. Environmental Health Prospects 80:85-99.

Cella, N., B. Groner and N. E.Hynes. 1998. Characterization of Stat5a and Stat5b homodimers and heterodimers and their association with the glucocortiocoid receptor in mammary cells. Mol. Cell. Biol. 18:1783-1792.

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

Cochran, B. H., J. Zullo, I. M. Verna and C. D. Stiles. 1984. Expression of the c-fos gene and fos-related gene is stimulated by platelet-derived growth factor. Science 226:1080-1082.

Gallie, D. R. 1998. A tale of two termini: a functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. Gene 216:1-11.

Gingras, A.-C., B. Raught and N. Sonenberg. 1999. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. 68:913-963.

Grange, T., C. N. de Sa, J. Oddos and R. Pictet. 1987. Human mRNA polyadenylate binding protein: evolutionary conservation of a nucleic acid binding motif. Nucleic Acids Research 5:4771-4787.

Hedrick, S. M., D. I. Cohen, E. A. Nielsen and M. M. Davis. 1984. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. Nature 308: 149-153.

Humphreys, R. C. and L. Hennighausen. 1999. Signal transducer and activator of transcription 5a influences mammary epithelial survival and tumorigenesis. Cell Growth and Differentiation 10: 685-694.

Inagawa, W. I., G. K. Bandyopadhyay and S. Nandi. 1990. Regulation of mammary epithelial cell growth in mice and rats. Endocrine Reviews 11: 494-523.

Joshi, K., J. A. Smith, N. Perushinghe and P. Monoghan. 1986. Cell proliferation in the human mammary epithelium. Am. J. Pathol. 124: 199-206.

Knight, C. H. and M. Peaker. 1982. Development of the mammary gland. J. Reprod. Fertil. 65: 521.

Knight, C. H. and C. J. Wilde. 1987. Mammary growth during lactation: Implications for increasing milk yield. J. Dairy Sci. 70: 1991-2000.

Knight, C. H., J. E. Hillerton, R. M. Teverson and A. Winter. 1992. Biopsy of the bovine mammary gland. Br. Vet. J. 148: 129-132.

Liu, X., G. W. Robinson, K. U. Wagner, L. Garrett, L. Wynshaw-Boris and L. Hennighausen. 1997. Stat5a is mandatory for adult mammary gland development and lactogenesis. Genes Dev. 11: 179-186.

Luo, G., G. An and R. Wu. 1994. A PCR differential screening method for rapid isolation of clones from a cDNA library. BioTechniques 16: 672-675.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65: 55-63.

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular cloning. A Laboratory manual. 2nd eds. Cold Spring Harbor Laboratory Press, New York.

Schroder, P., L. Meyer, T. T. Wheeler, H. - J. Thiesen and H. - M. Seyfert. 1998. Cloning and sequencing of the bovine STAT5A cDNA reveal significant sequence divergence with ovine. Biochimica et Biophysica Acta 1398: 99-105.

Seyfert, H. M., C. Pitra, L. Meyer, R. M. Brunner, T. T. Wheeler, A.

Molenaar, J. Y. McCracken, J. Herrmann, H. J. Thiesen and M. Schwerin. 2000. Molecular characterization of STAT5A- and STAT5B- encoding genes reveals extended intragenic sequence homologeneity in cattle and mouse and different degrees of divergent evolution of various domains. J. Mol. Evolution 50:550-561.

Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. PNAS, USA 77:5201-5205.

Topper, Y. J. and C. S. Freeman. 1980. Multiple hormone interactions in the developmental biology of the mammary gland. Physiol. Rev. 60:1049-1106.

Tucker, H. A. 1966. Regulation of mammary nucleic acid content by various suckling intensities. An. J. Physiol. 210:1209.

Tucker, H. A. 1987. Quantitative estimates of mammary growth during various physiological states: a review. J. Dairy Sci. 70:1958-1966.

Wang, M. Y., M. Cutler, L. Karinpour and K. C. Kleene. 1992. Nucleotide sequence of a mouse testis poly (A) binding protein cDNA. Nucleic Acids Research 20:3519.

Watson, C. J. and T. G. Burdon. 1996. Prolactin signal transduction mechanisms in the mammary gland: the role of the Jak/Stat pathway. Rev. Reprod. 1:1-5.

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30

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Staphylococcus aureus

superantigen   (Staphylococcal

Enterotoxin : SE)   .   *S.*

aureus

.   *S. aureus*

SE   multiplex polymerase chain reaction (nPCR)

,   *S. aureus*   toxin

typing   .

S. aureus   SE

SEC   ,   SEC

SEC

.

.

,

.

,

.

.

,

.

,

, -interferon nitogen

.

Natural Resistance Associated Macrophage Protein (Nramp)

,

.

,

antigen presenting cell (APC) T

. , Nramp Nramp ,

Mycobacteria, Brucella, Salmonella, Listeria

phagolysosome ,

.

Nramp1 mRNA reverse transcription-PCR

(RT-PCR) ,

Nramp1 mRNA ,

.

, major histocompatibility complex (MHC) class II

APC , dendritic B T

. MHC bovine lymphocyte antigen

(BoLA)

Th .

BoLA bovine chromosome 23 short arm , BoLA

class I, II, III antigen .

, BoLA class II BoLA

class II   ,   ,   ,

          . BoLA   DQ   DR   ,   *BoLA-DR* region   DRA
DRB   ,   DRA   monomorphic gene, DRB   polymorphic gene

                                                                          .

       DRB3   HLA (human lymphocyte antigen)-DRB1
                ,   (1 domain)
                                   , innate immunity   adaptive immunity
                         .   , BoLA DRB3.2 exon
                         .   *BoLA* DRB3.2 exon
       PCR   RFLP
       allele type   ,
       BoLA DRB3.2   type   BoLA
                      .

       ,   *S. aureus*
                                        SEC
                         ,

          ,
                           ,
                      .

2

1.   *Staphylococcus aureus*   prevalence

.

Multiplex polymerase chain reaction (PCR)
          Table 1   .

Table 1. Bacterial strains used in this study

Bacterial Strains	Toxin type	Reference
Staphylococcus aureus FRI 913	SEA, SEC, SEE and TSST-1	
Staphylococcus aureus MNHOCH	SEB	
Staphylococcus aureus MNDON	SEC	
Staphylococcus aureus RN2440	Recombinant SEC-bovine	
PMN403		
Staphylococcus aureus FRI 472	SED	
Staphylococcus aureus FRI 326	SEE	
Staphylococcus aureus FRI MN8	TSST-1	
Staphylococcus aureus RN4220	No toxin	
Staphylococcus epidermidis		ATCC 12228
Staphylococcus hyicus		NVRI*
Streptococcus uberis		ATCC 27958
Streptococcus agalactiae		ATCC 13813
Streptococcus dysagalactiae		ATCC 27957
Streptococcus pyogens		ATCC 21059
Clostridium perfrigens type A		ATCC 3629
Clostridium perfrigens type C		ATCC 3638
Escherichia coli O157:H7		U. S. A. **
Escherichia coli	LT producing strain	Denmark***
Escherichia coli	ST producing strain	Denmark***
Salmonella dublin		Denmark***
Yersinia enterocolitica		Denmark***

* *S. hyicus* was obtained from National Veterinary Research Institute, Korea.

** *E. coli* O157:H7 was obtained from *E. coli* reference center of Pennsylvania State University, Pennsylvania.

*** The strains were obtained from the International *Escherichia* and *Klebsiella* Centre of the Statens Serum Institute, Copenhagen, Denmark.

. *S. aureus*

8   19   (7.5× 105 cells/ml)
                    blood agar plate   37   18-24
    ,   377   *S. aureus*   . *S. aureus*   50%
egg yolk tellurite (Lab M, London)   Baird-Parker medium (BPM)
          37   48   .   catalase test,
coagulase test, 1% mannitol utilizatio test, termostable nuclease (TNase)
test   *S. aureus*   Vit
Gram-Positive Identification Card (Vit   Systems, Inc., Hazelwood, MO)
                    .

. DNA
S. aureus   Brain heart infusion (BHI) broth (Difco, USA)   37   8-24
              ,   (12,000 rpm, 3)   *S. aureus* cell
      .   50 unit   lysostaphin (Sigma, USA)   100 ug   protenase K
(Sigma, USA)   TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) 50 ul
              . 37   1.5   , phenol-chloroform-
isoamyl alcohol (PCI; 25:24:1, v/v)   nucleic acid   ,
      isopropanol   -70   30   .
(12,000 rpm, 10)   70%   2   vacuum-dry   RNase
(1 ug/ml)   D.W. 100 ul   37   1   .
DNA   spectrophotometer (Pharmacia Biotech, USA)   260 nm
              quality   -20   .

. Multiplex PCR (mPCR)
                  primer   Gene Bank (National Institute of Health, USA)
          sequence   design   ,   primer   oligonucleotide
sequence   Table 2   .
 mPCR   mixture   MgCl₂   10× PCR reaction buffer
(Promega,   USA) 2.5 ul,   400 uMd deoxynucleoside triphosphate mixture
(dNTP), 3 mM MgCl₂, 7.5% dimethyl sulfoxide (DMSO), *sea*, *seb*, *sec*, *sed*, *see*
primer   50 pmol, *tst* primer 100 pmol   template DNA 100 ng   D.W.
          volume   25 ul   . mPCR
                ,   .
mPCR   95   5   hot start   1 uint

Taq polynerase (Pronega)   , 95   1 , 56   (SEA, SEC, SED
prirer   )   50   (SEB, SEE, TSST-1 prirer   )   2 ,
72   1   30   ,   extension   72   5
     . PCR product   1.5%   agarose gel
  , ethidium bronide (EtBr)   .

Table 2. Nucleotide sequences of prirers used in nultiplex PCR

Gene	Prirers	'5 3' sequences	Location of gene	Product size (bp)
sea	SEA-1	TTGGAAACGGTTAAAACGAA	490-509	121 bp
	SEA-2	GAACCTTCCCATCAAAAACA	591-610	
seb	SEB-1	TCGCATCAAACTGACAAAGG	634-653	477 bp
	SEB-2	GCAGGTACTCTATAAGTGCC	1091-1110	
sec	SEC-1	GACATAAAAGCTAGGAATTT	676-695	257 bp
	SEC-2	AAATCGGATTAACATTATCC	913-932	
sed	SED-1	TAGATAAAGTTAAAACAAGC	354-373	318 bp
	SED-2	TAACTTACCGTGGACCTTC	652-671	
see	SEE-1	TAGATAAAGTTAAAACAAGC	491-510	169 bp
	SEE-2	TAACTTACCGTGGACCCTTC	640-659	
tst	TST-1	ATGGCTATATACATTCAATT	251-270	350 bp
	TST-2	TTTCCAATAACCACCCGTTT	581-600	

     . PCR products   sequencing
                    low nelting point agarose gel (Pronadisa,   USA)
elution   , pGEM T Vector System (Pronega)   cloning   . Cloning
PCR product   Quiagen column (Quiagen Co.)   prification   ,
ABI 377 DNA sequencer (Perkin Elner Co.)   dye ternination nethod
          sequencing

2. SEC nutant vaccine

   .
 5-6   25-30 g   BALB/c
                .

.

Staphylococcus aureus enterotoxin typing multiplex PCR

sec+   (No. 877)

.

. SEC   (SEC mutant)
1)   ,
Staphylococcal enterotoxin C toxin
          94   106
     SEC mutant protein (SEC)   .   SEC
      SEC   95   cysteine serine
     SEC-SER mutant protein (SEC-SER)   . SEC   SEC-SER
          Escherichia coli w3110 30 L   M9
               (LAPX 202BTG, Alfa-Laval)   4L
10 mM Tris buffer (pH 7.0)   .   Microfluidizer
(Microfluidics Crop., MA, USA)   8,000 psi   2
                          .

2)
           ammonium sulfate (Sigma, USA) 1.6 M   4
          (high speed centrifuge, J2-21M, BECKMAN, USA) 8,500
rpm   30   .
   3.5 M   ammonium sulfate   4
        .   4 L 10 mM Tris buffer (pH 6.5)
   , 300 kDa   , 300 kDa
               (filtrate)
10 kDa   (retentate)
   .   10 mM Tris buffer (pH 6.5)   ,
  800 umho   .

3)
10 mM Tris buffer (pH 6.5)
CM-sepharose   10 mM Tris buffer (pH 6.5)
      10 mM Tris buffer (pH 8.0)   .
0-200 mM NaCl   .   10 mM Tris

                    - 128 -

buffer (pH 8.0)   (DEAE-sepharose)
                            .   10 kDa
                  phosphate buffered saline (PBS)
                                          .

    .

  SEC   SEC-SER   (Emulsigen ISA-25 oil adjuvant)
3:1   0.375-375 ug   0.1
ml   .

    .

  nPCR   S. aureus   enterotoxin typing   sec+
                                    (No. 877)
                                      .   ,   brain heart
infusion broth (BHI, Difco, USA)   8,000× g   30
                              .   ammonium sulfate   52.5%
        4   10,000× g   30
              .   4
                                      .   Micro BCA protein assay
reagent (Bio-rad Co., USA)   5 ng/ml   4
                  .

    .

  SEC   SEC-SER   2   Table 1
Table 2   0.5 ng/ml   1 ml
                  .

    .

                              , Ficoll-Hypaque (D=1.086; Lympholyte-M)
        density-gradient centrifugation   .
    PBS   3   1× 107 /ml   .

    .

  ELISA   plate (Costar, USA)   coating buffer (Na₂CO3 1.5 g, NaHCO3 2.93 g,
D.W. 1,000 ml, pH 9.6)   SEC   well   100 ul (5 ug/ml)

4   .   washing buffer (PBST;
NaCl 8 g, KH₂PO4 0.2 g, Na₂HPO4 0.87 g, KCl 0.2 g, Tween 20 0.5 ml, D.W. 1
L, pH 7.2)   , washing buffer   bovine serum albumin (BSA, Sigma,
USA)   1%   well   100 ul   37   1
blocking   .   well   washing buffer   5-6
washing buffer   1:2,000   well   100 ul   37
    1   5-6   HRP conjugated anti-mouse IgG
(Sigma, USA)   4,000   well   100 ul   37   1
                    .   4-6   ,   2,2-azino-di-
(3-ethylbenzothiazoline-6-sulphonate) (ABTS, KPL, USA)   100 ul
        30   2.5 M H₂SO4   100 ul
    405 nm   .

    .   -IFN   IL-2 assay
                                    -IFN   IL-2 ELISA kit (Endogen, USA)
                                    . Anti-mouse   -IFN   anti-
mouse IL-2   coating   plate   mouse serum   1:10
well   100 ul   37   1   washing buffer
        5   .   -IFN   IL-2 standard
            405 nm   .

    .
                            276   Table 3   12   groups
(SEC mutant 2   0.374-375 ug   4   ,
adjuvant   )   1   3
    1   1 ml   1   1, 2, 3   blood
sampling   ,   -IFN   IL-2 assay   (3)
        1   .

Table 3. Experimental design for the determination of SEC or SEC-SER mutant antigen dose in mice

Group	No. of mice	Antigen (ug/head)	Post-inoculation				No. of mice challenged
			wk 0 (1st vacc.)	wk 1 (2nd acc.)	wk 2 (3rd vacc.)	wk 3	
SEC	23	0.375	-	5*	5*	5*	8
SEC	23	3.75	-	5*	5*	5*	8
SEC	23	37.5	-	5*	5*	5*	8
SEC	23	375	-	5*	5*	5*	8
Adjuvant	23	Adjuvant**	-	5*	5*	5*	8
Control	23	PBS***	-	5*	5*	5*	8
SEC-SER	23	0.375	-	5*	5*	5*	8
SEC-SER	23	3.75	-	5*	5*	5*	8
SEC-SER	23	37.5	-	5*	5*	5*	8
SEC-SER	23	375	-	5*	5*	5*	8
Adjuvant	23	Adjuvant**	-	5*	5*	5*	8
Control	23	PBS***	-	5*	5*	5*	8
	276	-	-	-	-	-	-

* No. of mice sacrificed

** Emulsigen ISA-25

*** Phosphate bufferd saline (pH 7.2)

.

3.75 ug   37.5 ug

,   156   Table 4   6   group

.

(3)   1   .

Table 4. Experimental design for immunogenicity of SEC or SEC-SER mutant antigen in mice

| Group | No. of mice | Antigen (ug/head) | Post-inoculation | | | | No. of mice challenged |
			wk 0 (1st vacc.)	wk 1 (2nd acc.)	wk 2 (3rd vacc.)	wk 3	
SEC	26	0.375	-	2*	2*	2*	20
SEC	26	3.75	-	2*	2*	2*	20
SEC-SER	26	0.375	-	2*	2*	2*	20
SEC-SER	26	3.75	-	2*	2*	2*	20
Adjuvant	26	Adjuvant**	-	2*	2*	2*	20
Control	26	PBS***	-	2*	2*	2*	20
	156	-	-	-	-	-	-

* No. of mice sacrificed

** Emulsigen ISA-25

*** Phosphate buffered saline (pH 7.2)

3. SEC mutant vaccine

.

2-3   25

(Table 5).

Table 5. Experimental design for immunogenicity of SEC mutant antigen in dairy cows

| Group | Antigen* (ng/head) | Adjuvant | N | Post-inoculation | | | | |
				wk 0 (1st vacc.)	wk 2 (2nd vacc.)	wk 6 (3rd vacc.)	wk 10	wk 14
Group 1	4	ISA-25**	5					
Group 2	4	CMC***	5		- Blood/Milk sampling			
Group 3	0.4	ISA-25	5		: wk 0, wk 2, wk 6,			
Group 4	0.4	CMC	5		wk 10, wk 14			
Group 5	Control	CMC	5					
Total	-	-	25	-	-	-	-	-

* Antigen : SEC-SER

** ISA-25 : Emulsigen ISA-25

*** CMC : Carboxyl methyl cellulose-Na (Sigma, USA)

.

SEC-SER mutant antigen 100 ㎍   150 ㎖   ,
Emulsigen ISA-25   magnetic stirrer
                          10 ㎖   . CMC adjuvant   control
          CMC   SEC-SER mutant antigen
    1-3 um   ,
                                        .   , protein   ,
endotoxin   QC test
  .
  , 5   1 ㎖
  , 7   .

  .
                      ㎖   4 ㎍, 0.4 ㎍   ISA-25
(Emulsigen)   CMC   3:1   1 ㎖
                      .

  .
                          (Table 5)
    ,
                      .

  .
  Fossomatic 4000 (Foss Electric Co. Denmark)
            .

  .
                              37   24
        , coagulase   , Baird-Parker   , DNase   ,
Voges-Proskauer   ,   mannitol   .

  .
1)
                                acid citrate dextrose (ACD ; sodium
citrate 22.0 g, citric acid 7.3 g, dextrose 24.5 g, D.W. 1,000 ㎖)

3:1   , 1,500 rpm   30
  . Buffy coat   37   0.87% Tris-buffered ammonium
chloride (Tris-NH₄Cl ; 0.01 M Tris, pH 7.2)   37
          5   .   1,500 rpm   10
                         , pellet   PBS   ACD   9:1   PBS-ACD buffer
2   .   pellet   RPMI 1640 (Sigma)
            histopaque (Sigma,   1.083)   1,500 rpm
   20   , histopaque
            .   PBS   3   PBS   , tryphan
blue   1× 107 /㎖
                  .

2)

        8   (Table 6).

Table 6. Monoclonal antibodies specific to bovine leukocyte
differentiation molecules

Molecules*	MAb**	Isotype of MAb	Cell type***
MHC Class I	H58A	IgG2a	Leukocyte
MHC Class II	H42A	IgG2a	Monocyte
BoCD2	BAQ95A	IgG1	T
BoCD4	CACT138A	IgG1	T helper, inducer
BoCD8	CACT80C	IgG1	T cytotoxic, suppressor
Surface IgM	PIG45A	IgG2b	B
TcR1-N12	CACT61A	IgM	N cell
GM1	DH59B	IgG1	Granulocyte/Monocyte

* Molecules : Bovine leukocyte differentiation molecules
** MAb : Monoclonal antibodies which specifically react to leukocyte
differentiation antigen
*** Cell type : Cells expressing molecules

3)   (Flow cytometry analysis)

 Conical bottom microplate   well   50 ul (15 ug/ml)

          ,   1× 107 /ml   100 ul   4

30   4   first washing buffer (PBS 450 ml, ACD 50 ml, 20%

NaN3 5 ml, gamma globulin free horse serum 10 ml, 250 mM EDTA 20 ml, 0.5%

phenol red 1 ml)   3   (2,000 rpm, 3 , 4)   ,

                                plate shaker   voltex mixer

          .   secondary antibody   ,   .

Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG+IgM

antibody (Caltag Lab Inc., San Francisco, USA)   200   well

100 ul   ,   4   30   , 4

sencondary washing buffer (first washing buffer   horse serum

)   3   .   2% PBS-formalin (38% formalin 20 ml,

PBS 980 ml)   200 ul/well

                 (4)   .   flow

cytometry   .

FACSCalibur   Cell Quest program (Becton Dickinson)

   .

4.

   .

 1988   5

            .   1

      2   .

                                1997   4

                              200,000   /ml

                              .

                                              500,000

/ml   .

   .

        ,   1× 107 cells/ml

   .

.

VMRD   (Pullman, USA)
(Table 7).

Table   7.   Monoclonal   antibodies   specific   to   bovine   leukocyte
differentiation molecules used to define the distribution of leukocyte
subpopulation from peripheral blood and mammary gland secretions

Molecules*	Cell type**	MAb***	Isotype of MAb
BoCD2	Pan T	BAQ95A	IgG1
BoCD4	T helper/inducer	CACT138A	IgG1
BoCD8	T cytotoxic/suppressor	CACT80C	IgG1
sIgM	B cell	Pig45A	IgG2b
N12	non T/non B	CACT61A	IgG1
ACT2	activated BoCD8	CACT26A	IgG1
ACT3	activated BoCD4	CACT114A	IgG2b
VC1-N1	non T/non B	BAQ4A9	IgG1
VC2-N2	non T/non B	B7A1	IgG1
MHC-class II			
DQ	APC****	TH81A	IgG2a
DR	APC	TH14B	IgG1
DP-like	APC	H42A	IgG1

* Molecules : Bovine leukocyte differentiation molecules
** Cell type : Cells expressing molecules
*** MAb : Monoclonal antibodies which specifically react to leukocyte
differentiation antigen
**** APC : Antigen presenting cell

.

4   200 ㎖
.   PBS-ACD-EDTA (PBS pH 7.2,
20% ACD, 20 mM EDTA)   .   10
2,500 rpm   30
PBS-ACD-EDTA   RPMI 1640 (Sigma)
histopaque (Sigma,   1.083)   PBS   3

. PBS   trypan blue
          2× 107 cells/ml   .
   .   (Flow cytometry
analysis)
                                    flow cytometry   .

    .
                                                  S. aureus   BHI
     18   , 2,000 rpm   20   .   PBS
          2   1%   HBSS   6× 108 cfu/ml
                  .

   .

     S. aureus   HBSS   1:5
         .   S. aureus (6× 108 cfu/ml) 0.1 ml   0.4 ml,
            (5× 106 cells/ml) 0.5 ml
       .   30, 60   50 ul   10 ml   10
                  ,   (SPC)   BHI (BBL,
USA)   ,   .
               .
          = [(-   ) /   ] × 100

   .   -Interferon
                          -interferon   50 ug/ml   ConA (Sigma,
USA)   50 ug/ml   PHA (Sigma, USA)   6   .   -interferon
                          sandwich ELISA   CSL   (Australia)
    Bovigam   . , microplate   well   80 pg/ml
                                          ELISA
   .   50 ul   well
    1   , 22   60   . 6
                          well   .
     100 ul   conjugate (horseradish peroxidase labelled anti-bovine
-interferon, CSL Co., Australia)   well   ,   60
            .   6   .

100 ul   well   , 1   22   (
)   30   .   50 ul

        450 nm   5   .   0.13   ,
    0.70   .

        .   nitogen
                    15%   fetal bovine serum (FBS, Sigma)
RPMI 1640 (Sigma, USA)   5 ml Tlr
    , 37   2   CO2   .   trypan
blue   well   1× 10€   96   well   U-
microplate   ,   nitogen   ConA (10 ul/ml, Sigma), PHA (50
ug/ml, Sigma)   PWM (10 ug/ml, Sigma)   100 ul   . 48
    ξ[H]-thymidine (ICN Radio chemicals, USA)   well   1uCi   18
                                    -liquid scintillation counter (Packard Model
1,600 TCR, USA)   ,   3
        .

    5.   Natural Resistance-Associated Macrophage Protein

    .
  5   , Nramp1 mRNA
                                                5
    3   5   .
                                    Nramp1
        11   ,   22   .

    .
                                                        ,
                                    .   Nramp
                LPS 20 ng/ml   PBMC-   ,
        RPMI 1640   5% CO2   37   6
            .

    . RNA
Guanidine HCl   ,

5,000 g   10   . 100 ul 3 M Sodium Acetate
(pH 5.2)   500 ul   .
   2   5,000 g   10
   . 100 ul   guanidine HCl   II
         2   5,000 g   10
                    .   2   50 ul   0.02 M EDTA (pH 8.0)
         chloroform:1-butanol (4:1)   .
                4 M   3 M   sodium acetate (pH 7.0)
      , 0.2%   SDS   0.05M EDTA (pH 8.0)   2
                  RNA   . RNA   RNA/DNA
Calculator (Pharmacia, LKB Mod. 80-2, USA)   260 nm
   .

   . Reverse Transcription-Polymerase Chain Reaction (RT-PCR)
1) cDNA
 cDNA   RT mixture   1 ug total RNA, AMV RT (15 U/ug RNA), 5 mM
MgCl₂, 1 mM dNTP, Oligo dT primer (0.5 ug RNA), 10mM Tris-HCl, 50 mM KCl,
0.1% Triton X-100, 20 ul D.W.   ,   42   30
         .

2) PCR
 PCR   primer   Table 8   *Nramp1*
18bp, 21bp   ,   -actin   PCR
               24bp, 27bp   . PCR   mixture (10 ul
RT product, 2 mM MgCl₂, 200 uM dNTP, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton
X-100, 1.25 U *Taq* Polymerase, 0.5 mM Primer pairs)   , PCR
      94   2   , 94   1.5 , 59   1   72
      2   35   .
 1.5%   agarose gel   , EtBr
       .   Advanced American Biotechnology Software RFLP (AAB
software, USA)   Image   . RT-PCR   image
         O.D.   .

Table 8. Preparation of priner for RT-PCR of *Nramp* and -actin

Priner		Sequence
Nramp1	sense :	5'CGTGGTGACAGGCAAGGA3'
	antisense :	5'CGAGGAAGAGGAAGAAGAAAG3'
-actin	sense :	5'ACGTCGCCTTGGACTTCGAGCAGG3'
	antisense :	5'GCTGCAAGCTCCACAGCGACGCCAGCA3'

6.   *BoLA LRE3*

  .

                5   ,
97   4
                                        13   .

  .

                                              2 ml PBS
            ,   microtube   -70   .

    .   DNA
 PBS   DNA   30 ul   100
ul proteinase K   (Signa, USA)   ,   4 ul   proteinase
K (4 ng/ml; Signa, USA)   37   2-3   . Proteinase
K   94   10   .

  . *BoLA LRE3*
BoLA LRE3 exon2   PCR

       .

1)   priner
 BoLA LRE3 exon2   priner   Table 9   *BoLA LRE3*
allele   , BoLA 30   24bp   priner
  , BoLA 31   antisenser   26bp   priner
              BoLA 31   priner   8bp   BoLA 32
25bp   priner   , PCR   heni-
nested   2   PCR   .

Table 9. Primers specific to *BoLA DRB3* locus used in this study

Primer	Sequence
BoLA30	5'-ATCCTCTCTCTGCAGCACATTTCC-3'
BoLA31	5'-TTTAAATTCGCGCTCACCTCGCCGCGT-3'
BoLA32	5'-TCGCCGCGTGCACAGTGAAACTCTC-3'

* Primers were synthesized using DNA/RNA synthesizer (ABI 392, USA)

2) Polymerase chain reaction   *BoLA-DRB3* exon 2

                    BoLA DRB3 exon2   PCR
Table 10   1   2   .
                    master mix   .

Table 10. Formulation of PCR mixtures

Round 1			Round 2		
DNA	20 ng	10 ul	DNA (1st product)	20 ng	10.5 ul
dNTPs	10 nM	5 ul	dNTPs	10 nM	5 ul
10× PCR buffer		5 ul	10× PCR buffer		5 ul
MgCl2	2.5 nM	1.5 ul	MgCl2	2.5 nM	1.5 ul
primer #30	0.5 uM	4 ul	primer #30	0.5 uM	4 ul
primer #31	0.5 uM	4 ul	primer #32	0.5 uM	4 ul
D.W		20.5 ul	D.W		20.5 ul
Total		50 ul	Total		50.5 ul

         20 ng   DNA   template   ,   50 nM KCl, 10 nM
Tris-HCl, 2.5 nM MgCl₂   PCR buffer   , 100 uM   dNTP
0.5 uM *DRB3*   primer,   1   unit   *Taq* polymerase
Thermocycler (Perkin Elmer, Gene Amp PCR system 2400, USA)
      . 1   94   4   denaturation   94   1   , 60
2   , 72   1   10   , 72   5   extension
  , 1   PCR product 10 ul   2   PCR template   .
2   94   4   denaturation   , 94   1 , 65   30 , 72
    30   25   72   5   extension   . 2%
agarose gel   *BoLA DRB3*   PCR
        .

3) PCR

 PCR   *Hae* III, *Xho* II   *Rsa* I   37   4
                        .   8%   polyacrylamide   gel
(Acrylamide/Bis, 19:1, BioRad Lab. USA)   Mini Protein (BioRad Lab.
USA)   80 V   75   4   .
   van Eijk   (1992)   allele   genotyping
   .

3

1.   *Staphylococcus aureus*   prevalence
  . Multiplex polymerase chain reaction (nPCR)

S. aureus   enterotoxin   group (SEA, SEC, and SED; SEB,
SEE, and TSST-1)   toxin gene   primer   nPCR
        .   , 95   5   hot start   1 uint
Taq polymerase (Promega)   , 95   1 , 56   (SEA, SEC, SED
primer   )   50   (SEB, SEE, TSST-1 primer   )   2 ,
72   1   30   ,   extension   72   5
    nPCR   .

  . Toxin typing   prevalence
                                131   S. aureus   nPCR
    36 (27.5%)   toxin gene
              ,   SEC (30.1%)   (Table
11).

Table 11. Toxin-typing and effects on inflammatory response of SEs and
TSST-1 from 131 *S. aureus*-infected mastitis milk samples by multiplex PCR
assays.

Toxin type	No. (%) of Strains	% of enterotoxigenic *S. aureus* isolates
Enterotoxigenic *S. aureus*:	36 (27.5)	100
SEA	6 (4.6)	16.7
SEB	1 (0.8)	2.8
SEC	11 (8.4)	30.1
SED	8 (6.1)	22.2
SEE	0 (0.0)	0
TSST-1	5 (3.8)	13.9
SEB+TSST-1	1 (0.8)	2.8
SEC+TSST-1	3 (2.3)	8.3
SED+TSST-1	1 (0.8)	2.8
Non-enterotoxigenic *S. aureus*:	95 (72.5)	-
Total Sum	131 (100)	-

2. SEC mutant vaccine

.

SEC mutant protein 2

1:1000

1   2   3   SEC-SER 3.75 ug
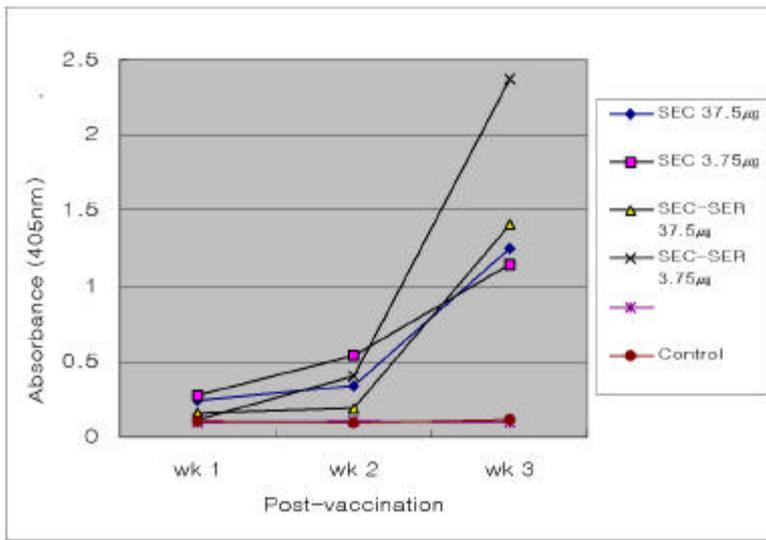
SEC-SER 37.5 ug   (Fig 1).

Fig 1. Serum antibody titers of mice vaccinated with SEC or SEC-SER mutant antigen determined by ELISA.

Ⅲ. Cytokine

1) γ-IFN

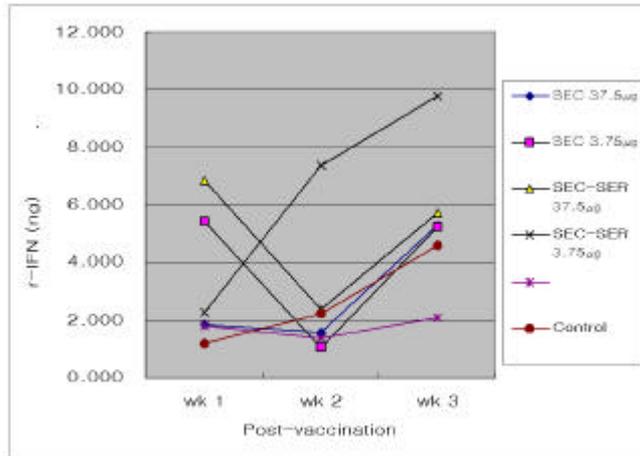SEC-SER 3.75 ug   1   2   3

            (Fig 2).

Fig 2. Production of γ-IFN in nice vaccinated with SEC or SEC-SER nutant
antigen determined by ELISA.

2) IL-2

SEC   1   2   3

        (Fig 3).

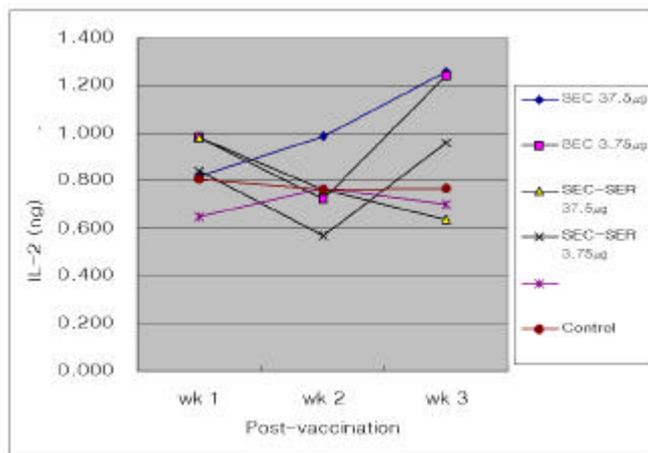

Fig 3. Production of IL-2 in nice vaccinated with SEC or SEC-SER nutant
antigen determined by ELISA

.

Mouse   , SEC mutant 2
    3. 75   37. 5 ug   ,
            SEC-SER 37. 5 ug   40%
                adjuvant   10   30 %
                    .   ,   80%   (Table
12).

Table 12. Mortality of mice vaccinated with SEC or SEC-SER mutant antigen
followed by SEC toxin challenge

Vaccines	Challenged with crude toxin*
SEC 37. 5 ug	1/10**
SEC 3. 75 ug	1/10
SEC-SER 37. 5 ug	4/10
SEC-SER 3. 75 ug	1/10
Adjuvant (Emulsigen)	3/10
Control (PBS)	8/10

 * 1LD50 (1. 1 ng/ml) of *S. aureus* No. 877 isolate containing *sec+*
 ** No. of mice died/No. of mice challenged

3. SEC mutant vaccine
    . SEC-SER mutant
                                                            (Group 5)   carboxy
methyl cellulose-Na (CMC ; Sigma, USA)   1   6
          , Group 2 (4 ng, CMC   )
       1   6   10
            .   50   /ml   ,   1
           10   , 14   2   , Group 3 (0. 4
ng, ISA-25   )   1   2
Group 4 (0. 4 ng, CMC   )   1   10   14
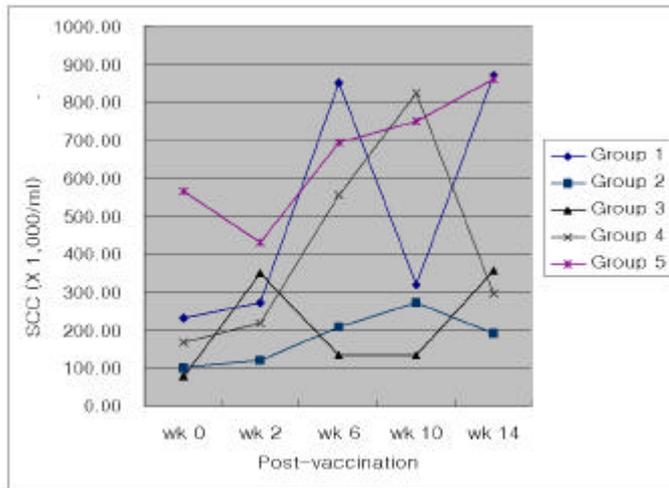   (Figs 4, 5).

Fig 4. Changes of milk somatic cell counts in dairy cows vaccinated with SEC-SER mutant antigen. Group 1 : SEC-SER 4 ng, ISA-25; Group 2 : SEC-SER 4 ng, CMC; Group 3 : SEC-SER 0.4 ng, ISA-25; Group 4 : SEC-SER 0.4 ng, CMC; Group 5 : CMC adjuvant
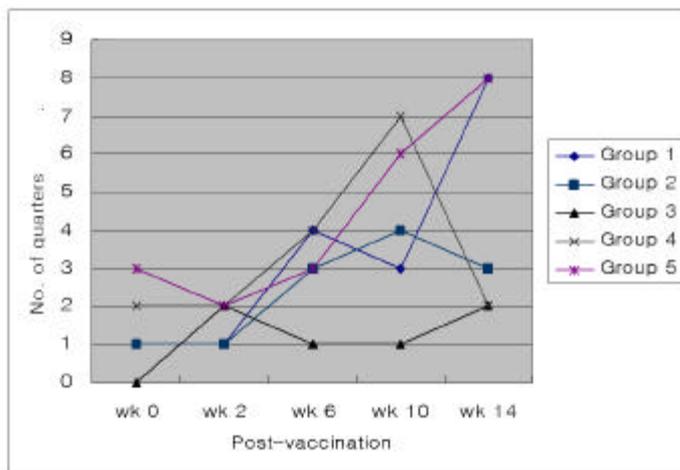

Fig 5. igure 5. Number of quarters of mammary gland over 500,000/ml somatic cell counts in dairy cows vaccinated with SEC-SER mutant antigen. Group 1 : SEC-SER 4 ng, ISA-25; Group 2 : SEC-SER 4 ng, CMC; Group 3 : SEC-SER 0.4 ng, ISA-25; Group 4 : SEC-SER 0.4 ng, CMC; Group 5 : CMC adjuvant

.

S. aureus Group 2 (4 ng,

CMC) (Group 5)

. 1 6

. coagulase

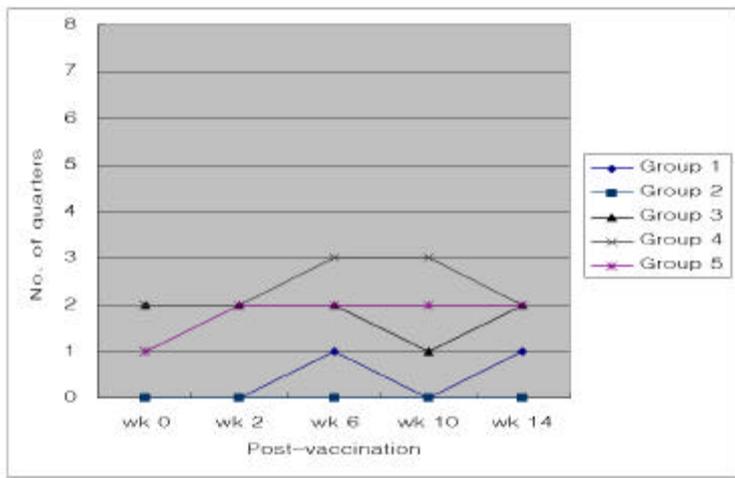negative staphylococcus (CNS) 6

. 1 10 14

Group (Fig 6-7).

Fig 6. Isolation of *S. aureus* from milk samples of dairy cows vaccinated with SEC-SER mutant antigen. Group 1 : SEC-SER 4 ng, ISA-25; Group 2 : SEC-SER 4 ng, CMC; Group 3 : SEC-SER 0.4 ng, ISA-25; Group 4 : SEC-SER 0.4 ng, CMC; Group 5 : CMC adjuvant
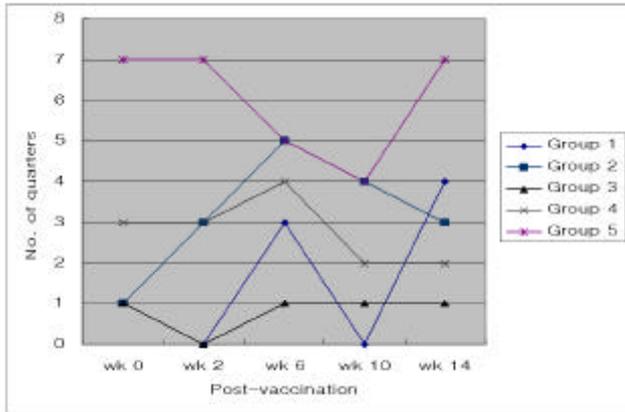
Fig 7. Isolation of Coagulase Negative Staphylococcus (CNS) from milk samples of dairy cows vaccinated with SEC-SER mutant antigen. Group 1 : SEC-SER 4 ng, ISA-25; Group 2 : SEC-SER 4 ng, CMC; Group 3 : SEC-SER 0.4 ng, ISA-25; Group 4 : SEC-SER 0.4 ng, CMC; Group 5 : CMC adjuvant

. SEC-SER

ELISA   Group   , Group 1 (4 ng, ISA-25   )   ,
   1   14   . Group 2 (4 ng, CMC   )   Group 4 (0.4 ng, CMC   )   ,
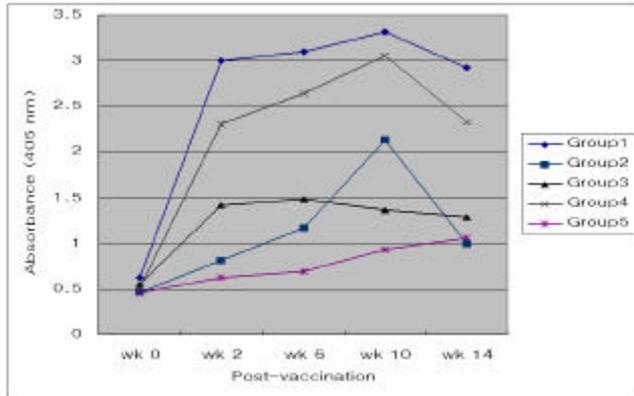1   10   (Fig 8).

Fig 8. Serum antibody titers of dairy cows vaccinated with SEC-SER mutant antigen determined by ELISA. Group 1 : SEC-SER 4 ng, ISA-25; Group 2 : SEC-SER 4 ng, CMC; Group 3 : SEC-SER 0.4 ng, ISA-25; Group 4 : SEC-SER 0.4 ng, CMC; Group 5 : CMC adjuvant

.

                                           1   , 2

    (1   2   ), 3   (1   6   )   1   10 , 14

    5   ,

(ISA-25, CMC)   Group 2 (4 ng,

CMC   )   .

           , 2   Total T   (BoCD2+), T

helper cell (BoCD4+), T cytotoxic/suppressor cell (BoCD8+)   T

                 1   6 (2   4)

  3   .   1   14

BoCD8+ T   BoCD2+   BoCD4+ T

                           .

NonT/NonB   BoCD2+   BoCD4+ T

           3

           .

monocyte   B   3

  (1   6)   .   B

                       , monocyte

  1   14

    .   1   macrophage/monocyte

     MHC class II molecule

                  .

4.

   .

Table 13, 14   .

                  MHC-DP-like, DQ   DR

    78.5% 59.8%,   68.0%   31.2% 31.2%,

21.6%   .   MHC-DP-like DQ

     35.1% 38.0%   43.0% 47.4%

, MHC-DR   42.3%,   42.5%   .

Table 13. Distribution of lymphocyte subpopulations of mammary gland secretion from mastitis-resistant and -susceptible cows analyzed by monoclonal antibodies specific to bovine leukocyte differentiation antigens using flow cytometry

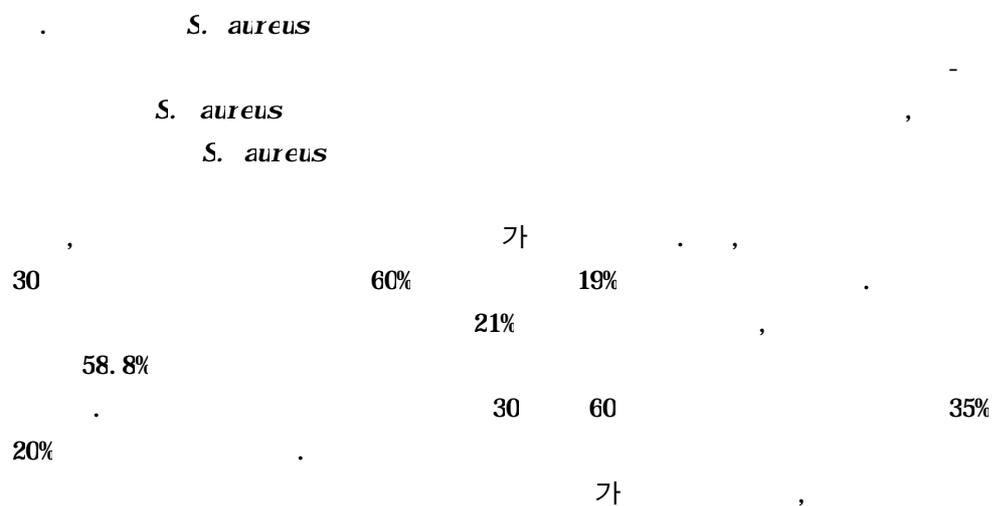| Bovine leukocyte differentiation antigen	Mean proportion of bovine leukocyte subpopulation in MGS[a](%)	
	Mastitis resistant (n=6)	Mastitis susceptible (n=6)
MHC-class II		
DP-like	78.5± 10.5[b]	31.2± 10.4
DQ	59.8± 11.4	31.2± 9.8
DR	68.0± 9.5	21.6± 12.5
BoCD2	38.2± 7.3	13.0± 8.7
BoCD4	27.9± 6.5	7.7± 7.5
BoCD8	8.6± 4.3	18.5± 8.3
Non T/Non B (N)	10.1± 4.7	3.4± 2.6
sIgM (B)	31.6± 9.3	15.7± 5.3
ACT2	5.8± 4.3	10.8± 6.4
ACT3	33.3± 9.7	19.0± 5.7
VC1-N1 (-TCR)	20.2± 6.7	14.5± 9.4
VC2-N2 (-TCR)	17.6± 8.4	8.5± 5.9
BoCD4/BoCD8 ratio	3.2	0.42

* MGS : mammary gland secretion

** Mean ± SD

Table 14. Distribution of peripheral blood lymphocyte subpopulation from mastitis-resistant and -susceptible cows analyzed by monoclonal antibodies specific to bovine leukocyte differentiation antigens using flow cytometry

Bovine leukocyte differentiation antigen	Mean proportion of bovine leukocyte subpopulation in PBL*(%)	
	Mastitis resistant (n=6)	Mastitis susceptible (n=6)
MHC-class II		
DP-like	35.1± 9.7**	43.0± 10.5
DQ	38.0± 6.8	47.4± 9.5
DR	42.3± 10.3	42.5± 9.8
BoCD2	31.4± 6.4	19.4± 6.2
BoCD4	15.4± 5.4	2.3± 2.6
BoCD8	6.1± 3.8	15.1± 5.4
Non T/Non B (N)	6.0± 2.4	2.3± 1.5
sIgM (B)	21.0± 3.7	34.4± 5.8
ACT2	7.9± 4.8	8.7± 2.8
ACT3	6.6± 5.7	11.2± 3.2
VC1-N1 (-TCR)	6.4± 2.5	5.8± 2.5
VC2-N2 (-TCR)	9.5± 4.5	7.7± 3.5
BoCD4/BoCD8 ratio	2.5	0.15

* PBL : peripheral blood leukocytes
** Mean ± SD

.   *S. aureus*

                                                                    -

          S. aureus   ,
            S. aureus

    ,   .   ,
30   60%   19%   .
                              21%   ,
    58.8%
        .   30   60   35%
20%   .

                                        ,

30   60   34.4%

20.2%   24.1%   11.2%   10%   .

.   -interferon

-interferon

,   O.D.   0.08   0.06

PHA   2.297

0.543   . ConA

-interferon   0.926   0.587

,   t

p<0.01   .

.   nitogen

ConA, PHA   PWM

nitogen   . 48

836,841 cpm   ,

nitogen   stinulation index (SI)

. Mitogen   SI

ConA   511, PHA   147, PWM   199   nitogen

(p<0.05).

(p>0.05) (Table 15).

Table 15. Mitogen-stinulated lymphoproliferative response of peripheral
blood lymphocytes from bovine nastitis-resistant and -susceptible cows by
stinulation with various nitogens

Mitogens	Resistant cows		Susceptible cows	
	cpm*	SI**	cpm	SI
Control	836± 233		841± 284	
ConA	426,952± 27,930	511	466,667± 96,168	555
PHA	123,201± 77,553	147	127,299± 56,905	151
PWM	166,366± 44,236	199	158,589± 47,305	190

* Data were expressed as nean counts per ninute (cpm)± SD

** Stinulation index (SI) = average cpm in experinental cultures/average
cpm in control cultures

5.   Natural Resistance-Associated Macrophage Protein

    . RT-PCR   Nramp1   mRNA
                                          mRNA
RNA   ,   RNA   RT mixture   cDNA
    . Nramp1   primer sense 18bp   antisense 21bp
    35cycle   PCR   Nramp1   cDNA
    .   221bp   Nramp1   (Fig
9).   Nramp1   Msp I
                                              221bp   ,
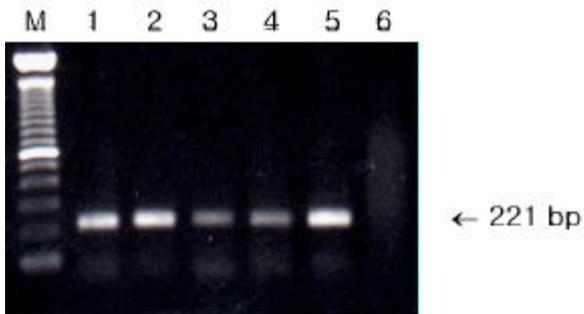   158bp   65bp   Nramp1   (Fig 10).

Fig 10. Determination of the Nramp1 mRNA for primer extension analysis was
subjected to the reverse transcriptase reaction with bovine monocytes (M :
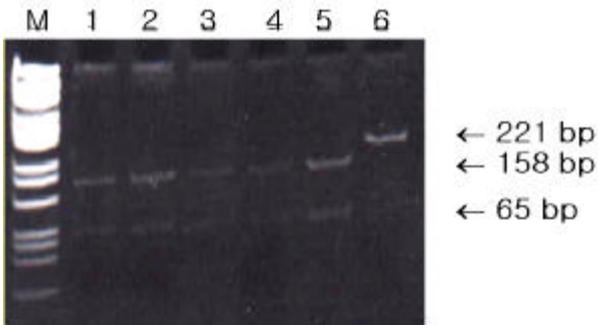marker, 100bp ladder, lanes 1 5 : Bovine monocytes, lane 6 : No cDNA).

Fig 11. Electroporetic analysis of RT-PCR products of Nramp1 gene after
digestion with Msp I (lane M : moleculer maker (pGEM), lanes 1 5 : RT-PCR
products of Nramp1 gene after digestion with Msp I, lane 6 : No
digestion).

. LPS   Nramp1 mRNA

                                        LPS   mRNA
RT-PCR   Nramp1   priner   PCR   .
            -actin   . Nramp1   LPS
                                                                  mRNA
            (Fig 12).
 Fig 13   ·   RT-PCR   LPS
                              1   5
, 6   10   3   . LPS
      0.D   500   2,000   , LPS
                                                                1,000
    0.D   ,   690   3,300
                        mRNA   .
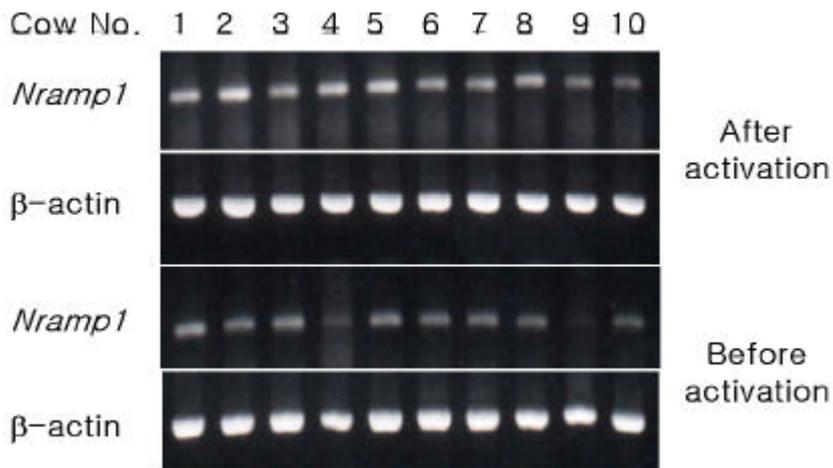   RT-PCR   Nramp1 mRNA
      .

Fig 12. Expression of Nramp1 mRNA in monocytes before or after activation
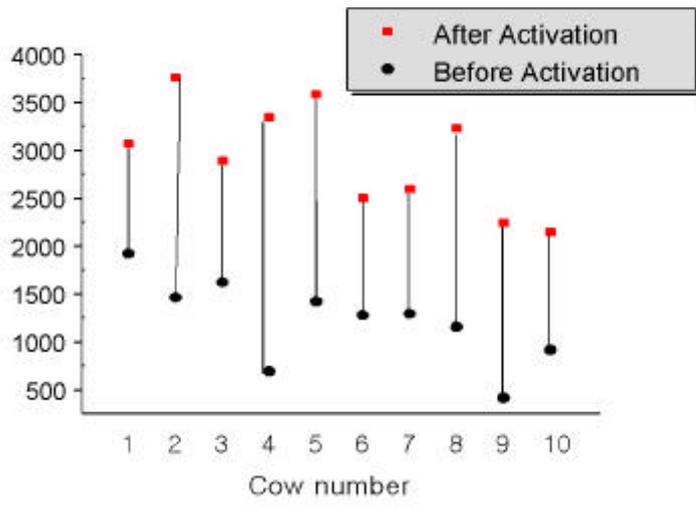with LPS from bovine(lanes 1 5 : infected cows, lanes 6 10 : uninfected
cows).

Fig 13. Optical density of Nramp1 mRNA expression of bovine monocytes
before or after activation with LPS.

.   Nramp1 mRNA
        Nramp1 mRNA   RT-PCR
                      11   LPS
        Nramp1   mRNA   Table 16
        .   , LPS   RT-PCR
        Nramp1 mRNA   .
                      1,1000.D   7,4000.D
    4,2000.D   ,   2000.D   63000.D   ,
    1,4900.D   2   ,   t
                                          (p<0.05).

Table 16. *Nramp1* mRNA expression in monocytes of bovine-mastitis resistant and -susceptible cows

Heads	Optical Density* (± SD)	
	Resistant cows**	Susceptible cows
1	5900	300
2	5500	1100
3	2000	6300
4	4400	4900
5	2800	500
6	5100	-1600
7	6900	200
8	2100	1400
9	-100	700
10	7400	1100
11	1100	800
Mean	4200± 2480	1490± 2200

* O.D (After activation - Before activation)

** p<0.05

6.   *BoLA DRB3*

. Hemi-nested PCR

                              DNA   PCR

       hemi-nested PCR   . 1   primer BoLA30   31

      10   , primer 31   8bp   primer 32   30

       , 1   10 ul   2   DNA template

       PCR   .   PCR   2%   agarose

gel   Fig 14   2 6   lane

284bp   .   DNA   ,
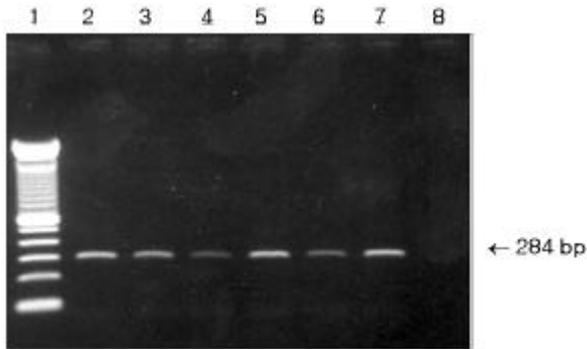
target DNA   PCR   .

Fig 14. Electrophoretic analysis of PCR amplified products of BoLA DRB3 exon2 gene on 2% agarose gel (lane 1: 100bp ladder DNA marker, lanes 2 6: cows, lane 7: +ve control, lane 8: -ve control).

. *BoLA* DRB3 PCR-RFLP

2   PCR   PCR   *Hae* III, *Xho* II   *Rsa* I

                       37   ,

   PCR   MHC Class   gene   RFLP   BoLA typing   .

Fig 15   *Rsa* I   lanes 2 4

     3   *Xho* I

lanes 5 7   5   3 , 6, 7   284bp

   . *Hae* III   2, 3

   .   *BoLA DRB3* *2* allele type   Fig 16

  3   a, b, c

          ,   allele type   .   *Rsa* I

             78bp, 54, 50, 39, 33   30bp   a

             , Fig 16   141bp, 39, 54

50bp   f   , 141bp, 93, 50bp   s   , 180bp   104bp

   n   a   s   19   .   *Xho* II

          fragments   199bp   85bp 2

       a , 219bp   65bp   b   a   f

          .   *Hae* III   fragments   167bp,

52   65bp   a   284bp   b   a   e

          .

a, b, c   Rsa I   f

, *Xho* II   a   *Hae* III   a   *BoLA DRB3.2* allele 8 type

, 19 type   s   , b   b   , 23

type   *Rsa* I, *Xho* II   *Hea* III   n   , b   a

. Fig 17   *Hae* III

5   lanes 2 4   167bp, 65   52bp   a

, 5, 6 lane   b   . Fig 18   *Rsa* I

lane 2   141bp, 54, 50

39   f   , lane 3   111bp, 104   69bp   n   , lane 4, 5

141bp, 90   50bp   q   line 6   141bp, 111   30bp

, Fig. 6   *Xho* II

lane 1, 4   5   199bp   85bp   a   ,

lane 2   3   112bp, 87   85bp   e   .

BoLA DRB3.2 allele type   .
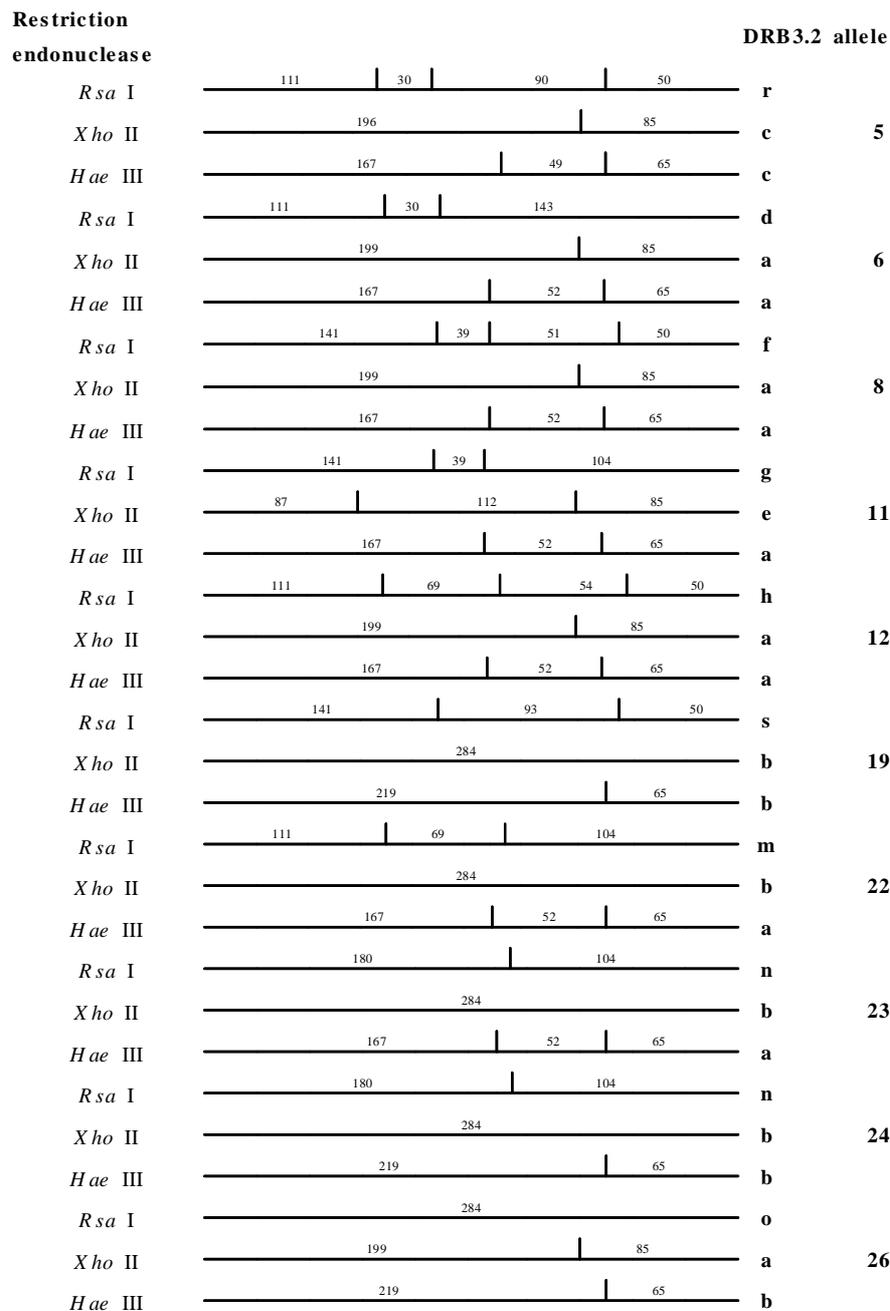
Fig 15. Representative determination of *BoLA DRB3* alleles analyzed by PCR-RFLP with *Rsa* I, *Xho* II and *Hae* III.
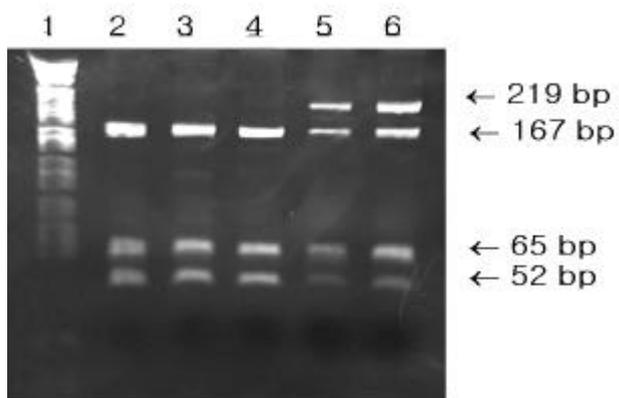
Fig 16. Allelic patterns of *EcLA DRE3* exon2 obtained by digestion with *Hae*III (lane 1 : naker, *Msp*I digest of pBR322, lanes 2 4 : a type, lanes 5 6 : b type)
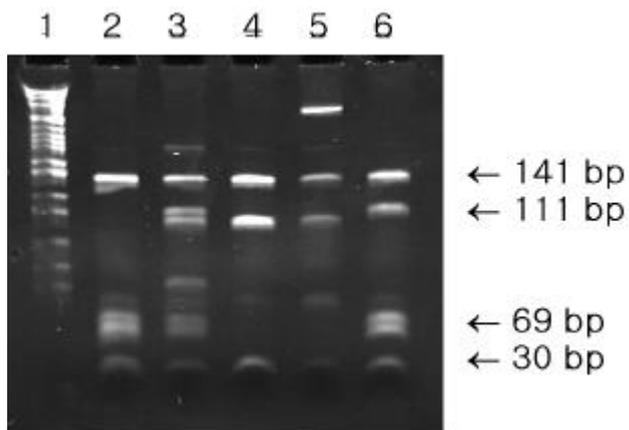

Fig 17. Allelic patterns of *EcLA DRE3* exon2 obtained by digestion with *Rsa*I (lane 1: naker, *Msp*I digest of pBR322, lane 2: e type, lane 3: m type, lanes 4 5: g type, lane 6: d type).
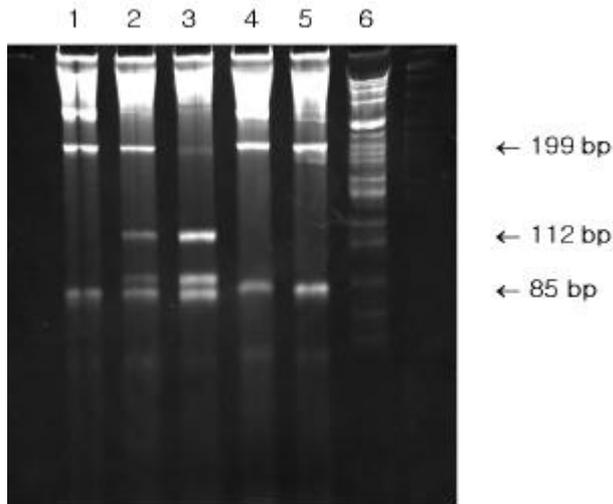
Fig 13. Allelic patterns of *BoLA DRB3* exon2 obtained by digestion with *Xho*II (lane 1, 4 5: a type, lanes 2 3: e type, lane 6: maker, *Asp*I digest of pBR322).

.   *BoLA DRB3.2* allele typing

          BoLA-DRB3 exon2   allele   typing   PCR-RFLP

                              DRB3.2 * 5type 2 , 6type 2 ,
8type 2 , 11type 3   *DRB3.2* * 12type   2   ,
          DRB3.2 * 6type   3 , 11type   2   12type
          , *DRB3.2* * 23type   *DRB3.2* * 24type   3
DRB3.2 * 26type   1   (Table 17).

                    DRB3.2 * 6type   11type   5   ,
DRB3.2 * 12type   3   ,
          DRB3.2 * 5, 8   22type   ,   *DRB3.2* *
19, 23, 24   26type   .

.   *BoLA DRB3.2* allele type

                              BoLA DRB3.2 allele type

                              DRB3.2 allele 5type
103,500 /ml   , 8type   64,000 , 22type   86,000
/ml   *DRB3.2* allele
   19type   327,000 /ml   , 22type   406,000 , 24type   265,000

, 26type 162,000 /㎖ . type 6, 11 12 type 449,000 /㎖, 251,000 298,000 /㎖ (Table 18).

Table 17. *BoLA DRB3* allele gene types of bovine mastitis-resistant and -susceptible cows

DRB3.2 allele	resistant	susceptible	No. of total
5	2	-	2
6	2	3	5
8	2	-	2
11	3	2	5
12	2	1	3
19	-	1	1
22	1	-	1
23	-	3	3
24	-	3	3
26	-	1	1
Total	12	14	26

Table 18. Milk somatic cell counts of *BoLA DRB3*2* allele groups

(× 1000 cells/㎖)

DRB3.2 allele	SCC*(± SD)	No. of cows
5	103.5± 38.9	2
6	449.4± 345.5	5
8	64± 4.2	2
11	251.2± 269	5
12	298± 376	3
19	327	1
22	86± 14.1	2
23	406.7± 172.1	3
24	265.5± 210	3
26	162	1
Total	274.8± 252.8	26

* SCC : Somatic cell counts

4

1.   377   *Staphylococcus aureus*
   toxin typing   27.5%   (Staphylococcal
enterotoxin; SE)   ,   Staphylococcal enterotoxin C (SEC)
                        .   (1990)
                                             133   *S. aurues*   88%
                                ,   SEC
   .   *S. aureus*   SEC
        SEC
      .

2. Superantigen (SAg)   *S. aureus*   (SE)
                    SEC   SEC-SER   , *S. aureus*
                                                                    SEC
         2   immunogen
         immunogenicity   , SEC   SEC-SER
            0.375 375 ug   3
      .   -IFN   control group   , 2
   1   2   -IFN   3
                  .
                  , cytokine
                      , 2   SEC-SER   ,
            3.75-37.5 ug   ,
                                          100   0.4-4 ng
            .

3.

         ,   MHC class II DP-like, DQ, DR   BoCD2,
BoCD4 T   , B
                        .

4.   *Nramp1*   ,
    nRNA
                              , *Nramp1*   nRNA
                        .   ,

nacrophage   *Nramp1*   *Nramp1*
                                                        .

5.   *BoLA DRB3.2* allele type
                        ,   *BoLA DRB3.2* allele
type   , 284bp   *BoLA DRB3.2* exon
                        .   *Rsa*I, *Xho*II, *Hae*III   3
                                                a, b, c
            3   *BoLA DRB3.2* allele type   .
                                BoLA DRB3.2   allele 5, 8, 22 type
            ,   *BoLA DRB3.2*   allele 23, 24 type
            .

6.

                        .

                              ,   .

1.   (SCI): 3   ,   : 3 (4   )

1) Ferens VA, Davis WC, Hamilton MJ, Park YH, Deobald CF, Fox L and
Bohach GA (1998) Activation of bovine lymphocyte subpopulations by
staphylococcal enterotoxin C. Infection and Immunity. 66(2):573-580.
2) Park YH, Yoo HS, Yoon JW, Yang SJ, An Jong Sam and Davis WC (2000)
Phenotypic and Functional Analysis of Bovine   lymphocytes. Journal of
Veterinary Science. 1(1):39-48.
3) Mullarky IK, Su C, Frieze N, Park YH and Sordillo LM (2001)

Staphylococcus aureus agr Genotypes with Enterotoxin Production Capabilities Can Resist Neutrophil Bactericidal Activity. Infection and Immunity. 69:45-51.

4) Davis WC, Hamilton MJ, Ferens W, Bohach GA and Park YH (1996) Role of Staphylococcal Enterotoxin C in the Pathogenesis of Mastitis. Korean Journal of Veterinary Public Health. 20(4):299-309.

5) Bohach GA, Ferens W, Deobald C, Davis WC, Hamilton MJ, Stauffacher CV and Park YH (1996) Interaction of Staphylococcal Enterotoxins with Immune Cells and Their Role in Animal Disease. Korean Journal of Veterinary Public Health. 20(4):337-348.

6) Sordillo LM, Park YH and Joo YS (1997) Role of Cytokines in the Pathophysiology and Control of Mastitis. Korean Journal of Veterinary Public Health. 21(3):261-270.

7)   C   (SEC mutant)
(Immunogenicity of Staphylococcal Enterotoxin C Mutant Antigen in Mice and Dairy Cow) -   , 2001

8)
(Phenotypic and Functional Analysis of leukocytes from bovine mastitis-resistant cows) -   , 2001

9)   Natural Resistance-Associated Macrophage Protein   (Expression of Natural Resistance-Associated Macrophage Protein gene in bovine mastitis-resistant cows) -   , 2001

10)   BoLA DRB3
(Chracteristics of BoLA DRB3 alleles in bovine mastitis-resistant cows)
-                , 2001

2.   :   4 ,   5

1) Park YH, Moon JS, Ku BG, Fox LK and Davis WC (1996) Downregulation of Immune Responses in the Mammary Gland Infected with Staphylococcus aureus by induction of gamma/delta Lymphocytes. Cytokines and the Type I Type II Paradigm International Symposium. Journal of Interferon and Cytokine Research. 16(8):672.

2)   ,   ,   ,   ,   ,   ,   (1997)
    Staphylococcus aureus   -lactamase   .
    . p 14

3)   ,   ,   ,   ,   ,   (1997) *Mastitis*

                            .   , p 54.

4)   ,   ,   ,   ,   , Mallad BA (1997) Diversity of phenotypic and genotypic expression in resistance to bovine mastitis.   . p 55.

5)   ,   ,   ,   , Davis WC, Bohach GA (1997) Toxin-typing of Staphylococcal enterotoxins A, B, C, D, E, and TSST-1 from mastitic milk using multiplex PCR.   , p 56.

6) Suppression of Bovine T Lymphocytes Responses by the Superantigen Staphylococcal Enterotoxin C (1997)

7) Yoon JW, Park YH, Jung SC, Lee SU, Davis WC and Bohach GA (1997) Toxin-typing of staphylococcal enterotoxins A, B, C, D, E, and TSST-1 from mastitis milk using multiplex PCR. 92nd American Dairy Science Association (ADSA) Annual Meeting. 80(1):171.

8) Lee JW, Park YH, Joo IS and Mallard BA (1997) Diversity of phenotypic and genotypic expression in resistance to bovine mastitis. 92nd American Dairy Science Association (ADSA) Annual Meeting. 80(1):193.

9) Lee SU, Park YH, Bohach GA, Yoon JW and Joo YS (1997) Alteration in peripheral blood monocuclear leukocyte subpopulation in cows vaccinated with staphylococcal enterotoxin C-mutant peptide antigen. 92nd American Dairy Science Association (ADSA) Annual Meeting. 80(1):193.

3.

1)   :   2000-007612   (2000   2   17)

                                  -      .

   ,

(Staphylococcal enterotoxin SEC-SER, expression vector and host cell, production method thereof, and manufacturing method of vaccine)

5

Amills M, Ramiya V, Norimine J and Lewin HA (1998) The major histocompatibility complex of ruminants. Rev Sci Tech. 17(1):108-120.

Barkema HW, Schukken YH, Lam TJGM, Beiboer ML, Benedictus G and Brand A (1998) Management practices associated with low, medium, and high somatic cell counts in bulk milk. J Dairy Sci. 81(7):1917-1927.

Barta O (1993) Immunologic techniques evaluating cells and theri functions. Veterinary Clinical Immunology Laboratory. Bar-Lab, Inc., USA B1.1-B3.29.

Barton CH, Whitehead SH and Blackwell JM (1995) Nramp transfection transfers Ity/Lsh/Bcg-related pleiotropic effects on macrophage activation: influence on oxidative burst and nitric oxide pathways. Mol Med. 1(3):267-279.

Belouchi A, Cellier M, Kwan T, Saini HS, Leroux G and Gros P (1995) The macrophage-specific membrane protein Nramp controlling natural resistance to infections in mice has homologues expressed in the root system of plants. Plant Mol Biol. 29(6):1181-1196.

Blackwell JM, Barton CH, White JK, Searle S, Baker AM, Williams H and Shaw MA (1995) Genomic organization and wequence of the human NRAMP gene : identification and mapping of a promoter region polymorphism. Mol Med 1(2):194-205.

Bohach GA, Fast DJ, Nelson RD, Schlievert PM (1990) Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. Crit Rev Microbiol. 17(4):251-272.

Bohach GA, Ferens W, Deobald C, Davis WC, Hamilton MJ, Stauffacher CV and Park YH (1996) Interaction of staphylococcal enterotoxins with immune cells and their role in animal disease. Kor J Vet Publ Hlth. 20(4):337-348.

Brown DH, Lafuse WP and Zwilling BS (1997) Stabilized expression of mRNA is associated with mycobacterial resistance controlled by Nramp1. Infect Immun. 65(2):597-603.

Cellier M, Shustik C, Dalton W, Rich E, Hu J, Malo D, Schurr E and Gros P (1997) Expression of the human NRAMP1 gene in professional primary

phagocytes: studies in blood cells and in HL-60 promyelocytic leukemia. J Leukoc Biol. 61(1):96-105.

Davis WC, Hamilton MJ, Park YH, Larsen RA and Wyatt CR. Ruminant leukocyte differentiation molecules. In : MHC, Differentiation Antigens, and Cytokines in Animals and Birds. Ed. O. Barta, BAR-LAB, Inc., Blacksburg, VA, pp47-70. Monographs in Animal Immunology.

Davis WC, MacHugh ND, Park YH, Hamilton MJ and Wyatt CR (1993) Identification of a monoclonal antibody reactive with the bovine orthologue of CD3 (BoCD3). Vet Immunol Immunopathol. 39(1-3):85-91.

Davis WC, Davis JE and Hamilton MJ (1995) Use of monoclonal antibodies and flow cytometry to cluster and analyze leukocyte differentiation molecules. Methods in Moleculary Biology. 45:149-168.

Feng J, Li Y, Hashad M, Schurr E, Gros P, Adams LG and Templeton JW (1996) Bovine natural resistance associated macrophage protein 1 (Nramp1) gene. Genome Res. 6(10):956-964.

Ferens WA, Bohach GA, Davis WC, Fox LK, Lee SU and Park YH (1998) Suppression of bovine T lymphocytes responses by the superantigens staphylococcal enterotoxin C. Kor J Vet Publ Hlth. 22(3):233-244.

Ferens WA, Davis WC, Hamilton MJ, Park YH and Bohach GA (1998) Activation of bovine lymphocyte subpopulations by staphylococcal enterotoxin C. Infect Immun. 66:573-580.

Gruenheid S, Cellier M, Vidal S and Gros P (1995) Identification and characterization of a second mouse Nramp gene. Genomics. 25(2):514-525.

Gruenheid S, Pinner E, Desjardins M and Gros P (1997) Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome. J Exp Med. 185(4):717-730.

Harmon RJ (1994) Mastitis and genetic evaluation for somatic cell count. J Dairy Sci. 77:2103-2112.

Harp JA, Kehrli ME and Hurley DJ (1991) Numbers and percent of T lymphocytes in bovine peripheral blood during the periparturient period. Vet Immunol Immunopathol. 28:29-35

Hogan KS, Smith KL and Hoblet KH (1989) Field survey of clinical mastitis in low somatic cell count herds. J Dairy Sci. 72:1547-1556.

Hurley DJ, Kensinger MH and Mastro AM (1990) An evaluation of the

mononuclear cells derived from bovine mammary gland dry secretions using leukocyte antigen specific monoclonal antibodies, light scattering properties and nonspecific esterase staining. Vet Immunol Immunopathol. 25: 177-193.

Jairath L, Dekkers JCM, Schaeffer LR, Liu Z, Burnside EB and Kolstad B (1998) Genetic evaluation for herd life in canada. J Dairy Sci. 81: 550-562.

Kehrli ME and Shuster DE (1994) Factors affecting milk somatic cells and their role in health of the bovine mammary gland. J Dairy Sci. 77: 619-627.

Kishi F, Tanizawa Y and Nobumoto M (1996) Structural analysis of human natural resistance-associated macrophage protein 1 promoter. Mol Immunol. 33(3):265-8.

Lammers A, Nuijten PJ, Kruijt E, Stockhofe-Zurwieden N, Vecht U, Smith HE, van Zijderveld FG (1999) Cell tropism of *Staphylococcus aureus* in bovine mammary gland cell cultures. Vet Microbiol. 67(2):77-89.

Larsen B, Jensen NE, Madsen P, Nielsen SM, Klastrup O and Madsen PS (1985) Association of the M blood group system with bovine mastitis. Anim Blood Groups Biochem Genet. 16(3):165-173.

Larsen RA, Monaghan ML, Park YH, Hamilton MJ, Ellis JA and Davis WC (1990) Identification and characterization of monoclonal antibodies reactive with bovine, caprine and ovine T-lymphocyte determinants by flow microfluorimetry. Vet Immunol Immunopathol. 25(2):195-208.

Lewin HA, Schmitt K, Hubert R, van Euk MJT and Arnheim N (1992) Close linkage between bovine prolactin and *BoLA-DRB3* genes: Genetic mapping in cattle by single sperm typing. Genomics. 13:44-48.

Lewin HA (1994) Host genetic mechanism of resistance and susceptibility to a bovine retroviral infection. Animal Biotechnology. 5(2):183-191.

Lewin HA, Russell GC and Glass EJ (1999) Comparative organization and function of the major histocompatibility complex of domesticated cattle. Immunol Rev. 167:145-158.

Lindberg PG and Andersson L (1988) Close association between DNA polymorphism of bovine major histocompatibility complex class I genes and serological BoLA-A specificities. Anim Genet. 19(3):245-255.

Lunden A, Sigurdardottir S, Edfors-Lilja I, Danell B, Rendel J and

Andersson L (1990) The relationship between bovine major histocompatibility complex class II polymorphism and disease studied by use of bull breeding values. Anim Genet 21(3):221-232.

Maillard JC, Renard C, Chardon P, Chantal I and Bensaid A (1999) Chracterization of 18 new *BoLA-DRB3* alleles. Animal Genetics. 20: 200-203.

Mamo W, Sandgren CH, Lindahl M and Jonsson P (1991) Induction of anti-phagocytic surface properties of *Staphylococcus aureus* from bovine mastitis by growth in milk whey. Zentralbl Veterinarmed 38(6):401-410.

Mamo W, Jonsson P, Flock JI, Lindberg M, Muller HP, Wadstrom T and Nelson L (1994) Vaccination against *Staphylococcus aureus* mastitis: immunological response of mice vaccinated with fibronectin-binding protein (FnBP-A) to challenge with *S. aureus*. 12(11):988-992.

Marrack P and Kappler J (1990) The staphylococcal enterotoxins and their relatives. Science 248:705-711.

Mejdell CM, Lie , Solbu H, Arnet EF and Spooner RL (1994) Association of major histocompatibility complex antigens [BoLA-A] with AI bull progeny test results for mastitis, ketosis and fertility in Norwegian cattle. Animal Genetics. 25:99-104.

Naessens J, Sileghem M, MacHugh N, Park YH, Davis WC and Toye P (1992) Selection of BoCD25 monoclonal antibodies by screening mouse L cells transfected with the bovine p55-interleukin-2 (IL-2) receptor gene. Immunology. 76(2):305-309.

Oddgeirsson O, Simpson SP, Morgan AL, Ross DS and Spooner RL (1988) Relationship between the bovine major histocompatibility complex (BoLA), erythrocyte markers and susceptibility to mastitis in Icelandic cattle. Anim Genet. 19(1):11-16.

Park YH, Fox LK, Davis WC, Hamilton MJ, Larsen RA and Okada K (1989) Flow cytometric analysis of the distribution of bovine leukocytes in peripheral blood and mammary secretions at different stages of lactation. Immunobiology Suppl 4, pp 81. 2nd International Veterinary Immunology Symposium, July 24-28.

Park YH, Fox LK, Davis WC, Hamilton MJ and Larsen RA (1989) Distribution of bovine leukocytes in peripheral blood and mammary secretions at different stages of lactation. Proc Soc Exp Biol Med 190:305.

Park YH, Fox LK, Hamilton MJ and Davis WC (1992) Bovine mononuclear
leukocyte subpopulations in peripheral blood and mammary gland
secretions during lactation. J Dairy Sci. 75(4):998-1006.

Park YH, Fox LK, Hamilton MJ and Davis WC (1993) Suppression of
proliferative response of BoCD4+ T lymphocytes by activated BoCD8+ T
lymphocytes in the mammary gland of cows with *Staphylococcus aureus*
mastitis. Vet Immunol Immunopathol. 36(2):137-51.

Park YH, Jung SC, Fox LK, Hamilton MJ and Davis WC (1993) Phenotypic and
functional characteristics of bovine mammary gland T lymphocytes with
Staphylococcus aureus infection. 88th annual Mtg. American Dairy Science
Assn. Univ. of Maryland. p187.

Park YH, Jung SC and Moon JS (1994) A subset of mammary gland   T
lymphocytes downregulates BoCD4 T lymphocyte response to Staphylococcus
aureus in cattle with intramammary infection. Kor J Immunol. 16:19-27.

Skamene E, Schurr E and Gros P (1998) Infection genomics: *Nramp1* as a
major determinant of natural resistance to intracellular infections.
Annu Rev Med. 49:275-287.

Sordillo LM, Park YH and Joo YS (1997) Role of cytokines in the
pathophysiology and control of mastitis. Kor J Vet Publ Hlth.
21(3):261-270.

van Eijk MJT, Strbart-Haynes JA and Lewin HA (1992) Extensive polymorphism
of the BoLA-DRB3 gene distinguished by PCR-RFLP. Animal Genetics.
23:483-496.

Vidal SM, Pinner E, Lepage P, Gauthier S and Gros P (1996) Natural
resistance to intracellular infections: *Nramp1* encodes a membrane
phosphoglycoprotein absent in macrophages from susceptible (Nramp1 D169)
mouse strains. J Immunol. 157(8):3559-3568.

Xu A, Clark TJ, Teutsch MR and Schook LB (1991) Sequencing and genetic
analysis of a bovine *IL2B* cDNA clone. Animal Genetics. 22(5):381-398.

Xu A, van Eijk MJ, Park C and Lewin HA (1993) Polymorphism in BoLA-DRB3
exon 2 correlates with resistance to persistent lymphocytosis caused by
bovine leukemia virus. J Immunol. 151(12):6977-85.

     ,   ,   . (1990)   *Staphylococcus aureus*   coagulase
        enterotoxin   .   . 14(1):15-19.

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