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Physiological state-dependent changes in transcription factor DNA-binding activities of the rabbit mammary gland

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Our earlier studies carried out *in silico* demonstrated that different transcription factors have their putative binding sites in the 5'-flanking regions of rabbit milk protein genes. Now we extended this study to include the experimental analysis of the transcription factors. This study of electrophoretic mobility shift assay (EMSA) of nuclear proteins showed for the first time the presence of AP1, CREB, Myb, and Sp1 transcription factors in the rabbit mammary gland. The abundance of all transcription factors studied in the mammary gland changed during pregnancy-lactation-weaning cycle. The DNA-binding activity of the Myb transcription factor correlated well with the level of milk protein gene expression. The affinity of Myb to its binding sequence is methylation-dependent. One of CRE-specific DNA-protein complexes was found only in nuclear protein from the lactating rabbits mammary gland. The amount of AP1 DNA-binding activity transiently decreased in the early involution. Transition from early to late involution was associated with the restoration of the AP1 activity, and with the decrease of the Myb and Sp1 protein-DNA binding activities.

KEY WORDS: casein genes / DNA-binding / rabbit / transcription factor

Gene expression is regulated mostly at the transcriptional level. The control of gene expression has a complex character and requires at least interactions of transcription factors and their binding sites in a gene promoter or enhancer. Mammary gland epithe-

lial cells are highly specialized for the synthesis of limited number of proteins during lactation and involution. Regulation of milk protein genes has common features: all of them are expressed only in the mammary gland epithelium during late pregnancy and lactation, their transcription is induced by lactogenic hormones – insulin, prolactin, and glucocorticoids, and repressed by progesterone. Transcription level of these genes is extremely high [Hobbs *et al*. 1982]. These features make mammary gland a good model for studying mechanisms of tissue- and stage-specific gene expression.

The main step on which regulation of gene expression occurs is the initiation of transcription. Minimum requirement for the initiation of gene transcription is binding of an appropriate set of transcription factors (TFs) to its 5'-upstream region. The combination of factors is determined by the regulatory sequences of a gene and each transcription factor recognizes DNA sequence to which it binds. Computer analysis of rabbits' milk protein gene promoters had shown potential binding sequences for various TFs. Their 5'-flanking regions contain putative binding sites for mammary gland-specific: MAF, STAT5, PMF, and for ubiquitous TFs: AP1, CREB, GR, NFI, NF-κB, Oct1, Sp1, YY1 [Malewski and Zwierzchowski 1995, Malewski 1998].

Over the last several years the regulation of expression of milk protein genes was extensively studied, and the number of transcription factors involved in their induction has been characterized. These factors include C/EBP, CTF/NFI, GR, MAF, PMF, STAT5, YY1 [Rosen *et al*. 1996, 1999]. Rabbits are small animals useful for experimental work, and simultaneously they are big enough for biotechnological industry. Recently, in the rabbit mammary gland the following TFs were studied: NFI, Oct1 [Malewski *et al*. 2002], STAT [Malewski and Zwierzchowski 1997, Tourkine *et al*. 1995] and MAF and PMF [Malewski and Zwierzchowski 1997].

The aim of this study was to analyse the DNA-binding activity of those TFs of which putative binding sites were identified previously [Malewski and Zwierzchowski 1995] in the rabbit milk protein gene 5'-flanking sequences. In the current study changes of these TFs in the mammary gland during pregnancy, lactation and involution were analysed with the electrophoretic mobility shift assay (EMSA). The specificity of DNA-protein complexes was analysed by titration with increasing amounts of appropriate unlabelled oligonucleotides.

Material and methods

Animals and tissues

Mammary glands of New Zealand rabbits at different physiological stages were used: day 15 and 25 of pregnancy, day 3 of lactation, and day 3 and 7 of involution (three animals per stage). Mammary gland samples were excised immediately after killing rabbits at the local abattoir. Mammary tissues were cleared from most adjacent muscles, fat and connective tissues, frozen at -25°C and stored at -75°C until use.

Preparation of nuclei

Nuclei were prepared from frozen tissues according to Wakao *et al*. [1992]. Equal samples of mammary gland tissues derived from 3-4 animals at the same physiological stage were pooled and used for preparation of cell nuclei. Mammary glands were homogenised in 10 volumes of buffer (10.0 mM HEPES-NaOH, pH 7.5; 10.0 mM NaCl; 0.1 mM EDTA; 0.1 mM EGTA; 1.0 mM DTT, 0.7 mM spermidine; 0.15 mM spermine; 0.2 mM PMSF and 0.1% NP-40) in a Polytron type homogenizer (KINEMATICA). The NaCl concentration was raised to 0.1 M by the addition of an appropriate volume of 5.0 M NaCl. After filtration through nylon nets, the homogenate was centrifuged at 1,800 g for 10 min. The pelleted nuclei were then washed once with buffer and pelleted again by centrifugation.

Extraction of nuclear proteins

Nuclei were suspended (0.125 v/w of the original tissue) in the buffer: 20.0 mM HEPES-NaOH pH 7.5; 600.0 mM NaCl; 0.2 mM EDTA; 2.0 mM EGTA; 1.0 mM DTT; 0.7 mM spermidine; 0.15 mM spermine; 0.1% NP-40; 0.2 mM PMSF; 25% (v/v) glycerol and incubated on ice for 40 min with gentle agitation. Then the nuclei were removed by centrifugation at 40,000 g for 20 min and the nuclear proteins were dialysed against 10v of buffer: 20.0 mM HEPES-NaOH pH 7.5; 50.0 mM NaCl; 2.0 mM EDTA; 1.0 mM DTT, 0.2 mM PMSF; 0.1% NP-40; 10% glycerol. The precipitate formed during the dialysis was removed by centrifugation at 40,000 g for 30 min. Extracts of nuclear proteins were divided into portions and stored at -75°C.

Preparation of probes

For measuring DNA-binding activities of AP1, CREB, and Sp1, oligonucleotides from the Transcription Factors Bandshift Kit (GIBCO-BRL) were used. Oligonucleotides for Myb were synthesised in the Polish Academy of Sciences Institute of Biochemistry and Biophysics, Warsaw (consensus sequences bolded and underlined):

AP1: 5'–GATCCTTCG**TGACTCA**GCGGGATCCTTCG**TGACTCA**GCGG–3'

CRE: 5'–GATC**TGAC**GTCATGAC**TGACGTCA**TGAC**TGACGTCA**TCA–3'

Myb: 5'-GTAGAGGCA**TAACGGTTGG**GTAGTGA–3'

Methylated Myb: 5'–GTAGAGGCA**TAAC^{M}GGTTGG**GTAGTGA–3'

Sp1:5'–ATCG**GGGCGGGG**ATCG**GGGCGGGG**ATCG**GGGCGGGG**–3'

Complementary oligonucleotides were annealed at room temperature. For labelling of the probes 2 pmoles of the double-stranded oligonucleotides with extended 5' ends were elongated with 6.0 U of Klenov fragment of DNA polymerase I, 1.0 mM of each of dATP, dGTP, dTTP and 50.0 mCi (1.85 MBq) of [α-^{32}P] dCTP (ICN). The

labelled probes were purified from unbound nucleotides using QIAquic Nucleotide Removal Kit (QIAGEN).

Electrophoretic mobility shift assay (EMSA)

EMSA analyses were performed according to Schmitt-Ney *et al*. [1991]. Twenty femtomoles (~50,000 cpm) of a labelled probe was incubated with 5 mg of nuclear proteins for 30 min. at room temperature in a 20-µl final reaction in buffer recommended by SantaCruz Biotechnology, Inc. (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1.0 mM DTT, 1.0 mM EDTA and 5% glycerol). Then, 2 µl of loading buffer (50% glycerol, 0.25% bromphenol blue) was added and the reaction mixture was loaded on a prerun 6% non-denaturing polyacrylamide gels in 0.25 × Tris-borate-EDTA (TBE). The electrophoresis was performed at room temperature at 100 V until the bromphenol blue had migrated to the end of gel.

The gels were dried in a BioRad (GEL DRYER 543) apparatus and subjected to radioautography with Kodak Screen. Autoradiograms were scanned with Bio-Rad Molecular Imager FX and densitometry was made with Quantity One programme (Bio-Rad).

Results and discussion

AP1 DNA-binding activity

Nuclear proteins derived from the rabbit mammary glands formed only one DNA-protein complex with the AP1 probe (Photo 1A). Competition experiments showed that this complex was specific (Photo 1B). The AP1 DNA-binding activity strongly increased on day 25 of pregnancy (as compared to day 15) and then continued to increase during lactation. The AP1 DNA-binding activity sharply decreased on day 3 of mammary gland involution, but later restored and on day 7 was comparable to that at day 25 of pregnancy.

CRE DNA-binding activity

Crude nuclear protein extracts from the mammary glands formed two DNA-protein complexes with the CRE probe (Photo 2A). The complex 1 appeared specific for lactation stage, while presence of the second complex 2 coincides with expression of milk protein genes on day 25 of pregnancy, lactation, and day 3 of involution. According to Malewski *et al*. [2002] the DNA-protein complex 1 was absent in mid pregnancy (day 15) and at late (day 7) involution of the mammary gland, when milk protein gene expression was not detected. The specificity of DNA-protein binding was estimated by competition with 10- and 50-fold excess of unlabelled probe. The DNA-protein complexes appeared partially specific as 50-fold competitor excess significantly reduced the binding of proteins to DNA (Photo 2B).

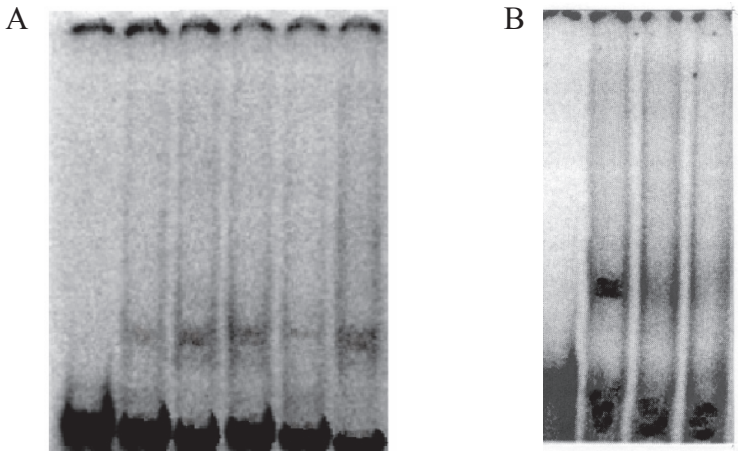

Photo 1A and 1B. DNA-binding activity of AP1 in the rabbit mammary gland nuclear extracts. **A**: Nuclear proteins derived from 15-day (P15) and 25-day (P25) pregnant, 10-day lactating (L), 3-day (I3) and 7-day (I7) post-weaning rabbit analysed by EMSA with labelled AP1 probe. **B**: Competition analysis – unlabelled AP1 probe was used as a competitor at 10× or 50× excess.

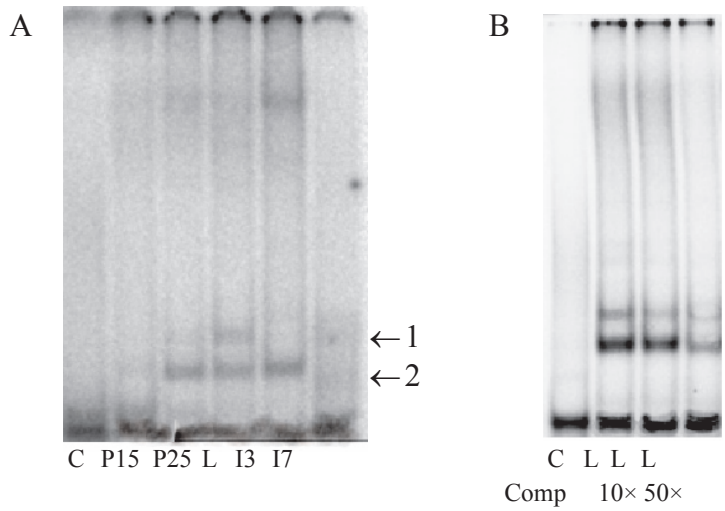

Photo 2A and 2B. DNA-binding activity of CREB in the rabbit mammary gland nuclear extracts. **A**: Nuclear proteins analysed by EMSA with labelled CRE probe. Arrows indicate DNA-protein complexes. **B**: Competition analysis with unlabelled CRE probe. Other details as in the legend to Photos 1A and 1B.

Myb DNA-binding activity

Myb binding site contains CG sequence that is often methylated in the genomic DNA. In the extracts of nuclear proteins derived from the rabbit mammary glands expressing milk protein genes (day 25 of pregnancy, lactation, and early involution) two DNA-protein complexes were found with the Myb probe (Photo 3A). Nuclear proteins were shown to bind more efficiently to methylated than to the unmethylated probes. Competition experiments showed that both complexes were the sequence-specific (Photo 3B).

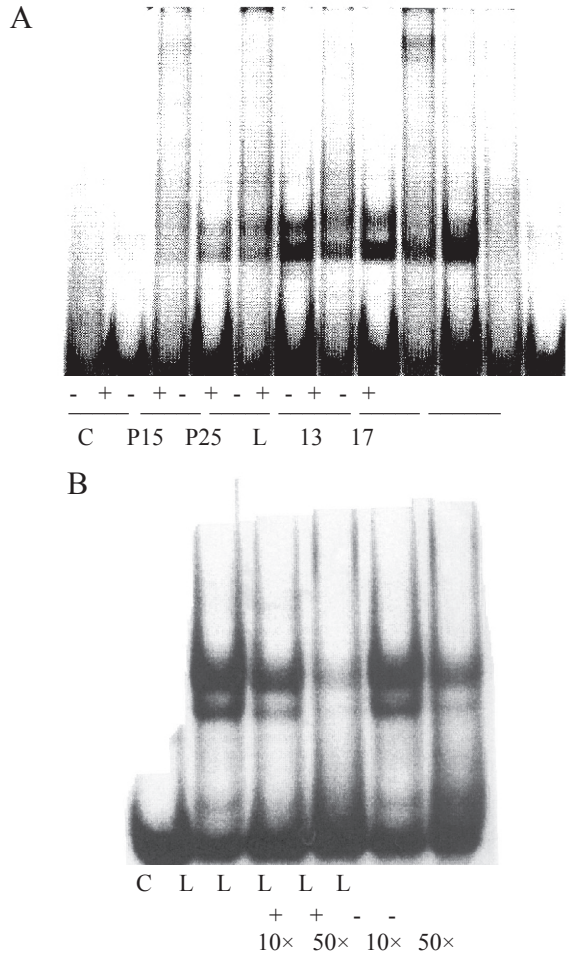

Photo 3A and 3B. DNA-binding activity of Myb in the rabbit mammary gland nuclear extracts. **A**: Nuclear proteins analysed by EMSA with labelled unmethylated (-) or methylated (+) probe. **B**: Competition analysis with unlabelled unmethylated (-) or methylated (+) Myb probe. Other details as in the legend to Photo 1A and 1B.

Sp1 DNA-binding activity

Sp1 binding activity was found in the mammary gland nuclear extracts at all investigated stages except late involution. Nuclear proteins formed single DNA-protein complex with the SP1 probes (Photo 4A). Amount of DNA-protein complex increased from middle of pregnancy, and reached a peak at day 3 of involution. During transition from early to late involution the DNA-binding activity was completely lost. Specificity of Sp1 DNA-protein complexes was confirmed by competition experiments (Photo 4B).

Over the last several years the transcription factors were extensively studied of the mammary glands of mice and rats, and the number of transcription factors involved in the induction of the milk protein genes has been characterized. However, very little is known about transcription factors in the rabbit mammary gland. Investigation of biological role of STAT5 showed significant differences in controlling lactation between rodents and ruminants [Wheeler *et al*. 2001].

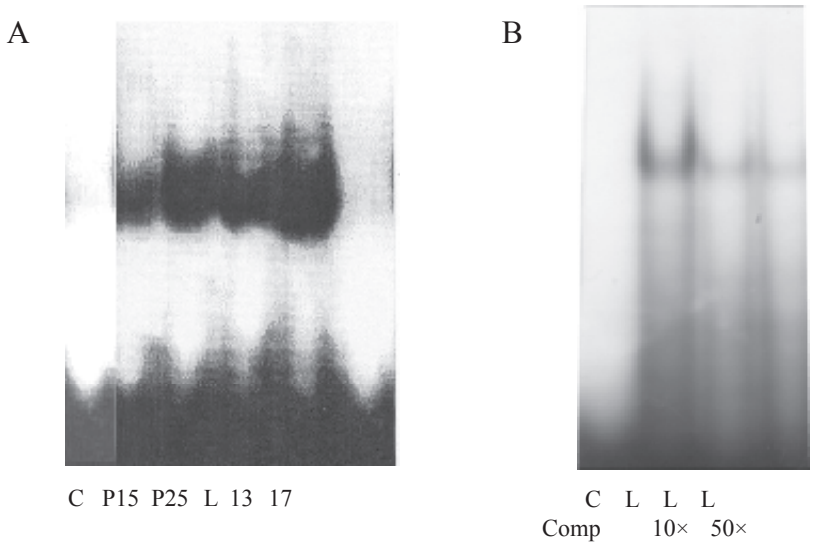

Photo 4A and 4B. DNA-binding activity of Sp1 in the rabbit mammary gland nuclear extracts. **A**: Nuclear proteins analysed by EMSA with labelled Sp1 probe. **B**: Competition analysis with unlabelled Sp1 probe. Other details as in the legend to Photo 1A and 1B.

AP1 is one of transcription factors that integrate system and local signals. Glucocorticoids and local signals regulate expression of fos and jun genes, which protein products make transcription factor AP1. In mice, the occlusion of one of nipple induces AP1 while level of AP1 in neighbour gland remains unchanged. Reversion of involution process in the mammary leadz to decrease of AP1 level [Marti *et al*. 1997]. AP1 was detected in the mouse mammary gland as the JunD homodimer and cFos/JunD

heterodimer [Marti *et al*. 1995]. This TF was detected in virgin mice, but it is especially abundant in the mammary gland during stage I of apoptosis. In the first 24-hours after pups withdrawal expression of c-jun gene transiently increased, later expression on the level of c-jun gene decreased, while an increase of c-fos gene expression was observed [Marti *et al*. 1994]. Expression of the third protein that makes AP1 transcription complex – Jun D - appeared during lactation and gradually increased during mammary gland involution [Jaggi *et al*. 1996].

In this study in the rabbit mammary gland detected was only one AP1-specific DNA-protein complex. Its amount increases during late pregnancy and remained at approximately the same level during lactation. Early involution is associated with its transient decrease.

Data about Myb transcription factor in the mammary gland are very scarce. In mice, knockout of the A-myb gene blocked proliferation of mammary epithelial cells during pregnancy, what accounted for decrease of milk duct branching and 60% decrease of the mammary gland weight [Toscani *et al*. 1997]. Myb is one of few of transcription factors found in the mammary gland of which binding sites can be methylated. Klepnauer [1993] reported that affinity of Myb transcription factor to its binding site is methylation-dependent. Myb-specific DNA-protein binding activity in the mammary gland of rabbit paralleled the activity of milk protein gene transcription. This might suggest that Myb could be important for milk protein gene expression. This study also showed that the affinity of protein(s) to methylated DNA sequence was much higher then to unmethylated sequences.

The Sp1-DNA complexes were detected in the mammary gland of virgin mice and its DNA-binding activity increased during pregnancy, and remained on the same level until day 10 of lactation. In nuclear extracts from involuting mammary gland Sp1 activity was not found [Geyamer and Doppler 2000]. In the rabbit mammary gland amount of the SP1 DNA-protein complexes progressively increased from day 15 of pregnancy to early involution and sharply decreased at late stage of involution.

The results presented here showed that AP1, CREB, Myb and Sp1 transcription factors are present in the rabbit mammary gland and are expressed at different levels of the lactation-reproduction cycle. Abundance of Myb transcription factor correlate well with the levels of milk protein gene expression. Only one of the CRE-specific DNA-protein complexes was found in the mammary glad of lactating rabbits. During early involution transient decrease of the AP1 DNA-binding activity was observed. Transition from early to late involution was associated with the restoration of AP1 activity, and decreasing of Myb and Sp1 DNA-protein binding activities.

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Zmiany aktywności wiązania z DNA wybranych czynników transkrypcyjnych z DNA w różnych stadiach fizjologicznych gruczołu mlekowego królic

S t r e s z c z e n i e

Nasze wcześniejsze badania prowadzone *in silico* wykazały obecność potencjalnych miejsc wiązania dla wielu czynników transkrypcyjnych w rejonie 5'-flankującym genów białek mleka. W niniejszej pracy analiza została poszerzona o doświadczalne zbadanie wiązania tych czynników z DNA. Stosując analizę EMSA do analizy białek jądrowych wydzielonych z gruczołu mlekowego w różnych stadiach rozwoju wykazaliśmy po raz pierwszy obecność u królic czynników transkrypcyjnych AP1, CREB, Myb i Sp1. Ilość białek wiążących się do sekwencji rozpoznawanej przez Myb koreluje z aktywnością transkrypcji genów białek mleka; powinowactwo wiązania Myb zależy od metylacji sekwencji DNA. Ilość czynników transkrypcyjnych w gruczole mlekowym zmienia w cyklu ciąża - laktacja - zasuszanie. Jeden z kompleksów DNA-białko, wiążących się do sekwencji CRE jest obecny w gruczole mlekowym tylko w okresie laktacji. Podczas inwolucji gruczołu mlekowego obserwowano przejściowe zmniejszenie ilości białek wiążących się do sekwencji rozpoznawanej przez czynnik transkrypcyjny AP1. W późniejszym okresie inwolucji ilość tego kompleksu powraca do poprzedniego poziomu, zmniejsza się natomiast poziom kompleksów DNA-białko tworzonych przez Myb i Sp1.