Differences in the Abundance of Nuclear Proteins in the Bovine Mammary Gland Throughout the Lactation and Gestation Cycles

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ABSTRACT

The mechanisms that control the synthesis of milk protein are not fully understood and could well involve undiscovered proteins within the mammary gland. A search for such proteins in high salt extracts of nuclei that had been isolated from bovine mammary tissues was undertaken using two-dimensional electrophoresis on large format gels. The sensitivity of the procedure was sufficient to detect the transcription factors Sp1 and NF-1 by Coomassie blue stain; over 300 proteins were routinely detected. Analysis of mammary tissue taken from 5 nonlactating cows in midpregnancy, 5 cows in late lactation, and 4 cows in early involution revealed five proteins where relative abundance was altered with stage of lactation or reproductive cycle. Four of these proteins were identified by Western blotting or amino acid sequencing as lactoferrin, annexin II, vimentin, and heavy-chain immunoglobulin. Analysis of proteins after further enrichment of the extracts by heparin-Sepharose affinity chromatography revealed an additional protein that was substantially more abundant in samples from lactating cows. This 90-kDa protein did not react with anti-Stat5 antibodies. Conceivably, one or more of these six proteins could play a role in the lactational function of the bovine mammary gland.

(**Key words**: lactation, bovine mammary gland, vimentin, annexin)

INTRODUCTION

Synthesis of milk by the mammary gland is under the complex control of local and systemic hormones and other factors that influence milk output (14, 16). The expression of milk protein genes is also dependent on the extracellular matrix that interacts with a complex on the cell surface that contains β-integrin and causes changes in cytosolic and cytoskeletal proteins (17, 18). Regulation of milk output within the gland by locally produced factors is evidenced by the correlation between milk production within a gland and the amount of milk removed from that gland (3). In recent years, response elements of lactogenic hormones have been mapped within the promoters of milk protein genes, and, in some cases, the proteins that mediate these lactational signals are known (8). Despite these advances, many aspects regarding the control of milk production are not completely understood, particularly the molecular mechanisms within mammary epithelial cells by which most of the extracellular signals are transduced and the mechanism by which these signals interact with one another. Control of mammary function likely involves additional, perhaps undiscovered, nuclear proteins. In this study, we sought to detect and identify such proteins by analysis of nuclear extracts from bovine mammary tissue using two-dimensional electrophoresis on large format gels.

MATERIALS AND METHODS

Tissue Collection and Preparation of Nuclear Extracts

Udders were obtained from Friesian dairy cows at the Ruakura slaughterhouse (Hamilton, New Zealand). The cows had been culled from a research herd and were of various ages and stages of lactation when slaughtered (Table 1). Pieces of mammary tissue were excised from the center of the left front quarter, avoiding tissue from the cisternal region, and snap-frozen in liquid nitrogen within 10 min of removal. High salt extracts of nuclei were prepared using the method of Dignam (6) in which a relatively crude preparation of nuclei (pellet produced from cen-

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TABLE 1. Lactation state, age, and pregnancy status of cows at slaughter.

Cow	Lactation state	Age	Pregnancy status
1	Not lactating	2	3 mo
2	Not lactating	2	6 mo
3	Not lactating	2	6 mo
4	Not lactating	2	6 mo
5	Not lactating	2	6 mo
6	7 mo	8	Not pregnant
7	7 mo	10	Not pregnant
8	7 mo	11	Not pregnant
9	7 mo	4	6 mo
10	7 mo	7	6 mo
11	Not milked in 11 d	3	Not pregnant
12	Not milked in 11 d	7	6 mo
13	Not milked in 8 d	3	6 mo
14	Not milked in 8 d	7	6 mo

trifugation of cell lysate at 1000 × *g*) was extracted with salt. The frozen tissue (30 g) was ground with a mortar and pestle under liquid nitrogen and then homogenized for 30 s in 100 ml of a low salt buffer [10 m*M* HEPES (pH 7.9), 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 1 m*M* phenylmethylsulfonyl fluoride, and 1 m*M* sodium orthovanadate] containing 0.5% (vol/vol) NP-40 using a tissue mincer (Ultra-Turrax; Janke & Kunkel, Staufen, Germany). The homogenate was filtered twice through cheesecloth. Nuclei were extracted in 0.3 *M* KCl. Typically, 6 to 10 mg of nuclear extract were obtained from 30 g of tissue and ranged in concentration from 0.5 to 1.5 mg/ml.

Subcellular Fractionation

Subcellular fractionation was performed essentially as has been described previously (7). Briefly, mammary tissue was homogenized as described previously, and nuclei and cell debris were pelleted by centrifugation at 1000 × *g* for 10 min. The supernatant was then centrifuged at 100,000 × *g* for 60 min to produce cytosolic (supernatant) and membrane plus organelle (pellet) fractions. To prepare extracts of nuclei that were enriched further than those prepared by the Dignam (6) method, the pellet that resulted from centrifugation at 1000 × *g* was resuspended in buffered 0.25 *M* sucrose and centrifuged through a cushion of buffered 2.2 *M* sucrose at 70,000 × *g* for 70 min. Both sucrose-purified preparations and crude nuclei preparations were extracted with 0.3 *M* KCl as described previously.

Electrophoresis and Densitometry

One-dimensional SDS-PAGE was performed as described by Laemmli (12). Samples to be analyzed were lyophilized and dissolved in sample buffer to a final concentration of 2 mg/ml. Two-dimensional electrophoresis was performed using an adaptation of the method of O'Farrell (15) essentially as described previously (22). Samples to be analyzed were lyophilized and dissolved in 0.2 ml of the sample buffer used in two-dimensional electrophoresis and were subjected to both isoelectric focusing and nonequilibrium pH gradient electrophoresis using gels cast in 400- × 3.5-mm i.d. tubes. The gels contained 1% (wt/vol) ampholytes (pH 3.5 to 10) and 1% (wt/vol) ampholytes (pH 5 to 7) (LKB, Uppsala, Sweden). The gels were extruded, equilibrated for 2 h at 20°C, then either stored at –70°C or loaded immediately onto 400- × 400- × 0.75-mm SDS gels containing 12% (wt/vol) acrylamide. Electrophoresis was carried out overnight at 25 mÅ per gel. Gels were stained for 1.5 h in Coomassie blue G, destained in 25% (vol/vol) methanol/5% (vol/vol) acetic acid, and then dried. Densitometry was performed on photographic images of dried gels containing the p36, p56, and p90 proteins using a scanning laser densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Heparin-Sepharose Chromatography

Heparin-Sepharose affinity chromatography was performed using a batch procedure as was previously described by Roy et al. (19). Aliquots of extracts of crude nuclei containing 5 mg of protein in approximately 3 ml of buffer A [20 m*M* HEPES-NaOH (pH 7.9), 1 m*M* EDTA, 100 m*M* KCl, and 1 m*M* DTT] were mixed with 0.15 ml of packed volume heparin-Sepharose CL-6B (Pharmacia, Uppsala, Sweden). The beads were washed, and the bound protein was eluted with buffer B (0.7 *M* KCl in buffer A) as described by Roy et al. (19). Proteins enriched with heparin-Sepharose were dialyzed overnight against water, assayed for protein using the method of Bradford (2), lyophilized, and taken up in SDS-PAGE sample buffer (12).

Amino Acid Sequencing

The two-dimensional protein spots and protein bands that were subjected to amino acid sequencing were treated in one of two ways. In the first method, the gels were electroblotted to nitrocellulose and stained for protein with Ponceau S; then, the protein of interest was cut out and digested with trypsin as described by Aebersold (1). In the second method, the gels were stained with Coomassie blue, and the protein spot or band was cut out; proteolysis was then