BRIEF COMMUNICATION

Binding of nuclear proteins to the bovine α-lactalbumin gene promoter

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The production of milk during lactation requires transcriptional activation of milk protein genes. This is facilitated by the binding of nuclear factors to the gene promoter. The DNA binding activity of some of these factors appears to be controlled by lactogenic hormones, although many aspects of this process are not understood. Determining the precise molecular mechanism of transcriptional control and which nuclear factors are involved will facilitate the manipulation of milk composition. Recently, a transcription factor called Stat5 has been shown to be required for the activation of the rat β-casein gene (Schmitt-Ney *et al.*, 1991), the sheep β-lactoglobulin gene (Burdon *et al.*, 1994) and the rat whey acidic protein gene (Li and Rosen, 1995). This protein is activated during late pregnancy and throughout lactation in the mammary gland of mice (Schmitt-Ney *et al.*, 1992). We have used the Stat5 DNA recognition sequence in this study to investigate the activity of Stat5 in the bovine mammary gland.

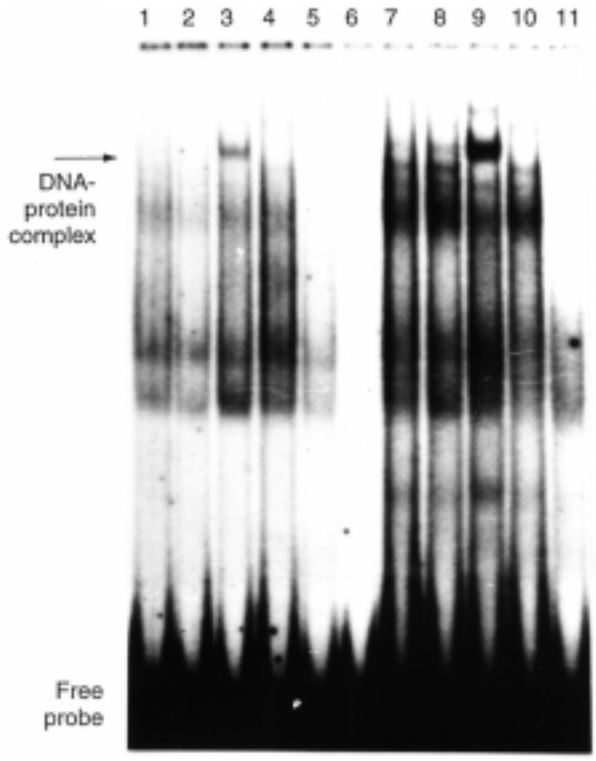
The whey protein, α-lactalbumin, is a key determinant in the production of lactose, the major osmotic component in milk. Understanding the mechanism of regulation of this protein will facilitate the manipulation of the water content in milk. We are determining which regions of the α-lactalbumin gene promoter are important for controlling transcription and identifying nuclear factors that interact with these regions. Our previous analysis has revealed that nuclear factors bind to at least three broad regions of the α-lactalbumin promoter. In this paper we report the analysis, using gel shift assays, of protein binding within one of these regions, a 70bp fragment at -300 to -370 relative to the transcription start site.

Mammary glands and livers were obtained after slaughter from animals at different stages of lactational development i.e. virgin and mid-pregnant cows, cows at mid-late lactation and cows with involuting udders. Biopsy samples were obtained from the secretory tissue of udders from cows in mid lactation using a previously described procedure (Farr *et al.*, 1994). Tissue samples were snap frozen in liquid nitrogen. Nuclei were prepared based on a combination of two procedures (Roy *et al.*, 1991; Current Protocols, 1993). Between 0.5g and 30g of frozen tissue was manually crushed, thawed in 2 volumes of a 0.01M Hepes buffer (pH 7.9) containing 0.01M KCl, then minced with an ultraturrex homogeniser. The homogenate was filtered through cheesecloth, and the cells collected in the filtrate were sheared in the presence of 0.5% NP-40 with a Potter-Elvehjem homogeniser using a teflon pestle. Nuclei were pelleted by centrifugation at 1200g for 10 minutes and washed twice in the buffer containing the 0.01 M KCl, once with 0.5% NP-40 and once without. Nuclear proteins were extracted by one of two ways; either as an ammonium sulphate precipitation (Schmitt-Ney *et al.*, 1991), or as a high salt (0.35M KCl) extract (Current Protocols, 1993). The nuclear extracts were then dialysed for 2-4 hours against a 0.02M Hepes buffer (pH 7.9) containing 0.1M KCl. Aliquots were frozen in liquid nitrogen and stored at -70°C. Both the Stat5 recognition sequence and the 70bp α-lactalbumin promoter sequence fragments were labelled by a Klenow polymerase fill-in reaction of overhanging 5' ends. Binding reactions were done essentially as outlined for the Gel Shift Assay System from Promega (Madison, WI). Unlabelled competitor oligos and antibodies for supershifts were added to the reactions prior to labelled DNA addition. Samples were run on a 0.5X TBE/6% acrylamide gel and bands visualised by autoradiography.

Nuclear proteins, which were extracted from bovine mammary glands at different stages of lactational development by the two methods, were analysed by gel shift assays (Figure 1). For both nuclear protein extraction methods, Stat5 DNA binding activity was present only in the tissue taken from the lactating cow (lanes 3 & 9) and was absent in tissue taken from nonlactating cows (virgin, lanes 1 & 7; pregnant, lanes 2 & 8; involuting, lanes 4 & 10). No Stat5 DNA binding activity was observed with the liver extracts (lanes 5 & 11).Work performed with extracts from murine mammary glands has revealed similar patterns (Schmitt-Ney *et al.*, 1992). There is a distinct difference in the intensities of the retarded bands depending on which method is used in the extraction procedure. The proteins which were extracted by the ammonium sulphate precipitation method (lanes 1-5) had a decreased band intensity compared with the proteins extracted by the high salt (0.35M KCl) extraction method (lanes 7-11). This result is not isolated to this study alone, as other probes and protein extracts from different species which we have assayed have given similar results (data not shown).

Mammary biopsy samples were taken from two udder quarters of each of three lactating cows. One quarter of

FIGURE 1: Binding of bovine nuclear proteins to the Stat5 sequence



Lanes 1-5 show binding of nuclear proteins extracted by the ammonium sulphate method.

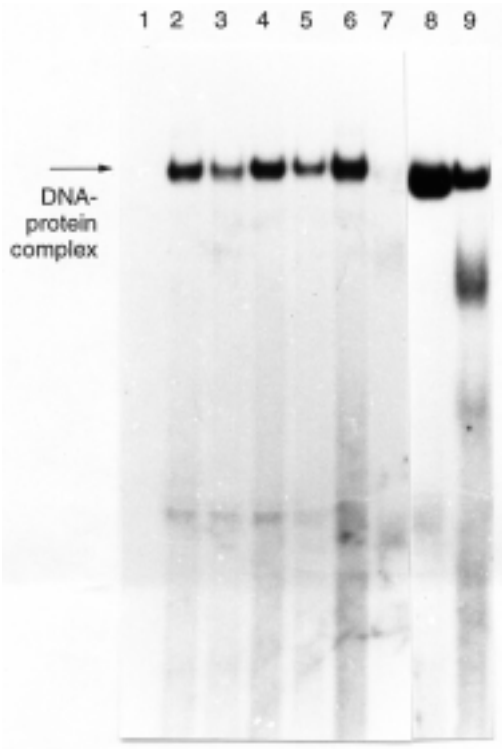
Lanes 7-11 show binding of proteins derived from a high salt extraction method.

Lanes 5 & 11 show a binding reaction with nuclear extracts from the liver of a lactating cow.

Lane 6 is the Stat5 probe alone.

Extracts are derived from udders of cows at different development stages as follows: lanes 1 & 7, virgin; lanes 2 & 8, pregnant; lanes 3 & 9, lactating; lanes 4 & 10, involuting.

FIGURE 2: Binding of bovine nuclear proteins at different times after milking.



Lane 1 is the Stat5 probe alone. This probe was used in the following binding reactions.

Lanes 2-7 show binding of proteins derived from mammary gland biopsy samples of three cows (lanes 2-3, 4-5 and 6-7 respectively) taken either 4 hours (lanes 2, 4 and 6) or 28 hours (lanes 3, 5 and 7) after milking.

Lane 8 shows the binding activity of mouse nuclear proteins.

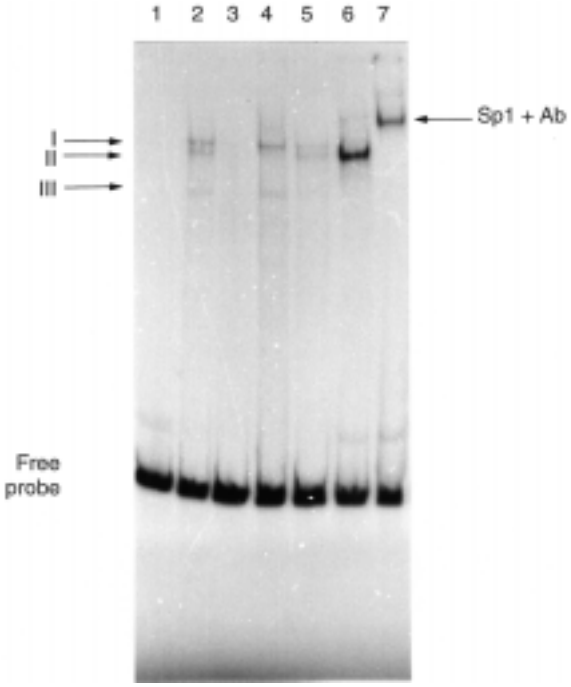
Lane 9 shows the binding activity of sheep nuclear proteins.

each cow had last been milked 4 hours and the other 28 hours before sample collection. Nuclear proteins were isolated using the high salt (0.35M KCl) extraction method and analysed for binding to the Stat5 probe using a gel shift assay (Figure 2, lanes 1-7). Nuclear extracts from mammary glands of a lactating mouse (lane 8) and a lactating sheep (lane 9) were also analysed. There was a much reduced DNA binding activity in the samples taken 28 hours post milking compared with the 4 hour post milking samples for all three cows. This suggests that the Stat5 DNA binding activity decreases when the gland is not being milked or suckled. The intensity of the band in the mouse sample is much stronger than that for both the cow and the sheep. This may be due to several factors including a higher affinity of mouse nuclear proteins than bovine or ovine nuclear proteins to the rat β-casein Stat5 sequence, and a greater abundance of Stat5 protein in the mouse extracts.

The 70bp fragment of the α-lactalbumin promoter was analysed in a gel shift assay using nuclear proteins derived from a lactating bovine mammary gland (Figure 3). Binding of bovine nuclear proteins to this fragment results in the appearance of three retarded bands (lane 2, I, II and III). None of these bands were competed off when an excess of unlabelled DNA containing Ap1, Ap2, TFIID, Oct1, CTF/NF1, GRE, CREB, NF-kB or Stat5 consensus binding sites were added (data not shown). However an excess of unlabelled oligo containing the Sp1 binding site competed off all three of these bands (lane 3). One of these three bands (II, the lower band in the doublet) was supershifted after addition of anti-Sp1 antibody to the binding reaction (lane 4). An excess of unlabelled 70bp fragment used as competitor DNA in the reaction caused a decrease in binding of all three bands (lane 5). Purified Sp1 protein added to the 70bp fragment resulted in a single band having the same mobility as the lower band in the doublet (II, lane 6), and the addition of an anti-Sp1 antibody to this binding reaction resulted in the expected supershift (lane 7). These results strongly suggest that the transcription factor Sp1 interacts with the bovine α-lactalbumin promoter at a site within the region -300 to -370 relative to the transcription start site.

We have demonstrated by gel shift assays that two different methods of nuclear protein extraction produce proteins that have different DNA binding activities. We have also demonstrated that in the case of lactating bovine mammary tissue, the time of sample collection after milking greatly influences how active nuclear proteins are.

FIGURE 3: Gel Shift Assay showing the binding of nuclear proteins to the α-lactalbumin promoter.



Lane 1 is the 70bp fragment alone. This probe is used in lanes 1-7.

Lane 2 shows the DNA-protein complexes formed (I, II and III) following nuclear protein addition. The bovine extract is used in lanes 2-5.

Lane 3 has the addition of an unlabelled Sp1 competitor oligo.

Lane 4 has the addition of an anti-Sp1 antibody to the reaction.

Lane 5 shows the addition of an unlabelled 70bp competitor oligo.

Lane 6 shows the addition of a purified Sp1 protein to the 70bp fragment.

Lane 7 is similar to lane 6, but has an anti-Sp1 antibody addition to the reaction.

Determining which method to use and which sample to collect in order to prepare good quality nuclear extracts can greatly affect the generation and interpretation of results. The binding of a nuclear protein, from the mammary gland of a dairy cow, to the Stat5 probe during lactation, but not during non-lactation, suggests that the Stat5 protein may be involved in the induction and/or maintenance of the lactation state in cows. Further studies are underway in our laboratory to define the role of Stat5 in cattle. Another transcription factor, Sp1, has been shown in this study to bind to a specific region of the α-lactalbumin promoter. It will be interesting in future studies to see what effect this particular factor, in conjunction with other factors, will have on the transcriptional control of the α-lactalbumin gene.

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