Conserved region of the rat α -lactalbumin promoter is a target site for protein binding *in vitro*

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Nuclear extracts from mammary glands of lactating rats contain DNA binding protein(s) with high affinity to the region from –125 to –85 of the rat α -lactalbumin promoter. The protected sequence is part of a mammary conserved sequence described by Hall *et al.* [(1987) *Biochem. J.* 242, 735–742]. Comparison of the DNA protein-binding sites in the 5' flanking sequence of the rat α -lactalbumin and mouse whey acidic protein genes suggests that the consensus sequence 5'-TGGCARNNWCKKC-3' is the protein(s) recognition site. This binding site is part of a highly conserved region [consensus sequence, TGGCAGSCTCGGCST-(G)YTCTCTCT(NTG)TGGCARA] present in the promoter of four whey protein genes.

INTRODUCTION

Our understanding of the control of milk protein gene expression is based on experiments with the whole animal, mammary gland explants and isolated epithelial cells (Hall & Campbell, 1986; Hobbs *et al.*, 1982; Lee *et al.*, 1985; Rosen *et al.*, 1985; Topper & Freeman, 1980; Topper *et al.*, 1984). Such studies have shown that peptide and steroid hormones, and cell-cell and cell-substratum interactions, as well as other factors, modulate the expression of milk proteins. Milk protein genes are of interest because they provide an attractive system for studying hormonal and developmental gene regulation (Vonderhaar & Bhattacharjee, 1985) and they also have potential applications in biotechnology (Clark *et al.*, 1987; Gordon *et al.*, 1987).

As a necessary step towards understanding the mechanisms and sequences involved in the regulation of milk protein gene expression, several genes have been cloned and sequenced (Jones *et al.*, 1985; Yu-Lee *et al.*, 1986; Qasba & Safaya, 1984; Hall *et al.*, 1987; Campbell *et al.*, 1984). Comparison of the 5' flanking regions of seven milk protein genes has revealed a highly conserved structural element [consensus sequence: RGAAGRAAA-(N)TGGACAGAAATCAA(CG)TTTCTA], extending from nucleotide –110 to –140 upstream of the transcription initiation site (Hall *et al.*, 1987). Although it has been suggested that this conserved sequence might be involved in the regulation of milk protein gene expression, its functional significance has not yet been determined by transient transfection experiments. However, experiments with transgenic animals have demonstrated that the 5' flanking regions of the mouse whey acidic protein (WAP) gene (Andres *et al.*, 1987; Gordon *et al.*, 1987; Schoenenberger *et al.*, 1988) and the rat β -casein gene (Lee *et al.*, 1988) can target the expression of a transgene predominantly to the lactating mammary gland, and that a sheep β -lactoglobulin gene can be expressed accurately in mice (Simons *et al.*, 1987).

There is evidence suggesting that numerous protein factors participate in directing RNA polymerase to transcriptional start sites by binding to promoter elements (Dynam & Tjian, 1985). The mammary conserved region is located just upstream of the TATA box in seven milk gene promoters and could be the target for specific nuclear proteins. In this report, we have used DNAase I-footprinting assays to analyse the binding of nuclear proteins to the mammary conserved sequence in the promoter of the rat α -lactalbumin gene. Nuclear proteins from mammary glands of virgin, pregnant and lactating rats recognize part of this mammary conserved sequence and the protected region is part of a 34 bp sequence highly conserved among four whey protein genes.

MATERIALS AND METHODS

Materials

Restriction endonucleases, the Klenow fragment of DNA polymerase I and T4 polynucleotide kinase were purchased from New England Biolabs, Beverly, MA, U.S.A. Alkaline phosphatase from calf intestine was obtained from Boehringer Mannheim. [α ³²-P]dATP, dGTP, dCTP and [γ ³²-P]ATP were purchased from Amersham International. All other chemicals were the highest grade available.

Recombinant plasmids and isolation of DNA fragments

The plasmid pB α -LA 2.5 (Qasba & Safaya, 1984) was used to obtain fragments from the 5' flanking region of the rat α -lactalbumin gene. The *Sst*I–*Kpn*I (–354 to +24) fragment from the mouse WAP gene had been cloned into the polylinker of pUC18 (Lubon & Hennighausen, 1987). Plasmid pBSTN1 containing a fragment overlapping the chicken β -globin gene promoter (–303 to +167) was a gift from Beverly Emerson and Gary Felsenfeld. The *Hind*III–*Fok*I (–335 to –15) fragment from the rat β -casein gene and

Abbreviations used: WAP, whey acid protein; NF1, nuclear factor 1; CTF, cellular transcription factor.

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These sequence data have been submitted to the EMBL/Genbank Data Libraries.

the *AvaII-HincII* (−337 to +220) fragment from the rat α -casein gene were isolated from plasmids that were generously provided by Jeff Rosen. DNA fragments were isolated from low-melting agarose gels.

Preparation of nuclei and nuclear extracts

Mammary glands were dissected from virgin, 12-day-pregnant and 8-day-lactating rats and immediately frozen in liquid nitrogen. Nuclei were isolated by pulverizing the frozen tissue in liquid nitrogen. Pulverized tissue was resuspended in 10 vol. of 0.3 M-sucrose in buffer A (10 mM-Hepes at pH 7.9, 10 mM-KCl, 1.5 mM-MgCl₂, 0.1 mM-EGTA, 0.5 mM-dithiothreitol, 0.5 mM-phenylmethanesulphonyl fluoride and 2 μ g/ml each of antipain, leupeptain and pepstatin A) and homogenized using a glass-teflon homogenizer (1500 rev./min, 10–12 strokes), with 2–3 additional strokes in the presence of 0.5 % NP-40. The optimal conditions for homogenization were monitored using a phase-contrast microscope. The homogenate was filtered through Miracloth (Calbiochem) and crude nuclei were pelleted by centrifugation at 1200 *g* for 10 min. The nuclei were resuspended in 0.3 M-sucrose in buffer A, layered on to 1.7 M-sucrose in buffer A and centrifuged in the HB4 rotor (Sorvall) at 12500 rev./min for 1 h. The pellet was washed in 0.3 M-sucrose in buffer A and nuclei were pelleted by centrifugation at 1200 *g* for 10 min. They were used immediately for protein extraction. Nuclear extracts were prepared using a modification of the procedure of Dignam *et al.* (1983). Nuclei were homogenized in 2.5 pelleted-nuclei vol. of 400 mM-NaCl, 10 mM-Hepes/KOH at pH 7.9, 1.5 mM-MgCl₂, 0.1 mM-EGTA, 0.5 mM-dithiothreitol, 10 % (v/v) glycerol and 0.5 mM-phenylmethanesulphonyl fluoride, using an all glass Dounce homogenizer (10 strokes, B pestle) and stirred slowly for 30 min, followed by centrifugation for 1 h at 100000 *g* using a type 70Ti rotor. After dialysing the supernatant for 3–4 h against 50 vol. of 20 mM-Hepes/KOH at pH 7.9, 100 mM-KCl, 0.1 M-EDTA, 0.5 mM-dithiothreitol, 20 % glycerol, and 0.5 mM-phenylmethanesulphonyl fluoride, the extract was cleared by centrifugation at 25000 *g* for 15 min. The extract was frozen in small aliquots in liquid nitrogen and was stored at −70 °C. The HeLa cell nuclear extract was prepared as described previously (Hennighausen & Lubon, 1987). The protein concentration in extracts was determined by the dye-binding method of Bradford (1976), using the Bio-Rad kit, and was 5–10 mg/ml.

DNAase I footprinting

The DNAase I-footprinting assay consisted of a 15 min preincubation at 4 °C in a 25 μ l reaction mixture containing crude nuclear proteins, 0.3 ng of the labelled DNA template, 1 μ g of poly(dI-dC), 20 mM-Hepes at pH 7.9, 50 mM-KCl, 1 mM-MgCl₂, 0.01 mM-EDTA, 0.5 mM-dithiothreitol and 10 % glycerol. In the DNAase I-footprint analysis with nuclear factor 1 (NF1), 200 ng of NF1 purified from HeLa cells (Rosenfeld & Kelly, 1986) was incubated with 0.3 ng of probe and 200 ng of poly(dI-dC). After incubation, 1 μ l (50 ng) of a DNAase I (Pharmacia) solution was added and the reaction was allowed to continue for 30 s and was stopped by the addition of 100 μ l of termination buffer [1 % Sarkosyl, 100 mM-NaCl, 100 mM-Tris/HCl (pH 8), 10 mM-EDTA, 10 μ g calf thymus DNA/ml]. DNA digestion products were purified by phenol/chloroform extractions,

followed by ethanol precipitation, and analysed on 8 % polyacrylamide/8 M-urea gel, followed by autoradiography.

RESULTS

Mammary gland nuclear extracts were assayed for sequence-specific DNA-binding activity to the rat α -

Fig. 1. DNAase I protection and competition analysis

The 207 bp *Sau3A-BamHI* (−202 to +5) fragment from the rat α -lactalbumin promoter was labelled at the 5' end of the *BamHI* site, incubated with 25 μ g of nuclear proteins from mammary gland of lactating rats and digested with DNAase I in the absence or presence of a 20-fold molar excess of additional competitor DNA fragments. Lane M, Maxam and Gilbert (A/G) sequencing reactions of the labelled fragment; lane C, DNAase I digestion in the absence of nuclear proteins; lane 1, DNAase I digestion in the presence of nuclear proteins and absence of specific competitor DNA; lanes 2–7, protection in the presence of the following competitor DNAs: *Sau3A-BamHI* (lane 2); *Sau3A-FokI* (−202 to −119) of the rat α -lactalbumin gene (lane 3); *HindIII-FokI* (−335 to −15) of the rat β -casein promoter (lane 4); *AvaII-HincII* (−337 to +220) of the rat α -casein gene (lane 5); *SstI-KpnI* (−354 to +24) of the mouse WAP promoter (lane 6); and *BstNI-BstNI* (−303 to +167) fragment of the chicken β -globin gene. The protected regions are indicated by closed rectangles, numbers represent the distance in base pairs upstream from rat α -lactalbumin cap site, and the asterisk indicates a protein-induced DNAase I hypersensitive site.

Fig. 2. DNAase I-footprint analysis of the binding of nuclear proteins from the mammary gland of virgin, pregnant and lactating rats to the α -lactalbumin promoter

The rat α -lactalbumin *Sau3A*–*Bam*HI (–202 to +5) promoter fragment was labelled at the 5' end of the lower (a) or upper strand (b). The autoradiographs display the footprint pattern of probes in the absence of protein (C, control), and in the presence of 25 μ g of extract from the mammary gland of lactating (L, lanes 1), pregnant (P, lanes 2) and virgin rats (V, lanes 3). Binding activities that protected the probes are indicated by closed rectangles. Numbers represent the distance in base pairs upstream from the rat α -lactalbumin cap site. Maxam and Gilbert (A/G) sequencing markers (lane M) of the fragment were used to locate the protected region. The asterisk indicates a protein-induced DNAase I hypersensitive site.

lactalbumin promoter by DNAase I footprinting. This assay is sensitive and allows detection in crude nuclear preparation of nanogram quantities of specific DNA-binding proteins. Fig. 1 shows the results of a footprint assay with extract from lactating mammary gland. The α -lactalbumin promoter fragment, containing the mammary conserved sequence, was labelled at the *Bam*HI site located 5 bp downstream of the transcriptional start site. One major protected region was observed from –124 to –79 on the lower strand (Fig. 1) and from –125 to –85 on the upper strand (Fig. 2). This region partially overlaps with the mammary conserved sequence. To verify specificity of protein binding the footprint reactions were carried out in the presence of different DNA fragments. Protection of the α -lactalbumin promoter upstream region was competed by a 20-fold molar excess of the unlabelled α -lactalbumin *Sau3A*–*Bam*HI fragment (Fig. 1, lane 2), and less intensively by a fragment from the promoter of mouse WAP gene (Fig. 1, lane 6). The *Sau3A*–*Fok*I fragment from the α -lactalbumin promoter and DNA fragments containing

Fig. 3. DNAase I-footprint analysis of the binding of nuclear proteins from HeLa cells to 5' flanking sequences of the rat α -lactalbumin gene

(a) The 207 bp *Sau3A*–*Bam*HI (–202 to +5) fragment of the rat α -lactalbumin promoter was labelled at the 5' end of *Sau3A* site and was digested with DNAase I in the presence of 25 μ g of the nuclear proteins from the HeLa cells (lane 1) or in the absence of proteins (lane C). (b) The *Eco*RI–*Ava*I (–1245 to –943) fragment from the 5' region of the rat α -lactalbumin gene was labelled at the 5' end of *Eco*RI site and digested in the presence of 25 μ g of the nuclear proteins from the mammary gland of lactating rats (lane 1) or in the absence of protein (lane C). (c) Probe described in (b) was digested with DNAase I in the presence of 25 μ g of nuclear proteins from the HeLa cells (lane 2) or in the absence of protein (lane C). Lanes M, Maxam and Gilbert (A/G) sequencing markers of the labelled fragments. The protected regions are indicated by closed rectangles. Numbers indicate the position relative to the site of transcription initiation.

promoter sequences of the rat α - and β -casein, and chicken β -globin genes did not compete well. This suggests that the observed binding to the rat α -lactalbumin promoter is specific and that the promoter of mouse WAP gene contains sequences which can recognize this binding activity.

We then investigated whether differences in binding to the α -lactalbumin promoter could be detected with nuclear extracts from virgin, pregnant and lactating mammary glands. Fig. 2 shows that the α -lactalbumin promoter sequences from –124 to –79 (lower strand) and –125 to –85 (upper strand) were protected by nuclear extracts from the three stages of mammary gland development. These results suggest that factor(s) binding to the upstream region of the α -lactalbumin promoter are present in mammary gland tissue at different stages of development.

DNAase I footprint analyses of the α -lactalbumin promoter with HeLa cell nuclear extracts did not result in a clear protection over the region from –125 to –85 (Fig. 3). In additional control experiments with an *Eco*RI–*Ava*I (–1245 to –943) fragment from the 5' flanking region of the rat α -lactalbumin gene, the

Fig. 4. DNAase I protection using nuclear factor 1 (NF1)

The *Sau3A*–*Bam*HI promoter fragment was labelled at the 5' end of the lower (lanes 1–3) or upper (lanes 4–6) strand. Lanes 1 and 4 show the A/G sequence ladder of the respective fragment; lanes 2 and 5 show the DNAase I-digested fragment in the absence of protein; lanes 3 and 6 show the DNAase I-digestion pattern of the fragments upon incubation with 200 ng of purified NF1. Rectangles indicate the protected region and the numbers show the distance in base pairs from the start site of transcription.

sequence between –1062 and –1040 was protected by nuclear proteins from lactating mammary gland and HeLa cells (Figs. 3b and 3c). This region has apparent sequence similarities to the binding site of cellular transcription factor (Sp1). Nuclear proteins from lactating mammary gland, but not from HeLa cells, protected additional sequences from –1004 to –970 (Fig. 3b).

Since the protein binding sites from –125 to –85 match part of the consensus recognition sequence for CTF/NF1 proteins (Jones *et al.*, 1987), we investigated whether purified NF1 could bind to this sequence in the α -lactalbumin promoter. Purified NF1 from HeLa cells protected this sequence in a similar fashion to proteins in the mammary gland extract (Fig. 4), suggesting that the binding activities in the nuclear extracts from mammary gland share characteristics with NF1.

DISCUSSION

The mammary conserved sequence is located in a similar position in the promoters of all seven milk protein genes and might be involved in their regulation (Hall *et al.*, 1987). Expression of the rat α -lactalbumin changes during gestation and lactation (Qasba *et al.*, 1982), and prolactin (Perry & Oka, 1984), insulin (Topper *et al.*,

Fig. 5. Comparison of whey protein sequences

(a) Comparison of the protein-binding sites in the rat α -lactalbumin and mouse WAP gene promoters. Sequences of the promoter and additional binding sites in the 5' flanking region (H. Lubon & L. Hennighausen, unpublished results) of the rat α -lactalbumin and mouse WAP gene (Lubon & Hennighausen, 1987) were aligned according to the best fit to the motif TGGCA, and grouped by best homology. The bases that match the consensus sequence are marked by * in each binding site. The numbers indicate the positions relative to the sites of transcription initiation. (b) Identification of the consensus sequence present in several whey protein genes. The binding sites from the 5' flanking sequences of the rat and human α -lactalbumin and mouse and rat WAP genes were aligned with the introduction of a few gaps (-). * Indicates bases that match the consensus sequence. Gaps were counted as signal mismatch. The numbers indicate the positions relative to the sites of transcription initiation. S = G or C, Y = C or T, R = A or G, K = G or T, W = A or T. hLA, human α -lactalbumin; rLA, rat α -lactalbumin; mWAP, mouse whey acidic protein; rWAP, rat whey acidic protein.

1984) and glucocorticoids (Ono & Oka, 1980; Ono *et al.*, 1981, Quirk *et al.*, 1986; Terada *et al.*, 1983) play a role in its expression. We therefore determined whether this conserved sequence in the α -lactalbumin promoter is a target for nuclear proteins isolated from mammary glands at different stages of development. We were not successful in detecting specific interactions of nuclear proteins with this sequence using different established and modified protein-blotting procedures (results not shown). However, DNAase I-footprinting experiments allowed us to show sequence-specific interactions of mammary gland nuclear proteins with the –125 to –85 region of the rat α -lactalbumin promoter, which contains at least two binding sites with a similar sequence motif, TTGGCAG. Mammary gland nuclear extracts from virgin, pregnant and lactating animals provided a similar protection pattern. At this stage we cannot assume that similar binding also occurs *in vivo*. For example, it has been shown that factors which recognize the MMTV promoter *in vivo* only after hormone stimulation are

already present in the uninduced cells and can bind to DNA *in vitro* (Cordingley *et al.*, 1987; Cordingley & Hager, 1988). Binding of nuclear factors to a promoter can alter chromatin conformation, which may be detected for some genes by DNAase I digestion of nuclei (Elgin, 1981). Two DNAase I hypersensitive sites at -4.1 and -5.5 kb, and at least two additional sites of weak hypersensitivity at -0.15 and -0.35 kb in the 5' flanking region of the rat α -lactalbumin gene have been mapped and were shown to be present whether or not the gene was actively expressed (H. Lubon & L. Hennighausen, unpublished observations). Within the limits of our mapping, there seems to be no clear inducible DNAase I-hypersensitive site around the conserved region of the promoter which binds nuclear protein *in vitro*. Changes of transcriptional activity have been observed without any changes in DNA-binding properties of specific transcription factors. This leads to the conclusion that regulation might be exerted by a mechanism which does not simply involve altered DNA-binding activity (Fisch *et al.*, 1987; Prywes & Roeder, 1986; Treisman, 1986).

The mouse WAP gene promoter, which binds specifically to mammary gland nuclear proteins (Lubon & Hennighausen, 1987), competes for factor binding to the rat α -lactalbumin promoter. This suggests that the mammary conserved sequence shared by the two genes is recognized by proteins with similar binding capacities. The protected regions of the rat α -lactalbumin and mouse WAP gene promoters share a consensus sequence containing a TGGCA motif (Fig. 5a), which matches part of the consensus recognition sequence for CTF/NF1 proteins (Jones *et al.*, 1987). It is therefore possible that CTF/NF1-like binding activity in mammary gland extract is involved in the protection of the rat α -lactalbumin and mouse WAP gene promoters. Moreover, the binding proteins were enriched in mammary gland extract because we could barely detect binding activity in HeLa cell nuclear extract. However, that NF1 purified from HeLa cells bound to the TGGCA motif in the α -lactalbumin promoter demonstrates that the observed binding activity was not mammary gland specific. Since CTF/NF1 proteins activate viral DNA replication and transcription of cellular genes, it can be suggested that the proteins binding to the rat α -lactalbumin and mouse WAP gene promoters are members of a family of DNA binding proteins that recognize and regulate a set of promoters.

Alignment of the protected regions in the rat α -lactalbumin and mouse WAP gene promoters with the 5' flanking regions of the human α -lactalbumin and rat WAP genes, according to the best fit to the TGGCA motif, revealed significant similarities (Fig. 5b). The average homology to the derived 34 bp consensus sequence was 82.3%. The first 24 bp of this consensus sequence display an even higher homology, 89.4%, and correspond to the region reported to be conserved among milk protein genes (Hall *et al.*, 1987). However, strong similarity was not observed between casein genes and the consensus sequence derived for the whey protein genes, which is in agreement with the competition assays. Further studies in transgenic animals will help to define the role of the mammary conserved sequence in the regulation of milk protein gene expression.

In conclusion, we have shown that nuclear factor(s) from the mammary gland of virgin, pregnant and lactating rats recognize the mammary gene conserved

sequence in the rat α -lactalbumin promoter, and that the region of protein binding is highly conserved in the four whey protein genes.

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