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Temporal profiles of appearance of DNase I hypersensitive sites associated with the ovine β-lactoglobulin gene differ in sheep and transgenic mice

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Abstract The ovine milk protein β-lactoglobulin is expressed in a distinct temporal pattern during lactogenesis. This expression pattern is reflected in the temporal profile of appearance of DNase I hypersensitive sites (HS) associated with the β-lactoglobulin gene in the mammary gland. Specifically, HSIV and HSV are present prior to the first major increase in expression, which occurs at mid-pregnancy, while HSI displays the converse profile, being detected after mid-pregnancy and during lactation. The extent of DNase I digestion at HSIII, encompassing the promoter region, reflects the level of β-lactoglobulin expression. In transgenic mouse mammary chromatin, β-lactoglobulin transgenes display the same set of DNase I hypersensitive sites as in sheep mammary chromatin. The temporal profile, however, differs from that seen in sheep: notably, HSIV and HSV are detected during lactation. The fact that β-lactoglobulin transgenes lacking HSIV and HSV are expressed but display a reduced transcription rate per integrated copy is compatible with a functional role for these regions. This suggests that HSIV and HSV may increase the likelihood of high-level transgene expression.

Key words Chromatin · Mammary gland · Ovine · Transgene

Introduction

The bulk of mRNA present in the lactating mammary gland comprises transcripts encoding casein and whey milk proteins (Mercier and Vilotte 1993). The major ruminant whey protein is β-lactoglobulin, of which no homologue has been found in rodent milk (Jenness and Sloan 1970). The chromatin structure of the ovine β-lactoglobulin gene reflects its expression status. No DNase I hypersensitive sites (HS) are present in the liver, where the gene is not expressed, while two distinct sites are present in the lactating sheep mammary gland; HSI and HSIII. A further weak site, HSII, is variably detected (Whitelaw et al. 1992). We have utilised transgenic mice to functionally identify the elements involved in regulating β-lactoglobulin expression. Efficient, position-independent expression of the ovine β-lactoglobulin gene can be achieved with a 7.0-kb transgene which contains only 408 bp of 5′-flanking sequences (Whitelaw et al. 1992). Although this promoter region, defined by HSIII, is essential for expression (Whitelaw et al. 1992) and capable of targeting expression to the mammary gland (Archibald et al. 1990; Webster et al. 1995), additional downstream sequences are required for efficient expression in transgenic mice (Webster et al. 1995, 1997; Whitelaw et al. 1991, 1992).

In virgin sheep, β-lactoglobulin expression is barely detectable. Then as pregnancy proceeds, β-lactoglobulin expression gradually increases, with the first major increase occurring at mid-pregnancy (110th day). Expression continues to increase through parturition to reach maximal levels during lactation (Harris et al. 1991). DNase I hypersensitivity at HSIII parallels this expression profile, with minimal susceptibility to cleavage being observed before mid-pregnancy while substantial cutting is seen thereafter and during lactation (Whitelaw 1995). The current study extends the analysis of the chromatin structure encompassing the ovine β-lactoglobulin gene to gain further insight into the regulation of β-lactoglobulin expression. Specifically, the profile of DNase I hypersensitivity of the β-lactoglobulin gene was determined during mammary gland development. Intronic HS present prior to the first major increase in β-lactoglobulin expression at mid-pregnancy are absent from ovine lactating mammary chromatin. They are, however, detected in β-lactoglobulin transgenes in lactating mouse mammary chromatin. These intronic HS

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are required for β-lactoglobulin expression, since removal of the introns at which these HS form reduces the transcription potential in vivo.

Materials and methods

Isolation of nuclei and DNase I digestion

Frozen tissue samples were ground in the presence of liquid nitrogen, homogenised in a Dounce homogeniser (Wheaton) in 0.12 M sucrose, 50 mM KCl, 10 mM NaCl, 0.1 mM spermine, 0.4 mM spermidine, 5 mM mercaptoethanol, 1 mM EDTA, 0.5 mM EGTA, 0.1 mM PMSF, ImMDTT, 0.05% Triton X-100, 10 mM TRIS-HCl pH 7.9, 0.05% NP-40 and filtered through several layers of miracloth (Cambridge Bioscience). Nuclei were pelleted by centrifugation at 1000 rpm for 10 mins (Jouan CR3000). All steps were carried out at 4° C. The nuclear pellet was resuspended in 500 µl of digestion buffer (0.3 M sucrose, 60 mM KCl, 2 mM EDTA, 2 mM EGTA, 15 mM NaCl, 5 mM $MgCl_2$, 15 mM TRIS-HCl, pH 7.5). Digestion was initiated by the addition of DNase I (Promega) in 5 mM $CaCl_2$, 1 mM $MgCl_2$, and was allowed to continue at 37° C. Reactions were stopped by the addition of EDTA, to 20 mM, and nuclei were lysed with SDS (final concentration 1%). Samples were incubated at 37° C overnight with 2 mg/ml proteinase K. DNA was isolated by sequential phenol and chloroform extraction and finally precipitated with ethanol (Sambrook et al. 1989). DNA was prepared, digested with the appropriate restriction enzyme, subjected to agarose gel electrophoresis and blotted to Zeta-probe membranes (Bio-Rad). Southern blots were hybridised (Church and Gilbert 1984) with random oligo-primed probes High Prime Boehringer Mannheim.

HS in ovine chromatin (samples prepared as described in Whitelaw 1995) were detected using *Hin*dIII digestion as described previously (Whitelaw et al. 1992). This was not possible for the BLG/45 transgene (Simons et al. 1987; Whitelaw et al. 1992), since this contains an additional *Hin*dIII site (introduced during subcloning). Therefore, an *Eco*RI-*Nco*I β-lactoglobulin-specific probe was used to indirectly end-label *Nco*I-restricted samples. The *Nco*I site lies 260 bp 5′ to the *Hin*dIII site. This probe was chosen since it would generate a fragment of about 2.0 kb if HSIII was present, which is similar in size to that produced by *Hin*dIII digestion of ovine samples. Blots were scanned on a PhosphorImager (Molecular Dynamics) using Image Quant software or subjected to X-ray autoradiography (Kodak). No hypersensitive sites were revealed by DNase I digestion of naked DNA from these nuclei.

Fig. 1 Detection of DNase I hypersensitive sites (HS) around the ß-lactoglobulin gene in ovine mammary chromatin. Nuclei were isolated from sheep mammary tissue at various stages of differentiation and digested with DNase I prior to restriction with *Hin*dIII. Fragments generated by cleavage at HS were indirectly end-labeled using a ß-lactoglobulin-specific *Pvu*II-*Hin*dIII fragment as probe. The extent of DNase I digestion is shown above each lane. The major HS are indicated

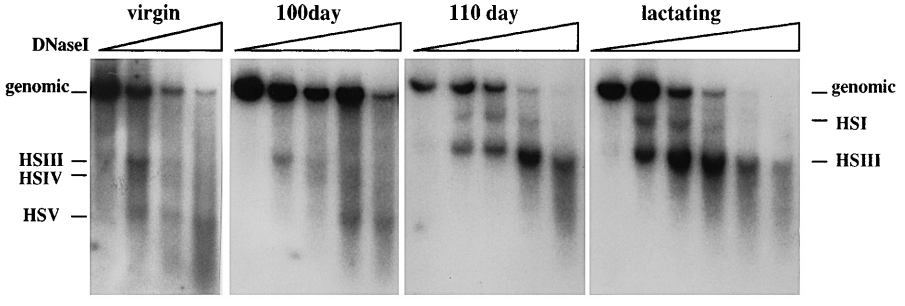
Nuclear run-on transcription assay

Nuclei were resuspended in 100 µl of 50% glycerol, 75 mM NaCl, 0.5 mM EDTA, 20 mM TRIS-HCl pH 7.9, 0.85 mM DTT, 0.125 mM PMSF, 50 U/µl RNasin. To this mixture was added: ATP, GTP and CTP (each to 0.5 mM); S-adenosylmethionine (in 1 mM H_2SO_4) to 0.25 mM; KCl to 100 mM; magnesium acetate to 2.5 mM, together with 125 µCi of [α-^{32}P]UTP (Amersham PB10163), and incubated at 30° C for 30 min. The elongation reaction was terminated and RNA isolated by mixing with RNAzol B (Biogenesis). A 10-µg aliquot of linearised plasmid DNA was diluted to 200 µl in H_2O, NaOH was added to 0.3 M and the mixture was incubated at 65° C for 60 min. The DNA targets were neutralised by adding an equal volume of 2 M ammonium acetate and then immobilised on a nitrocellulose membrane. Hybridisation and treatment of membranes were as described above for Southern blots. Blots were scanned on a PhosphorImager (Molecular Dynamics) using Image Quant software and hybridisation signals were normalised to that of ribosomal RNA.

Results

Temporal profile of appearance of HS in the region encompassing the ovine β-lactoglobulin gene

As expected for a milk protein gene (Mercier and Vilotte 1993), β-lactoglobulin expression increases slowly during pregnancy to reach maximal levels during lactation (Harris et al. 1991). Chromatin from lactating sheep mammary tissue reflects the high level of β-lactoglobulin expression by the presence of HS within its 5′-flanking sequences (Whitelaw et al. 1992). To extend this earlier analysis, nuclei were isolated from virgin, pregnant and lactating sheep mammary tissue and digested with DNase I. Subsequent indirect end-labeling (Wu 1980) of *Hin*dIII-restricted chromatin identified various HS (Fig. 1) which appear in distinct temporal patterns (see Fig. 3). In the undifferentiated virgin gland, HSIII, encompassing the promoter region, is just detectable. This reflects the low level of β-lactoglobulin mRNA detected in the mammary gland at this stage (Harris et al. 1991). In addition, two novel sites are detected 3′ to HSIII. These HS are localised in intron A (HSIV), approximately 700 bp downstream of HSIII, and intron B (HSV), approximately 1500 bp downstream of HSIII. These HS are maintained until the first major increase in β-lactoglobulin expression. Then, at about the 110th day of pregnancy, HSI appears and HSIII increases to become the dominant site of DNase I digestion, while HSIV and HSV disappear. This pattern is then retained



through the second half of pregnancy and into lactation. The weak and variable HSII (Whitelaw et al. 1992) was not detected in this study.

HS within β-lactoglobulin transgenes

Mice that are transgenic for β-lactoglobulin express the transgene in a position-independent manner, predominantly in the mammary gland, and in the appropriate temporal pattern (Harris et al. 1991; Whitelaw et al. 1992; Farini and Whitelaw 1995). This expression profile is exemplified by the transgenic mouse line BLG/45, which carries 17 copies of a genomic β-lactoglobulin transgene including 4.2 kb of 5′-and 1.9 kb of 3′-flanking sequences (Simons et al. 1987; Whitelaw et al. 1992). To determine whether the HS detected in ovine mammary chromatin was present within β-lactoglobulin transgenes, nuclei were isolated from lactating mammary tissue of BLG/45 transgenic mice, treated with DNase I and the digested chromatin was restricted with *Nco*I. Analysis of BLG/45 chromatin showed that HSI and HSIII were present. HSI is not essential, since removal of the sequences at which this HS appears has no effect on the efficiency of β-lactoglobulin transgene expression (Whitelaw et al. 1992) and does not affect expression in vitro in cell transfection studies (Demmer et al. 1995). In addition, the two intronic HS were detected in lactating BLG/45 mammary chromatin (Fig. 2). This pattern was confirmed in a further five lines (data not shown), indicating that the presence of HSIV and HSV during lactation was not the consequence of a position effect, i.e. not solely influenced by the site of transgene integration.

Our previous failure to detect the intronic HS in lactating mouse mammary chromatin was presumably due to the limited extent of DNase I titration in the earlier study (Whitelaw et al. 1992). Thus, the pattern of HS encompassing β-lactoglobulin transgenes in lactating mouse mammary chromatin appears to resemble a combination of the various temporally regulated HS seen in the ovine mammary gland during pregnancy and lactation (Fig. 3). In this regard, it is interesting to note that in transgenic mouse mammary chromatin, no HS were detected in the virgin state, with HSI, HSII, HSIII, HSIV and HSV all first appearing during late pregnancy (Whitelaw 1996).

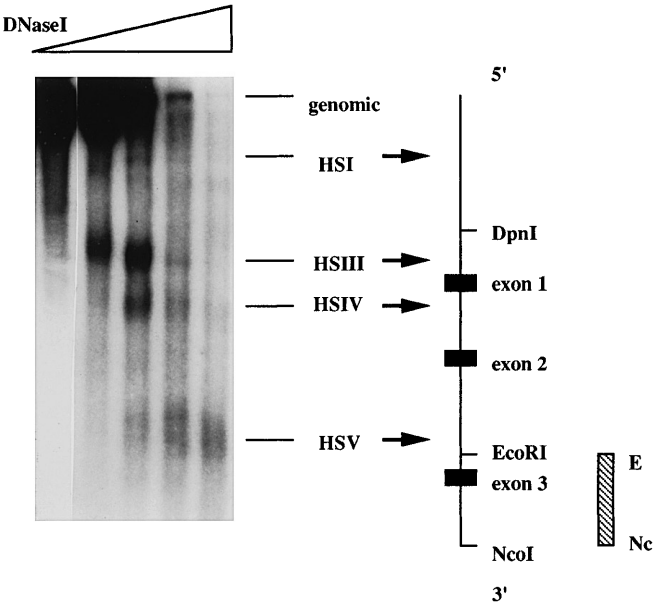


Fig. 2 Identification of HS in ß-lactoglobulin transgenes. Nuclei were isolated from lactating BLG/45 transgenic mouse mammary tissue and digested with DNase I prior to *Nco*I restriction. The extent of DNase I digestion is shown above each lane. Fragments generated by HS were indirectly end-labeled using a ß-lactoglobulin-specific *Nco*I-*Eco*RI fragment as probe, and the positions of HS are indicated in the adjacent diagram

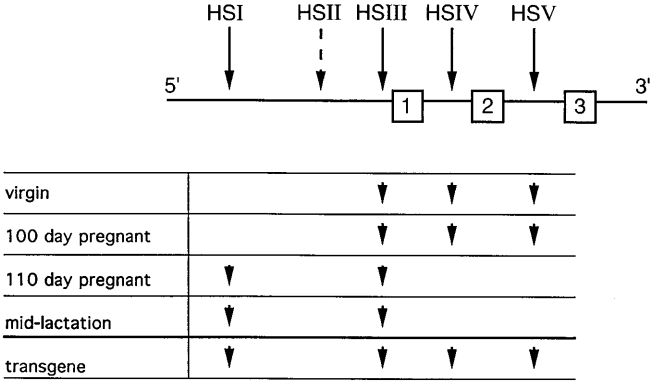


Fig. 3 Diagram of the HS profiles. The ovine ß-lactoglobulin gene is depicted schematically with exons represented as *numbered boxes*. The locations of HS are indicated by the *arrows* (detection of HSII is erratic). The patterns of HS detected at various stages are indicated by *arrowheads* below the diagram. For comparison, the pattern of HS present in actively expressed ß-lactoglobulin transgenes in lactating mouse mammary chromatin is also shown

Removal of intronic DNase I hypersensitive sites reduces transcription potential

Previous studies have indicated that intronic sequences are required for efficient β-lactoglobulin transgene expression (Whitelaw et al. 1991; Webster et al. 1997). In particular, expression of a transgene which lacked introns A and B (BLGΔAB), and thus the sites at which HSIV and HSV arise, was impaired compared to the wild-type BLGΔDp transgene which contains all six introns. Specifically, the BLGΔAB transgene was expressed, on average, at one-half to one-third the level of the wild-type BLGΔDp transgene (Webster et al. 1997). To investigate the impairment of expression associated with removal of HSIV and HSV, two lines each of BLGΔDp and BLGΔAB transgenic mice were identified for further analysis. These lines represent relatively high-copy-number integrants and display relatively high-level β-lactoglobulin expression. Nuclear run-on assays were

performed to determine whether removal of HSIV and HSV sequences affected the rate of transcription per transgene copy (Table 1). Relating the run-on signal to transgene copy number, the two-fold higher mRNA levels observed for the BLGΔDp transgene compared to BLGΔAB transgene correspond to a comparably greater transcription rate. This indicates that the removal of HSIV and HSV sequences from β-lactoglobulin transgenes reduces the rate of transcription per transgene copy.

Discussion

An early priming step may be required for the assembly of an active transcription complex on the promoter (Jimenez et al. 1992; Jenuwein et al. 1993; Jagle et al. 1997). For some genes, this becomes manifest in the temporal pattern of appearance of HS during differentiation (Jagle et al. 1997). HS are considered to represent regions that allow enhanced access to nuclear transcription factors (Elgin 1988; Gross and Garrard 1988) either at a nucleosome-free region (Lu et al. 1993) or in a nucleosomal context (McPherson et al. 1993). In most cases however, the actual molecular mechanisms leading to the formation of a HS still remain unclear, although it may represent a multistep process (Jenuwein et al. 1993).

In the chromatin encompassing the ovine β-lactoglobulin gene, temporal changes in organisation are clearly evident. Interestingly, both the appearance and disappearance of HS are detected. Initially, prior to the first major increase in β-lactoglobulin expression, two HS (HSIV and HSV) are present downstream of HSIII. Then, concomitant with increasing β-lactoglobulin expression, the two downstream HS disappear and HSI appears. We have shown previously that the sequences underlying HSI are not required for function (Whitelaw et al. 1992). Throughout the whole differentiation process, HSIII, which encompasses the promoter, is present: its prominence, however, increases in parallel with β-lactoglobulin expression. This distinctive temporal profile points to a functional role for HSIV and HSV in the establishment of β-lactoglobulin expression. Conceivably, this role could be suppressive or potentiating in character. If suppressive, the presence of HSIV and HSV early in pregnancy would prevent β-lactoglobulin expression and, indeed, β-lactoglobulin expression is minimal until these HS are lost at mid-pregnancy. Although this possibility cannot be discounted, it is nevertheless unlikely, since there is no need to invoke such a mechanism given the absence of the appropriate mammogenic or lactogenic stimuli at this stage of mammary differentiation (Mercier and Vilotte 1993; Osborne et al. 1995). Alternatively, HSIV and HSV could function in a positive manner to facilitate β-lactoglobulin expression. More specifically, since β-lactoglobulin expression is minimal at the stage when these HS are present, HSIV and HSV may function to prime the promoter (Jimenez et al. 1992; Jenuwein et al. 1993; Jagle et al. 1997) for the second phase of lactogenesis, when the lactogenic stimuli are present.

Genomic β-lactoglobulin constructs which contain at least 408 bp of 5′-flanking region are expressed in a position-independent manner, in that all lines express β-lactoglobulin at a level related to transgene copy-number (Whitelaw et al. 1992). Although β-lactoglobulin transgene chromatin displays the same HS as detected at the endogenous ovine β-lactoglobulin gene, the temporal pattern of their appearance differs. The full set of HS are either present or absent, with no HS being detectable in early pregnancy (Whitelaw 1996) while HSI, HSIII, HSIV and HSV are all present during lactation. The reason for this difference between sheep and transgenic mice is unclear. It could reflect an evolutionary divergence between mouse and sheep transcription factors; however, both sheep and mouse nuclear extracts show the same relative affinity for STAT5 (Watson et al. 1991), the main induced lactogenic transcription factor (Watson and Burdon 1996; Hennighausen et al. 1997). Alternatively, it may be a consequence of the multicopy nature of these transgenes. If so, and if HSIV and HSV do reflect a priming step for β-lactoglobulin expression,

Table 1 Comparison of the rates of transcription of the transgenes BLGΔDp and BLGΔAB

Transgenic line	Transgene copy number[a]	Steady-state RNA level[b]	RNA per transgene copy	Relative transcription rate[c]	Transcription rate per transgene copy
BLGΔDp/39	30	129	4.3	100[d]	3.3
	30	120	4.0	91	3.0
BLGΔDp/46	18	101	5.6	69	3.8
	18	93	5.2	60	3.3
BLGΔAB/10	16	50	3.1	29	1.8
	16	44	2.8	25	1.6
BLGΔAB/73	12	25	2.1	18	1.5
	12	23	1.9	16	1.3

[a] Determined by Southern analysis
[b] Mid-lactational steady-state RNA levels were determined by Northern analysis
[c] Nuclei were isolated from mid-lactation mammary tissue, and run-on transcription was assayed; run-on transcripts (approximately 5×10^6 cpm) were then hybridised and normalised as described in the text
[d] The transcription rate for the first BLGΔDp/39 mouse was set to 100

their presence at β-lactoglobulin transgenes may indicate that not all copies are transcriptionally fully active.

How could intronic HS facilitate β-lactoglobulin expression? We have considered two possible mechanisms to account for this. Potentially, HSIV and HSV might represent target sequences for transcription factors whose role is to stabilise HSIII. For example, the transcription factors interacting at HSIII might bind only transiently, cycling on and off during the transcription cycle (Eadara et al. 1996), resulting in the formation of a transient and unstable transcription factor complex. In this scenario, the formation of HSIV and HSV would function to stabilise the transcription factor complex at HSIII, perhaps by looping out the intervening DNA as proposed for the prolactin gene (Gothard et al. 1996) and invoked for the interaction of the β-globin locus control region with the various globin promoter elements (Wijgerde et al. 1995). Alternatively, a constitutive but weak transcription factor complex may be formed on the β-lactoglobulin promoter in the absence of the intronic HS. In this scenario, factors interacting at HSIV and HSV could function to modify the underlying nucleosomal chromatin structure in such a way as to increase the likelihood of HSIII formation, i.e. facilitate the establishment of nuclear factor access at the β-lactoglobulin promoter. In this regard, parallels can be drawn with the adenosine deaminase gene facilitator sequences, which participate in a chromatin structure transition that is necessary for DNase I hypersensitivity (Aronow et al. 1995).

Chromatin reorganisation must, at least partially, involve the remodelling of nucleosomes. In this regard, introns have been proposed to stimulate nucleosome alignment (Liu et al. 1995), and nucleosomal positioning can have a dramatic influence on access of transcription factors to their cognate binding sites within chromatin (Archer et al. 1991; Li and Wrange 1995). The glucocorticoid receptor is one of the transcription factors which can bind to its cognate recognition sequence within a nucleosomal context (Li and Wrange 1995) and may then be able to recruit chromatin remodelling activities (Farrants et al. 1997). With regard to the β-lactoglobulin gene, several half glucocorticoid receptor elements [TGT(T/G)C(T/C)] (Welte et al. 1993) are present within the sequences underlying HSIV and HSV, and glucocorticoids can stimulate expression of the gene (Burdon et al. 1994, Donofrio et al. 1996; Webster et al. 1997). At least in vitro, however, glucocorticoid enhancement of BLG expression does not seem to function through HSIV and HSV (Webster et al. 1997). Alternative chromatin elements include the matrix attachment regions, which can in some cases extend the region of accessible chromatin around an enhancer (Jenuwein et al. 1997). There is, however, no evidence that HSIV and HSV can function as matrix attachment regions (data not shown) and the sequence within introns A and B is not particularly AT rich.

There are now several examples of efficiently expressed genes that are regulated by a number of co-operating elements (e.g. Bonifer et al. 1994; Li and Stamatoyannopoulos 1994; Aronow et al. 1995; Jones et al. 1995; Ellis et al. 1997); however, the mechanisms involved remain to be characterised. The β-lactoglobulin gene appears to fall into this category, in that multiple, co-operating elements are required for its efficient expression.

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