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# Hormonal Influences on β-Lactoglobulin Transgene Expression Inferred from Chromatin Structure

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The major milk whey protein of ruminants is β-lactoglobulin. Transgenic mice which carry genomic fragments of ovine β-lactoglobulin express the transgene at high levels in the mammary gland. Using DNaseI as a probe for transcription complex formation in chromatin, the temporal induction pattern of β-lactoglobulin in transgenic mice has been addressed and compared to the known hormonal profiles during pregnancy. Prior to the 9th day of pregnancy no obvious hypersensitivity to DNaseI digestion at the β-lactoglobulin promoter was evident. From the 9th day of pregnancy through to lactation, the β-lactoglobulin promoter displays DNaseI hypersensitivity. These results support the hypothesis that placental lactogens are the major lactogenic influence from mid-pregnancy to parturition. 

The major genes expressed in the mammary gland encode the milk proteins (1). Expression of these genes is regulated by a complex association of hormones, growth factors and both inter- and extra-cellular interactions (2). This heterogeneous group of proteins comprise mainly of the caseins and wheys. The most abundant whey protein of ruminants is β-lactoglobulin. Lactogenesis, the expression of milk protein genes, occurs in two phases (3). Stage one occurs prior to parturition, during pregnancy. The hormonal influences required for expression of β-lactoglobulin during pregnancy are still poorly defined, although placental lactogens have been implicated (4,5). The second stage of lactogenesis occurs after parturition, during lactation, and prolactin is required for maximal expression of β-lactoglobulin (6). Prolactin activates β-lactoglobulin transcription through the transcription factor Stat5 (7,8) previously termed milk protein binding factor (9). In the present study, using DNaseI as a probe for transcription complex formation, the temporal induction pattern of β-lactoglobulin transgenes during the first stage of lactogenesis has been addressed and compared to the known hormonal profiles during pregnancy.

## MATERIALS AND METHODS

Mammary samples were obtained from BLG/45, BLGΔDp/39 and BLGΔDp/46 transgenic mice. The generation and characterisation of these mice has been described before (10,11). These mice carry approximately 17, 30 and 18 copies, respectively, of the genomic β-lactoglobulin transgenes. The BLG/45 transgene comprises the entire transcription unit plus 4.3 kilobase pair (kb) of 5′ and 1.9 kb of 3′ flanking sequences. The BLGΔDp transgene is similar except that it contains only 0.4 kb of 5′ flanking sequences. Both of these transgenes are expressed at high-levels (10,11), predominantly in the mammary gland (12). DNaseI digestion of chromatin was as previously described (10) with slight modifications (4). DNA samples were restricted with H*ind*III and specific β-lactoglobulin fragments were indirectly end-labeled with an E*co*RI-H*ind*III probe, positions 2050-3097 (13). The transgenes arrays analysed in these experiments give several genomic fragments with H*ind*III which presumably reflect the complexity of each array. Since the murine genome does not contain a β-lactoglobulin homologue there is no background hybridisation signal.

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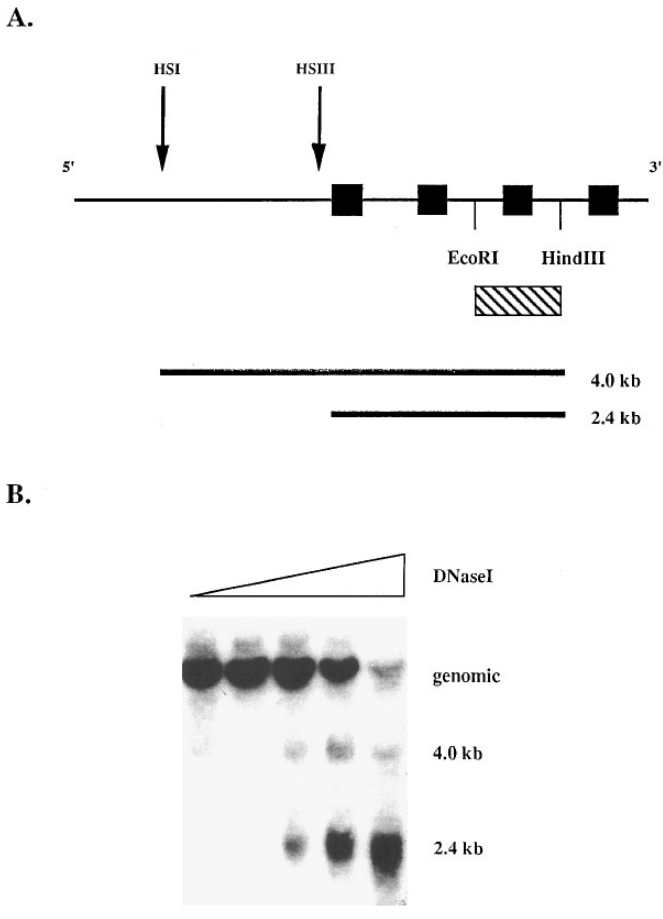


**FIG. 1.** DNaseI hypersensitivity encompassing the ovine $\beta$-lactoglobulin promoter in sheep. (A) The relative positions of DNaseI hypersensitive sites $HS_I$ and $HS_{III}$ within the region upstream of the first exon of the ovine $\beta$-lactoglobulin gene are shown as arrows. Exonic sequences are shown as filled boxes and the probe used is depicted as a hatched box. (B) Nuclei were isolated from lactating sheep mammary and digested with increasing amounts of DNaseI. The extent of DNaseI digestion is indicated by the triangle above the respective lanes. The subsequently purified genomic DNA was analysed by Southern blot after restriction with H*ind*III and probed with a E*co*RI-H*ind*III $\beta$-lactoglobulin specific probe.

## RESULTS

*DNaseI hypersensitivity of the ovine $\beta$-lactoglobulin 5′ flanking region.* The pattern of DNaseI hypersensitivity on the 5′ flanking region of the endogenous ovine $\beta$-lactoglobulin has been determined previously (4,10). The first site lies about 2.0 kb upstream of the first $\beta$-lactoglobulin exon, while the much stronger site encompasses the proximal promoter region (Figure 1). These sites have been termed $HS_I$ and $HS_{III}$ respectively. A very weak site of variable reproducibility has also been detected between these two major sites (10).

To determine if a similar pattern of nuclease hypersensitivity was present on $\beta$-lactoglobulin transgenes the chromatin structure of BLG/45 transgenic mice was analysed. These transgenic mice carry a 10.0 kb genomic $\beta$-lactoglobulin fragment which includes the sequences encompassed by $HS_I$ and $HS_{III}$. All BLG/45 mice express $\beta$-lactoglobulin abundantly in the mammary gland and expression follows the expected temporal expression profile, that of gradually increasing expression through pregnancy to reach maximal levels during lactation (10). Nuclei

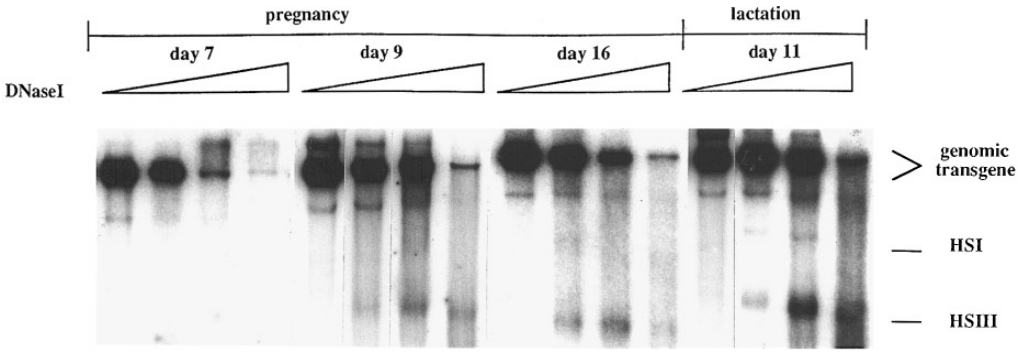


**FIG. 2.** Appearance $HS_I$ and $HS_{III}$ during pregnancy in BLG/45 transgenic mice. Nuclei from 7, 9, 16 day pregnant and 11 day lactating mammary tissue from BLG/45 transgenic mice. Chromatin was digested with increasing amounts of DNaseI and the subsequently purified genomic DNA analysed by Southern blot after restriction with H*ind*III and probed with a E*co*RI-H*ind*III $\beta$-lactoglobulin specific probe. The extent of DNaseI digestion is indicated by the triangle above the respective lanes. H*ind*III restriction of BLG/45 genomic DNA generates a transgene unit fragment of 10.9 kb and one slighly smaller fragment of weaker intensity which may represent a junction fragment. The DNaseI hypersensitive sites are as described in Fig. 1.

were isolated from mid-lactation BLG/45 females and after light treatment with DNaseI, the digested genomic DNA was isolated and restricted with H*ind*III (Figure 2). A 2.4 kb fragment corresponding to DNaseI digestion at $HS_{III}$ is clearly visible in lactating mammary chromatin. A further fragment of 4.0 kb, which is weaker in intensity, is also visible and corresponds to $HS_I$. This pattern is comparable to that of the endogenous $\beta$-lactoglobulin gene in sheep (Figure 1).

*Temporal pattern of DNaseI hypersensitivity on $\beta$-lactoglobulin transgenes.* To determine the temporal pattern of DNaseI hypersensitivity on $\beta$-lactoglobulin transgenes, nuclei were isolated at various time points from BLG/45 female transgenic mice. After light treatment with DNaseI, the digested genomic DNA was isolated and restricted with H*ind*III (Figure 2). A fragment corresponding to $HS_{III}$ is first visible at the 9th day of pregnancy. This state of DNaseI hypersensitivity is maintained, through late pregnancy (day 16), until lactation where an increase in the degree of DNaseI hypersensitivity is observed. In addition, $HS_I$ is predominantly detected during lactation. The temporal expression profile of $\beta$-lactoglobulin transgenes has been described previously (10,12,14). Expression of $\beta$-lactoglobulin is just detectable during early pregnancy, with levels dramatically increasing from day 10 onwards, to peak during lactation. Thus, the degree of DNaseI hypersensitivity encompassing the $\beta$-lactoglobulin promoter region corresponds to the levels of expression during pregnancy and lactation. Specifically, the appearance of $HS_{III}$ just precedes the first major increase in $\beta$-lactoglobulin expression. Secondly, $HS_I$ is only detected during the second stage of lactogenesis, i.e. after parturition. The role of this element in regulating expression of $\beta$-lactoglobulin is unclear since its deletion does not have an obvious effect on expression in transgenic mice (10), and only a slight effect on expression *in vitro* (8).

Transgenic mice carrying the BLG$\Delta$Dp transgene were also analysed. This transgene, although containing only 408 bp of 5′ flanking sequences, expresses $\beta$-lactoglobulin in a similar manner to that detected in BLG/45 mice (10). $HS_{III}$ is clearly visible in lactating mammary nuclei isolated from BLG$\Delta$Dp mice, and was first detected at the 10th day mid-pregnancy (Figure 3). This transgene does not include the region encompassing $HS_I$. Thus, BLG/45 and BLG$\Delta$Dp mice display a similar temporal pattern of DNaseI hypersensitivity appearance with respect to $HS_{III}$.

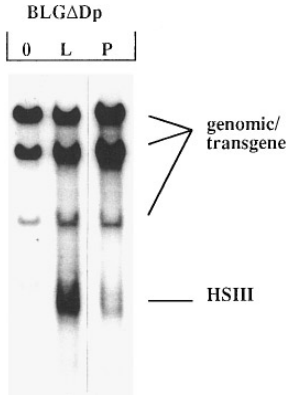


**FIG. 3.** $HS_{III}$ hypersensitivity in BLGΔDp transgenic mice. Nuclei were isolated from BLGΔDptransgenic mice and digested with increasing amounts of DNaseI. The subsequently purified genomic DNA was analysed by Southern blot, after restriction with H*ind*III, and probed with an E*co*RI-H*ind*III $\beta$-lactoglobulin specific probe. Lanes: 0, no DNaseI digestion; L, lactating mammary sample; P, 10 day pregnant mammary sample. The DNaseI hypersensitive sites are as described in the legend to Fig. 1.

## DISCUSSION

Expressed genes often display a distinct pattern of hypersensitivity to nuclease digestion. This is thought to represent alterations to the nucleosomal array, which are usually due to the sequence-dependent interaction of transcription factors (15). In the lactating mammary gland the ovine $\beta$-lactoglobulin promoter resides within a strong nuclease hypersensitive site (4,10). This site correlates with the presence of three binding sites for the prolactin-induced transcription factor Stat5 (7,8,9). Mutational analysis of these binding sites indicate that Stat5 is required for the enhanced expression of $\beta$-lactoglobulin transgenes during lactation (6). Thus, the strong DNaseI hypersensitive encompassing the proximal $\beta$-lactoglobulin promoter correlates with the formation of a hormonally-induced transcription complex. Using this argument, the appearance of $HS_{III}$ at the 9th day of pregnancy implies the formation of a transcription complex on the $\beta$-lactoglobulin promoter region at this stage of mammary development.

The major lactogen present during lactation is prolactin (3). Although detected during the first few days of pregnancy, prolactin levels drop dramatically on the 9th day of pregnancy, only rising again at parturition (16). Thus, prolactin is unlikely to be involved in the formation of a transcription complex on the $\beta$-lactoglobulin promoter during pregnancy. Prolactin is a member of a large family of cytokines which include other potential lactogens, i.e. growth hormone and placental lactogen (3). Although growth hormone levels (17) do appear to correlate with changes in DNaseI hypersensitivity, the levels are not sustained through to parturition. Furthermore, in rodents the ability of this hormone to activate whey milk proteins *in vitro* is still unclear (5,18). The temporal pattern of $HS_{III}$ appearance, however, does correlate well with the serum profiles of the placental lactogens (19) which have lactogenic properties *in vitro* (18). This correlation between the appearance of overt DNaseI hypersensitivity, increasing mRNA levels and increasing level of the serum placental lactogens levels the hypothesis that placental lactogen-II is the major lactogen during pregnancy (18). In the lactating mammary gland, enhanced $\beta$-lactoglobulin expression correlates with the presence of Stat5 (6). Stat5 binding activity has been detected during the latter part of pregnancy, particularly from day 12 onwards (20), correlating with the temporal pattern of Stat5 mRNA during pregnancy (21). Thus, it is possible that the DNaseI hypersensitivity during pregnancy is (at least partially) due to the interaction of a form of Stat5 with its cognate DNA binding site with the $\beta$-

lactoglobulin promoter and, that this factor may be activated by placental lactogen-II. This is comparable to a similar study in sheep, where changes in chromatin structure during the first stage of lactogenesis parallel ovine placental lactogen levels (4) and further supports the hypothesis that placental lactogens are the major lactogenic influence from mid-pregnancy to parturition.

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## REFERENCES

1. Mercier, J. C., and Vilotte, J. L. (1993) *J. Dairy Sci.* **76,** 3079–3098.
2. Streuli, C. H. (1993) *Seminars Cell Biology* **4,** 203–212.
3. Forsyth, I. A. (1994) *Exp. Clin. Endocrinol.* **102,** 244–251.
4. Whitelaw, C. B. A. (1995) *Biochem. Biophys. Res. Comm.* **209,** 1089–1093.
5. Thordarson, G., Villalobos, R., Colosi, P., Southard, J., Ogren, L., and Talamantes, F. (1986) *J. Endocrinol.* **109,** 263–274.
6. Burdon, T. G., Maitland, K. A., Clark, A. J., Wallace, R. M., and Watson, C. J. (1994) *Mol. Endocrinol.* **8,** 1528–1536.
7. Burdon, T. G., Demmer, J., Clark, A. J., and Watson, C. J. (1994) *FEBS Letters* **350,** 177–182.
8. Demmer, J., Burdon, T. G., Djiane, J., Watson, C. J., and Clark, A. J. (1995) *Mol. Cell. Endocrinol.* **107,** 113–121.
9. Watson, C. J., Gordon, K. E., Robertson, M., and Clark, A. J. (1991) *Nucl. Acids Res.* **19,** 6603–6610.
10. Whitelaw, C. B. A., Harris, S., McClenaghan, M., Simons, J. P., and Clark, A. J. (1992) *Biochem. J.* **286,** 31–39.
11. Simons, J. P., McClenaghan, M., and Clark, A. J. (1987) *Nature* **328,** 530–532.
12. Farini, E., and Whitelaw, C. B. A. (1995) *Mol. Gen. Genet.* **246,** 734–738.
13. Harris, S., Ali, S., Anderson, J., Archibald, A. L., and Clark, A. J. (1988) *Nucl. Acids Res.* **16,** 10379–10380.
14. Harris, S., McClenaghan, M., Simons, J. P., Ali, S., and Clark, A. J. (1991) *Dev. Gen.* **12,** 299–307.
15. Gross, D. S., and Garrard, W. T. (1988) *Annual Rev. Biochem.* **57,** 159–197.
16. Markoff, E., and Talamantes, F. (1981) *Biol. Reprod.* **24,** 846–851.
17. Talamantes, F., Soares, M. J., Colosi, P., Haro, L., and Ogren, L. (1984) *in* Frontiers and Prolactin Secretion: A Multidisciplinary Approach (Mena, F., and Valverde, C. Eds.) pp. 31–41. Academic Press, New York.
18. Goujon, L., Allevato, G., Simonin, G., Paquereau, L., Le Cam, A., Clark, J., Neilsen, J. H., Djiane, J., Postel-Viney, M. C., Edery, M., and Kelly, P. A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 957–961.
19. Soares, M. J., Colosi, P., and Talamantes, F. (1982) *Endocrinol.* **110,** 668–670.
20. Schmitt-Ney, M., Happ, B., Ball, R., and Groner, B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3130–3134.
21. Liu, X., Robinson, G. W., Gouilleux, F., Groner, B., and Hennighausen, L. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 8831–8835.