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Multiple Copies of β-Lactoglobulin Promoter Do Not Function as LCR

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Increasing the number of transcription factor binding sites within a construct can enhance expression. In an attempt to create a synthetic locus control region for mammary expression, we have generated β-lactoglobulin-reporter constructs with multiple copies of the cluster of transcription sites normally located within the proximal promoter. These constructs were functionally tested by stable transfection of mammary epithelial cells *in vitro* and in transgenic mice *in vivo*. Rather than enhancing expression, multimerisation of the promoter region acted neither *in vivo* nor *in vitro* to enhance expression. Indeed, its presence reduced expression. This failure to enhance expression was reflected in the inability of this region to form a DNaseI hypersensitive site autonomously in mammary chromatin *in vivo*. It is implicit from our study that not all combinations of transcription factor binding sites will enhance transcription.

Key Words: **biotechnology; chromatin; DNaseI; mammary; mouse; transcription; transgene.**

The primary control for most genes is exerted at the level of transcription. It is not surprising, therefore, that genes have evolved complex regulatory domains that harbour binding sites for tissue-specific and ubiquitous transcription factors to ensure appropriate expression. At a simplistic level, these control regions can be separated into two regions. First, the core promoter resides just upstream of the transcriptional start site and harbours DNA sequence elements (1, 2) that direct the formation of the basal transcription complex containing RNA polymerase (3). Distal to this, usually but not exclusively further 5′, are found the binding sites for activators (4). Where these binding sites reside in clusters, that are functionally transferable as a unit, they are termed enhancers. Enhancers are key regulatory components controlling spatial and temporal aspects of gene expression. For most enhancers both tissue-restricted and ubitiquous factors are required to interact for function. This is now thought to result in a large, termed the enhanceosome (5), which tend to form on short segments of DNA (~100 bp or less). The assembly of an enhanceosome is highly cooperative and promotes high levels of transcriptional synergy. This results from the multiple and synergistic interactions that occur within the enhanceosome and the multicomponent RNA polymerase II complex (6).

An enhancer functions primarily by promoter derepression *in vitro* (7) and this appears to manifest in an increased probability that a gene will be transcribed *in vivo* (8, 9). Thus, an increased probability of transcription can be correlated with the sequential addition of transcription factor binding sites. In studies using transgenic mice, dominantly acting transcription control elements have been identified which increase the likelihood of expression (10). The best example is that of the human β-globin locus control region (LCR). This LCR comprises of a series of DNaseI hypersensitive sites, with each site harbouring a dense cluster of DNA binding sites for transcription factors (4, 11). Again supporting a relationship between the number of transcriptional binding sites and increased likelihood of expression.

The β-lactoglobulin gene is expressed specifically in the lactating mammary gland. This strict spatial and temporal expression pattern is regulated by a complex interaction of hormones, the extracellular matrix and cell–cell interactions (12). We have previously shown that the proximal β-lactoglobulin 5′-flanking region contains all the necessary sequences to direct the appropriate expression profile in transgenic mice (13). It contains a cluster of binding sites for transcription factors including: STAT5, NF1, NFκB, AP2 (14–17). This is reflected in the strong expression a lactation-

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dependent, mammary-specific DNaseI hypersensitive site (HSIII) that encompasses this region (18). Therefore, in many ways this region resembles known enhancers. In particular, although interacting with a different set of transcription factors, it is structurally very similar to the INFβ enhancer as reported by Thanos and Maniatis (5).

It is now recognised that a series of transcription regulators can synergistically modify each other's activity though combinatorial interactions (6). Furthermore, increasing the number of transcription factor binding sites available to activate a gene can enhance the likelihood of expression of that gene (8–10). In an attempt to enhance expression *in vivo*, we have evaluated whether multimerising the cluster of transcription sites within the β-lactoglobulin proximal 5′-flanking region can alter the equilibrium between transcriptional repression and activation acting on a transgene.

MATERIALS AND METHODS

Plasmids. The parental β-lactoglobulin driven chloramphenicol acetyl transferase (CAT) reporter construct pBJ58 has been described before as BCΔDp (13). To standardise plasmids, the β-lactoglobulin-CAT sequences from pBJ58 were subcloned into pBluescript to generate pBC. To generate the synthetic LCR plasmids a 376 bp region of the β-lactoglobulin 5′-flanking sequence was amplified and tandemly-ligated by utilising PCR primers with 5′-terminal restriction enzyme sites with cohesive ends: *Bam*HI for forward primer BLGA (5′-CTCGGATCCCTTCACCCAA-3′) and *Bgl*II for reverse primer BLGB/2 (5′-GGTAGATCTAGGAGGCG-CATGCAGG-3′). The primer pair was used to amplify a region of the β-lactoglobulin proximal 5′-flanking region from nucleotides 3803 to 4179 (accession no. X68105), which included most of the functionally defined promoter (13) but excluding the TATA-box, from the plasmid pSS1tgSX (18). The amplified fragment was digested with *Bam*HI and *Bgl*II, ligated with an excess of insert (20:1 or 10:1), re-digested and cloned into the BamHI site of pBluescript. *Bam*HI/*Bgl*II ligated sites do not cleave with *Bam*HI, therefore *Bam*HI/*Sca*I digests determined the orientation and number of fragments in the array. Subsequent rounds of cloning allowed a tandem array of fragments to be built. SURE2 cells (Stratagene) were used in the transformations to prevent recombination within the arrays. Arrays that contained fragments in both directions were discarded. The β-lactoglobulin-CAT sequences were excised from pBJ58 as a 1.3 kb *Kpn*I/*Hind*III fragment and cloned into the equivalent sites 5′ to the multimerised sequences to generate; pBC1R, pBC2F, pBC3F, pBC4F.

HC11 cell transfection. Transfections were carried out in HC11 mouse mammary epithelial cells as described before (19). The protein extract concentrations were established using the Pierce BCA assay kit and CAT assays were performed as previously described (20).

Transgenic mice. BCΔDp mice have been described before (13). The BCB transgene was isolated as a *Kpn*I–*Not*I fragment from pBC2F for pronuclear microinjection. Of the 125 BCB offspring produced, 13 were positive by Southern blot, with only 8 transmitting the transgene to their progeny. G_1 mid-lactation female mice were analysed for CAT expression. The SAP transgene comprised only proximal 5′ flanking sequences, defined by *Ava*I (3319) and *Sph*I (4161) subcloned from pSS1tgXS into ptgpoly (21). This region is slightly larger (5′) than that multimerised in the pBC constructs to facilitate chromatin analysis. A 770 bp *Hind*III–*Eco*RI fragment was isolated for microinjection. Of the 116 SAP offspring produced, 27 were positive by PCR using forward primer JWSAP1 (5′-CCCAGCCACGCGGTTCCCGGAATCC-3′) and reverse primer JWSAP2 (5′-CCTAACTGGGCAGGTGCTTHCAGAGCC-3′). The two highest transgene copy-number transmitting lines were selected for analysis. Nuclei were isolated from midlactation mammary tissue from G_1 female mice and analysed for DNaseI hypersensitivity as described previously (18).

RESULTS

It has been shown that the inclusion of additional transcription factor binding sites within a construct can enhance the probability of expression (7–9). The proximal β-lactoglobulin 5′-flanking region, constituting the functionally defined promoter (13, 15, 18) contains a variety of transcription factor binding sites (14–17). Sufficient sequence information resides within this region to direct the correct spatial and temporal expression profile in transgenic mice (13). In an attempt to enhance expression we asked whether multiplying the number of transcription factor binding sites *en masse* could functionally act as a synthetic LCR. We posed three criteria on this putative synthetic LCR. Could the multimerised sequences increase expression of a β-lactoglobulin-CAT plasmid *in vitro* or the probability of its activation in transgenic mice *in vivo*. In addition, we determined whether the proximal 5′-flanking sequences could direct the formation of a DNaseI hypersensitive site autonomously.

The basic β-lactoglobulin-CAT plasmid (pBC) contains 408 bp of proximal 5′-flanking sequences, the first 35 bp of the untranslated region of exon 1 linked to the CAT gene to enable biochemical detection of expression. A series of four pBC plasmids (Fig. 1) based on the basic plasmid and containing multiple copies of the promoter inserted 3′ to the CAT gene were constructed. The multimerised region contained the proximal 5′-flanking sequences up to but not including the TATA-box. Four constructs were tested: pBC1R contained one copy in the reverse orientation to the CAT reporter; pBC2F contained two copies in the forward orientation; pBC3F contained three copies in the forward orientation; pB42F contained four copies in the forward orientation. The multimerised sequences were inserted downstream of the reporter gene to reflect the common aspect of the successful construct designs previously reported to increase the probability of expression (7–9). Note, since the fragment will be isolated from the plasmid backbone before microinjection to generate transgenic mice, this arrangement will result in the multimerised sequences being locate adjacent to the promoter in an head-to-tail transgene array *in vivo*.

To determine whether the multimerised sequences could enhance expression *in vitro*, mouse mammary HC11 cells in which the β-lactoglobulin gene is normally active (19), were stably transfected with the pBC

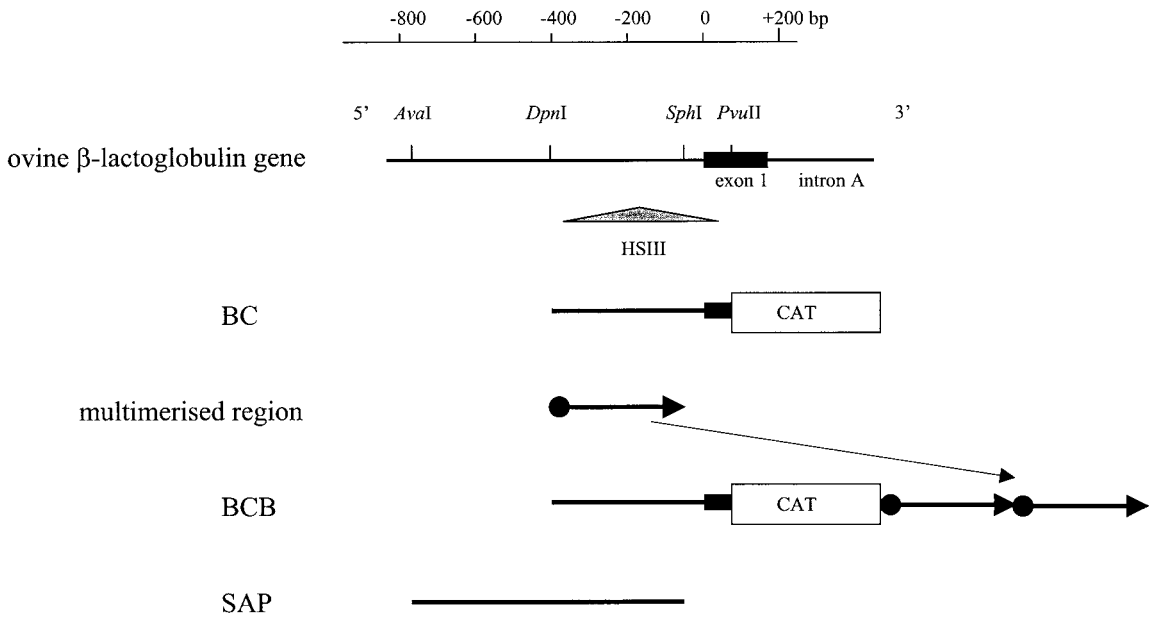

FIG. 1. Diagram of β-lactoglobulin 5′ region and derived transgenes. The proximal 5′-flanking region of ovine β-lactoglobulin gene is shown below scale bar in bp with relevant restriction sites. The major mammary-specific DNaseI hypersensitive site is shown as large triangle (HSIII: 18). BC: parental β-lactoglobulin-CAT construct (previously termed BCΔDp: 13). BCB: one of series of synthetic-LCR containing constructs that contained two additional copies of the multimerised 5′-flanking region inserted downstream of the CAT reporter gene (also called BC2F). SAP: transgene that containing only 5′-flanking sequences (upstream of TATA) without a linked transcription unit.

series of plasmids. A total of five transfections were performed and assayed for CAT expression. The inclusion of multiple copies of the proximal 5′-flanking region did not enhance expression levels (Fig. 2). To the contrary, the multimerised sequences reduce CAT expression, with the reduction in expression level reflecting the number of copies of the multimerised sequences.

In a parallel experiment BCB transgenic mice were generated using pBC2F which contained two addition copies of the β-lactoglobulin 5′-flanking sequences. The intention was to determine if the multimerised sequences could enhance expression *in vivo*. Eight BCB transgenic lines were produced and analysed for CAT activity in the lactating mammary gland. Paralleling the failure to enhance expression *in vitro*, no evidence of CAT expression was observed in any of the BCB transgenic lines (Table 1). This expression frequency was essentially the same as that seen for the parental basic β-lactoglobulin-CAT transgene were only one out of eight lines expressed the transgene (13). Thus, the

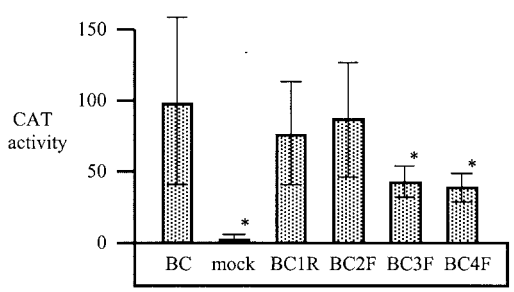

FIG. 2. CAT activity *in vitro*. CAT activity in protein extracts from HC11 cells stably transfected with pBC plasmids and mock (no DNA), expressed as the average percentage conversion of substrate to acetylated form per μg of extract per hour ($n = 5$). Student's paired *t*-tests between mean pBC and test plasmid with $P < 0.05$ shown by an asterisk.

TABLE 1

Transgene Expression Frequency *in Vivo*

Founder mouse	Transgene positive/ total G1 mice[a]	CAT expressing/ number mice tested[b]
11	7/15	0/2
18	0/4	—
22	2/20	ND
45	11/37	0/2
51	0/15	—
64	2/6	0/1
70	0/18	—
76	5/10	0/2
92	9/23	0/2
93	0/25	—
94	5/25	0/1
109	8/23	0/2
140	5/15	0/2

[a] Transgene positive mice were identified by PCR of tail DNA.

[b] CAT activity was analysed in mid-lactation mammary tissue of G1 female mice. In two lines only one positive G1 female mouse was obtained.

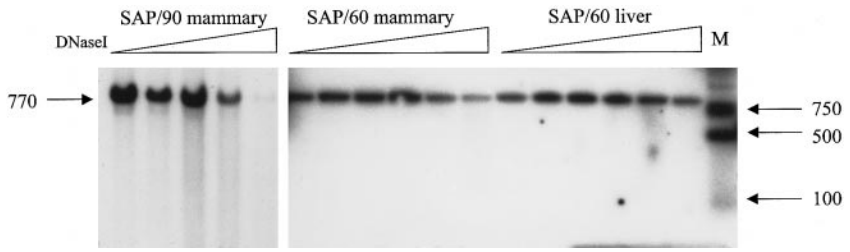

FIG. 3. Lack of DNaseI hypersensitivity associated with SAP transgene. The entire transgene fragment was used to probe Southern blots carrying DNaseI digested lactating mammary or liver tissue from SAP/60 and SAP/90 mice. Restriction with *Hind*III releases the 770 bp transgene unit fragment from genomic DNA. Genomic transgenic mouse DNA restricted with *Sac*I was used as a size marker (M) and to confirm that the desired DNaseI hypersensitive site derived fragment size (estimated to be between 400–700 bp) would be detected if present.

inclusion of additional copies of the proximal 5′-flanking region had no beneficial effect on transgene expression *in vivo*.

The failure of the multimerised sequences to act like an LCR and enhance expression *in vitro* or *in vivo* questioned the ability of the β-lactoglobulin proximal 5′-flanking region to direct the autonomous formation of a transcription complex. The β-lactoglobulin proximal 5′-flanking region in its normal context or when linked to its own transcription unit in transgenic mice resides in a mammary-specific DNaseI hypersensitive site reflecting the formation of a transcription complex at the promoter (18). It has been shown that transgenes comprising only HS2 of the β-globin LCR can direct the autonomous formation of a DNaseI hypersensitive site (22). To address whether the β-lactoglobulin proximal 5′-flanking region could autonomously form a DNaseI hypersensitive site in the absence of a linked transcription unit we generated SAP transgenic mice. Similar to the region multimerised in the BCB transgene, the SAP fragment comprises only the promoter region up to but not including the TATA-box. DNaseI digestion of nuclei isolated from SAP/60 transgenic mice indicated that there was no obvious difference in sensitivity between mammary and liver chromatin (Fig. 3). Preferential DNaseI digestion at the promoter (HSIII: 18) would have been reflected in the appearance of subgenomic bands specifically in mammary chromatin: no subbands were expected in the liver in which β-lactoglobulin gene is not active. The comparable DNaseI digestion observed for both mammary and liver chromatin suggests that the chromatin encompassing the SAP fragment is equally condensed in both tissues. A similar lack of hypersensitive sites was seen for a further two transgenic lines: SAP/19 (data not shown) and SAP/90 (Fig. 3).

This is in notable contrast to the efficient detection of hypersensitivity over the promoter in sheep mammary and genomic β-lactoglobulin mammary chromatin (18; data not shown). Since only three lines have been analysed, this does not exclude the possibility that this region could form a DNaseI hypersensitive site given the appropriate position-effect. However, it does imply that the proximal 5′-flanking sequences of the β-lactoglobulin gene are incapable of forming a DNaseI hypersensitive site autonomously.

DISCUSSION

The functional β-lactoglobulin gene promoter resides within the proximal 408 bp of 5′-flanking sequences and contains sufficient sequence information to direct tissue and temporally specific expression of a reporter gene in transgenic mice (13). Within this region there are several, closely clustered transcription factor binding sites (14–17) which are encompassed by the major mammary gland specific DNaseI hypersensitive site associated with the gene (18, 23). Nevertheless, this promoter region is susceptible to repressive position-effects *in vivo* (13).

The addition of transcription factor binding sites can increase expression *in vitro* (7–9) and enhance activation *in vivo* (10). In this study, we have evaluated whether multimerisation of the cluster of transcription factor binding sites within the proximal promoter can improve expression of β-lactoglobulin based constructs. Unexpectedly, rather than facilitating expression, the multimerised sequences acted neither *in vitro* nor *in vivo* as a synthetic LCR and, if anything, their presence appeared to silence expression. Certainly in HC11 cells, the number of multimerised copies present inversely correlated with the expression level. Further, from the transgenic mice data, although the difference between one or no expressing lines is marginal at best, it is possible that a similar reduction in the likelihood of transcriptional activation may also be occurring *in vivo*. Thus, the multimerised sequences may actually augment the formation of a repressed state. If this is indeed what is happening in these constructs, it may be that transcription elements become targets for repression when they fail to form the appropriate complexes required for gene activation. In this scenario, rather than gene or tissue-specific repressors of transcription and these exist for β-lactoglobulin (15), it is

more likely that more general mechanisms are involved (24).

This failure to enhance expression was reflected in the inability of this region to form autonomously a DNaseI hypersensitive site in mammary chromatin *in vivo.* This promoter region resides within a strong mammary hypersensitive site in sheep and in transgenic mice carrying genomic β-lactoglobulin transgenes (18, 23). Therefore, the efficient formation of a transcription complex at the promoter presumably requires β-lactoglobulin sequences that are outwith the promoter. The need for the synergistic interaction of sequences distributed throughout a gene for the establishment of a DNaseI hypersensitive site is a reappearing concept. For example, analysis of chicken β^{A}-globin transgenes demonstrated that the enhancer and promoter must cooperate to generate open chromatin (25). This is consistent with the co-operative mode of action of LCR elements (26) and models for the efficient expression of transgenes (27).

The inability of additional copies of the β-lactoglobulin proximal 5′-flanking region to function autonomously and enhance expression is compatible with the hypothesis that for transcriptional synergy, transcription complexes require to abide by rules of 'stereo-specificity' (6). It has been proposed that for efficient enhanceosome interaction with the basal polymerase II transcription machinery, demanding structural organisational rules have to be followed (5). Since the multimerised sequences in our constructs were derived from the promoter they were intended to interact with, we had considered it likely that they would functionally cooperate. Given, however, the lack of functional interaction between these sequences and the β-lactoglobulin promoter driving the CAT gene in our construct, the mere presence of the relevant transcription factor binding sites is obviously insufficient to enable stereo-compatibility.

From an applied perspective, it is implicit from our study that not all combinations of transcription factor binding sites will be effective in enhancing transcription. Indeed, in a screen to identify muscle-specific promoters from a library of random-assembled transcription factor binding sites, only 2% of the recombinants had an enhanced expression potential (28). Given that transcription factors must interact with chromatin template (29) perhaps it will be only through a better understanding of this environment that synthetic regulatory elements can be efficiently designed.

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