BLG-e1 – A novel regulatory element in the distal region of the β-lactoglobulin gene promoter

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Abstract **β-Lactoglobulin (BLG) is a major ruminant milk protein. A regulatory element, termed BLG-e1, was defined in the distal region of the ovine BLG gene promoter. This 299-bp element lacks the established *cis*-regulatory sequences that affect milk-protein gene expression. Nevertheless, it alters the binding of downstream BLG sequences to histone H4 and the sensitivity of the histone–DNA complexes to trichostatin A treatment. In mammary cells cultured under favorable lactogenic conditions, BLG-e1 acts as a potent, position-independent silencer of BLG/luciferase expression, and similarly affects the promoter activity of the mouse whey acidic protein gene. Intragenic sequences upstream of BLG exon 2 reverse the silencing effect of BLG-e1 in vitro and in transgenic mice.**

Keywords: Milk protein; Mammary gland; Regulatory element; Promoter; Acetylation

1. Introduction

Milk-protein gene expression is subjected to complex regulation which coordinates it with distinct stages in mammary gland development. Lactogenesis is induced by polypeptide and steroid hormones during the second term of pregnancy and is mediated by cell–cell and cell–extracellular matrix (ECM) interactions.

Specific *trans*-acting factors, such as signal transducer and activator of transcription 5 (Stat5), glucocorticoid-responsive element (GRE), and nuclear factor 1 (NF-1), which recognize *cis*-acting DNA motifs located in close proximity to the transcriptional start sites of casein, whey acidic protein (WAP) and β-lactoglobulin (BLG) genes, have been implicated in the regulation of milk-protein gene expression at specific developmental stages. Their combined action also confers mammary specificity [1]. Much less is known about the location and function of regulatory elements in the distal regions of milk-protein gene promoters. Distal regulatory elements have been reported for mouse WAP [2], rabbit αS1 casein [3], human β-casein [4] and bovine β-casein [5,6] genes. Their regulatory mechanism has not been fully identified, but appears to require the activation of one or more of the recognized enhancers, located near the transcription-initiation sites. A possible epigenetic effect has been proposed for the BCE-1 element in the bovine β-casein distal promoter, assuming modulation of histone acetylation in mediating the ECM response [6]. Acetylation of lysine residues in the N-terminal tail of histones H3 and H4 reduces their positive charge and weakens their affinity to DNA, which, in turn, facilitates transcription-factor binding [7].

Whereas caseins are the major milk proteins, BLG could be considered the most characteristic of ruminants, due to its absence from human and murine milk and its highest level in ruminant whey [8]. The ovine BLG gene has been cloned, sequenced and expressed at high levels in the mammary gland of transgenic mice [9–11]. Three major DNase-1 hypersensitive sites have been identified in the promoter region between −1800 and +100 bp from the transcription-initiation site. An additional, less characterized site has been located at −2500 bp [10,12]. Although hypersensitive sites usually indicate the presence of *cis*-regulatory elements, only the 400-bp proximal region of the BLG promoter has been studied in detail [12–14].

Here, we identified and studied the function of β-lactoglobulin element 1 (BLG-e1), a unique regulatory element located in the distal region of the BLG promoter, in CID-9 mammary cells cultured under favorable lactogenic conditions and in transgenic mice. We report that BLG-e1 affects acetylation of histone H4 and its binding to downstream BLG promoter sequences. Depending on the presence or absence of intragenic sequences upstream of exon 2, BLG-e1 serves as either an essential element or a silencer of BLG promoter activity.

2. Materials and methods

2.1. Construction of recombinant BLG/luciferase genes

BLG/luciferase hybrid gene constructs were prepared to study the activity of the BLG regulatory sequences. The first set of hybrid genes (Fig. 1) was based on vector p904 [15–17]. In the course of its construction, BLG sequences between the first part of exon 2 and the middle of exon 6 (including the TAG termination codon) were deleted and replaced with the luciferase gene, followed by the SV40 polyadenylation (PA) region, both from the pGL2-basic commercial vector (Promega, Madison, WI). To avoid biphasic expression, the natural BLG ATG translation-initiation codon as well as the second potential initiation codon in BLG exon 1 were converted into non-initiating ATT and ATC sequences, respectively. The resulting p904 vector is composed,

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Abbreviations: BLG, β-lactoglobulin; BLG-e1, β-lactoglobulin element 1; ECM, extracellular matrix; GRE, glucocorticoid-responsive element; HNF, hepatocyte nuclear factor; Stat5, signal transducer and activator of transcription 5; NF-1, nuclear factor 1; TEF, transcription enhancer factor; TSA, trichostatin A; WAP, whey acidic protein

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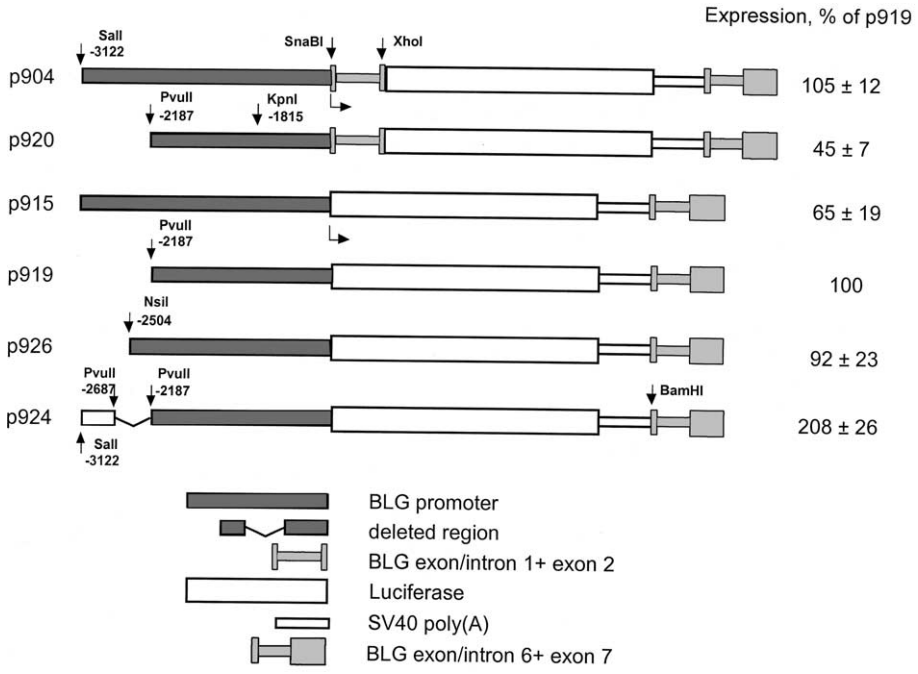

Fig. 1. Silencing of BLG/luciferase gene expression by a distal region in the BLG promoter depends on intragenic sequences residing near the transcription start site. Representation of BLG/luciferase vectors used to stably transfect CID-9 cells, and their relative activities. Luciferase cDNA was inserted in the first exon of the BLG gene (p915, p919, p926 and p924) or in the second exon of the BLG gene (p904 and p920). All constructs are terminated with BLG 3′-flanking sequences.

therefore, of 3.1 kb of the BLG promoter, 0.8 kb of exon 1/intron 1 and a small part of exon 2. The luciferase reporter is inserted into BLG exon 2 and followed by part of BLG exon 6/intron 6 and 1 kb of the BLG 3′ flanking region (including the BLG PA site). The TATA box in the BLG promoter of this construct remains 889 bp upstream of the translation-initiation site.

To construct vector p920, p904 was shortened by 935 bp upstream of the *Pvu*II site. In vector p915, the pGL2 reporter was introduced into an artificial *Xho*I site generated in BLG exon 1 of construct p904, 40 bp downstream of the BLG TATA box. This manipulation resulted in the elimination of a 767-bp *Sna*BI–*Xho*I fragment containing sequences of BLG exon1/intron 1 and a small part of exon 2. To construct vector p919, p915 was shortened by 935 bp, as in the construction of p920. In vector p926, a distal 618-bp fragment from the BLG promoter of p915 was excised upstream of the *Nsi*I site. Vector p924 is a modification of p915, lacking a 500-bp *Pvu*II–*Pvu*II fragment downstream of position −2187 in the BLG promoter.

Vector p908 was the basis for the second set of BLG/luciferase vectors (Fig. 2). It is composed of only two components: 3122 bp of the BLG 5′ sequence and the luciferase/SV40 reporter gene. It is much shorter than p904, due to the absence of both the BLG exon1/intron 1/exon 2 fragment and the BLG 3′ flanking region. Here, the luciferase/SV40 reporter was linked to the BLG promoter at the *Xho*I site as described above for p915. To construct vector p925, a distal 935-bp fragment, downstream of the *Pvu*II site in p908, was excised. In vector p927, a 500-bp *Pvu*II–*Pvu*II fragment was excised from the BLG promoter of p908, as with the construction of p924. Vector p928 lacks a BLG 5′ distal fragment of 618 bp downstream of the *Nsi*I site in p908. Vector p929 results from the elongation of p925 by 254 bp downstream of the *Pvu*II site. This was performed in two steps. First, a 291-bp PCR product encompassing an artificial *Nhe*I site at its 5′ end was engineered, using p908 as the template and the following primers:

*Nhe*I

$^{-2439}$ 5′ AAG CAG CAT GGC ATg CTA GcG TGT G 3′

$^{-2148}$ 5′ GAG AGA GAC GGA AAT AAA GCA GC 3′

(positions of the primers in the BLG gene, Accession No. X68105, are indicated. Lowercase letters designate modified nucleotides used to engineer the restriction site). Second, the PCR product was digested with *Nhe*I and *Pvu*II, and the resulting 254-bp fragment was ligated upstream of the *Pvu*II site in the BLG promoter of p925. To generate vector p934, a 134-bp PCR product was amplified from the BLG promoter region immediately upstream of p928. With the following primers and p908 as a template, this product was engineered to contain an artificial *Mlu*I site at its 5′ end and *Nhe*I site at its 3′ end.

*Mlu*I

$^{-2622}$ 5′ TTC ATT ACg cGT AAC TGG TCT GTT C 3′

*Nhe*I

$^{-2489}$ 5′ TGC TGT GTG CTa gCA TGC ATG TCC 3′

After digestion with these enzymes, the resultant 118-bp fragment was ligated to the *Nhe*I site of p929. To generate vector p933, a 137-bp PCR product amplified from a region immediately upstream of the 5′ end of p934 and containing the same pair of artificial sites, *Mlu*I at its 5′ end and *Nhe*I at its 3′ end, was prepared, using the following primers and p908 as a template:

*Mlu*I

$^{-2733}$ 5′ GCA GGG AAG ACg Cgt CAC AAC CGG 3′

*Nhe*I

$^{-2596}$ 5′ TAT GAA CAG ACC AGc Tag CAG TAA TG 3′

After digestion with the appropriate enzymes, the resultant fragment encompassing 111 bp was ligated to the *Nhe*I site of vector p929. To construct vector p930, the most distal *Sal*I–*Bbs*I 405-bp fragment of the available BLG promoter was ligated to p929. To construct vector p931, a 320-bp PCR product encompassing the absent region of the BLG promoter in p930 (the predicted regulatory element) and containing artificial *Mlu*I and *Nhe*I sites was prepared using the following primers and p908 as a template.

*Mlu*I

$^{-2733}$ 5′ GCA GGG AAG ACg CgtCAC AAC CGG 3′

*Nhe*I

$^{-2413}$ 5′ CAC ACA CgC TAG cAT GCC ATG CTG C 3′

The PCR fragment was digested with these enzymes and the resultant 299-bp fragment was ligated to the *Nhe*I site at the 5′ end of p925.

Vector p932 (Fig. 3) was constructed to generate transgenic mice. A distal 1307-bp *Pvu*II–*Kpn*I fragment was excised from p920 and replaced with the 1922-bp *Sal*I–*Kpn*I fragment from the promoter of vector p930. The resultant vector lacks the 299-bp predicted regulatory element upstream of the artificial *Nhe*I site at position −2419.