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An International Journal Focusing on Gene Cloning and Gene Structure and Function

W. Szybalski – Editor-in-Chief

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Cloning and nucleotide sequence of the bovine β -lactoglobulin gene

(Recombinant DNA; mammary gland; cDNA clone bank; whey protein; retinol)

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SUMMARY

A clone coding for β -lactoglobulin A has been isolated from a cDNA bank constructed from poly(A⁺)mRNA isolated from the bovine mammary gland. Its nucleotide sequence codes for the β -lactoglobulin A, from amino acid residues Leu-11 to Ile-162, as based on the amino acid sequence reported by Braunitzer et al. [Z. Physiol. Chem. 354 (1973) 867-878]. In addition to the 455-bp coding sequence, our clone pB β L4-10 contains a 3'-nontranslated region of approx. 270 bp.

INTRODUCTION

Bovine β -lactoglobulin (β Lg) is a whey protein, found in the milk of animals including bovine species, sheep, deer, dogs, and pigs but not in humans (Pervaiz and Brew, 1985). It occurs in several different variants due to some genetic variations that exist in given animal species. It is a single polypeptide of 18 kDa which corresponds to 162 aa residues.

The complete amino acid sequence of bovine β Lg has been reported and genetic variations in the amino acid sequence have been identified (Creamer et al., 1983).

β Lg is a globular protein and the primary sequence reveals two disulfide bonds between aa residues 66–160 and 106–119 and a free cysteine group at 121. On the basis of extensive analyses and calculations, the secondary structure of β Lg is 15, 51, 17, and 17% α -helix, β -sheet, reversed turn and aperiodic structure, respectively (Creamer et al., 1983). In solution, above pH 4.0 β Lg exists as a dimer in which the monomers appear to associate at the respective α -helical segments (aa 65–75 and 129–143) and the Cys-121 groups are close to the electron dense region of association. Hence dimerization protects the thiol groups. The β -sheet peptide sequence containing Cys-121 is relatively apolar and in the native monomer, Cys-121 is buried below the surface of the molecule.

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Abbreviations: aa, amino acids(s); β Lg, β -lactoglobulin; bp, base pair(s); LB, Luria-Bertani (see Maniatis et al., 1982); nt, nucleotide(s); ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TBS, see MATERIALS AND METHODS, section a.

The tertiary structure has been determined from x-ray crystallographic studies at a resolution of 2.8 Å (Sawyer et al., 1985; Papiz et al., 1986) and modelled using computer graphic programs (J. Brady, Dept. Food Science, Cornell University, personal communication). It possesses two antiparallel β -sheets, two disulfide bonds and one free cysteine residue.

The function of β Lg is not clear especially since the milk of certain mammalian species appears to be devoid of it. Based upon its structural homology to retinol-binding protein, it is hypothesized that it may also bind and transport retinol (Papiz et al., 1986) to the neonate.

Although extensive information is available on the primary and tertiary structure of bovine β Lg, very little has been reported on the cloning and characterization of its gene. A partial cDNA clone of bovine β Lg has been isolated (Willis et al., 1982). The β Lg clone obtained was 550 bp and a limited amino acid sequence inferred from nucleotide sequencing corresponded to aa residues 109–122.

In this study we have isolated a cDNA clone encoding for 93% of the structural gene and its 3'-nontranslated region extending to the poly(A⁺) tail.

MATERIALS AND METHODS

(a) Identification of β Lg cDNA clone

cDNA was synthesized from bovine poly(A)⁺ mRNA isolated from mammary tissue of lactating cow, G-C tailed and cloned into the *bla* gene of pBR322 (Jimenez-Flores et al., 1987). This cDNA bank was screened using polyclonal anti- β Lg serum obtained by repeated intramuscular injection of β Lg (Sigma Chemical Co.) into Flemish Giant rabbits. Recombinant *Escherichia coli* RR1 colonies carrying the bovine cDNA bank were propagated overnight on LB agar containing tetracycline (15 μ g/ml) and replicated onto 150-mm nitrocellulose filters (Biorad Inc., Richmond, CA). After incubation for 4–6 h at 37°C the colonies were lysed by suspending the filters in chloroform vapors for 20 min. The lysed colonies on the filters were incubated for 60 min in 50 mM Tris · HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂ 3% gelatin, 1 μ g/ml of DNase

I, and 40 μ g/ml of lysozyme. The filters were then washed twice in TBS buffer (20 mM Tris, pH 7.5; 500 mM NaCl) for 5 min. A 1:1000 dilution of rabbit anti- β Lg antisera was added in TBS with 1% gelatin for 60 min and the filter was then washed twice with TBS. A goat anti-rabbit horseradish peroxidase conjugated antibody was then added for 60 min (1:3000 dilution in TBS + 1% gelatin) to detect the primary antibody- β Lg complex. After washing the filter, bound anti-rabbit antibody was identified using a color-developing reagent (HRP color-developing reagent, Biorad Inc., with 0.02% hydrogen peroxide).

(b) Nucleotide sequencing and Western-blot analysis

The nucleotide sequence was determined by the method of Sanger et al. (1977) using M13mp18 or M13mp19 vectors.

Total cellular protein from *E. coli* RR1 alone and carrying pB β L4-10 was separated using 0.1% SDS–12.5% PAGE (Laemmli, 1970) transferred to nitrocellulose (Towbin et al., 1979) and developed using rabbit anti β Lg serum as described before.

RESULTS

(a) Identification of β Lg cDNA clone

A cDNA bank containing approx. 6000 clones was obtained from poly(A)⁺ mRNA isolated from the mammary tissue of a lactating cow. The screening of an initial 500 clones using rabbit polyclonal anti- β Lg serum resulted in the identification of a immunoreactive clone designated pB β L4-10. pB β L4-10 contains an insert of approx. 800 bp and an internal *Pst*I site.

By virtue of its identification using polyclonal antibodies a fusion must exist between the N-terminal of β -lactoglobulin and β -lactamase encoded by the region 5' to the *Pst*I site. Western-blot analysis of *E. coli* RR1 carrying pBR322 and pB β L4-10 was performed to determine the size of the fusion protein (Fig. 1). Although some cross-reactivity (or non-specific binding) is observed as common bands in both pBR322 and pB β L4-10 a unique band of 36 kDa is observed in pB β L4-10 extracts. Given the

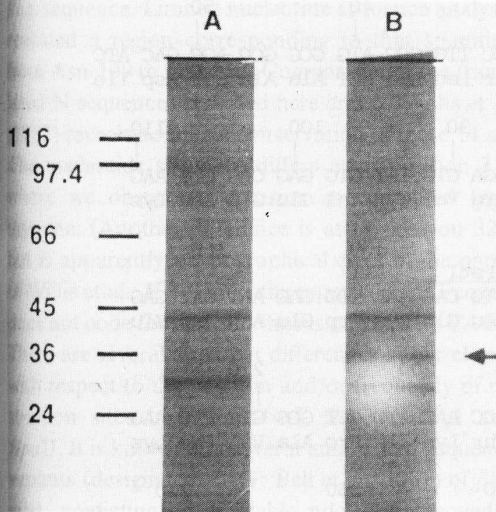


Fig. 1. Western-blot analysis of *E. coli* RR1 lysates using rabbit antiserum against β Lg. Total cellular protein from the respective *E. coli* RR1 strains were separated on a 0.1% SDS-12.5% PAGE in 25 mM Tris $\cdot$ HCl, pH 8.8 at 30 mA overnight. The proteins were electrophoretically transferred to a nitrocellulose filter in transfer buffer (25 mM Tris $\cdot$ HCl, 192 mM glycine; 20% methanol, pH 8.3) at 30 V for 12 h. After blocking in NET buffer (150 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Triton X-100, 50 mM Tris $\cdot$ HCl, pH 7.4) detection was performed using rabbit polyclonal antiserum against β Lg (diluted 1:1000). The antibody-antigen complex was visualized using a secondary goat antirabbit antibody conjugated to horseradish peroxidase (diluted 1:3000; BioRad Inc.) and the blot developed according to the manufacturer's instructions. Lanes: A, pBR322 (control); B, pBL4-10. The two bands common to both lanes are proteins which cross react with the anti- β Lg antiserum, the unique band present in lane B is the β Lg/ β lactamase fusion protein. The positions of protein markers (in kDa) are shown in the left margin. The protein M_r markers (Sigma Co.) used were β -galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa) and trypsinogen (24 kDa). The arrow indicates the location of the unique β Lg- β lactamase fusion protein.

size of the N terminus of β -lactamase to the *Pst*I site (20 kDa) approx. 16 kDa or 430 bp of the β -lactoglobulin-coding sequence should be present. β -lactoglobulin is 162 aa long (or a predicted 486 bp), therefore this clone should contain approx. 90% of the coding sequence for the mature protein.

A 600-bp *Pst*I insert from pBL4-10 was used to probe the cDNA bank for additional β Lg clones. Five colonies which hybridized to the 600-bp *Pst*I fragment were obtained and their plasmid inserts

confirmed by Southern-blot analysis. Subsequent nucleotide sequence analysis revealed that they were in fact shorter at the 5' end than pBL4-10 (see further).

(b) Nucleotide sequence

The sequence for the entire 800-bp cDNA insert in pBL4-10 was determined (Fig. 2). When translated, a single ORF of 452 bp or 151 aa was observed. This ORF corresponds to the published amino acid sequence of β Lg from Leu-11 to Ile-162. A 270-bp 3'-nontranslated region is located after the translational stop (nt 456) followed by a poly(A⁺) sequence which accounts for 36% of the predicted size of the full length message. A consensus polyadenylation site (nt 589; ATAAA) is also present upstream from the poly(A) sequence.

DISCUSSION

A cDNA clone coding for aa residues Leu-11 to Ile-162 of β Lg has been isolated from a bank constructed from bovine poly(A)⁺ mRNA. The deduced amino acid sequence is in complete agreement with the reported sequence for β Lg (Creamer et al., 1983). To date several cDNA clones coding for bovine mammary proteins have been identified including α_{s1} -casein, κ -casein (Stewart et al., 1984) and β -casein (Jimenez-Flores et al., 1987) and galactosyltransferase (Shaper et al., 1986). This is to our knowledge the first report on cloning of a virtually complete bovine β Lg sequence.

We were not successful in locating any clones that coded for the region located 5' to the Leu-11 codon. A 20-kDa protein was observed after in vitro translation of bovine poly(A)⁺ mRNA and subsequent immunoprecipitation with anti- β Lg antiserum (Yoshikawa et al., 1978). This suggests that pre- β Lg is approx. 2 kDa larger than the mature β Lg. The signal sequence of the ovine β Lg is 18 aa long and the sequence for the N terminus of the mature protein is almost identical to that of the bovine β Lg (Mercier et al., 1978).

Willis et al. (1982) reported the identification of a cDNA clone coding for β Lg from an Ayrshire cow. It is estimated that it codes for approx. 60% of the

```

      10      20      30      40      50
AT ATC CAG AAG GTG GCG GGG ACT TGG TAC TOC TTG GCC ATG GCG GCC AGC GAC ATC
  Ile Gln Lys Val Ala Gly Thr Trp Tyr Ser Leu Ala MET Ala Ala Ser Asp Ile
                                *
      60      70      80      90      100      110
TCC CTG CTG GAC GCC CAG AGT GCC CCC CTG AGA GTG TAT GTG GAG GAG CTG AAG
Ser Leu Leu Asp Ala Gln Ser Ala Pro Leu Arg Val Tyr Val Glu Glu Leu Lys
                                *
      120      130      140      150      160
CCC ACC OCT GAG GGC GAC CTG GAG ATC CTG CTG CAG AAA TGG GAG AAT GAT GAG
Pro Thr Pro Glu Gly Asp Leu Glu Ile Leu Leu Gln Lys Trp Glu Asn Asp Glu
      *
      170      180      190      200      210
TGT GCT CAG AAG AAG ATC ATT GCA GAA AAA ACC AAG ATC CCT GCG GTG TTC AAG
Cys Ala Gln Lys Lys Ile Ile Ala Glu Lys Thr Lys Ile Pro Ala Val Phe Lys
                                *
      220      230      240      250      260      270
ATC GAT GCC TTG AAC GAG AAC AAA GTC CTT GTG CTG GAC ACC GAC TAC AAA AAG
Ile Asp Ala Leu Asn Glu Asn Lys Val Leu Val Leu Asp Thr Asp Tyr Lys Lys
      280      290      300      310      320
TAC CTG CTC TTC TGC ATG GAG AAC AGT GCT GAG CCC GAG CAA AGC CTG GTC TGC
Tyr Leu Leu Phe Cys MET Glu Asn Ser Ala Glu Pro Glu Gln Ser Leu Val Cys
                                *
      330      340      350      360      370      380
CAG TGC CTG GTC AGG ACC CCG GAG GTG GAC GAC GAG GCC CTG GAG AAA TTC GAC
Gln Cys Leu Val Arg Thr Pro Glu Val Asp Asp Glu Ala Leu Glu Lys Phe Asp
                                *
      390      400      410      420      430
AAA GCC CTC AAG GCC CTG CCA ATG CAC ATC CCG CTG TOC TTC AAC CCA ACC CAG
Lys Ala Leu Lys Ala Leu Pro MET His Ile Arg Leu Ser Phe Asn Pro Thr Gln
                                *
      440      450      460      470      480
CTG GAG GAG CAG TGC CAC ATC TAG AGC CCG GGA GCC TTG GCC CTC TGG CGC GTG
Leu Glu Glu Gln Cys His Ile ---
      490      500      510      520      530      540
TCA TCC CCG CCT GCC CCA CAT GGG ACC AGG AGG AAC CCG GAC CAT ATA TCC CTG
      550      560      570      580      590
TGG ACC AGG CCC TCA GGC CCT CTG CGT CTG CTT GGG CGC TCT CTT GAT AAA CTA
      600
ACT GA (A)51
```

Fig. 2. Nucleotide sequence and derived amino acid sequence of ORF from pB β L4-10. The nucleotide sequence for pB β L4-10 corresponds to aa Leu-11 to Ile-162 of mature bovine β Lg. The nucleotide sequence was determined for nt 1-144 using the 160-bp *Pst*I fragment and for 550-bp *Pst*I fragment (nt 145-652) using the M13 -20 sequencing primer (New England Biolabs, Beverly, MA) and extended using synthetic oligodeoxynucleotide primers which bind to nt 353-368. Sequence analysis was performed using PC-Gen software (Intelligenetics Inc, Mountain View, CA). Asterisks mark the position of variable amino acids found in the different β Lg derivatives, i.e., β LgB (Creamer et al., 1983). The underlined region corresponds to the sequence reported by Willis et al. (1982).

β Lg sequence. Limited nucleotide sequence analysis revealed a region corresponding to that spanning from Asn-109 to Leu-122. Comparison of the translated N sequences reported here and by Willis et al. (1982) reveals complete conservation of these 14 aa. The nucleotide sequence differs at nt position 318 where we observe a cytosine as compared to a thymine. (Another difference is at nt position 327, but is apparently a typographical error in the paper of Willis et al., 1982, since the reported GAG codon does not code for Gln and thus is more likely CAG.) There are several apparent differences in our clones with respect to the position and/or frequency of restriction sites including *Ava*I, *Sau*3A, *Taq*I and *Hpa*II. It is known that several amino acid sequence variants (designated A-G; Bell et al., 1981) of β Lg exist, predicting considerable nucleotide sequence divergence.

Our cDNA clone most probably codes for β Lg A, as based on the presence of Asp-64 and Val-118 aa residues in the translated sequence (Braunitzer et al., 1973). Restriction polymorphisms would exist, for example, between β LgA and β LgC genes. The *Pst*I site (nt 141) present in our β LgA would be absent in the β LgC gene where the Gln-59 is replaced with a His residue, a substitution of C or T for G at nt 146. Furthermore, all of the aa substitutions in the β Lg variants can result from single nucleotide substitutions. A new kind of mutation occurring in β LgDr (symbol Dr indicates a β Lg variant isolated from *Bos indicus*, Droughtmaster) is the Asp-28 to Asn-28 change (Bell et al., 1981). The Asn-28 is glycosylated as the result of creating an Asn-X-Ser consensus glycosylation target, where the symbol X can be virtually any aa residue.

In conclusion, we have identified a cDNA clone coding for bovine β Lg. We are presently attempting to express this gene at high levels in *E. coli* using a synthetic oligodeoxynucleotide coding for the missing 11 aa residues. Once accomplished, we intend to study the conformation and thermal stability of this recombinant β Lg.

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