

SSCP polymorphism within a promoter of the bovine alpha S1 casein gene*

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(Received 29 July 1999; accepted 13 January 2000)

ABSTRACT

The detection of a new SSCP polymorphism within the promoter of the bovine alpha S1 casein gene is described. The fragment of alpha S1 casein gene promoter (218 bp) was PCR-amplified and then subjected to electrophoresis allowing the detection of SSCP polymorphism. Among 118 randomly chosen cows (Black-and-White) and 124 bulls (Black-and-White), we found two SSCP patterns, labelled R2 and R3, with respective frequencies of 0.05 and 0.95. The detected SSCP polymorphism may be useful in searching for associations between different SSCP patterns and gene expression, especially milk protein percentage and protein yield.

KEY WORDS: cattle, alpha S1 casein, polymorphism, SSCP

INTRODUCTION

Milk protein genes are potential quantitative trait loci (QTL) in dairy cattle (Lien et al., 1995; Velmala et al., 1995). Alpha S1 casein is a 199-amino acid phosphoglycoprotein secreted in epithelial cells of the mammary gland during lactation. Among 6 milk protein types, alpha S1 casein is the major milk protein (approximately 40% of total milk protein output) (Fox, 1992). The gene encoding bovine alpha S1 casein (CSN1S1) belongs to the cluster of four bovine casein genes located within a 250 kb fragment of chromosome 6 q31-33 (Rijnkels et al., 1997). The complete genomic sequence of bovine CSN1S1 has been characterised by Koczan et al. (1991). Six protein variants located within the coding part of bovine CSN1S1 gene are known (Fox, 1992; Erhardt, 1993). Apart from the cod-

* Supported by the State Committee for Scientific Research, Grant No 5 PO6D 04212

ing sequence, two other polymorphisms within regulatory regions of CSN1S1 gene have been reported: in the CSN1S1 promoter (Koczan et al., 1993) and 3' untranslated region (Perez et al., 1994).

In this study we tried to find if any further polymorphisms in the promoter of bovine CSN1S1 gene exist. Mutations located in this regulatory region might potentially influence gene transcription and the amount of milk protein secreted in the mammary gland by the cow.

MATERIAL AND METHODS

One hundred and eighteen cows (Black-and-White) and 124 A. I. bulls (Black-and-White) chosen randomly from 3 herds and several insemination stations were included in the analysis. Genomic DNA was isolated from somatic cells (milk), leukocytes (blood) and sperm (semen).

To isolate DNA from milk, 25 ml of milk was taken from each cow and then washed 3 times in PBS buffer. Purified somatic cells as well as 50 μ l of purified leukocytes (Lee et al., 1997) isolated from 2 ml of blood were then subjected to DNA isolation using a Wizard Genomic Purification Kit (Promega). Minor modifications to isolation of DNA from semen were applied (Kamiński and Zabołewicz, 1998).

The 218 bp fragment of the CSN1S1 gene was amplified using PCR. Briefly, the PCR mix consisted of: 2.5 μ l 10x PCR buffer, 2.0 μ l of 25 mmole MgCl₂, 2.0 μ l dNTP-mix (2 mmole each), 0.2 μ l of primer L218 and 0.2 μ l of primer R218 (100 nmole each), 1 U Taq polymerase (Promega), 600 ng of DNA template and H₂O to a volume of 25 μ l. PCR thermal protocol: initial denaturation: 2 min 94°C followed by 35 cycles of: 30 sec/94°C, 30 sec/60°C, 30 sec/72°C and finished by 5 min in 72°C. Using the Primer 3 program available via the Internet (www.firstmarket.com) the following PCR primers were designed:

L218: 5' AATTTAGAACAATGCCATTCCA '3,

R218: 5' GATGGCAGACTTTTGCTTCC 3'.

Seven μ l of PCR products were digested with Hinf I (Promega), Dde I (Promega) and Mae III (Boehringer Mannheim) restriction enzymes following the producer's instructions. The PCR products and restriction fragments were electrophoresed in 1.5% agarose/ethidium bromide gel.

The SSCP technique (Orita et al., 1989) was optimised according to the principles described by Tadashi et al. (1993) and the guidelines published by Electrophoresis Technik GmbH (ETC) in a bulletin attached to precast gels and buffers commercially available from this producer. 3.5 μ l of PCR product mixed with 12.5 μ l of the denaturation solution (50 mmole NaOH, 1 mmole EDTA) and 1 μ l of loading buffer containing 0.25% bromophenol blue and 0.25% xylene cyanol were denatured for 12 min at 85°C, rapidly chilled in an ice block and then loaded onto precast

polyacrylamide CleanGel 36S (ETC). The samples were electrophoresed in a Multiphor II Electrophoresis System (Amersham-Pharmacia Biotech). A thermostatically controlled refrigerated circulator (MultiTemp III, Amersham-Pharmacia Biotech) was used to maintain the gel at a constant temperature (6°C). Electrophoresis was performed in Delect gel buffer (ETC) under the following conditions: 300 V, 20 mA, 10 W for 10 min, 475 V, 30 mA, 20 W for 50 min and 550 V, 30 mA, 20 W for 60 min. The gels were stained using a Silver Staining Kit (Promega). The patterns of DNA bands were observed and photographed in a GDS7500 System (UVP).

RESULTS

Using PCR amplification, we were able to obtain the PCR product of the desirable 218 bp size that was a fragment of the promoter of the CSN1S1 gene (from nucleotide numbered +3 to -215 according to Koczan et al., 1991). The identity of the PCR product was confirmed by restriction enzyme analysis with the use of Dde I and Hinf I enzymes (data not shown).

Using the PCR-SSCP method we found a polymorphism observed as the appearance of two distinctly different SSCP patterns that we labelled R2 and R3. Pattern R3 showed four bands and pattern R2 was characterised by five bands (one additional band, migrating fastest) (Figure 1). Among 118 randomly chosen cows (Black-and-White) and 124 bulls (Black-and-White), we found R2 and R3 SSCP patterns with frequencies 0.05 and 0.95, respectively.

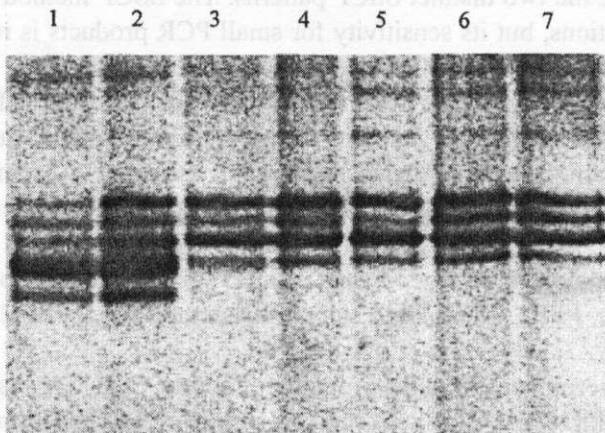


Figure 1. Detection of single stranded conformation polymorphism (SSCP) within 218 bp PCR products derived from promoter of the alpha S1 gene.

Two different SSCP patterns R2 and R3 are shown in the following lanes:

Lanes 1 and 2 – R2 (five bands); Lanes 3, 4, 5, 6, 7 – R3 (four bands)

DISCUSSION

We found a DNA polymorphism within the 5' flanking region of the CSN1S1 gene. We choose the part of the CSN1S1 gene containing the core promoter because this region is commonly considered as the most important for transcription regulation in Eucaryota genes. This region contains common regulatory motifs, like the TATA box, CAAT sequence and GC box as well as other consensus sequences for different transcription factors (Lewin, 1997; Papavassiliou, 1997).

According to the theory of the SSCP method (Orita et al., 1989), DNA polymorphism is detected when the electrophoretic mobility of single-stranded DNA bands is reproducibly different (a so-called SSCP pattern appears, defined as a constant combination of number and position of single-stranded DNA bands).

The number of bands and their position in the gel very clearly show two distinct SSCP patterns that can be interpreted as the occurrence of DNA polymorphism.

Resolution of SSCP bands needs to be optimised, in particular, the pH of the buffer, time and temperature of electrophoresis, and properties of the gel matrix. In the preliminary studies, different manually prepared as well as precast polyacrylamide gels and buffers were tested. The best results were achieved using CleanGel 36S and Delect buffer (ETC). Sometimes the gels showed additional weak bands comprised of reannealed double-stranded DNA or metastable "ghost" bands that are the effect of incompletely denatured single strands of DNA. But this did not obscure the two distinct SSCP patterns. The SSCP method is not able to detect all mutations, but its sensitivity for small PCR products is relatively high (85-95%) (Sheffield et al., 1993).

Milk as a source of genomic DNA turned out to be moderately effective. Out of 118 milk samples 95 yielded genomic DNA of different quality but suitable for effective PCR amplification. Unsuccessful DNA isolations were most probably due to too low a number of somatic cells in the milk and other unidentified factors (Lipkin et al., 1993).

The remaining cows were genotyped using DNA isolated from purified leukocytes (Lee et al., 1997). These DNA samples gave the best yield and specificity of PCR product.

DNA polymorphism within the promoter of bovine CSN1S1 was first reported by Koczan et al. (1993). They amplified the region of 310 bp (from -274 to +36) and found substitution of A for G in position -175. Occasionally this mutation is located in the Mae III recognition sequence, which is present in animals carrying the B allele of the CSN1S1 protein. We used Mae III to digest the 218 bp PCR product obtained from several cows phenotyped as BC and BB and found no dif-

ference in RFLP patterns. This means that the R2/R3 polymorphism is not located within the Mae III recognition sequence and can be considered as a new mutation. The Mendelian inheritance of R2 and R3 SSCP patterns was analysed within two sub-families (each comprising 20 progeny of two bull carriers of R3 and R2).

Analysis of the progeny of the R3 bull carrier confirmed its Mendelian segregation. The analysis of the R2 bull carrier's progeny was surprising. We found only one out of 20 daughters showing the same genotype as the father. To exclude the suspicion that what we were analysing was not a family, we analysed another multi-allele locus, namely the fragment of 5' region of the beta-lactoglobulin gene (Kamiński and Zabolewicz, 1998). The results confirmed the family status of the sire (R2 bull carrier) and its daughters. All these results led us to conclude that we may be observing an unknown mechanism of natural elimination of R2 animals. Unfortunately, in the analysis we were not able to include the sons of the R2 bull carrier. That could have explained if this phenomena is dependent on sex.

So far, polymorphisms within regulatory fragments of bovine milk protein genes have been found only in kappa-casein (Schild et al., 1994; Kamiński, 1996), beta-lactoglobulin genes (Wagner et al., 1994; Kamiński and Zabolewicz, 1998) and alpha-lactalbumin (Bleck and Bremel, 1993a; Kamiński, 1999).

Our results are the first step in evaluating the importance of the detected polymorphism. We hope that the mutation in the regulatory regions of milk protein genes may influence its expression. This hypothesis was proved by Bleck and Bremel (1993b) for the promoter of bovine alpha-lactalbumin, by Lum et al. (1997) for the beta-lactoglobulin promoter as well as for human cytochrome P450IIE1 (Hayashi et al., 1991), human haptoglobin 2-I gene (Grant and Maeda, 1993) or lately, human tumour necrosis factor (Knight et al., 1999). The potential importance of variation within regulatory sequences of bovine CSN1S1 gene was proved by Perez et al. (1994) who found the insertion of the LINE sequence in the 3' untranslated region of this gene. They suggest that this insert reduces the transcriptional rate and/or decreases the stability of mRNA of CSN1S1.

CONCLUSIONS

A new SSCP polymorphism within the promoter of the bovine CSN1S1 gene was found. The R2 SSCP pattern is very rare. Because alpha S1 casein is a major milk protein, we assumed that the sequence variation within the promoter of CSN1S1 may potentially influence gene expression and overall amount of protein in milk – presently the most important criterion in dairy cattle selection.

ACKNOWLEDGEMENTS

We would like to thank Prof. Krzysztof Walawski for valuable discussion as well as for help in sample collection. We also wish to thank Elżbieta Wójcik and Anna Ruść for excellent technical assistance. We are grateful the breeders from OSH Osięciny, OHZ Pawłowice, RSP Lubiń and A. I. Station in Bydgoszcz for the help in sample collection.

REFERENCENCES

- Bleck G.T., Bremel R.D., 1993a. Sequence and single-base polymorphisms of the bovine alpha-lactalbumin 5'-flanking region. *Gene* 126, 213-218
- Bleck G.T., Bremel R.D., 1993b. Correlation of the alpha-lactalbumin (+15) polymorphism to milk production and milk composition of Holstein. *J. Dairy Sci.* 76, 2292-2298
- Erhardt G., 1993. A new alpha S1 casein allele in bovine milk and its occurrence in different breeds. *Anim. Genet.* 24, 65-66
- Fox P.F., 1992. *Advanced Dairy Chemistry. Volume 1. Proteins.* Elsevier Science Publishers Ltd., London and New York
- Grant D.J., Maeda N., 1993. A base substitution in the promoter associated with the human haptoglobin 2-1-modified phenotype decreases transcriptional activity and responsiveness to interleukin -6 in human hepatoma cells. *Amer. J. Hum. Genet.* 52, 974-980
- Hayashi S., Watanabe J., Kawajiri K., 1991. Genetic polymorphisms in the 5' flanking region change transcriptional regulation of the human cytochrome P450IIE1 gene. *J. Biochem.* 110, 559-565
- Kamiński S., 1996. Dde I RFLP at the 5' region of bovine kappa-casein gene. *J. Appl. Genet.* 37, 173-178
- Kamiński S., 1999. Identification of Sdu I polymorphism within 5' flanking region of bovine alpha-lactalbumin gene. *Anim. Sci. Pap. Rep.* 17, 23-27
- Kamiński S., Zabolwicz T., 1998. SSCP polymorphism within 5' region of bovine beta-lactoglobulin (LGB) gene. *J. Appl. Genet.* 39, 97-102
- Knight J.C., Udalova I., Hill A.V.S., Greenwood B.M., Peshu N., Marsh K., Kwiatkowski D., 1999. A polymorphism that affects OCT-1 binding to the TNF promoter region is associated with severe malaria. *Nat. Genet.* 22, 145-150
- Koczan D., Hobom G., Seyfert H.M., 1991. Genomic organisation of the bovine alpha S1 casein gene. *Nucl. Acid Res.* 19, 5591-5596
- Koczan D., Hobom G., Seyfert H.-M., 1993. Characterisation of the bovine alpha S1 casein gene C-allele based on a Mae III polymorphism. *Anim. Genet.* 24, 74
- Lee H., Morse S., Olsvik Q., 1997. *Nucleic Acid Amplification Technologies.* Biotechniques Books. Eaton Publishing, pp. 54-55
- Lewin B., 1997. *Genes VI.* Oxford University Press, Oxford
- Lien S., Gomez-Raya L., Steine T., Fimland E., Rogne S., 1995. Associations between casein haplotypes and milk yield traits. *J. Dairy Sci.* 78, 2047-2056
- Lipkin E., Shalom A., Khatib H., Soller M., Friedmann A., 1993. Milk as a source of deoxyribonucleic acid and as a substrate for the polymerase chain reaction. *J. Dairy Sci.* 76, 2025-2032

- Lum L.S., Dovč P., Medrano F.J., 1997. Polymorphism in bovine β -lactoglobulin promoter and differences in the binding affinity of activator protein-2 transcription factor. *J. Dairy Sci.* 80, 1389-1397
- Orita M., Iwahara H., Kanazawa H., Hayashi K., Sekiya T., 1989. Detections of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Nat. Acad. Sci. USA* 86, 2766-2770
- Perez M.J., Leroux CH., Bonastre A.S., Martin P., 1994. Occurrence of a LINE sequence in the 3' UTR of the goat alpha S1 casein E encoding allele associated with reduced protein synthesis level. *Gene* 147, 179-187
- Papavassiliou A., 1997. *Transcription Factors in Eucariyotes*. Springer-Verlag, Heidelberg (Germany)
- Rijnkels M., Kooiman P.M., de Boer H.A., Pieper F.R., 1997. Organisation of the bovine casein gene locus. *Mamm. Genome* 8, 148-152
- Sheffield V.C., Beck J.S., Kwitek A.E., Sandstrom D.W., Stone E.M., 1993. The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions. *Genomics* 16, 325-332
- Schild T.A., Wagner V., Geldermann H., 1994. Variants within the 5'-flanking regions of bovine milk protein genes: k-casein encoding gene. *Theor. Appl. Genet.* 89, 116-120
- Tadashi H., Buzard G.S., Calvert L.J., Weghorst C.M., 1993. "Cold SSCP": a simple, rapid and non-radioactive method for optimized single-strand conformation polymorphism analyses. *Nucl. Acid Res.* 21, 3637-3642
- Velmala R., Mantysaari E.A., Maki-Tanila A., 1995. Casein haplotypes and their association with milk production traits in the Finnish Ayrshire cattle. *Anim. Genet.* 26, 419-425
- Wagner V.A., Schild T.A., Geldermann H., 1994. DNA variants within the 5'-flanking region of milk-protein-encoding genes. II. The β -lactoglobulin-encoding gene. *Theor. Appl. Genet.* 89, 121-126

STRESZCZENIE

Polimorfizm SSCP w obrębie promotora genu bydłowej alfa S1 kazeiny

Opisano wykrycie nowego polimorfizmu SSCP (single stranded conformation polymorphism – polimorfizm konformacji pojedynczych łańcuchów) w obrębie promotora genu bydłowej alfa S1 kazeiny. Za pomocą metody PCR namnożono fragment promotora genu alfa S1 kazeiny liczącego 218 par zasad, a następnie poddano go elektroforezie umożliwiającej wykrycie polimorfizmu konformacji pojedynczych łańcuchów DNA. U losowo wybranych 118 krów i 124 buhajów rasy czarno-białej stwierdzono występowanie dwóch wzorców SSCP, nazwanych R2 i R3 o frekwencji, odpowiednio 0,05 i 0,95.

Wykryty polimorfizm może być użyteczny w poszukiwaniu związków między różnymi wzorcami SSCP, a ekspresją genu alfa S1 kazeiny, szczególnie procentową zawartością białek mleka oraz wydajnością białka w mleku.