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Heterologous Introns Enhanced Expression of Human Lactoferrin cDNA in Mouse Mammary Epithelial Cells

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Abstract: The expression of a recombinant human lactoferrin is reported in mouse HC11 mammary epithelial cells. Expression of human lactoferrin (hLF) was achieved by placing its cDNA under the control of the bovine β-casein gene. To improve the hLF expression level in a cell culture system, two artificial introns were also introduced to construct expression vectors. One intron was a hybrid-splice signal consisting of bovine β-casein intron 1 and rabbit β-globin intron II. The other intron was a DNA fragment spanning intron 8 of the bovine β-casein gene. The hybrid intron moderately elevated hLF expression, whereas intron 8 alone did not express any detectable amount of hLF as judged by Northern and Western blot analyses. When the two introns were used together they contributed to a synergistic elevation of hLF expression. These data indicate that artificial introns on both sides of the hLF cDNA were necessary to increase expression of cDNA.

Key words: Bovine β-casein promoter, human lactoferrin, hybrid intron.

The name lactoferrin originally derives from the molecule's identification as the protein in milk associated with iron (Lönnerdal *et al.*, 1976). It is a member of a family of transferrins which also includes transferrin and ovotransferrin (Aisen and Listowsky, 1980). The transferrins are widely distributed in extracellular fluids of vertebrates. Each is composed of a single polypeptide chain (approximately 700 amino acids) weighing approximately 80 kDa, and has two iron binding sites located on the N- and C-terminal halves, respectively (Metz-Boutigue *et al.*, 1984). Human lactoferrin (hLF) is abundant in secretions, such as milk, seminal fluid, tears, sweat, nasal, and genital secretions (Masson *et al.*, 1966). The affinity of lactoferrin for iron is high (Kapp $\sim 10^{20}$). This affinity, coupled with its ability to retain iron over a broad pH range, imparts important biological properties, including transport of iron through the stomach to the small intestine for release to brush border cells and sequestering of iron, thus making it unavailable for support of microbial growth (Cox *et al.*, 1979; Hu *et al.*, 1990). This antibiotic effect can also be exerted through the mechanism of direct binding to the outer membrane of Gram-negative bacteria (Ellison *et al.*, 1988).

The levels of lactoferrin in human colostrum can reach up to 6 g/l and can decrease to 1 g/l in mature milk (Lönnerdal *et al.*, 1976). The levels of lactoferrin in bovine milk are lower ranging from 0.02 to 0.2 g/l (Masson and Heremans, 1971). It has been suggested that these differences may account at least in part for the decreased susceptibility of breast fed infants to iron deficiency anemia and microbial diarrhea relative to infants fed on commercial formula based on bovine milk.

A few species have been employed as hosts for production of hLF. A recombinant vector containing the hLF cDNA sequence was introduced into baby hamster kidney cells in culture (Stowell *et al.*, 1991). hLF was expressed under the metallothionein promoter by Zn^{2+} induction. hLF expression in the filamentous fungi *Aspergillus oryzae* has also been described (Ward *et al.*, 1992). In this system hLF was expressed and secreted into the growth medium at levels up to 25 mg/l. Transgenic mice were generated producing hLF in their mammary glands (Platenburg *et al.*, 1994). Regulatory sequences from the bovine αS1-casein gene were used to target hLF expression in mammary glands. However, the expression level was relatively low (0.1~36 μg/ml).

To direct a high level expression of hLF to mouse and cow mammary gland, three expression vectors have been developed by combining the regulatory regions of bovine β-casein and the coding region of hLF.

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Intron sequences were also used since they often enhance the efficiency of expression (Palmiter *et al.*, 1991). In this paper hLF expression level of the three vectors is compared using HC11 cells which were originated from a murine mammary epithelial cell.

Materials and Methods

Construction of bovine β-casein/hLF fusion genes

The bovine β-casein gene was isolated from a library of bovine genomic DNA fragments. The cloned DNA contained 13 kb of the 5′ flanking region as well as 3.5 kb of the 3′ flanking region. The restriction map of the transcribed region and its partial nucleotide sequences agreed well with published data (Bonsing *et al.*, 1988).

Expression of human lactoferrin (hLF) was achieved by placing its cDNA under the control of the bovine β-casein gene. The first non-coding exon and its 2 kb long 5′-flanking region as well as 170 bp of the first intron of the bovine β-casein gene were isolated from the cloned DNA by PCR amplification. A hybrid splicing signal sequence comprising the 3′-splice site of rabbit β-globin intron II was also prepared by PCR. *Bam*HI and *Sac*II sites were introduced into the ends of the PCR primers. A synthetic DNA linker containing the bovine β-casein signal sequence and the first two amino acids of hLF was ligated to the *Eag*I-*Eco*RI digested hLF coding sequence. Another synthetic DNA linker containing the stop codon of hLF was ligated downstream of the hLF cDNA. A DNA fragment spanning exons 8 and 9 of the bovine β-casein gene was placed downstream of the hLF cDNA followed by the SV40 polyadenylation signal. The resulting vector was named pBL1 (Fig. 1A). pBL1 was then digested with *Not*I and *Sfi*I, and the larger fragment was electroeluted, made blunt-ended, and self-ligated to construct pBL2 (Fig. 1B). pBL3 containing the hLF signal sequence instead of the bovine β-casein signal sequence was constructed as follows (Fig. 1C): 2 kb of the bovine β-casein promoter and the hLF cDNA were amplified by PCR, then digested with *Sac*I-*Sac*II and *Sac*II-*Not*I, respectively, and replaced the *Sac*I-*Not*I fragment of pBL1.

Transfection of HC11 cells

HC11 mammary epithelial cells were grown in RPMI 1640 medium (GibcoBRL) supplemented with 10% heat-inactivated fetal calf serum (GibcoBRL), 5 μg/ml of insulin (Sigma), 10 ng/ml of epidermal growth factor (Sigma), and 50 μg/ml of gentamicin (Sigma). The cells were transfected with 10 μg of bovine β-casein/hLF expression plasmid and 1 μg of plasmid pSV2neo by the calcium phosphate precipitation method (Graham and van der Eb., 1973). After selection with G418 (Sigma), several hundred resistant colonies were pooled, expanded, and used for hormone induction. Hormone induction was performed by the addition of 0.1 μM dexamethasone (Sigma) and 5 μg/ml of ovine prolactin (Sigma) with insulin but without epidermal growth factor (Sigma).

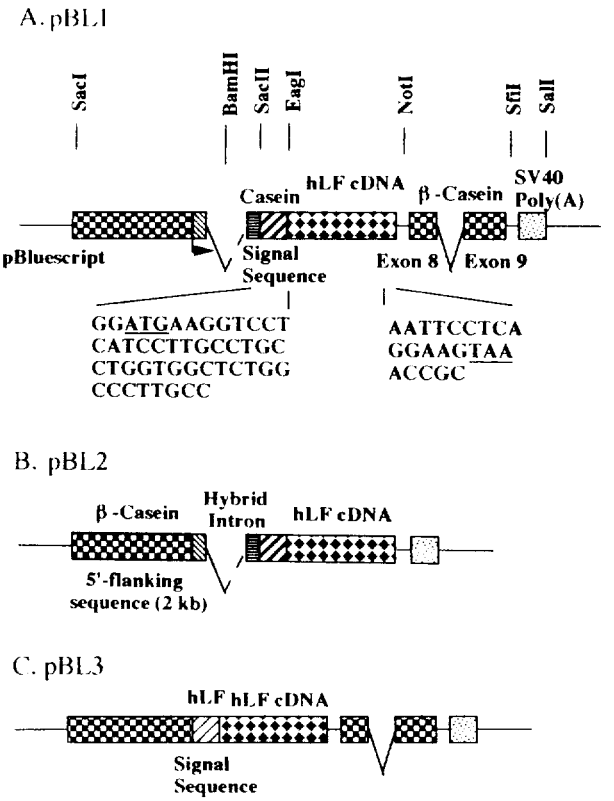

Fig. 1. Schematic diagram of bovine β-casein/hLF expression vectors. (A) components for the construction of pBL1 vector-5′-flanking sequence, exon 1, signal sequence, and exon 8 and 9 of bovine β-casein gene, intron II of rabbit β-globin gene, coding region of human lactoferrin cDNA, and poly(A) signal of SV40 DNA-are indicated. The sequences spanning the β-casein/hLF junctions are shown. The transcriptional initiation site (→) and the translational start site (ATG) of bovine β-casein gene, and the translational stop codon (TAA) of hLF are indicated. The scheme to construct the vectors is described in Materials and Methods. (B) pBL2 is constructed by *Not*I and *Sfi*I digestion of pBL1 and re-ligation of the larger fragment. (C) pBL3 is same as pBL1 except that it has the signal sequence of hLF and that it does not have the 5′ intron.

Northern blot analysis and RT-PCR

Total RNA from cultured cells was isolated as described by Sambrook *et al.* (1989). For Northern blotting, RNA was separated on a 1.0% agarose-formaldehyde gel and transferred to a nylon membrane (Boehringer Mannheim). Blots were probed with a 1.2 kb *Sma*I-*Eco*RI restriction fragment of hLF cDNA labeled by

the nick translation method.

For RT-PCR, first-strand cDNA was synthesized from 30 μg of total RNA using AMV reverse transcriptase (Promega). Two μl of an aliquot was used for PCR. Primers 1 (5'-GGGATTCAGCTCCTCCTTCA-3') and 2 (5'-CCACCGCGGTATAGTGAGTCGTATTA-3') were used to check the splicing of the 5' intron. Primers 3 (5'-AATTCCTCAGGAAGTAAAACCGC-3') and 4 (5'-CCCGGCCTTGAAGGCCTATGCTAATGTTGAAT-3') were used to check the splicing of the 3' intron.

Western blot analysis

To detect hLF in the culture medium, 500 μl samples were concentrated using an ultracentrifuge filter (Xpertex, P.J. Cobert Associates, Inc.). Protein pellets were resuspended in electrophoresis sample buffer (2% SDS, 10% glycerol, 0.08 M Tris-HCl, pH 6.8, 2 mM EDTA, 0.1 M DTT, 0.01% bromophenol blue) and denatured at 95℃ for 10 min before loading onto a 8% denaturing polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Polyclonal rabbit anti-hLF antiserum (diluted 1 : 1,000 in 1% BSA) prepared in this laboratory was used to detect hLF. Bound antibody was detected by addition of alkaline phoshatase-conjugated goat anti-rabbit IgG (Bio-Rad).

Results

Construction of hLF expression vectors

The 5'-flanking region, exon 1, and exon 8 and 9 regions of the bovine β-casein gene cloned in this study did not show any differences when compared with published sequences (Bonsing *et al.*, 1988). The construction schemes of the plasmids are outlined in Fig. 1. In pBL1 and 2, intron II of the rabbit β-globin gene (van der Berg *et al.*, 1978) was used as a hybrid splice signal. Rabbit β-globin gene intron II facilitates efficient splicing of expressed transcripts in the pSG5 eukaryotic expression vector (Stratagene). It was confirmed by sequencing analysis that the exon region of the rabbit β-globin gene had no translational start codon (ATG). Translation of the pBL1 and pBL2 vectors, therefore, has to start at the first exon of the bovine β-casein gene as it does in the native β-casein gene.

pBL2 was designed to detect the effect of splicing at the 3' side of hLF cDNA on the expression level. To analyze the effect of the hybrid splice signal at the 5' side of hLF cDNA, the sequence was removed from pBL1 and the hLF signal sequence was used to replace the bovine β-casein signal sequence.

Expression of bovine β-casein/hLF fusion genes in HC11 cells

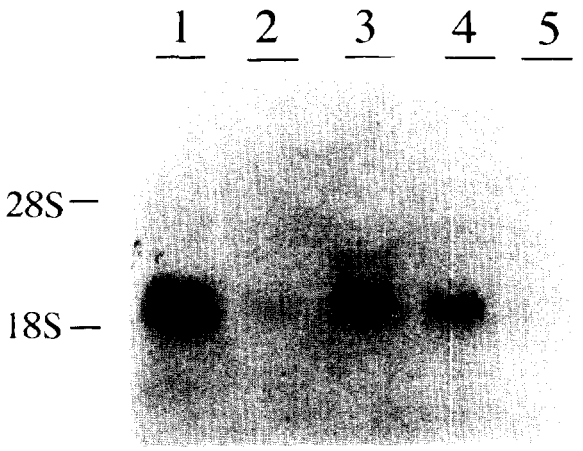

Fig. 2. Comparison of hLF RNA levels in HC11 cells stably transfected with hLF expression vectors. An autoradiogram of a Northern hybridization analysis of total RNA (30 μg per lane) from transfected HC11 cells hybridized with a ^{32}P-labeled hLF cDNA (1.2 kb *Sma*I-*Eco*RI fragment) is shown. HC11 cells were transfected with hLF expression vector drived by mouse mammary tumor virus (MMTV) promoter (lane 1); pCAT-Basic Vector (lane 2, Promega); pBL1 (lane 3); pBL2 (lane 4); pBL3 (lane 5).

Northern blot analysis was performed to compare the expression levels of three hLF expression vectors. Another hLF expression vector was constructed as a control using a mouse mammary tumor virus promoter which is known to drive a high expression level of foreign proteins in animal cell culture systems. These plasmids were co-transfected with pSV2neo to confer antibiotic resistance. After G418 selection, at least 100 colonies were pooled and cultured. All transfected cell pools contained a similar number of recombinant hLF genes, as determined by Southern blot hybridization of genomic DNA (data not shown). Northern hybridization of the total RNAs with hLF cDNA as a probe detected a single message species of the same size (approximately 2.5 kb, Fig. 2). This probe also cross-hybridized with endogenous mouse lactoferrin RNA from the HC11 cells transfected with pCAT-Basic vector, but the signal was weak (Fig. 2, lane 2). Of the three β-casein/hLF expression vectors, pBL1 showed the highest level of transcript (Fig. 2, lane 3) and pBL2 showed an intermediate level (Fig. 2, lane 4). However, pBL3 did not express any transcript (Fig. 2, lane 5).

To confirm the correct splicing of the transcripts, RT-PCR was performed using specific sets of primers (Fig. 3). PCR using the pBL1 vector as a template DNA was also done to indicate the size of the unspliced forms (Fig. 3, lanes 7 and 8). When the sizes were compared, PCR products from the RNA of transfected HC11 cells were smaller than products from the pBL1 vector itself by the size of the spliced out DNA seg-

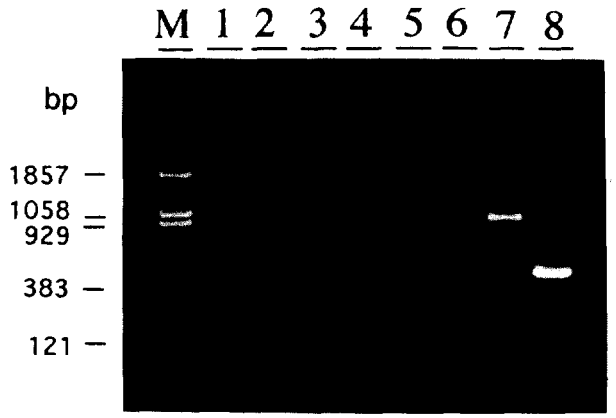

Fig. 3. RT-PCR analysis of total RNAs isolated from stably transfected HC11 cells. RT-PCR was performed from total RNAs isolated from HC11 cells stably transfected with pCAT-Basic Vector (lanes 1 and 2); pBL1 (lanes 3 and 4); pBL2 (lane 5); pBL3 (lane 6). Lanes 7 and 8 are PCR products from pBL1 DNA itself. Primer 1 (5'-GGGATTCAGCTCCTCCTTCA-3') and 2 (5'-CCACCGCGGTATAGTGAGTCGTATTA-3') were used for PCR of lanes 1, 3, 5, and 7. Primers 3 (5'-AATTCCTCAGGAAGTAAAACCGC-3') and 4 (5'-CCCGGCCTTGAAGGCCTATGCTAATGTTGAAT-3') were used for PCR of lanes 2, 4, 6, and 8. Lane M is a pBR322 DNA molecular weight marker digested with *Bst*NI.

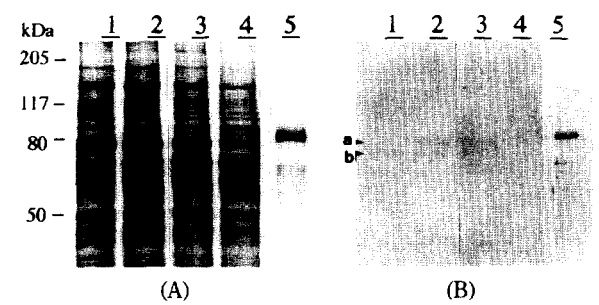

Fig. 4. SDS-polyacrylamide gel electrophoresis (A) and Western blot analysis (B) of proteins secreted into growth media of HC11 transfectants. Lanes: 1, HC11 cells stably transfected with pCAT-Basic Vector; pBL1 (lane 2); pBL2 (lane 3); pBL3 (lane 4). Lane 5 is 1 μg of human lactoferrin (Sigma). Band a is 80 kDa recombinant hLF and band b is a 75 kDa protein.

ment. This result indicates that hLF RNA was expressed and spliced correctly in the HC11 cells. The larger fragments appearing in lanes 4 and 6 of Fig. 3 are unspliced forms of the 3′ intron in the transcript.

Secretion of hLF from HC11 cells transfected with bovine β-casein/hLF fusion genes

To test whether the expressed hLF was successfully secreted into the growth medium, the proteins in the medium were isolated. A polyclonal antibody raised against purified human lactoferrin (Sigma) recognized by Western blotting, two polypeptides of which size were approximately 80 kDa (Fig. 4, band a) and 75 kDa (Fig. 4, band b). The smaller band appeared on every sample including HC11 cells transfected with pCAT-Basic vector. Therefore, it seems that this band is due to non-specific binding of the polyclonal antibody against a serum protein. In HC11 cells transfected with pBL1 or pBL2, an 80 kDa polypeptide co-migrating with the purified human lactoferrin appeared. However, from the culture media of HC11 cells transfected with pBL3, no detectable amount of hLF was found, which was expected after Northern blot analysis. The concentrations of hLF in the culture media were determined by an enzyme-linked immunosorbent assay (ELISA). HC11 cells transfected with pBL1 produced a higher level of hLF polypeptide (7.5~680 ng/ml) than pBL2 (3.0~120 ng/ml), as did in RNA level.

Discussion

Regulatory elements of eukaryotic gene transcription comprise the promoter element, intron sequences, and mRNA polyadenylation signal. In this study three different DNA constructs were made in order to promote the expression of human lactoferrin in the epithelial cell line of mouse mammary gland. All fusion DNA constructs contained the 2 kb 5′ flanking region of the bovine β-casein gene, hLF cDNA, and the SV40 poly(A) signal. The sequence elements responsible for rat and mouse β-casein gene expression have been studied with transgenic mice (Lee *et al.*, 1988; Lee *et al.*, 1989), by transfection of primary mammary epithelial cells (Yoshimura and Oka, 1990) and in a mammary cell line (Doppler *et al.*, 1990; Doppler *et al.*, 1989). These studies have suggested that approximately 500 bp of the 5′-flanking sequence of the mouse and rat β-casein gene is sufficient for tissue-specific and hormonally induced expression. Especially in the region from −305 to −15, several stretches of nucleotides are highly conserved in all calcium sensitive caseins sequenced, including bovine β-casein (Groenen *et al.*, 1992). The 2 kb of the 5′-flanking sequence of bovine β-casein used in this study was therefore expected to properly express hLF in HC11 cells.

In addition to the 5′ expression regulation sequences, the expression vectors developed in this study had recombinant intervening sequences which interrupt the transcribed but untranslated 5′ and/or 3′ region of the gene. Although many cDNAs are efficiently expressed from vectors lacking splicing signals, introns are normally built into expression vectors since they often enhance the efficiency of expression. There appears to be a strong requirement for introns for expression of foreign genes in transgenic mice (Palmiter *et al.*, 1991).

The 5′ intervening sequence used in this study contained a hybrid intervening sequence. Such hybrid inter-

vening sequences contain a 5′ RNA splice signal and a 3′ RNA splice signal from intervening sequences of different sources. Particularly preferred are 3′ splice signal sequences containing portions of the sequence which include immunoglobulins and T-cell antigen receptors as well as a repertoire of the major histocompatibility complex (MHC) genes (Wieringa *et al.*, 1983).

A 3′ splice signal sequence of rabbit β-globin intron II was used in this study. When 2 kb of the 5′ expression regulation sequence from the β-casein gene was used with a β-casein/β-globin hybrid intervening sequence, hLF expression was promoted (in the case of pBL 1 and pBL2 in Fig. 2). For a high expression level it is preferable to use a 3′ intron as well as a 5′ intron because pBL1 (which has both introns) showed a higher level of hLF RNA and protein than pBL2 (which has only the 5′ intron). pBL3 having no introns did not show any detectable amount of hLF expression by Northern blot or Western blot analyses. These results can be explained as follows. First, the 5′ intron is critical for hLF expression, so only the vectors which had the site (pBL1 and pBL2) could express hLF. Second, the hLF signal sequence in pBL3 was not processed properly to secrete hLF into the culture medium. However, the absence of the transcript from pBL3 indicates that its lower expression was due to absence of the 5′ intron. The hLF expression level in the culture medium of HC11 cells transfected with pBL1 was 7.5 to 680 ng/ml, as determined by an ELISA. Considering that the β-casein promoter and the HC11 cells are from heterologous species, the expression level of hLF may be increased after further study. The use of a bovine mammary gland epithelial cell line which can differentiate in culture and subsequently express foreign genes, such as MAC-T cell line (Huynh *et al.*, 1991) might provide a higher expression level of hLF.

The vectors developed in this study, especially pBL1, induced hLF expression in the HC11 cells and can be used as expression vectors to produce foreign proteins in transgenic animals.

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