—Research Note—

A Comparative Study of the 5' Flanking Sequences of the α S1- and β -Casein Genes: Human and Other Mammalian Species

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Abstract. The 5' flanking region (1.2 kb) and the first exon and intron (1.5 kb) of the human α S1-casein (*CSN1*) gene, and the 5' flanking sequence (2.0 kb) of the human β -casein (*CSN2*) gene were cloned and sequenced. Strong sequence homology was observed between the 5' flanking regions of the casein genes of humans and other mammals. Several transcriptional regulatory elements (GRE, C/EBP, YY1, STAT5) identified in the rat β -casein gene were conserved in both human casein genes.

Key words: Milk protein genes, Yeast artificial chromosome, Bubble-vector PCR, Sequence homology, Promoter.

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Caseins are the major components of mammalian milk and are the primary source of amino acids, calcium and phosphate for the suckling infant. Caseins consist of so called calcium sensitive caseins (α S1-, α S2-, β -) and κ -casein. Calcium sensitive caseins are phosphorylated and bind calcium within micelles for delivery to the infant. κ -Casein is thought to stabilize these micelles.

The casein genes of several mammalian species have already been cloned and sequenced [1]. The promoters of these genes have been analyzed in detail to study their tissue-, stage-, sex- and hormone-specific expression [1–3].

The cDNA sequences of human α S1-, β - and κ -casein have been reported [4–6]. No sequence data of human α S1-casein (*CSN1*) gene is available. The human β -casein (*CSN2*) gene has been isolated, but its 5' flanking sequence was not included [7]. Recently, we isolated five yeast artificial chromosome

(YAC) clones containing the entire human casein gene family and found that the casein genes appear to be clustered within 300 kb [8]. The casein gene family was localized to chromosome 4q21.1 by fluorescence *in situ* hybridization analysis. Chromosomal fragmentation analysis of the human casein genes indicated that distance between the *CSN1* and *CSN2* genes is approximately 10 kb [8].

In order to characterize the 5' flanking sequences of casein genes, we isolated, sequenced and compared these regions of the human *CSN1* and *CSN2* genes with casein genes of other mammalian species.

Materials and Methods

PCR cloning and sequence strategy is illustrated in Fig. 1. YAC clone yhCEPH 748F3 [8, 9] (Research Genetics, AL, USA) was used to isolate *CSN1* and *CSN2* genes in the present study. The bubble-vector PCR method was employed to isolate the 5'

Fig. 1. Cloning and sequencing strategy of the 5' flanking regions of *CSN1* (A) and *CSN2* (B) genes. The boxes represent exons. PCR primers (short arrows) used for cloning of casein gene fragments are shown. H, *HaeIII*; P, *PvuII*; R, *RsaI*; S, *SphI*.

Table 1. Oligonucleotide sequences of PCR primers

Primer	Sequences
α S1-1	TCAACACAACCTTGCTTCTCT
α S1-2	AGGCCTGGCAAGAGCAACAG
α S1-10	CTGTATGTAAACGCAGTACC
α S1-12	AGCCCAAGTCTGGAGAGAAG
β CA-6	AAGCTACTTTGAAATACATGG
CSN2EX1B	CTACGTTCTTCTATAAGCTC
G	TCGCTAAGAGCATGCTTGCCAATGCTAAGC
H	CCTCTCCTGTGCTAAGAGCATGCTTGCCA

The locations and orientations of primers are indicated in Fig. 1.

flanking regions of the *CSN1* and *CSN2* genes [10] by using α S1-12 and α S1-10 as primers for the *CSN1* gene, and CSN2EX1B and β CA-6 as primers for the *CSN2* gene. The nucleotide sequences of PCR primers are listed in Table 1. The locations and orientations of primers are indicated in Fig. 1. The DNA of yhCEPH 748F3 was digested with *PvuII*, and vectorette was ligated [10]. The ligated products were amplified by PCR using primers, G and α S1-10 for the *CSN1* gene, and G and CSN2EX1B for the *CSN2* gene (Table 1 and Fig. 1). The PCR products were further amplified through nested primers, H and α S1-12 for the *CSN1* gene, and H

and β CA-6 for the *CSN2* gene. The sizes of the amplified products representing the 5' flanking regions were 1.2 kb for the *CSN1* gene and 2.0 kb for the *CSN2* gene. In addition, the first exon to the second exon (1.5 kb) of the human *CSN1* gene was amplified by the PCR method using the primer pair α S1-1 and α S1-2, that were designed from exon 1 and 2, respectively, by comparisons with human *CSN1* cDNA and the bovine *CSN1* gene [4,11]. These PCR products were ligated to pT7Blue (Novagen, WI, USA). The cloned fragments were digested with *RsaI* or *HaeIII*, subcloned to pBlue-script (Stratagene, CA, USA) and sequenced using an ABI 373 DNA sequencer (Perkin Elmer, CT, USA).

Results and Discussion

The nucleotide sequences of the human *CSN1* and *CSN2* genes were shown in Figs. 2 and 3, respectively. The potential transcription start site of the human *CSN1* gene was determined from that of the bovine *CSN1* gene [11]. Typical TATA boxes were located at positions -33 and -30 upstream of the transcription start sites of the human *CSN1*

		ctgatgcttg	tctcagcctc	ttaaataaat	cacagtgaat	-1141
aggaaacact	agtaagttgt	tgaattgat	gtgatgtagt	cagtaaggaa	gacactaact	-1081
aaagagaact	ttaatatgga	aaagtacatc	tcttctgata	cccaatctga	aattaaccag	-1021
tctgaaatgt	gtgtaaccc	agaataatgc	ccttttcata	tgactgtgaa	aaggctgtaa	-961
atatcattac	tattttttta	tttcttaaaa	tatatagtat	gattcttttc	ctgtaaaaaa	-901
gcaaagtcgc	agtcaacaac	aattttttaa	aacctctct	attacagata	ttttcctcag	-841
gatatagtat	ttttctacat	tgtataatga	tagttaacat	aaaggaacag	agtctgggat	-781
aggagagagg	catttttcatt	tctttccttt	atttactttt	aaaaaacagg	aaatcagagg	-721
tatggcataa	ttttccaaaa	tctttttaa	ttaaataat	tgaataaata	ttacttctaa	-661
ttagtcaatg	ttataatttg	ttaaataaat	actatcacia	ccaatatgcc	taaaagaaa	-601
caaacaaaaa	attgtctgct	gtagcttaaa	tatcaccaaa	acacatagga	cagttggata	-541
tccttctctg	aaaaaaagat	cagagcatat	acagtataag	ttaatccata	aagcagatag	-481
tcacaataaa	taataagagga	caagctgcat	tttaaaaggt	aaataaatga	tgaattcttt	-421
taatggcctg	gtacactgat	agaaattgga	acgtttctaa	gacctattt	tgtcccagg	-361
atttccatta	taggaattga	atttttcaaa	agatatacag	agaattttat	taatatgttc	-301
ctgtcaaat	atcccttcaa	cataaaaatca	tgtttttata	atagccataa	atttggaaat	-241
ttttgtattt	taatttgcaa	taatgccact	ttatttcttg	tgtaatctgt	tatcatagca	-181
tgaatcactc	cctgttga	atttctcagaa	tttctcggga	aaggaattgg	acagaaaatt	-121
agtttcctat	ttgggagaat	tcttagaatt	taaataaacc	tattgggtga	actgaacca	-61
caaaattagc	attttcta	taccacattt	aaatagcgta	aagcaaagag	atcgccatc	-1
ATCTTGCTCA	TCAACACAAC	TTGCTTCTCT	CCAGACTTGG	GCTTAAGta	ctgctttac	60
atacagcaaa	attgctatca	ttttacatta	tctaattctct	ttctcccact	gtttactaag	120
tatatattgta	ccatttccat	atgtttgcaa	atattaaaa	ttcttatgat	tttctggcac	180
cttaaaaaatc	cttttgcaaa	ttctgatacc	attttacagc	tttaggtgat	gaatcaatac	240
agttttttaa	aaataccaaa	tcattttttg	ataattttgt	aactaaaata	tattttttaa	300
gttgatacat	aatttcagta	gaggcttggg	gtaagcta	ttaaattatt	tttaccta	360
tctatgagaa	gttggttatgc	aaataatttc	ttatttttgt	aattaccata	gcaaaaacca	420
tttctagcag	aatagttcaa	ggccattttt	gtttcatgtc	atttgggcaa	caaaattaac	480
tctacaatgt	gaatatctat	tatagataat	tagctattga	tggctacttt	gtaatgatta	540
ttaaatttag	atattcagaa	agacacaaat	agtaaattta	tttgggttca	aaaatatgcc	600
ttttttatta	acacatttag	attctaaaat	ttgtagactg	aagcaatata	tataaatgtc	660
agcatttgta	tgttgattaa	gggtggaaca	aatagtatgt	gtgacctgat	tttttttcta	720
atgattttctc	tggaaacat	tttccagta	catgtggatg	tccatatttc	ggggttcttg	780
caatttaaatg	aaactctgga	aatcaaatat	aagacatgtc	aatattttga	ttctatccaa	840
ttcaatcatc	tttatatatt	tatgtcatga	atattccaat	gtagcaaaac	acgaagttat	900
ggctagattc	tttattttaa	aattgaaaga	gatcaaatagc	ttttacataa	cgtgcagaca	960
ggattttttca	tgaagactct	aaaagaaaag	aatgctctca	aatagattta	aaagagaagc	1020
tcatttttcaa	aacttttcaa	taaaatttaa	ttccagttct	cagtggattt	aactttattg	1080
cccacttttag	aaaaatctgat	ttgtcatcta	ggaggaaaag	tgttttcttt	tttcaattgt	1140
attttcaaat	tagcacttct	ttatttctct	aagccatatt	attacattaa	gaacaaaatg	1200
ttatgtttta	aagtgttagt	gaagaaaaat	gtttgtaaga	aattagtatt	tctaataaat	1260
ataagaacta	agaaaaataa	ctagaatggg	aaacattgat	attgagtagt	aaagttttta	1320
aaatctttaag	tttataaaaa	ttcacattac	agctcctcaa	atcagcctac	ttttatcata	1380
atttgcctct	ttgttgaaaa	atcaagaatt	tttttcagga	aacaaattta	acgtaataat	1440
atctaactct	ttcttttttg	ttctcttaca	tagGCTCTGA	TAACCATGAG	GCTTCTCATT	1500
				M R	L L I	
CTCACCTGTC	TTGTGGCTGT	TGCTCTTGCC	AGGCCCT			1536
L T C	L V A V	A R P	K L			

Fig. 2. Nucleotide sequence of the 5' flanking region and the first exon and intron of the human *CSN1* gene. Exon sequences are shown in capital letters. The deduced amino acid sequence is also shown. A TATA box is underlined. The putative transcription start site was assigned the number +1. The nucleotide sequence data will appear in the DDBJ, EMBL and Genbank nucleotide sequence databases with the accession number D85424.

and *CSN2* genes, respectively. The human *CSN1* gene had a short exon 1, which had an untranslated region, and a 1.5 kb intron 1 followed by exon 2, which had an initial Met codon. These nucleotide structures were the same as the *CSN1* genes of other mammalian species. The exon-intron boundary of the human *CSN1* gene followed the GT/AG rule and was highly homologous with the consensus sequences [12].

Human *CSN1* and *CSN2* genes sequences characterized in this study show high homology with

those of other mammalian species, including cattle [11, 13], goats [14], mice [15], rabbits [16, 17], rats [18, 19] and sheep [20] and possess 60–71% and 64–73% homology with those of other mammalian species. Furthermore, the nucleotide sequences of the regions between positions -200 and -1 of human *CSN1* and *CSN2* genes are highly conserved with those of other mammalian casein genes. This region of the human *CSN1* gene shows over 80% homology with those of rabbit and bovine *CSN1* genes. The same region of the human *CSN2* gene

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ctgtg tagcatttca -1981
ctgttgacct cacttgcttc ctctagcaat gcaggatatac aaggagagga agtggattct -1921
gtactctctt attcttgata tcgcagggttt tttttttcct atttcctttt gctataccag -1861
ccctgtataa agaaaggata ctggaatttt gttttgtaca attttctttc tcacttgata -1801
ctctttccct agagaccttc acttactcct ttgaaggagg aaattacctt taaaaattat -1741
actcatgatt atttcttgag tatcacattt aattttaaac atctctctga caccttcaaa -1681
tttaccacgt acaagggttt actcagtggt tcatttctaa tttcagcctg gtttcatatt -1621
ttgattttata acatggctaa cttttcagtc actcaggcat aatatattaa ttcatttccc -1561
caatttttca catttctcgt atttttaatga gaagtttgct tttttattgc ctaagatgcc -1501
caggaccatt tccctacttg agatcatcat aatgtctgtc ttaggctact gcaataacat -1441
aacataacaa cttcctttac taggttacct gatctgtgtg cataaagtca tgaaatccaa -1381
gaaattttac aatccatttt tctttcaaat ctctaataata tttgcttcca ctgaattatg -1321
tcacagtgcg tgctcgacat tcacagtcct ccatgatctg attctatcaa acctctgcaa -1261
ttgtaacttg caatgtatgc cctctgaata tcctcaaact ggtaggttgc tctttcagtt -1201
cctgtgtttt cttcagccaa aatatctatc cccacttaac catctcgcaa aattctactc -1141
attctctaa gctcaactaa aagagcagtt ccttaatggc aagctcccct gccacacaca -1081
gcacaggag cctacttttc caattcaggg ataattaaat tgcacatatt tcaaccaaat -1021
gtacatttga tagataaatt ttataaaaaa ttatcagaag gttaaaacat gggctctgag -961
actcacatgt gatcaaacag aatcacatgt agtcagacag atctgagtc aactggtatc -901
tttgccatta acaatatggg tttagcgaag ttacttaacc ttttggcctc tctgttttct -841
caacctaaat atgaagagga gagctacctc atgatgttct tgtgcagatt atacaagata -781
atatctttgc ttagtgcttg gcaaaagggt agaactaagc aaaaaaaccc atcattaata -721
ttatcataat tatgcttgac ctgaaaagag ataatacaata ttaaaaggta aataaaactc -661
cgtgataact tgcaaaattt ttattacaga ttactgaag attttgccta taccagccac -601
ccagattccc tcttcaagtt attccattac agaggaaagt ttatttatct tgtgtacaaa -541
atatttgttt taactaaatt attttttttt ccaaatcaag tggccctggg agaaacagtt -481
tgccctacat aactaagcta ccatcatgga ctgtttatct tgcagtttc ttcattttcag -421
cataaagcac taacaactgc ttaggttcac atgaactgt ggctttgtg aagccctgtg -361
tatatctagc gttcacatag aattttccca caactgaaat ctcaaggccc cactaggccc -301
taaagagacc taattatgtc cataataact tggggacagg gccagagtat cactgtcctc -241
cagtcattgt cttttgttat tgaataattg catttctctg acaggtttct tttatcaact -181
tgtggattgt tcccttgcta taatgatgcc cagaatttct ggggatattg aataggcaga -121
aatcattttc taatcatgtg gacttcttgg aattaaagct cgctattgat cttatttcaa -61
atcacaaaat tagtgtgtca ttaaatatag tatataaaca gtcacagaag ctgatggacc -1
ATCATCCATC CAGCACTACC TTCACTTCTC TTCAATCTTG GAAAAACGgt aagaatttta 60
gatacaaat tggtgtatct tctcctaacc atgtatttca aagttagctt 109

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Fig. 3. Nucleotide sequence of the 5' flanking region of the human CSN2 gene. Exon sequences are shown in capital letters. A TATA box is underlined. The putative transcription start site was assigned the number +1. The nucleotide sequence data will appear in the DDBJ, EMBL and Genbank nucleotide sequence databases with the accession number D85380.

			1/2 GRE	C/EBP	YY1	STAT5	
rat	CSN2	-154	TGTC	CCCCAGAATT	TTCTTGGG	AAAG-AAAATAG-AAG-AAACCATTTTCTAATCATGTGAAC	TTCTTGGGAATT-AAG -81
mouse	CSN2	-157	...A...	T.....-C.....A...-.....C...AG.....G...			-83
rabbit	CSN1	-158	CTCT..T.....-.....G..G..CTG.AT.GA-..TTA...C...T.TGA.A...-.....A....T..A				-85
rabbit	CSN2	-157	..CT.....G.....T...GAGT...-..T.....A.....G..G.....G..A				-83
sheep	CSN2	-157	..CT.....G.....C..A.G.....G...-..TT.....C.....CAG.T...AA.....C..A				-82
goat	CSN2	-157	..CT.....G.....C..A.....G...-..TT.....C.....CAG.T...A.....C..A				-82
bovine	CSN1	-159	CTCT..TT.....G..G..CTG.AC.GAAC.TTG...C...TGTGA.-AG.A...A.....T..A				-84
bovine	CSN2	-161	..CT.....T.G.....C..A.....G...-..TT.....C.....CAG.T...A.....C..A				-87
human	CSN1	-162	AAATT.T.....C.....-G..T.G.AC..A...TT.G...CT..TTG.GAG.A...A....T..A				-87
human	CSN2	-157	..ATG.....G.....T.-TGT....GC...-..T.....G.....A...				-83

Fig. 4. Comparison of transcription factor binding region of casein genes among several mammalian species. Dots indicate nucleotides identical to the rat CSN2 gene sequence. The nucleotide sequences of these genes are aligned, and the nuclear protein binding sites identified are indicated above.

shows over 75% homology with those of cattle, goat, rabbit, rat and sheep. Nucleotide sequences of further upstream region show less homology.

The region between position -154 and -81 of the rat CSN2 gene has been reported as the binding region for several transcription factors, including glucocorticoid hormone responsive element (GRE),

CCAAT -enhancer binding protein (C/EBP), yin yang 1 (YY1) and signal transducers and activators of transcription 5 (STAT5) [3]. This region is considered to mediate hormonal and tissue-specific induction for the rat casein gene. The 5' flanking regions of the casein genes of other mammalian species were aligned with that of human CSN1 and

CSN2 genes (Fig. 4). The binding regions of GRE, C/EBP, YY1 and STAT5 were conserved among casein genes. STAT5 is thought to be crucial for prolactin regulation of rat CSN2 gene expression [21], while a zinc-finger transcription factor, yin yang 1 (YY1), acts as a repressor in casein gene expression [22]. CCAAT enhancer-binding proteins (C/EBPs) are members of a family of heat-stable transcription factors that contain a leucine zipper motif [23]. Interaction between the glucocorticoid receptor and C/EBP is thought to facilitate dissociation of YY1, thereby enabling the binding of prolactin-activated STAT5 [3].

In the bovine CSN2 gene, another transcription motif, bce1, is located in an upstream region between -1562 and -1613 and has been reported to mediate both hormonal and cell-substratum regulation on bovine CSN2 gene expression [24].

The region between -3442 and -3385 in the rabbit CSN1 gene has been reported to be responsible for prolactin induction of reporter genes [25].

The distal 5' flanking region of the bovine and rabbit gene also seems to contain the STAT5, C/

EBP and GRE binding sites. We did not find clear homology of the distal transcription factor binding regions in the 5' flanking region of the CSN1 and CSN2 genes. These distal elements may be located further upstream of the human CSN1 and CSN2 gene sequenced in the present studies.

The possible cis-acting elements were identified in the 5' flanking regions of the human CSN1 and CSN2 genes in the present study. Although that transcription factors such as YY1 and STAT5 potentially bind to the promoters of the human casein genes and regulate gene expression in the same fashion as the rat gene, the function of these elements in the regulation of transcriptions needs to be confirmed experimentally.

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