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Genomic organization and chromosomal localization of the human casein gene family

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Abstract Five yeast artificial chromosome (YAC) clones containing the human casein gene family were isolated and characterized to study the control mechanisms for the expression of these genes. Partial restriction analysis in conjunction with the chromosomal fragmentation method and fluorescence in situ hybridization (FISH) analysis were performed to construct a detailed physical map of the casein gene family and to determine the chromosomal localization of these genes. The isolated YAC clones 748F3, 750D11, 882G11, 886B3 and 960D2 were 1.2 Mb, 860 kb, 800 kb 1.5 Mb and 1.5 Mb in size, respectively. The clones 748F3, 882G11, 886B3 and 960D2 contained the entire casein gene family, while the κ-casein gene was absent in 750D11. The human αS1-, β- and κ-casein genes were found to be closely linked and arranged in the order αS1-β-κ. The distance between αS1 and β, and between αS1 and κ was approximately 10 and 300 kb, respectively. The β-casein gene was oriented in the opposite direction to the αS1- and κ-casein genes. The casein gene family was localized to chromosome 4q21.1 by FISH analysis.

Introduction

Caseins are the major components of human milk, and are the primary source of amino acids, calcium and phosphate for the suckling infant. The human caseins include αS1- and β-casein, which are the substrates for protein kinase and precipitate in the presence of calcium (so-called calcium-sensitive caseins), and κ-casein, which prevents the precipitation of the other caseins by calcium through micelle formation (Groenen and van der Poel 1994).

The casein genes of several other animal species have been cloned and sequenced. The promoters of these genes have been analyzed in detail to study their tissue-, stage-, sex- and hormone-specific expression (Mercier and Vilotte 1993; Groenen and van der Poel 1994). These casein genes have been mapped to the same chromosomal region and are thought to compose a single multigene family (Mercier and Vilotte 1993). In cattle, four casein genes, αS1, αS2, β and κ are closely physically linked within a region of 200 to 300 kb (Ferretti et al. 1990; Threadgill and Womack 1990).

In humans, αS1-, β- and κ-casein cDNAs have been reported and the β-casein gene has been isolated (Lonnerdal et al. 1990; Bergstrom et al. 1992; Hansson et al. 1994; Johnsen et al. 1995), but the organization of the complete casein gene family has not been reported. The localization of the β-casein gene has not been clearly resolved. Using human-hamster somatic cell hybrids, Menon et al. (1992) presented evidence that the human β-casein gene is on chromosome 4pter–q21. In contrast, Kost et al. (1994) performed in situ hybridization using a DNA fragment encoding β-casein and recorded a peak of grains over band p3 on chromosome 1.

Yeast *Saccharomyces cerevisiae* artificial chromosome (YAC) vectors have provided the means for isolating large DNAs of up to several megabases (Burke et al. 1987). We have isolated YACs containing the genes for human αS1-, β-, and κ-casein and used partial restriction endonuclease digestion and chromosomal fragmentation to determine the genomic arrangement of these genes. The casein genes were mapped on human chromosomes by fluorescence in situ hybridization (FISH).

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Materials and methods

Media and yeast strains

The yeast strains YPH925 and CGY2516, and the plasmid vector pBP103 were obtained from The American Type Culture Collection (Smith et al. 1990; Pavan et al. 1991; Spencer et al. 1994). Synthetic complete (SC), drop-out and 5′-fluoroorotic acid (5′-FOA) medium were prepared according to Strathern et al. (1993). CGY2516 was plated on an SC plate containing 60 mg/l canavanine, and canavanine-resistant colonies were selected.

Polymerase chain reaction (PCR)

The PCR was performed in a 25-μl volume containing 3 pmol of each of the primers, 0.25 U of *Taq* polymerase (Boehringer) and 200 nM each of dNTPs and 1 × accessory buffer (Boehringer) in a DNA thermal cycler (Perkin-Elmer Cetus) for 40 cycles of 30 s each at 94°, 55° and 72° C. Oligonucleotide primers for PCR listed in Table 1 were synthesized based upon the previously reported nucleotide sequence of the β-casein gene, and αS1- and κ-casein cDNAs (Bergstrom et al. 1992; Hansson et al. 1994; Johnsen et al. 1995).

YAC library screening and DNA preparation

Human YAC libraries (CEPH library A and B, Research Genetics) were screened by the PCR method (Green and Olson 1990) using the primers CSN2EX8A and CSN2EX8B (Table 1). Yeast strains were grown in YPD or SC-Ura-Trp medium. Yeast chromosomal DNA was prepared in agarose plugs (InCert agarose, FMC Bio-Products) by the lithium dodecyl sulfate method (Johnston 1993). For PCR analysis, a yeast chromosomal DNA plug was melted in ten volumes of sterilized water at 68° C for 10 min and 1 μl of this solution was used for 25 μl of PCR.

Table 1 Oligonucleotide sequences of polymerase chain reaction (PCR) primers

Primer	Sequence
αS1-1	TCAACACAACTTGCTTCTCT
αS1-2	AGGCCTGGCAAGAGCAACAG
αS1-3	CTTTCCAGCAGCTCAACCAA
αS1-4	CCACTGTAGCATGACGTTAT
αS1-5	TTTATTGTCTAAATTTCAGT
αS1-6	AAATCTCTGTCACTGCACAA
CSN2EX1A	AACAGTCACAGAAGCTGATG
CSN2EX1B	CTACGTTCCTTCTATAAGCTC
CSN2EX6A	ACTGTCTACACTAAGGGCAG
CSN2EX8A	TGAGACGAGACAGATAAGCT
CSN2EX8B	TTGAACTTCTGTGGTACTAG
BCSN1A	TAAGGATCCTGTCAAGCAGTGAGGAAT
BCSN1B	TATGGATCCTGAGTCACAGGGTAGATCTG
CSN10EX4AA	CTGAGTTAGGCCACATGC
KCSN1A	TAAGGATCCTTGCCAGCTCAACTACTG
KCSN1B	TAAGGATCCGGTTTCAGCAAGTCCTGCGT
KCSN2A	TGCCATGAGAATGATGAAAG
KCSN2B	CATAATATGTGCGAGGCACA
KCSN3B	GTTGGTGTAGGTTCAACAGT
KCSN4A	TCCTGCCACTGAACCAACGG
KCSN4B	CTGCGTTGTGTTCTTTGATA

Fluorescence in situ hybridization

The human chromosomal spread was prepared from the phytohemagglutinin-stimulated blood lymphocytes of a healthy donor after thymidine synchronization and bromodeoxyuridine incorporation by the method of Takahashi et al. (1991). Probe labeling and in situ hybridization were carried out according to Lichter et al. (1988). Fluorescence signals were imaged using a Zeiss Axioskop epifluorescence microscope equipped with a cooled charge-coupled device camera (Photometrics, PXL 1400) (Okumura et al. 1994).

Partial restriction analysis of YAC

DNA in an agarose plug was digested with various restriction enzymes including *Asc*I, *Csp*I, *Not*I, *Mlu*I, *Nru*I, *Pvu*I, *Sal*I, *Bss*HII and *Sfi*I at 0.01, 0.1 1 and 20 U for 1 h. Pulsed-field gel electrophoresis (PFGE) was carried out on CHEF-DRII (Bio-Rad) using 1% agarose (Bio-Rad) in 0.5 × TBE at 6 V/cm and 14° C for 24 h with a switch time of 60–120 s. DNA transfer and hybridization were performed according to Sambrook et al. (1989). DNA was transferred to nylon membrane Hybond N+ (Amersham). A 1.4-kb *Pvu*II-*Sal*I fragment and a 2.1-kb *Pvu*II-*Eco*RI fragment isolated from pBR322 were used as hybridization probes for the YAC right and left arms, respectively. The DNA probes for milk protein genes were prepared by the PCR method using a human mammary gland cDNA library (Clontech Laboratories) as a template. The primers used were αS1-1 and αS1-6 for αS1 casein (950 bp), BCSN1A and BCSN1B for β-casein (620 bp), and KCSN1A and KCSN1B for κ-casein (650 bp; Table 1). The DNA was labeled with [^{32}P]dCTP using the Rediprime labeling system (Amersham).

Fragmentation of YACs

YACs were transferred to *kar1*$^{-}$ strain YPH925, and canavanine-resistant CGY2516 successively according to Spencer et al.. (1994). CGY2516 was used for auxotrophic markers and copy number amplification (Smith et al. 1990) to purify YAC DNA from yeast chromosomes in our other experiments. Acentric fragmentation vectors for YACs containing milk protein genes were constructed as described below. Exon sequences of human αS1- and κ-casein genes were predicted from other mammalian sequences (Alexander et al. 1988; Johnsen et al. 1995). The casein YAC DNA was amplified by PCR with primer pairs αS1-3–αS1-4 (151 bp), CSN2EX6A-BCSN1B (326 bp) and CSN10EX4AA-KCSN3B (150 bp). The amplified products were cloned into pT7Blue (Novagen). The inserts were recovered from the plasmid by *Eco*RI-*Sal*I and *Bam*HI-*Xba*I digestion and ligated into the *Eco*RI-*Sal*I and *Bam*HI-*Xba*I sites of pBP103 (Pavan et al. 1991). This plasmid contained a telomere and a *HIS3* gene to enable construction of fragmentation vectors in both orientations. The resulting fragmentation vectors were denoted pBP-αS1R and pBP-αS1L for the αS1-casein gene, pBP-bR and pBP-bL for the β-casein gene, and pBP-kR and pBP-kL for the κ-casein gene.

The fragmentation vectors were digested with *Sal*I and transformation was carried out by the lithium acetate method (Gietz and Woods 1993). The transformants were plated on SC-His 5′-FOA plates. Colonies were transferred to SC-Trp-His plates and chromosomal DNA was prepared from the selected colonies. YAC length was determined by PFGE and Southern hybridization analyses. Absence of the flanking regions of the sequences used in chromosomal fragmentation was determined by the PCR method using the primers: αS1-1 and αS1-2 for 5′ αS1-casein, αS1-5 and αS1-6 for 3′ αS1-casein, CSN2EX1A and CSN2EX1B for 5′ β-casein, CSN2EX8A and CSN2EX8B for 3′ β-casein, KCSN2A and KCSN2B for 5′ κ-casein, and KCSN4A and KCSN4B for 3′ κ-casein (Table 1).

Results

Isolation of human YAC clones

YAC clones, 748F3 (1.2 Mb), 750D11 (860 kb), 882G11 (800 kb), 886B3 (1.5 Mb) and 960D2 (1.5 Mb), were isolated by screening human YAC libraries with PCR using primers for the β-casein gene (Fig. 3). By PCR and Southern blot analyses, clones 748F3, 882G11, 960D2 and 886B3 were found to contain the αS1- and κ-casein genes in addition to the β-casein gene, while clone 750D11 contained only the αS1- and β-casein genes (Fig. 3).

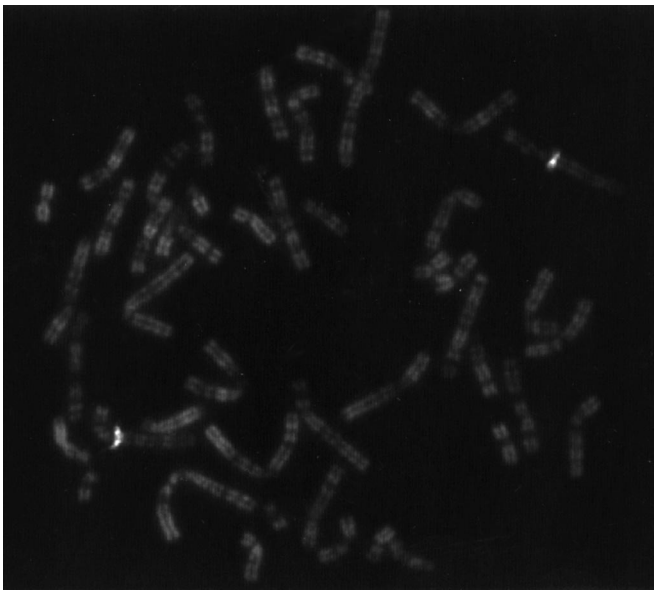


Fig. 1 Chromosomal localization of the human casein gene family by fluorescence in situ hybridization (FISH) using the yeast artificial chromosomal (YAG) clone 748F3 as a probe. Typical fluorescence dots were observed on chromosomal band 4q21.1

Chromosomal localization of isolated YACs.

A FISH analysis was carried out to determine the chromosomal localization of the casein genes using YAC clones 748F3 and 750D11. YAC DNA from 748F3 hybridized to band 21.1 of the long arm of chromosome 4 (Fig. 1). A FISH signal for 750D11 was detected on another chromosomal band, in addition to 4q21.1, indicating that 750D11 was chimeric.

Structure of YAC containing the casein gene family

DNA from clones 748F3 and 750D11 was partially digested with *Asc*I, *Csp*I, *Not*I, *Mlu*I, *Nru*I, *Pvu*I, *Sal*I, *Bss*HII and *Sfi*I, and 882G11 DNA was digested with *Not*I, *Pvu*I, *Sal*I, *Bss*HII and *Sfi*I. The digested YAC DNAs were separated by PFGE and transferred to nylon membranes, followed by Southern hybridization with YAC left and right arm, αS1-, β- and κ-casein probes (Fig. 3).

The locations and orientations of the casein genes in the YAC clones were analyzed by the chromosomal fragmentation method using casein gene exon sequences for homologous recombination and the presence of the flanking sequences was determined by the PCR method (Pavan

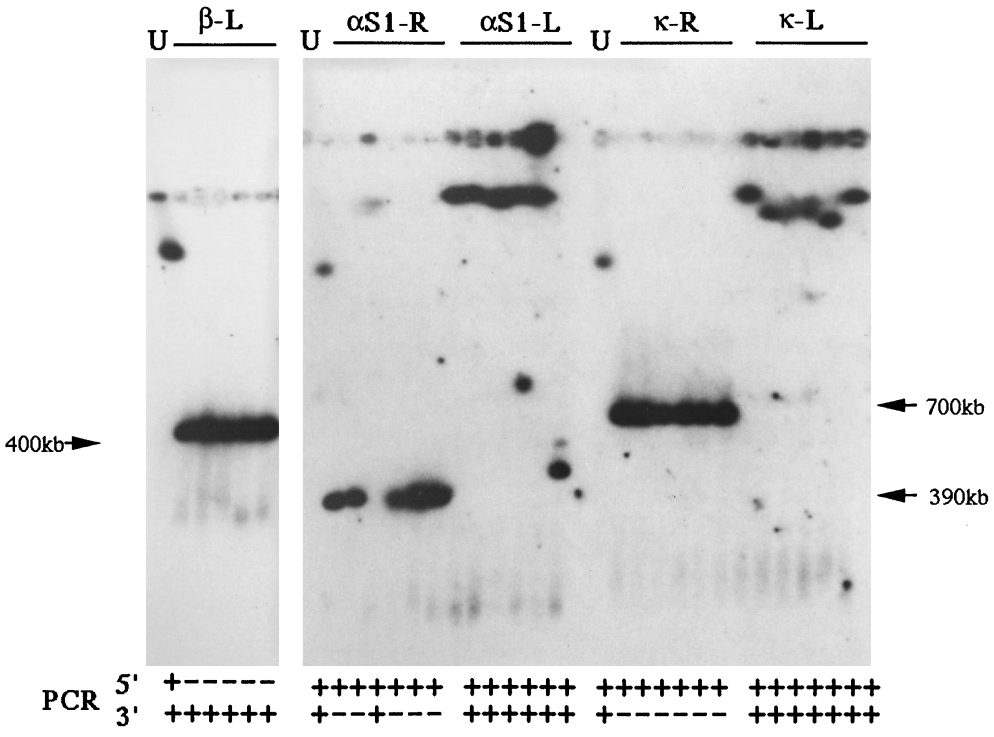


Fig. 2 Southern blot and PCR analyses of recombinant clones derived from YAC clone 748F3. DNA of recombinant clones (His+, Trp+, Ura–) was analyzed by PFGE and Southern blotting using the YAC left-arm probe. PCR was performed to screen recombinant clones for the presence of 5′- or 3′-flanking sequences. The fragmentation vectors used for transformation are αS1-R, αS1-L, β-R, β-L, κ-R and κ-L. (*R* indicates the orientation left to right arm of YAC and *L* indicates the opposite orientation). No transformant was obtained from experiments using vector β-R. (*U* denotes an unmodified 748F3)

et al. 1991). Results of fragmentation analysis are summarized in Table 2. Clones 748F3 and 960D2 were fragmented by pBP-αS1R, pBP-αS1L, pBP-βR, pBP-βL, pBP-κR and pBP-κL. Clone 750D11 was fragmented by pBP-αS1R, pBP-αS1L, pBP-βR and pBP-βL, and clone 886B3 by pBP-βR and pBP-βL. The results of fragmentation for 748F3 are shown in Fig. 2. In the β-casein gene, all five independent clones of 748F3 fragmented by pBP-bL had 400-kb bands and lacked the 5′ sequences of the β-casein gene. Five of six clones fragmented by pBP-αS1R and all six clones fragmented by pBP-κR were 390 and 700 kb in size, respectively (Table 2, Fig. 2), and they all lacked the 3′-flanking sequence of the β-casein gene. No clones were obtained by fragmentation with pBP-βR, and very few clones were obtained by fragmentation with pBP-αS1L and pBP-κL. These fragmented clones showed various insert sizes, indicating aberrant illegitimate recombinations (Fig. 2). Clone 750D11 was fragmented to 680 kb by pBPαS1R and to 700 kb by pBPβL. Clone 886B3 was fragmented to 1 Mb by pPBβR. Clone 960D2 was fragmented to 370 kb by pBPαS1L, 380 kb by pPBβR, and 100 kb by pBPκL. Results from Southern blotting analysis using cDNA probes were consistent with those from the chromosomal fragmentation of clones 748F3 and 750D11. The insert orientation of 960D2 and 886B3 was found to be opposite to that of 748F3, 750D11 and 882G11. This was confirmed by analysis by partial restriction enzyme digestion (data not shown). The orientations of the αS1- and κ-casein genes were identical, whereas the β-casein gene was in the reverse orientation to these two genes. The distance between the αS1- and β-casein genes is approximately 10 kb and the distance between αS1- and κ-casein is approximately 300 kb (Fig. 3).

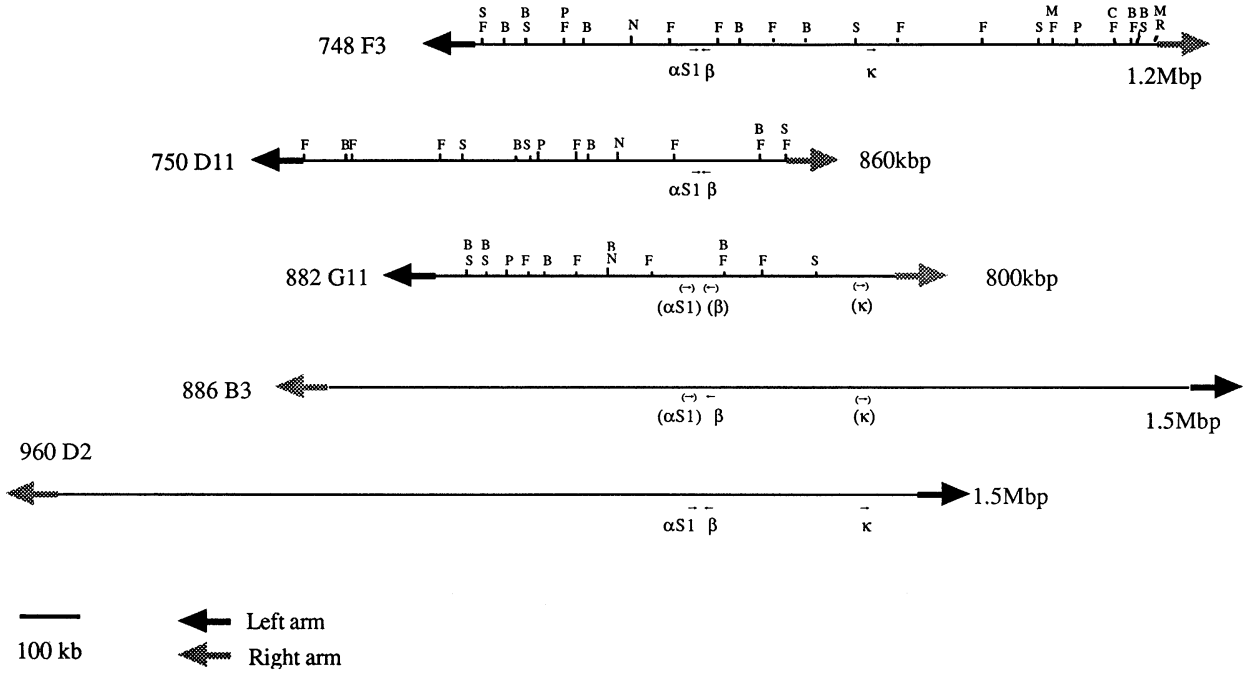
Table 2 Summary of fragmentation analysis. YAC clones were fragmented by the vectors pBPαS1R, pBPαS1L, pBPβR, pBPβL, pBPκR and pBPκL. The lengths of fragmented YAC clones were analyzed by pulsed-field gel electrophoresis (PFGE) and are shown in kilobases. (*R* orientation left to right arm of YAC, *L* reverse orientation, *NA* not analyzed, – vectors that could not give proper recombination)

YAC clones	Fragmentation vector					
	pBPαS1		pBPβ		pBPκ	
	R	L	R	L	R	L
748F3	390	–	–	400	700	–
750D11	680	–	–	700	NA	NA
882G11	NA	NA	NA	NA	NA	NA
886B3	NA	NA	1000	–	NA	NA
960D2	–	370	380	–	–	100

Fig. 3 Summary of partial restriction digestion and fragmentation analysis. YAC DNA partially digested with a restriction enzyme was separated by PFGE and transferred to a nylon membrane. The membranes were hybridized with YAC left-arm, right-arm and cDNA probes. 748F3 and 750D11 were digested with *Asc*I, *Not*I *(N)*, *Mlu*I *(M)*, *Csp*I *(C)*, *Pvu*I *(P)*, *Nru*I *(R)*, *Sal*I *(S)*, *Bss*HII *(B)* and *Sfi*I *(F)*. 882G11 was digested with *Not*I *(N)*, *Pvu*I *(P)*, *Sal*I *(S)*, *Bss*HII *(B)* and *Sfi*I *(F)*. There is no *Asc*I restriction site in 748F3 and no *Asc*I, *Csp*I and *Nru*I site in 750D11. Results of chromosomal fragmentation obtained in the present study (Fig. 2, Table 2) were added to the restriction map. The orientations and locations of the casein genes are indicated by *arrows*. Possible orientations and locations of caseins genes are indicated in *parentheses*

Discussion

The casein genes are thought to be members of a single multigene family in several animal species, based on previous experiments with somatic cell hybrids in mice (Gupta et al. 1982), restriction fragment length polymor-



phism analysis in pigs and sheep (Leveziel et al. 1991; Archibald et al. 1994), and FISH in rabbits (Gellin et al. 1985). In cattle, four casein genes, αS1-, β-, αS2- and κ-casein, have been demonstrated to be physically linked in this order within a region of 200 or 300 kb (Ferretti et al. 1990; Threadgill and Womack 1990). In the present study using five YAC clones, the human αS1-, β- and κ-casein genes have also been demonstrated to be closely linked within a region of 300 kb.

It has been reported that YAC inserts may sustain deletions, rearrangements or chimerism. Some minor differences in restriction sites were seen among 748F3, 750D11 and 882G11. It is not known whether these differences are reflected by minor deletions or by rearrangement of the inserts of the YAC clones. There was no large deletion or rearrangement at least between the αS1- and κ-casein genes because the orientations of and distances between the casein genes in these YAC clones were the same.

Naora (1986) has proposed that "territorial effects" influence the transcription of clustered genes on the same DNA strand. These effects are dependent on gene size and intergenic distance. Chromosomal fragmentation analysis of the human casein genes indicated that the distance between the αS1- and β-casein genes is approximately 10 kb. Since we have determined that the αS1- and β-casein genes are on different DNA strands, both genes are able to be expressed at the same time according to Naora's proposal (Naora 1986; Naora et al. 1988). This is consistent with the fact that both αS1- and β-casein appear in human milk at the same time.

The chromosomal localization of the human β-casein gene has been unclear in previous studies: the human β-casein gene was localized to 4pter–q21 using hamster-human somatic cell hybrids (Menon et al. 1992) or 1p3 by in situ hybridization (Kost et al. 1994). Our present study clearly suggests that the human β-casein gene along with the human αS1- and κ-casein genes is located at band q21.1 of chromosome 4.

Remote transcription regulatory elements such as a locus control region (LCR) in the β-globin genes (Grosveld et al. 1987) and matrix attachment regions (MARs) have not been found for the milk protein genes. Since we demonstrated that one of the isolated YAC clones (748F3 1.2 Mb), which contained the entire casein gene family, was not chimeric, the milk-specific LCR and MARs that regulate the expression of milk proteins may be included in the YAC insert. Further studies are required to elucidate the presence of these elements.

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