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Molecular cloning of the full-length cDNA encoding the human calbindin-D_{9k}

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The full-length cDNA encoding the human calbindin-D_{9k} (CaBP-9k) has been cloned using reverse transcription/PCR methodology with rat- and bovine-derived primers and intestinal RNA. A core product, and both a 5′ and 3′ product encompassing the full-length cDNA were obtained. The clones include coding region for 79 amino acids, 57 nucleotides 5′- and 159 nucleotides 3′-non-coding region, and a poly(A) tail. The deduced protein sequence is homologous to other mammalian CaBPs. Northern analysis revealed the mRNA in human duodenum to be about 600 nucleotides in length. Expression levels in adult human tissue were substantially lower than in child, rat or porcine intestine.

Calbindin; Vitamin D; Cloning; PCR

1. INTRODUCTION

The vitamin D-dependent calcium-binding protein, calbindin-D_{9k} (CaBP-9k) is a cytosolic protein of molecular weight 9,000 [1]. The CaBP-9k belongs to a family of calcium-binding proteins which includes such proteins as calmodulin, parvalbumin, troponin C, and S100 protein [2]. CaBP-9k is found in the mammalian intestine [3], placenta [4], uterus [5], and kidney [6]. Its exact function is unknown. The intestinal CaBP-9k is vitamin D-dependent and its expression correlates with calcium transport activity [7]. A possible role in calcium transport in placenta and kidney has been postulated in a vitamin D-independent fashion [2,6]. Uterine CaBP-9k is transcriptionally regulated by 17β-estradiol [8,9] with a putative function in control of myometrial activity via intracellular calcium. The rat [10], murine [11], bovine [12], and porcine [13] CaBP-9k amino acid sequences are known, as are the cDNAs for rat [14] and bovine [15]. Neither the amino acid sequence nor a cDNA sequence for the human CaBP-9k has been determined. A small molecular weight calcium-binding protein has been detected in human intestine by a calcium-binding technique [16] and has subsequently been partially purified [17]. Antibodies generated against the rat CaBP-9k do not cross-react with this human protein [18]. A rat cDNA probe does not detect CaBP-9k in human intestinal RNA (unpublished observation). In order to identify and study the significance of the human CaBP-9k, reverse transcription/PCR experiments for cDNA cloning were performed using rat bovine derived primers. Generation of a PCR product allows synthesis of a human-specific cDNA probe as well as primers for further amplification. This approach enables us to determine the primary structure of the human CaBP-9k and to isolate the corresponding gene.

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2. MATERIALS AND METHODS

2.1. *RNA preparation/Northern blot analysis*

The human tissues were collected from autopsies or received from the National Disease Research Interchange (NDRI, Philadelphia, PA). The protocol was approved by the University of British Columbia Ethics Committee. Total RNA was isolated by the guanidinium isothiocyanate/CsCl procedure [19,20]. Poly(A)$^+$ RNA was purified by oligo-dT cellulose chromatography (Pharmacia, Baie d'Urfe, Quebec, Canada). For Northern blot analysis, 10 μg of poly(A)$^+$ or 20 μg of total RNA was separated on a 1% denaturing agarose gel. Standard procedures for Northern blot analysis were used. Probe synthesis was carried out by random primed labeling using [α^{32}P]dCTP.

2.2. *cDNA synthesis*

cDNA was synthesized from human duodenal total RNA using Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT; 20U/μg total RNA) in a reaction buffer containing 2.0 μg oligo-d$(T)_{12-18}$ (for core PCR; see Fig. 1) or 0.2 μg NotI-d$(T)_{18}$ primer (Pharmacia, for 3′ RACE PCR), 10 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 40 mM KCl, 50 mM DTT, 1 mM dNTPs; and 25 U RNAse inhibitor. The reaction mixtures (25 μl) were incubated at 42°C for 1 h, followed by 10 min at 95°C, and chilled on ice. A 1 μl aliquot of the reaction was amplified by PCR.

2.3. *Amplification of human CaBP-9k cDNA by PCR*

PCR amplification was performed by adding 1 μl aliquot of first strand cDNA reaction mixture to 24 μl buffer giving 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 50 μM dNTPs, and 100 pmol each of the appropriate PCR primers, and 1 U AmpliTaq DNA polymerase (Perkin-Elmer, Vancouver, B.C., Canada). The

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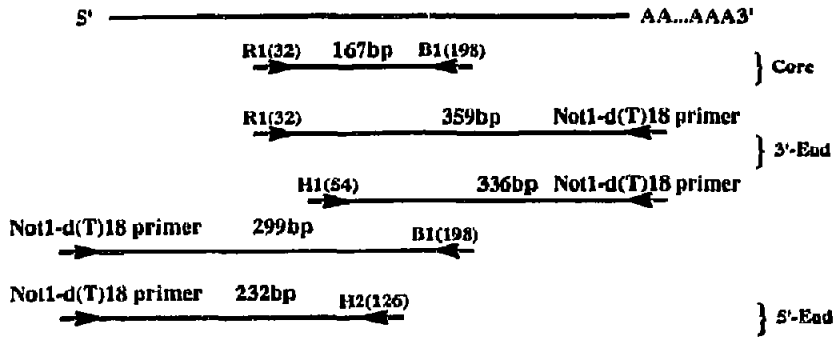

Fig. 1. Position of PCR primers on the rat CaBP-9k mRNA and sizes of amplification products. The prefixes indicate the source of the sequence from which the primer is derived (R=rat; B=bovine; H=human). Numbers in parentheses indicate nucleotide position on target sequence where the 5' end of the primer will anneal. Position 1 is the first nucleotide in the initiation methionine.

PCR primers (Fig. 1) were designed according to the published rat [14] and bovine [15] cDNA sequences. PCR amplification was performed using a Perkin-Elmer Cetus DNA Thermal Cycler. After an initial denaturation step at 96°C for 5 min, the following thermal cycle profile was used for generating a 167 base pair (bp) core PCR product: denaturation for 30 s at 96°C, 1 min cooling to 37°C, annealing of primers at 37°C for 30 s, 1 min heating to 72°C, extension of primers at 72°C for 1.5 min, 1 min heating to 96°C. After 30 cycles, an extension step at 72°C for 7 min was added. For 3' Rapid Amplification of cDNA Ends (RACE), first round PCR was performed as described above using NotI-d(T)$_{18}$ and R_1 primer [21]. A 1 μl aliquot of the first round PCR mixture was used for a second amplification generating a 3' RACE human PCR product (≈340 bp). Following succesful amplification of the core product a human primer H_1 was synthesized. Thirty cycles were performed using H_1 and NotI-d(T)$_{18}$ primers. Aliquots of the PCR reaction were analyzed by electrophoresis on 8% polyacrylamide and Southern blotting. Hybridization was carried out with a rat probe for detection of the human core product (≈167 bp). For analysis of the 3' RACE product, the human core product was used as a probe. Subsequently, the 5' RACE technique was used to amplify the 5' end of the human CaBP-9k cDNA. For priming the cDNA synthesis reaction, the bovine-derived primer B_1 was used. A homopolymer d(A) tail was added by incubation with terminal deoxynucleotidyltransferase. First round PCR was performed with NotI-d(T)$_{18}$ and B_1 primers. The product was reamplified with NotI-d(T)$_{18}$ and a human specific primer H_2, derived from the 3' RACE clone (see Fig. 1). The PCR conditions were as described for the 3' RACE step.

The PCR products were purified by MERMAID kit (Bio 101, La Jolla, CA, USA) and cloned into pUC19 after a filling-in reaction with Klenow fragment and blunt end ligation. Double-stranded DNA sequencing was performed using the dideoxy chain termination method with [^{35}S]dATP [22]. Nucleotide sequence comparison was performed with the program from the Genetics Computer Group of the University of Wisconsin-Madison.

3. RESULTS

3.1. *Cloning of the human CaBP-9k PCR products*

Reverse transcription followed by PCR was performed to clone the human CaBP-9k cDNA from duodenal tissue using an upstream primer identical to the rat RNA sequence (R_1) and a downstream primer complementary to the bovine RNA sequence (B_1). The strategy used for amplification is schematically shown in Fig. 1. A PCR product of the expected size (167 bp) was obtained. Hybridization of the Southern blotted PCR product with a radio-labeled rat cDNA probe confirmed the specificity of the human PCR product. Rat intestinal cDNA was used as a positive control in the PCR experiments. The rat probe produced a very weak signal after Southern analysis, while the rat product showed the expected intense hybridization (data not shown). The 167 bp product was cloned and sequenced, which confirmed the human origin of the product ruling out contamination by rat cDNA. Subsequently the Rapid Amplification of cDNA Ends (3' RACE) technique was performed using an upstream primer, R_1, and a downstream primer, NotI-d(T)$_{18}$, in first round PCR. The product was analyzed by Southern hybridization with the human core product. A band of correct size could be detected by autoradiography but was undetectable by ethidium bromide staining. A human specific primer H_1 was synthesized according to the sequence of the core product (Fig. 1). After a second round of amplification with the H_1 and NotI-d(T)$_{18}$ primers, the product could be generated in sufficient quantities to be visualized by ethidium bromide. Subsequently, the 5' RACE technique was used to amplify a cDNA fragment spanning part of the core product and the 5' end of the RNA. The 5' RACE amplification product was detectable by Southern analysis using the 3' RACE clone as probe. The size of the re-amplification product using the human primer H_2 and NotI-d(T)$_{18}$ was 232 nucleotides (Fig. 1) and contained a 74 base pair overlap with the core product. Both, the 3' and 5' RACE products were cloned into pUC19 and sequenced. The combined sequence representing the full-length cDNA was 456 nucleotides long. The sequence was aligned with the bovine [15], porcine (Jeung et al., Biol. Reprod., in press), and rat cDNA sequences [14] (see Fig. 2A). The cDNA contained a start codon at position 58 and a poly(A) tail preceded by the polyadenylation signal AATAAA (see Fig. 2B). Homology was calculated for the coding, and the 5' and 3' non-coding regions. Comparison of the human sequence revealed 87.1% homology to bovine, 90.4% to porcine, and 80.4% to the rat cDNA coding regions. Homologies within the 5' and 3' non-coding

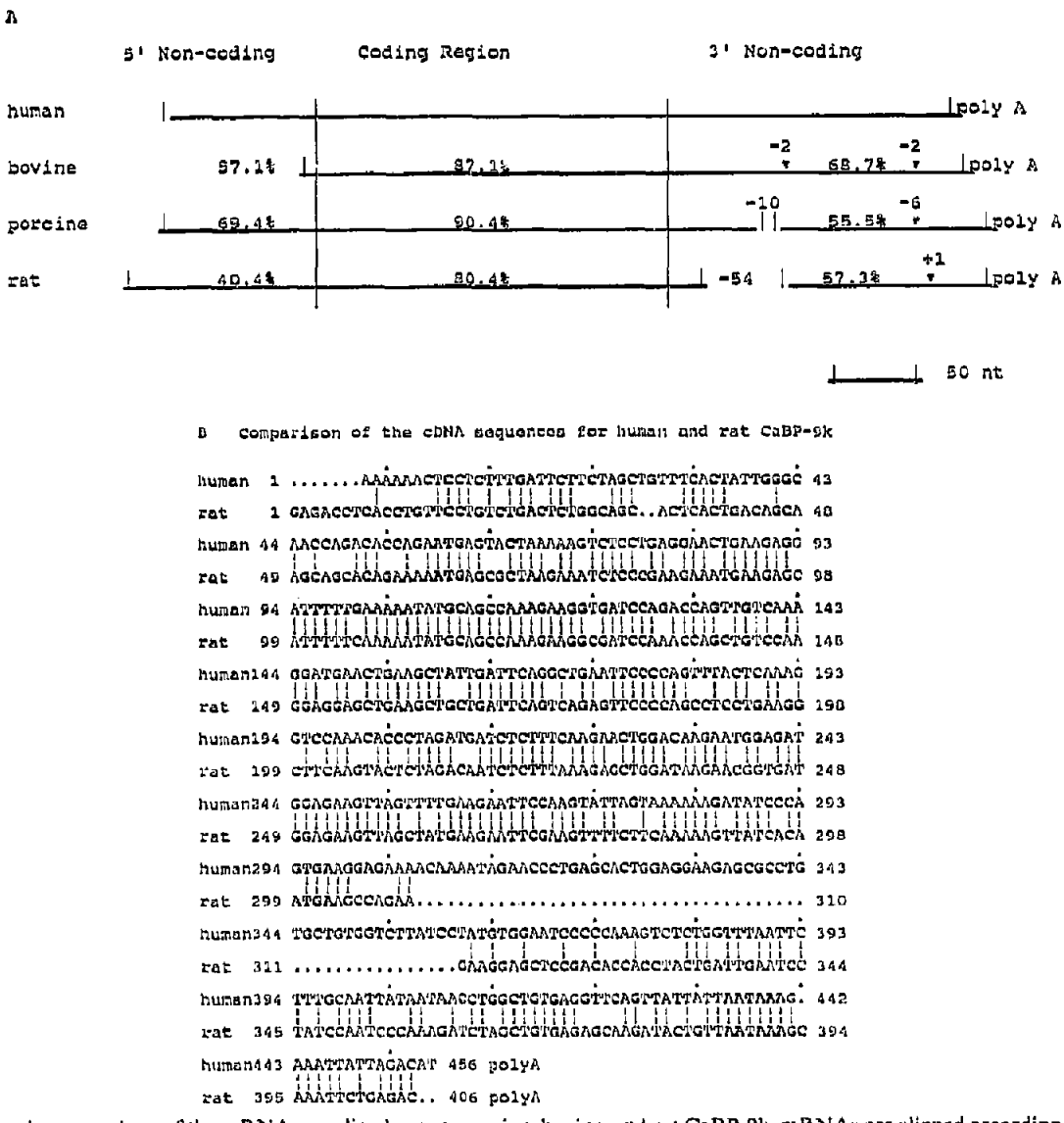

Fig. 2. (A) Schematic comparison of the mRNA encoding human, porcine, bovine, and rat CaBP-9k. mRNAs are aligned according to their coding regions and polyadenylation sites. Gaps in the 3' regions of bovine, porcine and rat sequences are indicated as negative numbers. One inserted nucleotide is located in the 3' region of the rat mRNA (+1). Note the 54 nucleotide gap in the 3' non-coding region of the rat mRNA. Nucleotide homologies to the human mRNA are indicated for the coding and non-coding regions. (Scale indicated, nt=nucleotide.) (B) Nucleotide sequence of the human CaBP-9k cDNA compared to rat cDNA sequence. The start and termination codons and the polyadenylation signals are indicated in bold.

regions were substantially lower. The human 3' non-coding region has 4, 16, or 53 additional nucleotides compared to bovine, porcine and rat, respectively. The derived amino acid sequence of the human clone was compared to the other known mammalian CaBP-9k sequences (Fig. 3).

3.2. *RNA analysis*

Northern analysis of human RNA was performed using the 3' RACE clone as probe. With 20 μg total RNA a weak signal was detectable in duodenum, but not in placenta, uterus, kidney, pectoral muscle, or brain (data not shown). Fig. 4 shows the autoradiograph of a Northern blot using 10 μg of human poly(A)$^+$ RNA from a 2.5-year-old child and a 32-year-old adult as well as 20 μg total RNA from rat duodenum. The human probe hybridized specifically to a single $\approx$600 nucleotide RNA species in human RNA. The level of CaBP-9k RNA was approximately 5-fold higher in the child duodenum than in the adult tissue (equal RNA loading was confirmed by probing with bovine β-actin, data not shown). When the same blot was probed with a rat CaBP-9k cDNA, a strong signal was detected only in the rat RNA. The human probe also hybridizes very strongly to porcine RNA (data not shown).

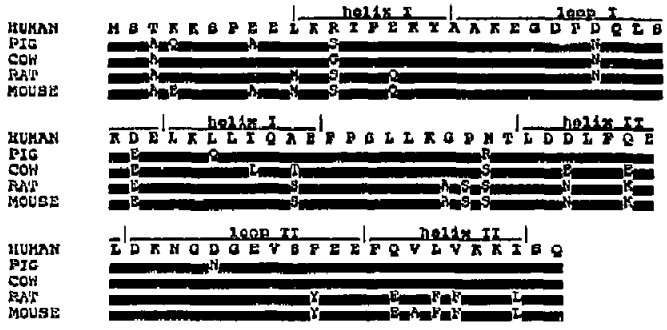

Fig. 3. Comparison of the amino acid sequences of mammalian CaBP-9ks. Only amino acids different from the human sequence are shown for pig, cow, rat, and mouse. Identity: human/pig 88.6%; human/cow 88.6%; human/rat 78.5%; human/mouse 75.9%.

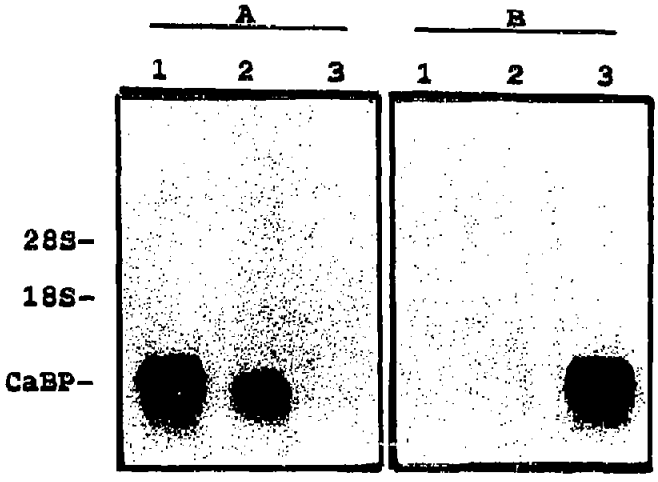

Fig. 4. Northern analysis of human and rat intestinal RNA. 10 μg of poly(A)$^+$ RNA from a 2.5-year-old child (1) and 32-year-old adult (2), and 20 μg of total RNA from rat intestine (3) were analyzed. The blot was hybridized with the isolated human (A) or with a rat (B) cDNA probe.

4. DISCUSSION

4.1. *Cloning of the human CaBP-9k PCR products*

This study confirms the expression of a human protein analogous to the rat CaBP-9k. In previous experiments we were unable to use a rat cDNA probe to detect CaBP-9k in human RNA, whilst detection was possible in porcine, bovine, and ovine mRNA (unpublished observation). Therefore, conventional screening methods with a suitable cDNA library were not feasible for cloning the human CaBP-9k. Instead, we used PCR methodology to amplify fragments of the human CaBP-9k mRNA. Application of the anchored PCR technique [21] generated a full-length cDNA clone. Homology between the rat and human cDNA sequences is lower than between rat and bovine or porcine CaBP-9k. The low homology combined with the difficulty to obtain high quality, non-degraded RNA from human intestine may explain the lack of detection of human CaBP-9k using a rat cDNA probe.

Interestingly, the human CaBP-9k mRNA has an extra 54 nucleotides inserted in the 3′ non-coding region, when aligned to the rat RNA. All of the 3′ non-coding region in the rat RNA is encoded in a single exon of the gene [23]. Therefore, different RNA splicing in humans seems unlikely. It is noteworthy that the mRNAs for bovine and porcine CaBP-9k have a similar but not identical inserted region compared to the rat sequence. The human CaBP-9k shows highest homology to the porcine and bovine proteins (88.6%), followed by rat (78.5%) and murine (75.9%) CaBP-9ks. The relatively high homology of the coding region was expected, especially in the region encoding the 17 C-terminal amino acids. This part of the second calcium-binding domain is identical to the porcine and bovine proteins, while the two rodent proteins differ in four positions in this region. There are four unique amino acids found in human at position 3 (threonine → alanine), 12 (arginine → serine or glycine), 30 (aspartic acid → glutamic acid), and 48 (asparagine → arginine or serine). The two loop regions of the calcium-binding domains reveal the highest amino acid conservation, while the helix and linker regions are more variable.

4.2. *RNA analysis*

Using the cloned human CaBP-9k cDNA as probe a single 600 nucleotide band was detected in duodenal RNA. The use of 10 μg poly(A)$^+$ RNA was necessary to detect a signal after overnight autoradiography, while amounts as low as 1 μg total RNA from rat intestine are sufficient for detection. It appears that the CaBP-9k RNA levels in the human tissue samples used were substantially lower than in rat and porcine material. This may explain the lack of CaBP-9k detection in tissues like placenta, uterus and kidney, where expression occurs in other species. Detection of CaBP-9k in these tissues may require purification of poly(A)$^+$ RNA or PCR techniques. Only limited information is available on the human CaBP-9k. Staun et al. [24] measured reported relatively high levels in 15-month-old children, where are comparable to levels found in rats [25]. With increasing age to 10 years, the levels dropped 10-fold to about 3 μg/mg protein. The normal level reported in vitamin D sufficient rats amounts to 30 μg/mg protein [15]. A decrease of CaBP-9k expression in aging rats has been reported previously [26]. High demand of intestinal calcium absorption during skeletal growth and mineralization is believed to be the reason for high expression during postnatal development. For Northern analysis in this study, adult tissue was used, which may explain the low CaBP-9k mRNA level.

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