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The 5′ end sequences and exon organization in rat regucalcin gene*

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Abstract

The 5′-flanking region of the gene for a Ca^{2+}-binding protein regucalcin was cloned from a rat genomic library which was constructed in lambda EMBL3 SP6/T7 vector. The genomic library was screened by using the radiolabeled probe with the 5′ region (0.5 kb) of rat regucalcin complementary deoxyribonucleic acid (cDNA). Positive clone had the 5.5 kb fragment which was hybridized with the 5′-probe. This fragment contained three exons (I–III) of the gene coding for a rat regucalcin. The nucleotide sequence of exons completely agreed with that of a rat regucalcin cDNA clone. A supposed translational initiation site existed in the exon II. Homology analysis showed that a putative transcription start site in the rat regucalcin gene was located at position 26 downstream from a TATA-box. Another upstream element, a CCAAT box-like sequence, was located at −170. Moreover, there were many regulatory elements (Hox, AP-1, AP-2 and AP-4) in the 5′-flanking region of the rat regucalcin gene. The organization of rat regucalcin gene seemed to be about 18 kb in size and consisted of seven exons and six introns. (Mol Cell Biochem **165:** 145–150, 1996)

Key words: regucalcin, calcium-binding protein, gene cloning, gene organization, 5′ end sequence

Introduction

Calcium ion (Ca^{2+}) plays an important role in liver metabolism [1, 2]. The Ca^{2+} action is amplified by calmodulin [3] and protein kinase C [4]. In recent years, it has been proposed that a novel Ca^{2+}-binding protein regucalcin, which exists in the cytoplasm of rat liver [5], may play an important role in the regulation of Ca^{2+} signaling [6]. Regucalcin can reverse the activatory effect of Ca^{2+} on various enzymes due to the binding of Ca^{2+} in liver cells [6]. Moreover, regucalcin has an inhibitory effect on Ca^{2+}/calmodulin-dependent enzyme activation, protein kinase C activation and Ca^{2+}-activated DNA fragmentation [7–9]. Presumably, regucalcin plays a role as a regulatory protein in Ca^{2+} signaling in liver cells.

Recently, we have determined the sequences of a cDNA encoding rat liver regucalcin and of the amino acid residues of complete regucalcin molecule [10]. The expression of regucalcin gene is demonstrated in human, mouse, bovine and chicken but not yeast [11]. Hepatic regucalcin mRNA expression is stimulated by the administration of calcium chloride in rats; the expression may be mediated through Ca^{2+}/calmodulin [12, 13]. Moreover, hepatic regucalcin mRNA expression is stimulated by hormone administration which can regulate liver metabolism [14–16], and it is inhibited by liver injury with chemical injection [17, 18]. Presumably, the expression of regucalcin mRNA is regulated by various factors.

More recently, we have determined the partial coding sequence (about 60% of open reading frame) and the entire 3′-untranslated region of the regucalcin [19]. These sequences are consisted of four exons in the rat regucalcin gene. It has been also demonstrated that the regucalcin gene is localized on rat chromosome Xq11.1–12 proximal end by using a genomic DNA fragment containing four exons as a probe [19].

*The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databeses with the following accession number D67071, D67069, and D67070.

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However, the characterization of the 5′-flanking region of regucalcin gene and its exon organization have not been clarified thus far. Therefore, the present study was undertaken to determine the complete exon organization of rat regucalcin gene. It was found that the rat regucalcin gene was consisted of seven exons and six introns, and that several consensus regulatory elements existed in the upstream of the 5′-flanking region of the gene.

Materials and methods

Materials

A genomic library of male Sprague Dawley rat in lambda EMBL3 SP6/T7 vector was obtained from Clontech Laboratories Inc. (Palo Alto, CA). Deoxycytidine 5′-[α-^{32}P] triphosphate (^{32}P-dCTP; 37TBq/mmol), charged nylon membrane (Hybond N$^+$) for screening of regucalcin gene, and adenosine 5′-[γ-^{32}P] triphosphate (^{32}P-ATP; 37 TBq/mmol) for DNA sequencing were purchased from Amersham (Buckinghamshire, UK). λ Phage vector (λ FIX II) for DNA cloning and host bacterial cells (*E. coli* SURE strain) were obtained from Stratagene (La Jolla, CA). Reagents (analytical grade) and enzymes were purchased from Sigma Chemical Co. (St. Louis, MO) and Toyobo (Tokyo, Japan).

Screening of the 5′ region of the rat regucalcin gene

A genomic library of male Sprague Dawley rat in lambda EMBL3 SP6/T7 vector (Clontech) was used for a screening of the 5′ region of the rat regucalcin gene. A 0.5 kb *Bam* HI fragment, covering one third of the 5′ part of the rat regucalcin cDNA (*Eco*RI-*Xho*I fragment, 1.6 kb) which had been cloned previously [10], was used as a probe. The probe was labeled with ^{32}P-dCTP by random primers with the DNA polymerase Klenow fragment [20]. Screening and hybridization were performed according to the standard procedure [21]. The phages of the genomic library were used to infect *E. coli* SURE, and genomic clones of the rat regucalcin were screened from 10^5 plaques by plaque hybridization using duplicate membranes. The membranes were prehybridized, and hybridized in buffer solution containing 5 × SSPE (1 × SSPE; 1.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 5 × denhardt's reagent (0.02% w/v each of bovine serum albumin, Ficoll, polyvinylpyrolidine) and 0.5% SDS with ^{32}P labeled regucalcin cDNA (0.5 kb fragment of the 5′ part) in a sealed plastic bag at 65°C for 18 h. After hybridization, the membranes were washed as follows: 2 × SSPE and 0.1% SDS at room temperature (twice, each for 15 min), followed by 0.1 × SSPE and 0.1% SDS at 50°C, and then the membranes were exposed to X-ray film for 12 h. Positive phage clones (1×10^6 phages) were obtained and purified.

Characterization of cloned genomic DNA

Phage DNA was amplified and the genomic insert characterized by digestion with restriction enzymes, followed by Southern hybridization analysis. Ten micrograms of genomic DNA extracted from the cloned λ phages were digested with various restriction enzymes, and the DNA was electrophoresed in 0.8% agarose gel. The separated DNA, according to size by electrophoresis, was transferred to nylon membrane by blotting with 20 × SSPE for 12 h [21]. The blot was hybridized to rat regucalcin cDNA, washed and exposed as described in the preceding paragraph. Restriction enzyme map of the genomic clone was made, and the subcloning of genomic DNA fragments (5.5 kb *Eco*RI-*Xho*I fragment and 3.5 kb *Eco*RI-B*am* HI fragment) were then performed using the pBluescript SK (Stratagene) plus plasmid vector.

DNA sequencing

Nucleotide sequencing was performed by the method of dideoxynucleotide termination [22]. The nucleotide sequences of the inserted fragments of plasmids were determined by using CircumVent thermal cycle dideoxy DNA sequencing kit (New England BioLabs) using ^{32}P-labeled primers. 5′-Terminal of synthetic oligonucleotides was labeled with γ-^{32}P-ATP with T_4 polynucleotide kinase [23]. The sequencing reaction was performed with Gene ATAQ Controller (Pharmacia). Both the strands were sequenced. The obtained sequences were assembled and analyzed by PC/GENE program (Inteligenetics).

Results

By Southern screening with regucalcin cDNA, four independent clones were obtained out of 1×10^6 phages. The restriction map of the 5′ part of the regucalcin gene and its localization in these clones is shown in Fig. 1. These clones were not hybridized with the downstream of the B*am* HI site. Also, these clones did not cover four 3′-most exons and their nearby regions. The 5.5 kb *Eco*-RI-*Xho* I fragment or the 3.5 kb *Eco*RI-B*am* HI fragment was hybridized with the 5′ end-probe (0.5 kb) of rat regucalcin cDNA. These fragments were cut out from λRCB2 or λRCB3, because both λRCB1 and λRCB3 contained the 3.5 kb *Eco*RI-B*am*HI fragment, and both λRCB2 and λRCB4 contained the 5.5 kb *Eco*RI-*Hho*I fragment. λRCB2 and λRCB3 were subcloned into the pBluescript SK+plasmid.

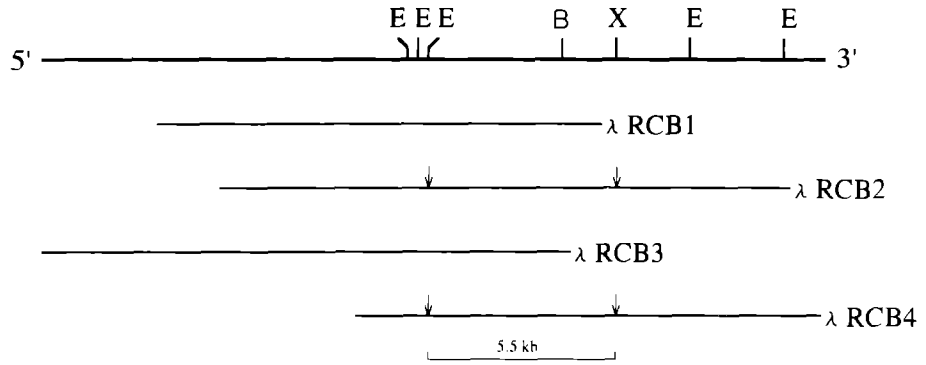

Fig. 1. Region and restriction sites in the inserts of genomic λRCB clones. *Eco* RI (E), *Bam* HI (B), and *Xho* (X) restriction sites are indicated. λRCB1 or λRCB3 covered the 3.5 kb *Eco*RI-B*am* HI fragment, and λRCB2 or λRCB4 contained the 5.5 kb *Eco*RI-*Xho* I fragment. These fragments were hybridized with the 5′ end-probe (0.5 kb) of rat regucalcin cDNA. The 5.5 kb region (indicated by arrowhead) between E and X in genomic λRCB2 was subcloned for the sequence of DNA. Also, the 3.5 kb *Eco*RI-B*am*HI fragment in genomic λRCB3 was subcloned for the DNA sequence.

The nucleotide and deduced amino acid sequences of the λRCB (2 and 3) contained the rat regucalcin gene, and the sequences are shown in Fig. 2. The nucleotide sequences of the three exons (I–III) identified with that of a rat regucalcin cDNA clone [10], and the exon/intron splice junction sequences were in accordance with the GT-AG rule for a splicing site [24]. The regucalcin gene contained several consensus sequences of regulatory elements for transcription (in the sequences of first paragraph of Fig. 2). A transcription start signal, a TATA-box [25], was present at position 26 upstream from the transcription start site. Homology analysis showed that a putative transcription start site in the rat regucalcin gene is located at 26 downstream from a TATA-box [25]. This was demonstrated by primer extension analysis. Another upstream element, a CCAAT box-like sequence [26], was located at –170. The sequence homologous to Hox, AP-1, AP-2 and AP-4 was found at –368, –361, –160 and –138, respectively [27–29].

The second and third exons are also shown in Fig. 2. The second exon was located from position 129–307 in the sequences of second paragraph of Fig. 2. The translational initiation site existed in this exon. The third exon was localized from position 99–281 in the sequences of third -paragraph of Fig. 2. The dinucleotide repeat was localized in the downstream intron.

The present sequences (exon I–III) of the regucalcin gene and the sequences (exon IV–VII) which were reported previously [19] were combined, and the structure of the gene with mRNA and protein of rat regucalcin is shown in Fig. 3. The junctions of the exon/intron were defined by comparison of the gene sequence with the known rat regucalcin sequence and by consideration of consensus eukaryotic splice function sequence. The 5′-untranslated region was encoded by exon I (429 bases) and 16 bases of exon II. The coding region was present from exon II–exon VII. The 3′-untranslated region was contained in exon VII. Translated region of the gene is interrupted by six introns, and a large intron (the third intron, > 9 kb) is present between exon III and IV. Therefore, the rat regucalcin gene was seemed to be more than 18 kb in size and was consisted of seven exons and six introns.

Discussion

Previously, we reported that a cDNA encoding rat liver regucalcin and the complete regucalcin molecule consists of 299 amino acid residues [10]. Recently, we isolated the rat regucalcin gene which contained four exons of the gene coding for a rat regucalcin [19]. These exons included the partial coding sequence (61.2% of open reading frame) and the entire 3′-untranslated region of the gene [19]. Moreover, chromosomal location of the rat regucalcin gene was determined by direct R-banding fluorescence *in situ* hybridization (FISH) method with the clone containing four exons. The regucalcin gene was localized on rat chromosome Xq11.1–12 [19]. In the present study, the 5′ end-flanking region of the regucalcin gene was isolated, and the nucleotide and the deduced amino acid sequences of the exon I–III of the regucalcin gene was determined.

The 5′ end-flanking region of the regucalcin gene contained several consensus sequences of regulatory elements for transcription. At the transcription start site, there is a tendency for the first base of mRNA to be adenine nucleotide (A), and the consensus sequence for the mRNA start site has been established (5′ Py Py C A Py Py Py Py Py 3′, Py:pyrimidine) [25]. The transcription start site in the regucalcin (TTCACACCT) was fitted to the consensus sequence. Furthermore, a common feature to eukaryotic genes is the TATA-box found about 30 nucleotides upstream of the initiation point of mRNA transcription [25]. In the rat regucalcin gene, such a sequence occurs in the anticipated position (–26) with respect to the putative transcription start site. Thus, we chose A as a position 1 of the nucleotide sequence of regucalcin gene.

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                                    -368 gaattcct gactgatctttttttcctgatagttttttttcttttctttt
                                           Hox    AP-1
-320 cttttcttttcttttcttttcttttctcttctcttctctt ctcttctcttctcttctcttctcctcttcttccttctc
-240 ccagttcactggtctttggcctaaagtccaagcatagaat gttctttgcctgtgggattcaaggctccctgccaaactgg
                                                                          CAAT-box
-160 cctcatgcaaggaagcaagatacagctaaggggggctgagt tcatccactgcagtggagctggaagtggagctctgtagac
      AP-2                    AP-4
- 80 gaatacattcaaggtcttagttctatcccagcactaccag tttaaaaaaaatacgtatacatcccagggaaattgcccttc
                                                          TATA-box
   1 ACACCTGCCATTGTCCGAATTGAAAATTAACATAGGTTGG GATTGGGGGCAGAGACAGGACTAGATCTTAATTCCAGGGC
  81 CTAGCACAGGTTAGGAAACTGTTCAACCTCAGCCATATAC CAAGCCTCTGGCTGTTAACTTAATTCAGTCTATTATCACT
 161 GATGAACACATTTCTAAAACTGGCTGCCTCCCCCCACCCC CAAGGAGATCAATGAATAAGGGAGAGGTGGCAGCATAAAA
 241 GGGTAACCTGCAGACACCCAGAGGGGCCACCAATGAGGAA GCTCCCCGCCCCTTGGAGGTCAGGGTGCTGGCATAAAAAG
 321 CACTCTCTGACTAGCAGGCTTGCCAGGAGTCATTACAACC CAGGTGTGGATGCTGGAGTGTTTCCTTTGTCTTCTATTTT
 401 AAAGATATCTTGAAAAAAACCTGTCACTGgtaagttgact gaagatgtctcatgtcatgtctttttttttttcatggcat
 481 gtcttatcagctctttctt

                    ..... ~ 0.9 kb .....

     cagggttgctgttaacctgtgatttgtgaaaactatactt gcatagtgctacacctcaaggtgtgcctgccatctgtact
     aaatttgttccatgtaattgccttacatttgttctctgtt tctctcagTCCTTTTCCTGCGACCATGTCTTCCATCAAGA
                                                                  M  S  S  I  K  I
                                                                  1
     TTGAATGTGTTTTAAGGGAGAACTACAGGTGTGGGGAGTC CCCTGTGTGGGAGGAGGCATCAAAGTGTCTGCTGTTTGTA
       E  C  V  L  R  E  N  Y  R  C  G  E  S   P  V  W  E  E  A  S  K  C  L  L  F  V

     GACATCCCTTCAAAGACTGTCTGCCGATGGGATTCGATCA GCAATCGAGTGCAGCGAGTTGGTGTAGgtcagtatgaaag
     D  I  P  S  K  T  V  C  R  W  D  S  I  S   N  R  V  Q  R  V  G  V  D
                                                                        55
     ctgagctcctttccttgcagggagccatcttttcccagtg caggggggtaatgtcaccctgtttgtcattgccctggatcc

                    ..... ~ 2.0 kb .....

     ctaaaaattactggatcctttcctaagagatgttacaaga cacaatgactctatgttgccgggccaccagagcttacctg
     ttaactttgcctctgcagATGCCCCAGTCAGTTCAGTGGC ACTTCGACAGTCAGGAGGCTATGTTGCCACCATTGGAACC
                       A  P  V  S  S  V  A   L  R  Q  S  G  G  Y  V  A  T  I  G  T
                       56
     AAGTTCTGTGCTTTGAACTGGGAAGATCAATCAGTATTTA TCCTAGCCATGGTGGATGAAGATAAGAAAAACAATCGATT
     K  F  C  A  L  N  W  E  D  Q  S  V  F  I   L  A  M  V  D  E  D  K  K  N  N  R  F

     CAATGATGGGAAGGTGGATCCTGCTGGGAGATACTTTGCT Ggtatgatttctttcttagttttgcctctatttgctatct
      N  D  G  K  V  D  P  A  G  R  Y  F  A   G
                                               116
     ttgaatcaatctctctctctctctctctctctctctctct ctctctctctctctctctttctctctctctctctctgtgt
     gtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgt t
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Fig. 2. Nucleotide sequence of rat regucalcin gene derived from genomic clones λRCB. Capital letters indicate exons (first paragraph = exon I, second paragraph = exon II, and third paragraph = exon III). Nucleotide numbers around exon I are given with the initiation site of transcription as +1. The putative Hox, AP-1, AP-2 and AP-4 elements, or CAAT-box and TATA-box are underlined. The sequences which were not determined are indicated by dashes with the approximate length. The amino acid sequence of the coding regions is shown as one-letter code (exon II and III). The numbers in the sequence refer to the positions of amino acids sequence predicted from rat regucalcin cDNA.

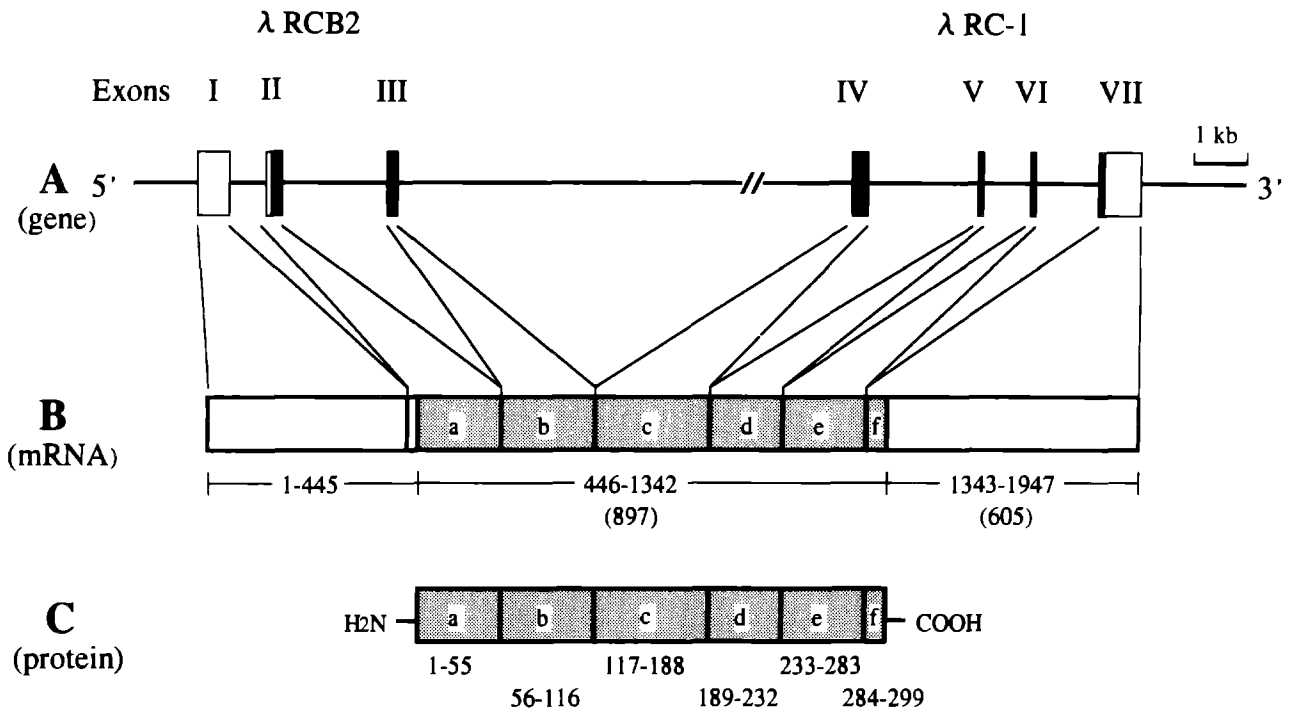

Fig. 3. Alignment of the exons for the rat regucalcin gene with the cDNA. Figure A shows the genomic organization of rat regucalcin gene.. The positions of exons shown as solid boxes are indicated in agreement with the protein coding regions, and the 5′- and 3′-untranslated regions are shown as open box. Introns are depicted by connecting lines. λRC-1 (exons IV-VII) was reported in our previous publication [19]. Figure B shows a diagram of the regucalcin cDNA from rat liver. Figure C shows the organization of amino acid residues of regucalcin.

Another upstream element, a CCAAT box-like sequence [26], was present at –170 in the 5′-flanking region of the regucalcin gene. A sequence homologous to Hox, AP-1, AP-2 and AP-4 was also found at –368, –361, –160 and –138 of the 5′-flanking region of the gene, respectively [27–29]. This suggests the existence of many regulatory elements for transcription of the regucalcin gene. Of these elements, the activator protein-1 (AP-1) transcription factor consists of either *Jun* homodimers or *Fos/Jun* heterodimeric complexes which bind the palindromic consensus sequence TGA(C/G)TCA [28]. The gene of AP-1 is a member of a group of immediate early genes (IEGs), which have the ability to be activated gene expression within short-term by a variety of extracellular stimuli. It is also known that the expression of *c-fos* can be induced by increase in intracellular calcium, which appears to act through the sequences of a cyclic AMP-responsive element (CRE) and a calcium responsive element (CaRE) [30]. A homologous to the consensus sequence, a AP-1 responsive element, was found at –361 in the upstream of the 5′ end of regucalcin gene. Previously, we reported that the expression of regucalcin mRNA in the liver was clearly stimulated at the early time point (within 30 min) after calcium administration in rats [12, 13]. Presumably, regucalcin mRNA expression is partly mediated through an AP-1 protein, which is bound at AP-1 consensus sequence in the regulatory region of the regucalcin gene. However, the existence of proteins which bind to the regulatory elements of regucalcin gene for transcription remains to be elucidated. The structure of the promoter may be strengthened with direct experiments. The regulatory mechanism for the regucalcin gene expression may be interesting.

From the present and previous investigations [19], the exon organization of rat regucalcin gene has been determined. As summarized in Fig. 3, the 5′-untranslated region of the regucalcin gene was encoded by the exon I (429 bases) and 16 bases of the exon II, and the coding region was present in from the exon II to the exon VIII containing the 3′-untranslated region. Thus, rat regucalcin gene consisted of seven exons and six introns with the size more than 18 kb. The regucalcin gene is demonstrated in human, mouse, rat, bovine and chicken but not yeast [11]. Also, regucalcin protein predominantly exists in the liver of rats among various species [31], although it is expressed at low levels in the kidney of rats. Thus, regucalcin is an unique Ca^{2+}-binding protein which can regulate Ca^{2+} signaling in liver cells [6–9].

In conclusion, it has been demonstrated that the organization of rat regucalcin gene consists of seven exons and six introns, and that the 5′-flanking region of the exon I contained several consensus sequences of regulatory elements for transcription.

Acknowledgements

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