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The organisation of the mouse chromogranin B (secretogranin I) gene

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A cosmid clone containing the gene for mouse chromogranin B (secretogranin I), a secretory protein found in secretory granules of most endocrine cells and neurons, was isolated and sequenced. The chromogranin B protein was found to be encoded by 5 exons which correspond to the cleaved signal peptide, the short N-terminal sequence preceding the disulfide-bonded loop structure, the disulfide-bonded loop structure itself, the large, variable region comprising ~90% of the protein, and the conserved C-terminal sequence. The promoter region of the chromogranin B gene is very GC-rich and contains a CATAA motif, a cAMP-responsive element and an Sp1 binding site.

Chromogranin; Secretogranin; Secretory granule; Gene structure; Promoter; Transcription factor

1. INTRODUCTION

The chromogranins/secretogranins (Cgs/Sgs) are a family of acidic secretory proteins [1,2]. A remarkable feature of these proteins is their occurrence in secretory granules of most peptidergic endocrine cells and neurons [1,2], which is more widespread than that of any known peptide hormone and neuropeptide. The Cgs/Sgs have therefore increasingly been used as markers of normal and neoplastic neuroendocrine cells (for reviews, see [3]). The molecular basis of the widespread, yet exclusively neuroendocrine, expression of these proteins is unknown.

The physiological function(s) of the Cgs/Sgs remain(s) to be established. Proposed functions include roles as: (i) precursors of biologically active peptides, in particular of those modulating regulated protein secretion in an autocrine and/or paracrine fashion [4–6]; (ii) modulators of the proteolytic processing of peptide hormone and neuropeptide precursors [7,8]; and (iii) helper proteins in the packaging of certain peptide hormones and neuropeptides into secretory granules and in the organisation of the secretory granule matrix [1,9]. Although in vitro data consistent with these roles have been reported [6,8,10–12], there is no direct in vivo evidence establishing a physiological role of the Cgs/Sgs. This may be partly due to the lack of an appropriate animal model which allows the manipulation of not only the extracellular but also the intracellular levels of these proteins.

During the past 4 years, the primary structure of the 3 established members of this protein family, chromogranin A (CgA) [13,14], chromogranin B (secretogranin I) (CgB) [15,16] and secretogranin II (SgII) [11,17], has been deduced from the sequences of cloned cDNAs. The comparison of the primary structure of these proteins revealed that CgA and CgB are more closely related to each other than either protein is to SgII [11,15,16]. In particular, CgA and CgB contain a homologous, disulfide-bonded loop structure near their N-termini and another homologous sequence at their C-termini [15,16]. In either protein, these two homologous domains are highly conserved despite considerable interspecies sequence variation in the remaining, major portion of the molecule. The protein sequence of both pre-CgA and pre-CgB appeared to consist of 5 parts: (i) a cleaved signal peptide; (ii) a short N-terminal sequence preceding the disulfide-bonded loop structure; (iii) the disulfide-bonded loop structure itself; (iv) a large, variable region; and (v) the conserved C-terminal sequence [15].

It would therefore be of interest to relate the sequence of these proteins to the exon-intron organization of the respective genes. The isolation and characterization of these genes would also be a first step towards understanding their expression and would provide the tools to investigate their function(s) in vivo. We present here the first report on the genomic organisation of a member of the Cg/Sg protein family, mouse CgB.

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Abbreviations: Cgs/Sgs, chromogranins/secretogranins; CgA, chromogranin A; CgB, chromogranin B; SgII, secretogranin II; CRE, cAMP-responsive element

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2. MATERIALS AND METHODS

2.1. *Cloning of the mouse CgB gene*

A cosmid library of 129/svSLcp mouse DNA was screened with a rat CgB cDNA probe [16], and positive clones were isolated and characterised further as described [18] including fine mapping of restriction enzyme sites. The orientation of the mouse CgB gene was determined by differential hybridization of DNA fragments specific for the 5′ or 3′ part of the rat CgB cDNA on Southern blots of cosmid DNA digested with various restriction enzymes.

2.2. *Sequence analyses*

The sequences of the promoter region, all exons and most parts of the introns were obtained by subcloning of restriction fragments into suitable vectors and sequencing using the dideoxy-chain-termination method.

2.3. *Primer extension*

Primer extension analysis was performed as described [19], using an oligonucleotide complementary to positions +50 to +69 of the mouse CgB gene (see fig.1B). RNA was isolated from mouse AtT-20 cells and mouse liver by standard procedures.

3. RESULTS AND DISCUSSION

3.1. *Cloning and sequencing of the mouse CgB gene*

When the human CgB cDNA [15] and the rat CgB cDNA [16] (kindly provided by Dr G. Sutcliffe) were used as probes on mouse genomic Southern blots, only the rat probe gave a clear signal (data not shown). The presence of single hybridizing fragments after digestion of mouse genomic DNA with various restriction enzymes suggested that mouse CgB is encoded by a single gene. Screening of an (amplified) mouse cosmid library led to the identification of 8 clones which were found to be identical by mapping of restriction enzyme sites. Further mapping of one of these clones (mcSgI-9c, fig.1A) using 12 different restriction enzymes suggested that it contained the complete mouse CgB gene.

To determine the exon-intron structure of this gene, restriction fragments containing CgB sequences, as identified by Southern blot analysis using the rat CgB cDNA as probe, were subcloned and sequenced as shown in fig.1A. The comparison of the nucleotide sequence with that of the rat CgB cDNA [16] allowed the definition of the exon-intron boundaries (fig.1B) which were consistent with the established consensus rules for donor and acceptor splice sites. The mouse CgB gene without the promoter region is ~12.1 kb long and is comprised of 5 exons and 4 introns (fig.1A,B). The length of the exons ranges from 47 bp (exon 2) to 1766 bp (exon 4), and that of the introns from 0.4 kb (intron 2) to 5.4 kb (intron 3). The 5 exons predict an open reading frame of 2031 nucleotides, encoding a protein of 677 residues.

3.2. *Relationship between exons and protein sequence*

Exon 1, besides specifying the 5′-untranslated region of the mRNA (62 nucleotides, fig.1B), encodes most of the signal peptide of pre-CgB (fig.2). (It cannot be excluded that concerning the 3′ acceptor splice site of intron 1 (aagCTGTCAG, fig.1B), CAG rather than aag serves as acceptor site; with respect to the 5′ donor splice site of intron 1, this would predict that in the sequence Ggtgagtg (Fig.1B) the second gtg would serve as donor site to retain the open reading frame. This alternative would result in a 19-residue signal peptide ending with -GDS (rather than -AVSS, see fig.2), with -D- being encoded by both exons 1 and 2, but would not affect the amino acid sequence downstream of the putative signal peptidase cleavage site.)

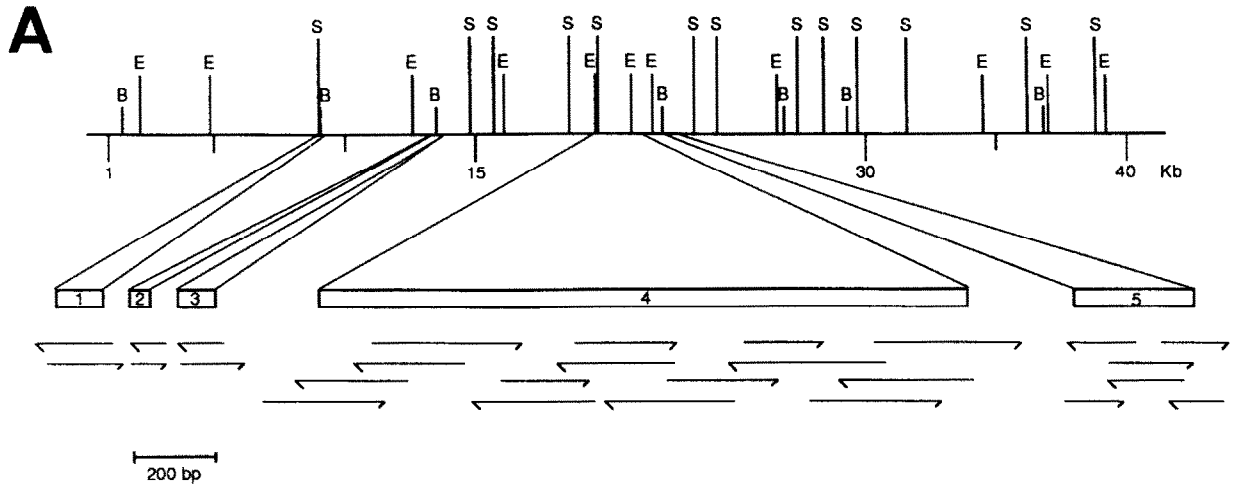
The small exon 2 encodes (besides the end of the signal peptide) all except the last 3 residues of the short N-terminal sequence which precedes the disulfide-bonded loop structure (fig.2) [15]. The latter and a further 6 residues, including the first dibasic site, are encoded by exon 3. The large exon 4 encodes essentially the entire variable region of CgB which comprises ~90% of the mature protein. Finally, exon 5 encodes the last dibasic site and the conserved C-terminal domain [15], and in addition specifies the 3′-untranslated region of the mRNA including the polyadenylation signal (fig.1B).

The major conclusion that can be drawn from these data is the remarkable correspondence between the previously suggested [15] domain structure of the CgB protein sequence and the organisation of the CgB gene into 5 exons. In particular, the fact that the disulfide-bonded loop structure and the C-terminal portion of CgB are each encoded by separate exons strongly suggests that these domains are indeed distinct units with presumably important, though as yet unknown, roles in the structure and function of this protein.

3.3. *Species comparison of the chromogranin B protein sequence*

A comparison of the predicted protein sequence of mouse CgB with that of human [15] and rat [16] CgB is shown in fig.2. Confirming and extending previous observations [15,16], the N- and C-terminal regions of this protein are highly conserved between species and are homologous to the corresponding regions in CgA. In contrast, the bulk of mouse CgB, between the disulfide-bonded loop structure and the conserved C-terminal domain, shows a remarkable degree of sequence variation not only to the corresponding human protein, but even to another rodent, rat, CgB. The tyrosine sulfation site previously identified in human [15] and rat [16] CgB is conserved in the mouse protein (Tyr-328) and is thus presumably biologically significant. In contrast, neither the -RGD- sequence at position 157–159 of rat CgB [16] nor the potential N-glycosylation site at position 278 of mouse CgB are conserved.

Mouse CgB contains 18 sites of two or more adjacent amino acids (fig.2). 13 of these sites, including the 11 C-terminal ones, are conserved in sequence position between mouse, rat and human CgB; 2 sites are slightly



B

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                                                                    *
gcccaccgcgccaCAGCTGCTCTGCGGAGCCCGCACCCGCCGAGCTCCTCCTACACTCAGCTGGCGAGAGGCACCATGCAGCCGGCTATG
CTCCTCGGCCTCCTGGGCGCAGCGGCTCTGGCCGgtgagtgggatt.....................................
(intron 1: 3.5 kb).....................................................................

tctccttcaaagCTGTCAGCTCAGCTCCAGTGGATAACAGGGATCACAATGAAGAAATGgtaagttgagat.................
(intron 2: 0.4 kb).....................................................................

gtcttctcatagGTGACTCGCTGCATTATCGAAGTCCTCTCAAATGCCCTATCCAAGTCCAGTGTTCCAACGATCACCCCTGAGTGCCGG
CAAGTCCTGAAGAAGAgtaagtgtctca.....................................................
(intron 3: 5.4 kb).....................................................................

ttttcattatagGTGGAAAAGAGGTCAAAGGTGAAGAGAAAGGTGAAAACCAGAACTCGAAGTTTGAAGTACGGTTGCTAAGAGACCCAG
CCGATGCCTCAGGAACCCGCTGGGCCTCCAGTAGGGAGGATGCAGGCGCTCCAGTAGAAGACTCTCAAGGCCAGACGAAGGTAGGCAATG
AGAAGTGGACAGAAGGAGGAGGACACAGCCGAGAGGGAGTGGATGATCAAGAGAGTCTCCGTCCCTCCAACCAACAAGCGTCCAAAGAAG
CAAAGATATACCATTCGGAAGAGAGGGTGGGAAAAGAGAGGGAGAAAGAAGAAGGCAAGATTTACCCAATGGGGGAGCACAGGGAAGATG
CTGGTGAAGAGAAGAAACACATTGAAGATTCTGGAGAGAAGCCGAACACCTTCTCCAACAAAAGAAGCGAGGCTTCCGCTAAGAAAAAAG
ATGAGTCAGTGGCCAGAGCAGATGCACACTCCATGGAGCTGGAGGAGAAGACACACAGTAGGGAGCAAAGCAGCCAGGAGAGTGGAGAGG
AGACAAGGAGGCAGGAGAAACCCCAGGAGCTTACCGACCAGGACCAGAGCCAGGAAGAATCCCAGGAAGGCGAGGAAGGTGAGGAAGGCG
AGGAAGGCGAGGAAGGCGAGGAAGACTCAGCCTCTGAAGTAACCAAACGCAGGCCCAGACACCACCACGGGAGAAGCGGGTCCAACAAGT
CCTCTTACGAAGGGCATCCTCTATCTGAGGAAAGGAGACCCTCTCCCAAGGAGTCCAAGGAGGCAGATGTGGCCACAGTCCGTTTAGGGG
AAAAAAGGAGTCACCATCTAGCTCACTACAGGGCTTCAGAAGAAGAACCTGAATATGGGGAAGAATCGAGAAGCTATCGGGGACTACAGT
ACCGGGGTAGAGGAAGCGAAGAGGACAGAGCCCCAAGGCCTCGGAGTGAGGAGAGTCAGGAAAGGGAGTATAAGAGAAACCACCCCGACT
CTGAACTGGAAAGCACAGCAAACAGACATGGTGAAGAAACCGAGGAAGAGAGGAGCTACGAGGGGGCAAATGGACGCCAACACAGAGGCA
GGGGGCGGGAGCCAGGAGCCCATTCTGCTCTTGACACCAGAGAAGAAAAAAGGCTCTTGGATGAAGGGCACTACCCTGTTCGCGAAAGCC
CAATAGATACGGCAAAAAGGTACCCACAAAGCAAATGGCAAGAGCAGGAGAAAAACTACCTCAACTATGGCGAGGAAGGGGACCAAGGGA
GATGGTGGCAGCAGGAAGAGCAGCTAGGACCGGAAGAAAGCAGGGAGGAAGTAAGGTTTCCAGACAGACAATATGAGCCCTATCCCATCA
CTGAAAAGAGGAAGAGGTTAGGGGCGCTGTTCAACCCGTACTTTGACCCTCTCCAGTGGAAGAATAGTGACTTTGAGAAAAGAGGCAACC
CAGATGACAGTTTTCTTGAGGATGAAGGTGAAGACAGAAATGGGGTGACCTTGACTGAGAAGAATTCCTTCCCAGAGTACAACTATGACT
GGTGGGAGAGAAGGCCCTTCTCAGAGGATGTGAATTGGGGATATGAGAAGAGAAGCTTTGCCAGGGCCCCTCAGCTCGACTTGAAACGGC
AATATGATGGAGTGGCCGAGTTGGACCAGCTGCTTCACTACAGGAAGAAGGCAGACGAATTTCCCGATTTCTACGACTCGGAGGAGCAGA
TGGGGCCTCACCAGGAGGCAAACGATGAAAAGGCCAGGGCTGACCAGAGAGTTCTGACGGCGGAAGAGgtacggtttg.............
(intron 4: 0,5 kb).....................................................................
                                                                                          *
tttctgtttcccagAAAAAGGAACTGGAGAACTTGGCTGCGATGGATCTGGAACTCCAGAAGATAGGGGAAAAGTTCAGCCAGCGGGGCT
GACAGTTGGAGAGACGAGCCTTTCACTGAAGGACACCATTGATTCACCCACAGGCAGAAAGCAGAATTTACTGTTTATTAAATGTTTGAT
GCAGTTGGAAATATCATTTATCTTTGCCAGAATGCTAATGCCATGTGACTAGCGTGACTTGTAGCGTATTCTTCCTGCAAAATAGATGTT
TCACGTGTCTGTGACAATGACCGTGTTACTGTTGTTGAAACACAAACAATAAAAAGATTTACCTGAAACaaagtccttttt.........
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Fig.1. The mouse CgB gene. (A) Localisation of exons 1–5 on the cosmid mcSgI-9c containing the mouse CgB gene. The sites of three restriction enzymes (B = *BglI*; E = *EcoRI*; S = *SstI*) as well as the orientation and length of the individual DNA sequences obtained (arrows) are indicated. (B) DNA sequence of exons 1–5 (capital letters) and of portions of the introns and the 5′ and 3′ adjacent sequences (small letters) of the mouse CgB gene. For the 5′ end of exon 1, see figs. 3 and 4. The 3′ end of exon 5 is defined by comparison with the 3′ end of the rat CgB mRNA [16]. The lengths of the 4 introns are indicated. The oligonucleotide used for primer extension (see fig.3) was complementary to the underlined nucleotides. Asterisks, start and stop codons of translation; open triangles, putative polyadenylation signal.

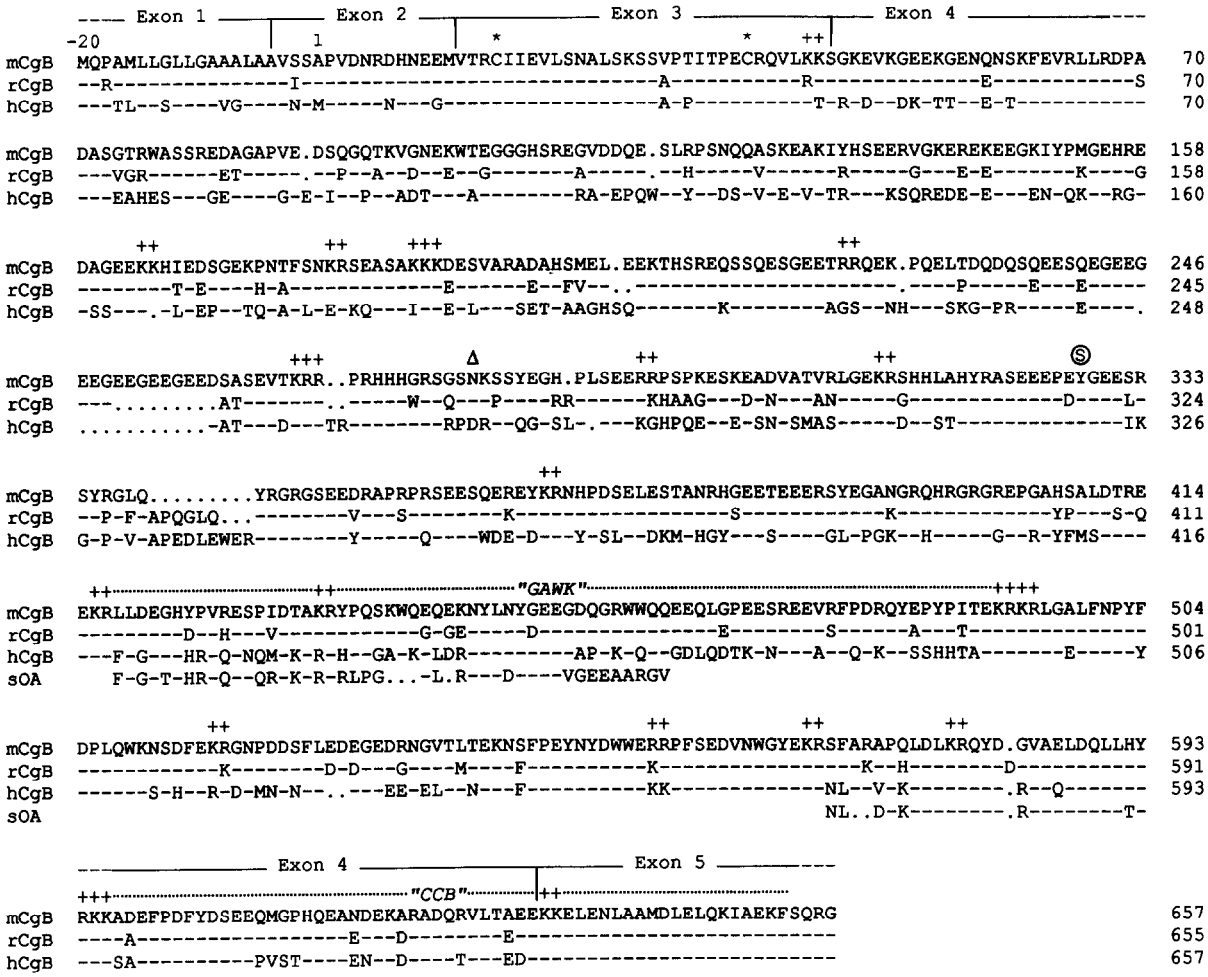

Fig.2. Relationship between exons and primary structure of mouse CgB and comparison of the mouse (mCgB), rat (rCgB) [16] and human (hCgB) [15] protein sequences. Gaps introduced for aligning the sequences are indicated by dots. Amino acid residues in rCgB and hCgB identical to the corresponding residues in mCgB are shown as dashes. Numbers on the right refer to the sequences positions of the respective mature, secretory protein; −20, beginning of signal peptide; 1, N-terminus of mature, secretory protein. *, cysteine residues; +, sites with two or more adjacent basic amino acid residues; Δ, potential N-glycosylation site; circled S, tyrosine sulfation site. The positions of the human CgB-derived peptides 'GAWK' [20,21] and 'CCB' [21] are indicated by the dotted lines. The sequences of peptides isolated from the ovine adrenal medulla (sOA) and presumably derived from ovine CgB are also given [21,27,28].

shifted in sequence position compared to human CgB, and 3 sites are conserved between mouse and rat CgB but absent in human CgB. The high degree of conservation of the di-, tri- and tetrabasic sites suggests that they are significant for the structure and/or the proteolytic processing of CgB. However, the question remains unanswered whether the presence and conservation of these sites suggests that CgB is a precursor of biologically active peptides. Two observations are worth mentioning in this regard. First, all of the 18 di-, tri- and tetrabasic sites are located either within the region encoded by the large exon 4 (16 sites) or directly adjacent to it (2 sites). Thus, if CgB should prove to be a precursor for biologically active peptides, most, if not all, of these peptides would be encoded by the same exon. Second, the sequence variation between rodent and human CgB in the region from which the peptide 'GAWK' [20,21] is derived is quite remarkable and higher than one might expect if 'GAWK' were a structurally conserved, biologically active peptide.

3.4. *The mouse CgB promoter*

To define the mRNA initiation site and subsequently the DNA sequence containing the CgB promoter, a primer extension experiment was performed. As shown in fig.3, a specific extension product was obtained only with RNA from AtT-20-cells (lane 1), a mouse neuroendocrine cell line which expresses CgB (data not shown), but not with RNA from mouse liver (lane 3), a non-neuroendocrine tissue. The same oligonucleotide used for primer extension was also used to sequence the mouse CgB DNA. Side-by-side analysis of the products of the primer extension and sequencing reactions on the same gel allowed the identication of the 5′ end of the mouse CgB mRNA as the nucleotide located 62 residues upstream of the ATG (fig.1B).

Fig.3. Mapping of the CgB mRNA start site by primer extension analysis. A labelled oligonucleotide, complementary to the nucleotide sequence from +50 to +69 (see fig.1), was annealed to 10 μg RNA from AtT-20 cells (lane 1), to 1 μg RNA from mouse liver (lane 2), to 10 μg RNA from mouse liver (lane 3), or to 10 μg of tRNA (lane 4). To determine precisely the endpoint of extension, a DNA sequencing reaction was performed with the same oligonucleotide and analysed on the same polyacrylamide gel. The endpoint of extension is indicated by the arrow.

The region preceding the mRNA initiation site (marked + 1 in fig.4) shows notable features. First, the 215 bp sequence shown in fig.4 is very GC-rich (75%).

```
-215  GATCTACAGC  ACCTGGCGTC  CCCGCATCCC  TGCCCGTGCC  TGCTGGGACC
                                                -134
-165  CTGCGCAAGG  CCGCACACAC  TGGCTGTGCT  TCCGCCCGCT  GGCGCCGCCT
                  -102
-115  CGGGGCTGCT  CCGTGACGTC  AGGGTCTGAG  CGAGGGGCGC  CCCAGTCGCC
                                              -31
-65   GCTCGCCCCC  GGGTACTGCA  CCCCGCCTGC  CCTTCATAAG  AGCCAGGCCT
                  +1
-15   GCGCCCACCG  CGCCACAGCT  GCTCTGCGGA  GCCGC.....
                  └──►
```

Fig.4. The mouse CgB promoter region. 215 base pairs of the putative mouse CgB promoter region are shown. The position +1 and the arrow indicate the mRNA initiation site, determined by primer extension (see fig.3). Typical promoter elements such as TATA box (CATAA, position −31), CRE box (TGACGTCA, position −102) and Sp1 box (CCGCCC, position −134) are underlined.

Second, consistent with the typical distance of about 30 bp between mRNA start site and TATA box, a CATAA sequence similar to that found in the β-globin gene [22] is present at position −31. Third, a cAMP-responsive element (CRE box) [23] and an inverted binding site for the Sp1 transcription factor [23] are found at positions −102 and −134, respectively. Other known binding sites for transcription factors could not be identified.

The presence of these elements suggests a functional promoter in this region. It is worth noting that the CRE box is present in the promoters of several other genes known to be expressed in neuroendocrine cells, such as parathyroid hormone [24], proenkephalin A [25] and somatostatin [26]. Since these genes are inducible by cAMP [26], it is tempting to speculate that the expression of mouse CgB may be stimulated by those extracellular factors that increase intracellular cAMP levels. A further comparison of these promoters shows that besides the CgB promoter, only the parathyroid hormone promoter contains an Sp1 binding site and none contains the TATA box in the form of a CATAA motif. In addition, we have noticed a correlation between the GC content of these promoters and the broadness of expression of these genes, the promoter of the proenkephalin A gene, which is expressed in a variety of endocrine cells and neurons, had a higher GC content (75%) than that of the parathyroid hormone gene (37%), which is expressed more selectively. It will be interesting to test the role of these elements, as well as others present in the ~4.5 kb of 5′ sequence of the cloned mouse CgB gene, in the widespread, yet specifically neuroendocrine, expression of CgB.

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