Isolation and partial characterization of the calcitonin gene in a lower vertebrate

Predicted structure of avian calcitonin gene-related peptide

S. Minvielle, M. Cressent, F. Lasmoles, A. Jullienne, G. Milhaud and M.S. Moukhtar

Unite 113 INSERM and UA 163 CNRS, CHU St. Antoine, 27 Rue Chaligny, 75571 Paris Cedex 12, France

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The gene coding for calcitonin was isolated and characterized in a non-mammalian vertebrate, chicken. Sequencing of the 3'-end of this gene revealed after the calcitonin coding exon, an intron followed by an exon coding for a calcitonin gene-related peptide, which displays significant amino acid sequence homology with mammalian CGRPs so far sequenced.

(Chicken) *Calcitonin gene-related peptide* *Amino acid sequence* *Nucleotide sequence* *Calcitonin gene*

1. INTRODUCTION

Calcitonin, the hypophosphatemic and hypocalcemic hormone [1,2], is involved in the regulation of calcium metabolism and is produced by the 'C' cells situated in the thyroid in mammals and in the ultimobranchial glands in submammalian vertebrates [3]. In mammals the calcitonin gene codes for two peptides: calcitonin and calcitonin gene-related peptide (CGRP) [4]. CGRP is a neuropeptide largely distributed in central and peripheral nervous system, where it plays the role of a neuromodulator (ingestive behavior, response to painful stimuli) [5]. This peptide also controls cardiovascular homeostasis [6]. The organization and sequence of the calcitonin gene in two mammals, man [7] and rat [8], have been established. Specific messengers for both molecules are generated by alternate splicing [4,9]. The sequence of CGRP in lower vertebrates remains unknown. Recently we have elucidated the sequence of calcitonin mRNA in a lower vertebrate: chicken [10]. The deduced amino acid sequence [11] reveals that the molecule is highly conserved among lower vertebrates, but that its sequence differs considerably from mammalian calcitonin. We have now isolated the calcitonin gene from a chicken genomic library and sequenced a large portion of its 3'-region. We report here the predicted amino acid sequence of an avian CGRP molecule differing from human and murine CGRPs by 4–6 amino acids. It thus appears that CGRP, in contrast to calcitonin, is a highly conserved molecule.

2. MATERIAL AND METHODS

2.1. *Screening of the chicken genomic library and Southern blot analysis*

A chicken genomic library constructed by inserting chicken DNA partially digested with *Mbo*I into phage λ L47.1 cleaved by *Bam*HI [12] was kindly provided by M. Ballivet. 0.6×10^6 recombinant phages were screened by plaque hybridization [13,14] using a [^{32}P]cDNA probe specific for avian calcitonin messenger [10]. A phage (L CG.21) was purified, as described by Maniatis et al. [14], from a plaque showing a high signal with the specific probe. DNA from this phage was subjected to restriction analysis using *Eco*RI, *Xba*I, *Hin*dIII, *Bgl*II and *Bam*HI, electrophoresed and transferred

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to nitrocellulose then probed with the labelled cDNA specific for calcitonin mRNA.

2.2. *Sequencing strategy*

A *Hin*dIII-*Hin*dIII 3300 bp fragment was subcloned into plasmid PUC 9, propagated in *E. coli* (TB1), the plasmid extracted, purified by CsCl centrifugation, the insert recovered and subjected to restriction analysis using *Acc*I, *Alu*I, *Bgl*II, *Dde*I, *Eco*RI*, *Hin*cII, *Pst*I, *Sau*3A, *Sph*I, *Sst*I and *Xmn*I. The restriction fragments were subcloned into M13 mp10, mp11, mp18 and mp19 [15]. Nucleotide sequences were determined by the dideoxy termination method [16]. A partially overlapping 3′ 3750 bp *Xba*I-*Xba*I fragment was subcloned directly in phage M13 mp19. By the sequential overlapping technique [17] we obtained clones and determined the sequence of the inserted DNA as above.

3. RESULTS

Fig.1 shows a Southern blot of a restriction analysis of L CG.21 probed with the calcitonin-specific cDNA. Several fragments show a high hybridization with this probe. The partial nucleotide sequence of fragment *Xba*I-*Xba*I reveals the presence of an open reading frame coding for a characteristic CGRP molecule (fig.2). The amino

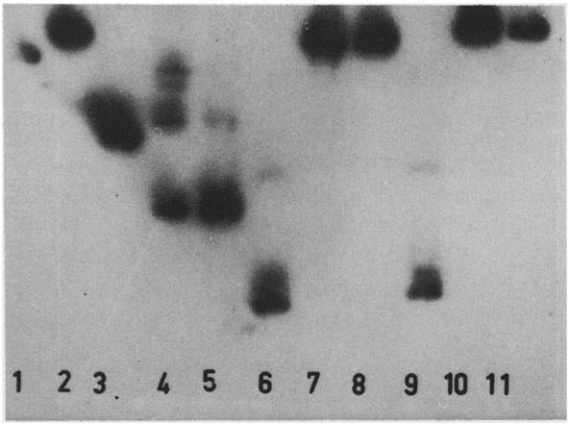

Fig.1. Autoradiography of Southern blot analysis of L CG.21 DNA digested with *Eco*RI (1), *Xba*I (2), *Eco*RI + *Xba*I (3), *Eco*RI + *Hin*dIII (4), *Hin*dIII (5), *Hin*dIII + *Xba*I (6), *Bgl*II (7), *Bgl*II + *Xba*I (8), *Hin*dIII + *Bgl*II (9), *Bam*HI (10), uncut (11) and hybridized with the avian calcitonin-specific cDNA.

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                                                    5'------aaactgacttttcttctcctttcaaaaagcagC
                                                                                            ▲

GTA ACA GCA CAG AAA AGG GCA TGC AAC ACA GCT ACC TGC GTG ACC
Val Thr Ala Gln Lys Arg Ala Cys Asn Thr Ala Thr Cys Val Thr

CAT CGT CTG GCA GAC TTC CTG AGC AGG TCA GGA GGA GTG GGC AAG
His Arg Leu Ala Asp Phe Leu Ser Arg Ser Gly Gly Val Gly Lys

AAC AAC TTT GTC CCA ACC AAC GTG GGC TCT AAG GCT TTC GGC AGG
Asn Asn Phe Val Pro Thr Asn Val Gly Ser Lys Ala Phe Gly Arg

CGA AGA AGA AGC GTT CAA ATA TAA GAA GCT GAA TGA CAA CAT GCC
Arg Arg Arg Ser Val Gln Ile Stop

TACgtaaatatattgcaagaagcatctcatgcagcaaccctttagt ------ 3'
  ▲
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Fig.2. Partial nucleotide sequence of the fragment *Xba*I-*Xba*I and predicted amino acid sequence characteristic for a CGRP molecule. Intron-exon boundaries are indicated (▲).

acid sequences of chicken CGRP and mammalian CGRPs [4,18–21] are compared (fig.3A). In contrast with calcitonin [22,23] (fig.3B) the amino acid sequence of CGRP is highly conserved.

4. DISCUSSION

We have isolated from a chicken genomic library a gene containing the calcitonin exon using a cDNA probe specific for calcitonin mRNA in this species. This gene also codes for a CGRP molecule; elucidation of the 3′-region of this gene reveals the presence of an exon coding for a typical 37-amino-acid CGRP molecule [21], preceded by a Lys-Arg cleavage site and followed by a typical cleavage and amidation site. Thus, as in mammals, the calcitonin gene in chicken also expresses two different peptides.

We have recently elucidated the sequence of calcitonin mRNA in a non-mammalian vertebrate, chicken, and deduced the amino acid sequence of the hormone [11]. The calcitonin sequence in lower vertebrates is highly conserved, for example chicken and eel calcitonins differ by only two amino acids.

Calcitonin in mammals shows considerable differences in amino acid sequence between artiodactyls on the one hand and primates and rodents on the other. Both types of mammalian hormones show a low homology with calcitonins from lower vertebrates. Our results suggested that the calci-

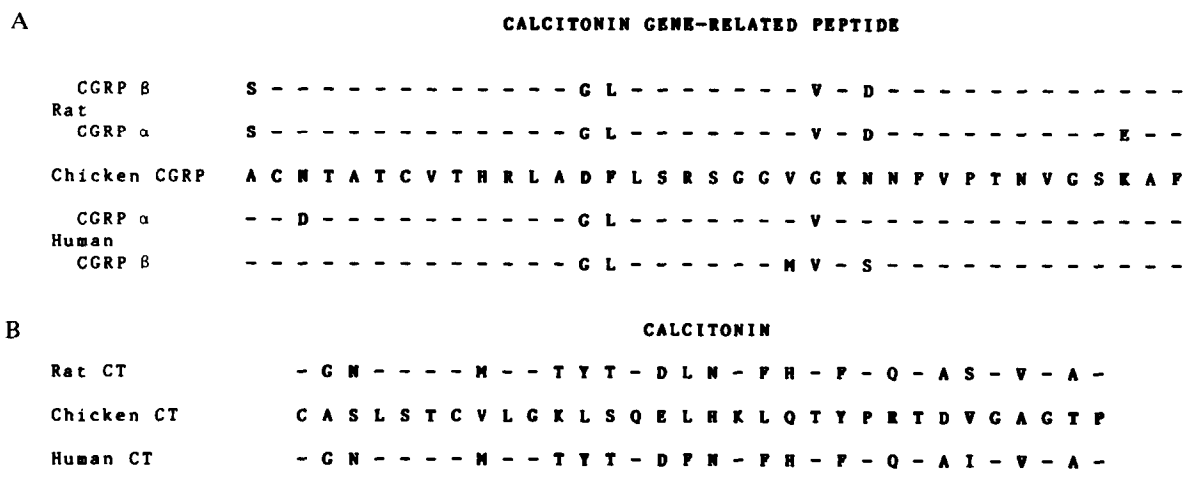

Fig.3. (A) Alignment of mammalian and avian CGRPs. Rat CGRP β [19], rat CGRP α [14], human CGRP α [21], human CGRP β [20]. (B) Sequence of chicken calcitonin compared with human and murine calcitonins. Rat CT [23], chicken CT [11], human CT [22].

tonin was highly conserved in lower vertebrates and that large modification in its sequence corresponded with the appearance of mammals [10,11].

In contrast to the calcitonin exon, the CGRP exon seems highly conserved. The high conservation of calcitonin sequence in non-mammalian vertebrates and the significant differences observed in mammalian vertebrates are in favour of a different physiological role for the hormone in these two types of vertebrates. This does not seem to be the case for CGRP. In fact, the high sequence homology between the avian and mammalian CGRPs favours the hypothesis that the physiological role of CGRP has not varied greatly during evolution and that this peptide plays a more primordial role than calcitonin.

The calcitonin gene is thus another interesting model for evolutionary studies as this gene expresses by alternate splicing two peptides, the rate of divergence of which varies considerably.

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