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Molecular cloning of a cDNA encoding the bombesin precursor in skin of *Bombina variegata*

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A cDNA library was constructed using poly(A)-rich RNA isolated from skin of the frog *Bombina variegata*. This library was screened with two oligo-nucleotides complementary to parts of the sequence of bombesin. The nucleotide sequence of one of the cloned cDNAs encoding a bombesin precursor is presented. The predicted polypeptide contains a single copy of the end-product. The bombesin sequence is preceded by a leucine residue suggesting an unusual type of precursor processing.

cDNA cloning; Bombesin precursor; Amino acid sequence; (Amphibian skin)

1. INTRODUCTION

About 20 years ago, bombesin and two related peptides termed ranatensin and alytesin were discovered in amphibian skin [1,2]. Several additional peptides belonging to the same family were subsequently isolated from skin of different frogs (reviewed in [3]). In experimental animals, these peptides cause an increase in blood pressure and have potent stimulatory effects on gastric acid secretion, enzyme secretion from the exocrine pancreas and contraction of the gall bladder. As had previously been shown for several other peptides from amphibian skin, the bombesin-like peptides were also later found to have counterparts in the gastrointestinal tract and the central nervous system of mammals. These are gastrin releasing peptide [4] and the neuromedins B and C [5,6]. Bombesin and other peptides belonging to this family have also been shown to act as growth factors for e.g. murine fibroblasts [7] and human small cell lung cancer cells [8].

Using cDNA cloning techniques, the structure of the precursors for ranatensin from skin of *Rana pipiens* [9], as well as human gastrin releasing peptide [10] and neuromedin B ([9], for review see [11]) could be elucidated. These are all derived from relatively small precursors containing a single copy of the end-product. Here we present the structure of a cloned cDNA encoding the precursor of bombesin in the skin of *Bombina variegata*. The structure of prepro-bombesin shows similarities to the precursor of ranatensin.

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2. EXPERIMENTAL

2.1. *Materials*

Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer Mannheim, Bethesda Research Lab., New England Biolabs, or Stratagene. All radiochemicals were from ICN Radiochemicals (Irvine, USA).

2.2. *RNA isolation and cDNA cloning*

B. variegata, 3–4 cm long, were caught in May in Lower Austria. Frogs were kept in water tanks at room temperature and fed with young crickets. Skin was prepared, immediately frozen in liquid nitrogen and stored at −70°C. Poly(A)-rich RNA was isolated as described earlier for skin of *Xenopus laevis* [12]. Double-stranded cDNA was prepared by standard procedures and inserted via GC-tailing into the *Pst*I-site of the Bluescript plasmid (Stratagene [13]).

2.3. *Screening of cDNA libraries and analysis of clones*

A cDNA library comprising about 25000 clones was sequentially hybridized [14] with the synthetic oligonucleotides 3′-GT(T/C)ACC-CGNCANCC and 3′-CCNGT(A/G)(A/G)ANTACCC, where N stands for all four nucleotides. These are the antisense oligonucleotides complementary to the segments Gln-Trp-Ala-Val-Gl(y) and Gly-His-Leu-Met-Gl(y), respectively, of the bombesin sequence and the glycine required for the formation of the carboxy-terminal amide. Positive clones were isolated and further characterized by cleavage with restriction endonucleases. Nucleotide sequences were determined using both the chemical degradation as well as the enzymatic method [15,16].

2.4. *Northern blot analysis*

Poly(A)-rich RNA from skin (3 μg) was fractionated in 1.2% agarose gels containing 0.8 M formaldehyde [17] and blotted directly on Nytran sheets (Schleicher & Schuell). The position of ribosomal RNAs used as standards was marked on the filters. Prepro-bombesin cDNA labeled by the primer extension method [18] was used for probing the Northern blots.

3. RESULTS AND DISCUSSION

A cDNA library prepared from skin of *B. variegata* was screened with two oligonucleotides complementary

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                                        ....CTCTCACAGCTCTGCGGTACTCACAGCTTTA

GACATGTCTGCGATTCCTCTGAACAGGATCCTGCCTCTAGGGTTCCTGCTGATTTTCTCC
   M  S  A  I  P  L  N  R  I  L  P  L  G  F  L  L  I  F  S

TTCATCTCTCTGTCCAGCTGCATGGAGTTCGTTGAAGATCCTAACAATCAGGGCGGTCTC
 F  I  S  L  S↑S  C  M  E  F  V  E  D  P  N  N  Q  G  G  L

AACCTGCAGCAGAGGCTGGGGAATCAGTGGGCAGTGGGTCACTTGATGGGTAAGAAGAGC
 N  L GlnGlnArgLeuGlyAsnGlnTrpAlaValGlyHisLeuMet G  K  K  S

CTGCAGGACACAGACTTTGAAGAGATGGAAAGTTTTGCTAAACGTAACGTTGAGAACATG
 L  Q  D  T  D  F  E  E  M  E  S  F  A  K  R  N  V  E  N  M

AAAGCAGAATCAGAAAGAGAGCTACGGCATGCACAGTTGGTAGTAAGGAACATCTTGGAG
 K  A  E  S  E  R  E  L  R  H  A  Q  L  V  V  R  N  I  L  E

CAGTATCTGAAGAATATGCAGAATTAGCAAAGAAATGTGTCTTCCTGTACATACAGAAAT
 Q  Y  L  K  N  M  Q  N  /

ATATTTGTGCCTGAGACATGGGACTTATTTTAAACATTCCAAAGTTTATTGTTTACAAAA

AAAAAACTGAATTCTAAAGACAATAAGAATTTTTCATTTATAATTGTAATTTAAGATCCA

TTTTCTAAATTTAAAGTATAAAAACAACTCATCCTCAGAGTATGTACGGAATATTTTTTC

TGACATTTTATGCAGTGTCTAACTAAAACCTGTGAATAAAAGTCATTCTTTGCAAA...
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Fig.1. Nucleotide sequence and deduced amino acid sequence of cloned cDNA coding for prepro-bombesin. Amino acids are abbreviated by the single letter code, except for the bombesin sequence, which is emphasized by using the three-letter code. The arrow marks the most likely site of signal peptidase cleavage. The poly-adenylation signal is underlined, the stop codon is marked as (/).

to parts of the mRNA for the bombesin precursor. Two dozen positive clones were picked and analyzed further. While differing in length at both the 5′- and 3′-ends, restriction mapping and partial sequencing indicated that their inserts were all transcribed from a single mRNA species. The sequence of the clone B-15 with the longest insert of 627 basepairs is shown in fig.1. This cDNA terminates with the poly-adenylation signal AATAAA and three adenines, which may thus be part of the poly(A)-tail. Most other clones terminated at the stretch of 10 adenines which occurs in the 3′-untranslated region (see fig.1). The single open reading frame present in clone B-15 encodes a bombesin precursor which comprises 107 amino acids. This predicted polypeptide starts with an initiating methionine and a signal peptide that most likely terminates at serine-24. The precursor contains one copy of the tetradecapeptide bombesin after which the typical prohormone processing sequence Gly-Lys-Lys is present. This is the site of endo- and exo-proteolytic processing as well as for the formation of the carboxy-terminal amide present in the end-product. The carboxy-terminal sequence contains one additional Lys-Arg, which is also a potential processing site.

Interestingly, in the precursor the bombesin structure is not preceded by a pair of basic amino acids but instead by a leucine residue. This suggests a rather unusual type of processing reaction by e.g. a chymotrypsin-like enzyme. Processing after leucine residues has also been observed in rat pancreas, where the C-peptide of pro-insulin is further hydrolyzed in vivo at a Leu-Ala bond [19]. Moreover, the liberation of rat relaxin from its precursor must also involve cleavage after a particular leucine residue [20].

Northern blot analysis of poly(A)-rich RNA from skin of *B. variegata* has shown the presence of a major mRNA species which hybridized with the labeled cDNA (fig.2). This mRNA contains about 700 nucleotides, which corresponds well with the size of the insert present in clone B-15. A second, minor component which comprises about 2000 nucleotides was also

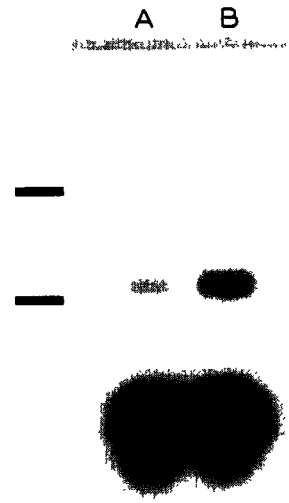

Fig.2. Northern blot of poly(A)-rich RNA from skin of *B. variegata*. (A) and (B) represent two different RNA preparations. The position of 18 and 28S ribosomal RNA is marked.

MSAIPLNRILPLGF***LLIFSFISLSSCMEFVEDPNNQGGLNLQQRLGNQWAVGHLM

MTTIPAIGILPIDFLTILLLFSFISHSVCVEFAEDAGELDKSNAFRRQVPQWAVGHFM

GKKSLQDTDFEEMESFAKRNVENMKAESERELRHAQLVVRNILEQYLKNMQN/

GKRSLSD*DTEQATMYSSRFVESTS//

Fig.3. Comparison of the sequences of prepro-bombesin (upper line) and prepro-ranatensin (taken from [9]). Identical amino acids are underlined. Two gaps marked (*) were introduced to maximize the homology. Stop codons are marked (/).

detectable. However, the corresponding cDNA has not been found in our library as yet. No positive signal could be observed when poly(A)-rich RNA from skin of *X. laevis* was used for the same Northern blot (data not shown).

The precursors for four members of the family of bombesin-like peptides are all encoded by fairly small mRNAs containing less than 800 nucleotides. After the signal peptide, only a small propeptide is found in the ranatensin [9] and bombesin precursors, while in case of the precursors of gastrin releasing peptide [10] and neuromedin B [9], the sequence of the mature peptide immediately follows the signal peptide. In all these precursors, only a single copy of the end-product followed by the common processing site Gly-Xaa-Xaa has been found, with Xaa being lysine or arginine. However, only the prepro-parts of the bombesin and ranatensin precursors show significant homology. These precursors are 47% identical in their amino acid sequence (see fig.3) and 58% in the common coding parts of their nucleotide sequences (data not shown), with the highest degree of similarity being present in the signal peptide and the end products. The bombesin precursor, however, contains an additional 27 amino acids at the carboxyl end. In the ranatensin precursor, a dibasic cleavage site is also present at the amino-terminal side of the end-product [9]. In prepro-bombesin, one of the arginine codons of this site is mutated to a glutamine codon. As a consequence, the above-mentioned hydrolysis of a leucine-glutamine bond must take place during the liberation of bombesin from its precursor. Processing enzymes have been detected in skin secretion of *X. laevis* [21–23]. It will be interesting to check whether this is also true in the case of *B. variegata* so that the search for this endoprotease specifically involved in the activation of the bombesin precursor could be initiated.

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