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Isolation and sequence of a cDNA encoding the precursor of a bombesinlike peptide from brain and early embryos of *Xenopus laevis*

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ABSTRACT **A cDNA encoding the precursor of a bombesinlike peptide was isolated from brain of *Xenopus laevis*. The predicted end product resembles neuromedin B, which was originally isolated from mammalian spinal cord. The mRNA for this precursor was also present in gastrointestinal tract and in ovaries. Moreover, it could be detected in early embryos (stage 2 and stage 10) of *X. laevis*. These findings suggest novel roles for peptides of the bombesin family in oocyte maturation and early amphibian development.**

Bombesin and ranatensin are related peptides originally isolated from skin of *Bombina bombina* and *Rana pipiens*, respectively (1, 2). Several peptides belonging to this family have since been detected in skin of other frog species (3, 4). As in other instances, homologues of these amphibian peptides were later found to be present in mammalian endocrine and neural cells also. Gastrin-releasing peptide (GRP) and its carboxyl-terminal decapeptide, formerly called neuromedin C, are both widely distributed in the nervous system and gastrointestinal tract as well as in normal and neoplastic lung (5–9). These peptides stimulate the release of hormones and enzymes (reviewed in ref. 3) and also act as potent mitogens on certain normal and tumor cells (10–12). Neuromedin B, another member of the family of bombesinlike peptides, was originally isolated from porcine spinal cord (13, 14). It is also present in many parts of the brain and the gastrointestinal tract, yet its distribution differs from that of GRP and its smaller fragments (6). Bombesin and related peptides bind to cellular receptors which belong to the class that is coupled to guanine nucleotide-binding proteins (15, 16). Present evidence indicates that in mammals at least two types of receptors with different affinities for GRP and neuromedin B exist (17–19).

By using molecular cloning techniques, the structures of the precursors for several of these peptides have been deduced (20–24). In particular, the cDNAs of the bombesin precursors from two species of *Bombina*, *B. variegata* and *B. orientalis*, have recently been analyzed (23, 24). The predicted precursor polypeptides contain a single copy of the biologically active end product. Using the cDNA cloned from skin of *B. variegata*, we have now isolated a cDNA encoding a homologous precursor from a library prepared from brain of *Xenopus laevis*.‡ The corresponding mRNA is also present in other tissues, and it is already expressed in early embryos of this frog.

MATERIALS AND METHODS

Materials. Restriction enzymes and modifying enzymes were obtained from Boehringer Mannheim, Bethesda Research Laboratories, and New England Biolabs; radiochemicals were purchased from NEN. Oligonucleotides were kindly supplied by G. Schaffner (IMP, Vienna).

RNA Isolation and cDNA Cloning. *X. laevis* tissues and embryos were prepared, immediately frozen on dry ice, and stored at −70°C. Total RNA was isolated by using guanidinium thiocyanate as described (25). To separate DNA from the RNA, a LiCl precipitation step was used instead of a CsCl gradient. Polyadenylylated RNA was obtained by chromatography over oligo(dT)-cellulose.

Screening of a cDNA Library and Analysis of Clones. Poly(A)-enriched RNA from brain of *X. laevis* was transcribed into cDNA by using oligo(dT) as primer and Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). The second strand was synthesized with polymerase I and RNase H (26). After methylation and the addition of *Eco*RI linkers, the cDNA was ligated with phage λgt11 arms (27). The DNA was packaged *in vitro* according to the supplier's recommendation. The library was amplified once on LB plates. As probe, we used the cloned cDNA from skin of *B. variegata* coding for preprobombesin (23), which was labeled by the random priming procedure (28). Hybridization was performed at 55°C for 16 hr in 100 mM sodium phosphate buffer (pH 7.0) containing 850 mM NaCl, 1 mM EDTA, 10× Denhardt's solution (1× = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 0.1% sodium dodecyl sulfate (SDS), and 100 μg of denatured salmon sperm DNA per ml. Filters were washed twice for 30 min at 50°C with 2× SSPE (1× = 0.15 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA)/0.1% SDS. Positive clones were isolated and subcloned in the Bluescript II plasmid (Stratagene). Nucleotide sequences were determined by the dideoxy chain termination method (29), using a Sequenase kit (United States Biochemical).

Northern Blot Analysis. Total RNA (10 μg) from different tissues or poly(A)-rich RNA (5 μg) from embryonic stages of the developing tadpole (30) were fractionated on 1% agarose gels containing 0.8 M formaldehyde (31). RNA was blotted onto nylon membranes (Schleicher & Schuell) and hybridized with labeled cDNA coding for the *X. laevis* neuromedin B precursor at 65°C. Filters were washed at the same temperature in 0.2× SSPE/0.1% SDS and then used for autoradiography.

PCR Analysis. Total RNA (10 μg) from ovaries, gastrointestinal tract, and stage 10 embryos was transcribed into cDNA, using oligo(dT) as primer. Two oligonucleotides marked in Fig. 1 were used for the PCR. From each RNA sample, fragments containing about 250 base pairs could be amplified. These were subcloned in the Bluescript II plasmid and sequenced.

RESULTS

Using mRNA isolated from brain of *X. laevis*, we prepared a cDNA library in phage λgt11 by standard procedures. As a

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Abbreviation: GRP, gastrin-releasing peptide.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. L01530).

probe we used the cloned preprobombesin cDNA from skin of *B. variegata* (23). Several positive phages were further purified and their inserts were subcloned in the Bluescript vector. Two clones were then sequenced, and the nucleotide sequence of the one with the larger insert is shown in Fig. 1. This cDNA comprises 650 nucleotides excluding the poly(A) tail. After the second ATG initiation codon, an open reading frame is present which can be translated into a polypeptide containing 120 amino acids. Of these, the first 29 have the typical features of a signal peptide. In the predicted polypeptide, the sequence of a decapeptide of the bombesin family is present, flanked by typical processing signals—namely, a pair of arginine residues at the amino-terminal side and the sequence Gly-Lys-Lys at the carboxyl-terminal side. This peptide terminates with the sequence His-Phe-Met-NH_2 and thus belongs to the ranatensin/neuromedin B group (3) of bombesinlike peptides (see Fig. 2). Since the mRNA encoding this peptide is present in brain but not in skin (see below), we propose to name it *Xenopus* neuromedin B.

Tissue Distribution. We used Northern blots to investigate the presence of the mRNA encoding the *Xenopus* neuromedin B precursor in various tissues. Total RNA was isolated from brain, skin, lung, liver, muscle, kidney, gastrointestinal tract, ovaries, and testis of *X. laevis*. An mRNA of size similar to that in brain was found to be present in intestine (see Fig. 3*A*). Surprisingly, a strong signal was also obtained with total RNA from ovaries. After separation of oocytes and follicle cells, the mRNA for the *Xenopus* neuromedin precursor was present in both fractions (data not shown).

Several other tissues, in particular skin and lung, do not contain significant amounts of this mRNA. It could also not be detected in lung from tadpoles (stage 53 to 63).

Developmental Northern Blots. We also performed Northern blots with RNA isolated from various developmental stages of *X. laevis*. As shown in Fig. 3*B*, a signal was detected in stage 2 and stage 10 embryos, which then disappeared, but was again found in feeding tadpoles (stage 45).

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                        ----------------------->
CTGGGGATGTCTGCTGTGCCTCTGACTAGGATGCTGCCGCTCAGGTTTCTCACTCACCTG
      M  S  A  V  P  L  T  R  M  L  P  L  R  F  L  T  H  L

CTGCTTCTCTCCTTCATCCCACTCTATTTTTGCATGGAATTTTCTGAAGATGCTAGAAAT
 L  L  L  S  F  T  P  L  Y  F  C  M  E  F  S  E  D  A  R  N

ATAGAAAAAATCAGGAGAGGAAATCAGTGGGCAATTGGGCATTTCATGGGTAAAAAGAGC
 I  E  K  I  R  R GlyAsnGlnTrpAlaIleGlyHisPheMet G  K  K  S

CTGCAAGACACATATAATCCATCTGAACAGGACATGGATTCAGAAGACTTTCGTCCAAGA
 L  Q  D  T  Y  N  P  S  E  Q  D  M  D  S  E  D  F  R  P  R

                  <-----------------
ATCATTGAAATGATAAGAGGCACCTTCCGGCAAGAGCCGATTAGAGCACTGTCGCCCAGA
 I  I  E  M  I  A  G  T  F  R  Q  E  P  I  R  A  L  S  P  R

AAGCAAGATGAAATACAGTGGATGTTAAAGAAGATCATGGATGATTATATTAAAACGACT
 K  Q  D  E  I  Q  W  M  L  K  K  I  M  D  D  Y  I  K  T  T

CAGAAGTAGCGAAGACACTGTTTCTCCTGTACATACAAAAATATATCTATTTGTGCCTGA
 Q  K  /

GATATACTACATATTTCACATATTCCAAAGGCTGTTGTTTACAAATAAAACCATAAAATG

CTTATGAAAATCTGAGTGTTTCAGTTCATACAATGATTTTGGAAGTAATTTTTTTACAAA

ATTGTTAAAGTAAAAAGCATCATCTATTTAGAATATGTACCGAGTGTTTTTTTCTGCCAT

TTGAACTTAGTGTTCTAAAGAAAACTGTGAATAAAAGCCATTCTTTACTCAT(polyA)
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FIG. 1. Nucleotide and deduced amino acid sequence of clone B5A from brain of *X. laevis*. The amino acid sequence is given in the single-letter code; only the sequence of the bombesinlike peptide is emphasized by using the three-letter code. The stop codon is marked (/), and the polyadenylylation signal is underlined. The sense and antisense oligonucleotides used for the PCR experiments are overlined.

```
1          G N Q W A I G H F M*
2          G N L W A T G H F M*
3       <Q V P Q W A V G H F M*
4  <Q Q R L G N Q W A V G H L M*
5          G N H W A V G H L M*
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FIG. 2. Sequence comparison of several bombesinlike peptides. 1, Neuromedin B (*X. laevis* brain); 2, neuromedin B (pig brain); 3, ranatensin (*Rana pipiens* skin); 4, bombesin (*Bombina bombina* skin); 5, GRP, carboxyl-terminal decapeptide (pig gastrointestinal tract). *, Carboxyl-terminal amide; <Q, pyroglutamic acid.

PCR Experiments. We wanted to test whether the mRNAs present in gastrointestinal tract, ovaries, and early embryos encode the same or related precursors of *Xenopus* neuromedin B as the one from brain. Accordingly, total RNA from these tissues was first transcribed into cDNA, using oligo(dT) as primer. This cDNA and the two oligonucleotides marked in Fig. 1 were then used for the PCR. The PCR products were cloned in the Bluescript plasmid and sequenced. Save for occasional point mutations apparently introduced by the *Taq* DNA polymerase, the cDNA sequences from gastrointestinal tract, ovaries, and stage 10 embryos were identical to the one determined for the cloned mRNA from brain. This indicates that the same gene is expressed in adult tissues and in early embryos.

DISCUSSION

By using the cloned cDNA of preprobombesin from skin of *B. variegata* as a probe, a cDNA encoding a homologous

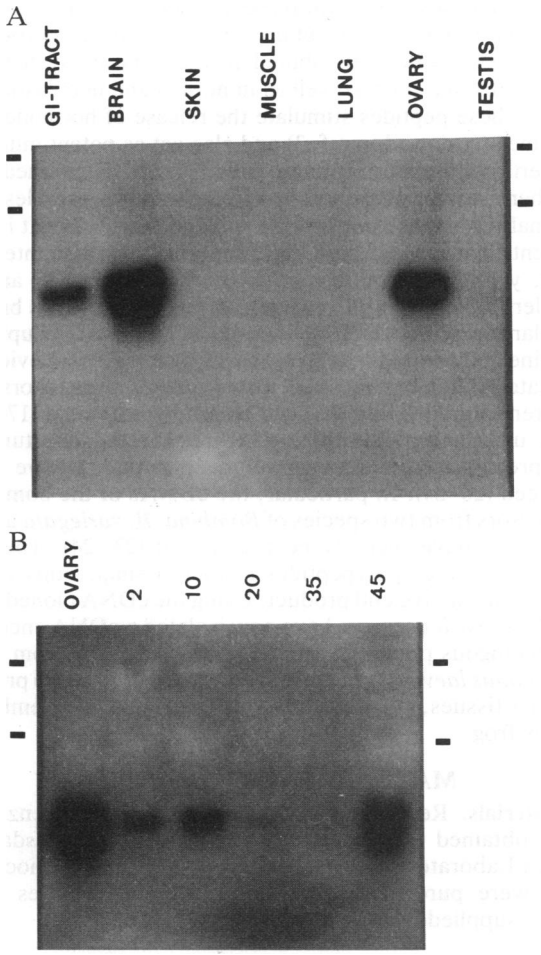

FIG. 3. Northern blot analysis of RNA from various tissues (*A*) and embryonic stages (*B*) of *X. laevis*. The positions of 18S and 28S rRNA are marked.

precursor could be isolated from an *X. laevis* brain cDNA library. This cDNA encodes a precursor polypeptide containing 122 amino acids, a size that corresponds to that of other known precursors of the bombesin family of peptides. The similarity between the cDNAs from brain of *X. laevis* and skin of *B. variegata* is highest in the sequence encoding the mature decapeptide and the region immediately following this part. Moreover, some similarity also exists in the central region of the signal peptides and at the amino end of the predicted propeptide. In all these parts, the *X. laevis* cDNA displays some, albeit weaker, similarity to the cDNA from skin of *Rana pipiens* encoding the ranatensin precursor (22). Conversely, apart from the segment encoding the final product, no significant homology between the *Xenopus* cDNA and the one encoding the human neuromedin B precursor (22) could be detected.

The predicted end-product encoded by this cloned mRNA from brain of *X. laevis* is a decapeptide with the structure Gly-Asn-Gln-Trp-Ala-Ile-His-Phe-Met-NH_2. As this sequence differs by only two residues from that of mammalian neuromedin B (see Fig. 2), we propose the name *Xenopus* neuromedin B for this peptide. A notable difference is found at residue 6, which is isoleucine in the *Xenopus* peptide, while all other known members of the bombesin family contain either valine or threonine at this position (3). The presence of bombesin- and ranatensinlike peptides in amphibian brain has earlier been demonstrated by immunocytochemistry (32). Moreover, a peptide closely related to mammalian GRP/neuromedin C has been isolated from brain of *Rana ridibunda* (33).

Northern blot analysis showed that the mRNA encoding the *Xenopus* neuromedin B precursor was also present, as expected, in gastrointestinal tract. This mRNA is, however, not expressed to any detectable extent in the skin of this frog. This is in line with the fact that peptides belonging to the bombesin family could not be detected in skin secretion of this species (34). In this respect, *X. laevis* differs from other amphibian species, for example *Bombina*, *Rana*, and *Litoria* species (3), in which, as already mentioned, bombesinlike peptides are major constituents of the skin secretion.

The Northern blot experiments also demonstrated the presence of relatively large amounts of the mRNA encoding the *Xenopus* neuromedin B precursor in ovaries. The presence of bombesinlike peptides in this tissue has not been reported for any other species as far as we know. Moreover, this mRNA is also present in small quantities as maternal message in stage 2 embryos—i.e., the two-cell stage. Apparently, it is then expressed early in development in midgastrula around stage 10. Subsequently, the mRNA disappears, only to reappear in late embryos after stage 45.

These findings prompt a number of questions. First, is this mRNA indeed translated and, if so, is the resulting preproneuromedin B then processed to yield the final product? It is known that early embryos contain many dormant mRNAs. Moreover, mature *Xenopus* oocytes are apparently incapable of processing precursors of peptide hormones (35). As was shown recently, these cells do not contain the mRNAs for the prohormone processing enzymes PC1/PC3 and PC2, which hydrolyze peptide precursors at pairs of lysine/arginine residues (36). Current evidence indicates that these cells have only the constitutive pathway of secretion and lack the regulated one. This may also be the case for early embryos at least up to the midblastula transition. The end-product neuromedin B, if present prior to this transition, would thus have to be generated at an early stage of oocyte development. It is also not clear whether at the gastrula stage, when rather high levels of the neuromedin precursor mRNA are detected, all the components for the transport, processing, storage, and secretion of the final product are present.

For the time being one can only speculate about the possible role of a bombesinlike peptide in early amphibian development. Based on the role of this family of peptides in mammals, two main possibilities exist. Such a peptide could be a growth factor required for the high rate of cell division ensuing after fertilization. Alternatively, a role as a secretagogue that triggers the release of stored components is conceivable. Control of cell proliferation by bombesin and related peptides has in fact been documented for a number of cell types (reviewed in ref. 37). In particular, such a peptide may act as a growth factor in fetal mammalian lung. In humans, the mRNA encoding GRP is transiently increased during fetal lung development (38). In addition, it was shown that bombesin can activate cell proliferation in otic vesicles from chicken embryos (39). It will be interesting to test whether a peptide of this family plays a similar role in early amphibian development.

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