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A cDNA from brain of *Xenopus laevis* coding for a new precursor of thyrotropin-releasing hormone

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Thyrotropin-releasing hormone (TRH) is found in large amounts in the skin of *Xenopus laevis*. In this tissue, 3 TRH precursor mRNAs can be detected of which the 2 more expressed encode almost identical proteins. However, Northern blot analysis of TRH precursor mRNAs in the brain of *X. laevis* revealed the existence of a new mRNA of about 1200 nucleotides which was present along with the larger TRH precursor mRNA identified in the skin. A cloned cDNA of a TRH precursor, corresponding in size to this new mRNA, was isolated and sequenced from a *Xenopus* brain λgt11 library. It encodes a precursor polypeptide which also contains 7 copies of TRH. However, at the amino acid level it differs by about 16% from the corresponding prepro-TRHs from skin. We have also attempted to characterize the gene encoding this prepro-TRH from *Xenopus* brain. Only the first and part of the second exon could be detected which are separated by an intron containing more than 8000 base pairs. Interestingly, the 5′-flanking region of this gene does not contain the characteristic promoter elements of the mammalian TRH genes suggesting marked differences in the regulation of their expression.

Thyrotropin-releasing hormone; Precursor; *Xenopus laevis*

1. INTRODUCTION

Thyrotropin-releasing hormone (TRH) has been identified in many regions of the mammalian brain [1–3] and in the gastrointestinal tract [4]. Like many other neuropeptides, TRH is also present in the skin of certain amphibians, sometimes in very high concentrations [5–7]. After the isolation of TRH [8,9], the mechanism of its biosynthesis remained unknown until, about 15 years later, a cDNA coding for the precursor of TRH was isolated from a cDNA library prepared from skin of *Xenopus laevis* [10]. Sequence analysis of cDNAs encoding the TRH precursors has since been carried out for several species [11–13]. They predict that the structure of prepro-TRH consists of several separate copies of the TRH progenitor sequence (Gln-His-Pro-Gly) each flanked by pairs of basic residues and linked together by one of several connecting peptides.

In mammals, TRH is a strong stimulator of thyrotropin (TSH) secretion by the anterior pituitary gland [14,15]. The expression of the hypothalamic TRH gene is negatively regulated by thyroid hormones [16,17]. Moreover, a thyroid hormone inhibitory element has been identified in the 5′-flanking region of rat [18] and human [12] prepro-TRH genes. A different situation is encountered in amphibians where TRH is not a physiological regulator of TSH secretion but is involved in the control of the melanotropic cells [19,20].

It would thus be of some interest to investigate the biosynthesis of TRH in amphibian brain. Here we show that 2 mRNAs for prepro-TRH are expressed in brain of *X. laevis*. Northern blot analysis, using different labelled fragments from *Xenopus* skin prepro-TRH cDNAs, suggested that the smaller of the 2 brain mRNAs was different from the ones present in skin. The sequence of this new TRH precursor as deduced from cloned cDNA is presented in this publication.

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2. EXPERIMENTAL

2.1. *Materials*

Restriction enzymes and DNA-modifying enzymes were obtained from New England Biolabs (Bethesda Research Laboratories, Stratagene) or Boehringer-Mannheim. All radiochemicals were purchased from the Radiochemical Center (Amersham, UK).

2.2. *RNA isolation and cDNA cloning*

RNA from *Xenopus* brain was isolated using guanidinium thiocyanate essentially as described [21] and passed over oligo(dT) cellulose to select for poly(A)+ RNA. cDNA was synthesized with MMLV reverse transcriptase with oligo(dT) as a primer according to the manufacturer's recommendations (Bethesda Research Laboratories). The second strand was synthesized by using RNase H and DNA polymerase I [22]. Double-stranded cDNA was further modified according to a standard protocol [23] and ligated with phage λgt11 arms (Promega). The ligation reaction was packaged in vitro (Stratagene) and the resulting library was amplified once on Luria-Bertani (LB) agar plates.

2.3. *Northern blot analysis*

RNA was separated by electrophoresis on 1.2% agarose gels con-

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taining 0.8 M formaldehyde essentially as described [24], blotted onto Nytran filters (Schleider & Schuell) and bound to the matrix by UV irradiation. The filters were hybridized as described by Church and Gilbert [25]. Fragments from clones L4 and C6 previously isolated from *Xenopus* skin [13] were labelled according to Feinberg and Vogelstein [26] and used to probe the blots.

2.4. *Screening*

From the cDNA library about 200,000 clones were plated and 2 sets of filter replicas were produced. One set of filter replicas was hybridized with the non-coding 3′ regions of clones L4 and C6, while the other set was hybridized with the coding region of clone 8/136 [10]. The hybridization was done in 750 mM NaCl, 100 mM sodium phosphate, pH 7, 10× Denhardt's solution, 0.1% SDS and 100 μg/ml denatured herring sperm DNA at 68°C overnight. Filters were washed at 65°C with 2× SSPE, 0.1% SDS and 0.2× SSPE, 0.1% SDS (2 × 20 min each), then they were exposed on Kodak XAR film. Positive phages were isolated, further characterized by cleavage with restriction endonucleases and subcloned into Bluescript vectors. Nucleotide sequences were determined using the enzymatic method [27]. Sanger sequencing was performed on double-stranded plasmid DNA by using a Sequenase kit and conditions recommended by the manufacturer (United States Biochemical Corp.).

2.5. *Genomic clones*

A genomic library from *X. laevis* was kindly supplied by Dr. I.B. Dawid. This library had been prepared from a partial *Mbo*I digest of erythrocyte DNA, which was then cloned into the *Bam*HI site of λJ-1 [28]. About 500,000 phages were screened with the coding region of the TRH cDNA. Phages giving a positive signal were purified and digested with appropriate restriction endonucleases. The resulting fragments were subcloned into the plasmid Bluescript (Stratagene) for sequence analysis.

3. RESULTS

3.1. *Pattern of TRH mRNAs in* Xenopus *skin*

To resolve the major TRH mRNAs expressed in *X. laevis* skin and brain, Northern blot analyses were performed. Using a TRH cDNA probe that recognizes TRH mRNAs, 3 species were separated on Northern blot (Fig. 1A). The 2 predominant mRNAs have approximate sizes of 1500 and 3000 nucleotides and these probably correspond to the inserts of clones L4 and C6 previously isolated in the skin [13]. An additional mRNA with respective apparent size of 1000 nucleotides was less abundant. Analysis of the Northern blot in Fig. 1A with a probe specific for the inserts of clones L4 and C6 revealed the 2 main bands (Fig. 1B). Hybridization with probe 3′-C6, which is specific only for the insert of clone C6, revealed a single band of about 3000 nucleotides (Fig. 1C). Thus, in the *Xenopus* skin, the 2 mRNAs corresponding to clone C6 and L4 are major expressed TRH mRNAs.

3.2. *Pattern of TRH mRNAs in* Xenopus *brain*

Northern blot analysis of *X. laevis* brain with TRH probe detected 2 bands of equal intensity (Fig. 1D), of which one has an identical size to the largest mRNA from skin. It was assumed to represent the mRNA corresponding to the insert of clone C6, because it hybridized with both the 3′-L4 and 3′-C6 probes (Fig. 1E,F). The other species containing about 1200 nucleotides was detected only with the TRH probe. This mRNA was not present in the skin.

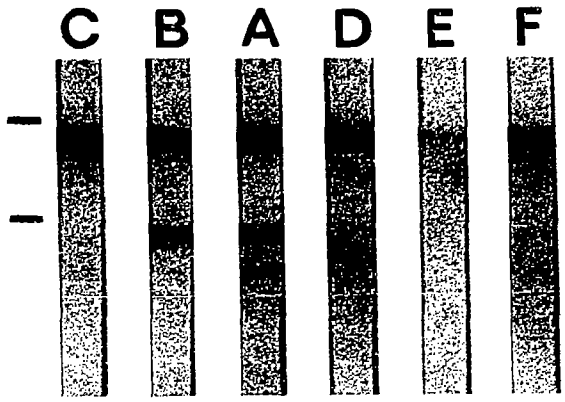

Fig. 1. Fractionation by Northern blot of *Xenopus laevis* TRH mRNAs extracted from skin (A, B and C) and brain (D, E and F). Using the TRH-cDNAs (named 8/136, L4 and C6) isolated from a *X. laevis* skin cDNA library, 3 fragments originating from the 3′-untranslated L4 cDNA (3′-L4 probe), the 3′-untranslated C6 cDNA (3′-C6 probe) and the coding region of the 8/136 cDNA (TRH probe) were labeled with α-^{32}P. To each lane 3 μg of poly(A)-rich RNA were applied. Migration of TRH-, L4- and C6-related mRNA species was detected by hybridization with TRH probe (A,D), 3′-L4 probe (B,E) and 3′-C6 probe (C,F). The exposure time was reduced from 12 to 3 h for lanes A, B and C. Horizontal bars indicate the position of 18 and 28 S ribosomal RNAs.

3.3. *Characterization of brain cDNA clones*

A λgt11 cDNA library constructed from *Xenopus* brain was then screened by the 3 probes derived from skin cDNAs. One clone (named bTRH), which only hybridized with a probe derived from the coding region of skin prepro-TRH cDNA, could be isolated. The nucleotide sequence of the insert in clone bTRH is shown in Fig. 2. The open reading frame encodes a TRH precursor which comprises 224 amino acids giving rise to a calculated M_r of 26,151. The predicted polypeptide starts with an initiating methionine and a signal peptide (as evident from the abundance of hydrophobic amino acids) that most likely terminates at serine 15 or 20. This precursor contains 7 copies of the TRH progenitor sequence (Gln-His-Pro-Gly) which are flanked on both sides by pairs of basic residues.

Upon partial sequence analysis of another brain cDNA clone (data not shown), which corresponded to the open reading frame of skin TRH cDNAs, it became clear that the 2 groups of predicted TRH precursors shown in Fig. 1, reflecting the existence of 2 different TRH mRNAs, are indeed present in the brain of *X. laevis*. Comparison of the nucleotide sequences of their open reading frames (Fig. 2) reveals several point mutations and small deletions/insertions. The overall degree of amino acid homology is only 84%, and all changes are exclusively localized within extra-TRH sequences.

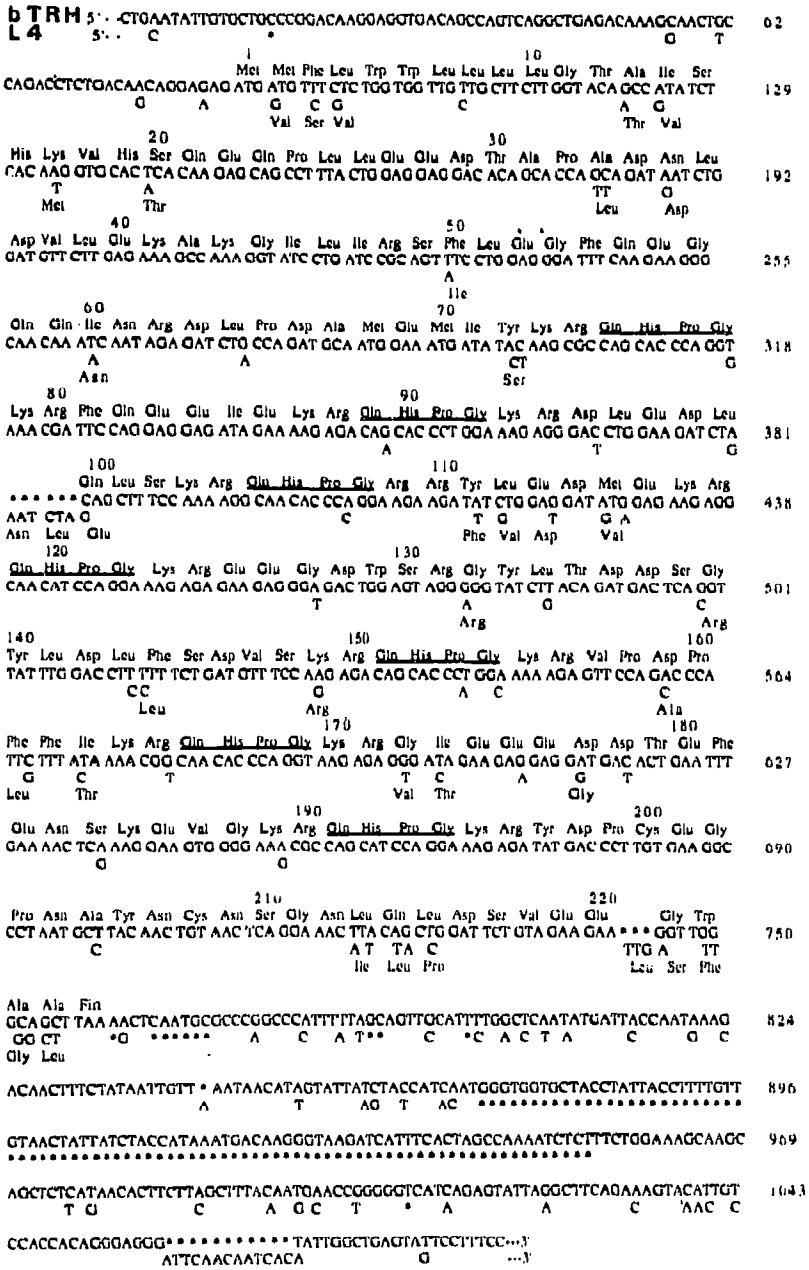

Fig. 2. Comparison of the nucleotide and the deduced amino acid sequences of brain (bTRH) and skin (L4) clones. The nucleotide and amino acid differences found in the L4 sequence are shown beneath the sequence of bTRH; absence of a nucleotide or an amino acid in the L4 sequence indicates that the L4 and bTRH sequences are the same at that position. Deletions are marked by asterisks. Amino acid residues are numbered from the putative start codon. The copies of TRH progenitor sequence (Gln-His-Pro-Gly) are underlined.

However, the structural organization of these predicted TRH precursors is identical.

3.4. *Partial characterization of the* Xenopus laevis *bTRH gene*

The *X. laevis* bTRH gene (Fig. 3) contains at least 2 exons separated by an intron of approximately 8000 bp. Exon 1 encodes the 5′-untranslated region, extending from the transcriptional origin to a site 5 bases upstream from the initiating methionine. The amino-terminal prepro-TRH is encoded by exon 2. Within the characterized protein-coding region, the nucleotide sequence of the genomic clone was identical with that of the bTRH cDNA. However, the genomic clone and the cDNA differed by 2 bases within the 5′-untranslated region. It is likely that these differences could represent either reverse transcriptase errors or polymorphisms. The 5′-flanking region revealed only a single TATA

gtatgcaacactcccattattaaagttaaacaacttccatcatagcatactaaccctaaccctatcca
gacatgacactgcacatggctagtgaatttgatatgtgttcagtacttgatgctcctcagaggtgaagc
aaatgcaaaaaaaaataaatacatattccctattactcgcacagtataacgcgaaagattagaataac
acccctatctatttgatctcagagcgcaaatcttacgtcagttgggctgaggaggttaagagtcccccc
tgaaatgctgcatttctgacatcaaagaggtgtttaaaaaaagcgaagacatatccctaactgcatcac
agggatctctataattggaccaggggcggtataaatcggggcggcagcagcataaagcagcacaga

↓

GCAGCACAGGGACACACTCTGAATATTGTGCTGCCCGGACAAGGCGGTGACAGCCA

GTCAGGCTGAGACAAAGIAACTGCCAGACCTCTGACAACAGgtaactgagccttcccct

ctatacictctgcaa····(8000 bp intron)····tgctatttaatcttctgtatattaaattacag

GAGAG *ATG* ATGTTTCTCTGGTGGTTGTTGCTTCTTGGTACAGCCATATCTCACAAG

GTGCACTCACAAGAGCAGCCTTTACTGGAGGAGGACACAGCACCAGCAGATAATCT

GGATGTTCTTGAGAAAGCCAAAGGTATCCTGATC····

Fig. 3. Partial nucleotide sequence of a *Xenopus laevis* gene encoding a brain prepro-TRH. Exons are shown in capital letters while intron and flanking sequences are in lower case letters. The putative transcriptional start site is indicated by an arrow. Two nucleotide changes in sequence from bTRH cDNA are indicated by underlining. The TATA box is shown in bold and the translational starting codon (ATG) in italics.

sequence as the typical consensus sequence of regulation.

4. DISCUSSION

The data presented in this communication show that different mRNAs for TRH precursors are present in skin and brain of *X. laevis*, respectively. In the former, 3 mRNAs could be detected, of which the 2 major ones, corresponding to the cloned cDNAs termed L4 and C6, have been characterized previously [13]. These 2 cDNAs have almost identical 5'- and coding sequences but differ considerably in their 3'-untranslated regions. Clones corresponding to the third mRNA present in skin have not yet been found. Conversely, only 2 major mRNAs could be detected in brain of this frog, of which the larger one probably corresponds to the cDNA clone, C6, isolated from skin. Surprisingly, a new TRH mRNA was found in brain. The insert present in clone bTRH codes for a prepro-TRH similar to the ones present in skin with 7 copies of the sequence Gln-His-Pro-Gly flanked by pairs of basic amino acids. However, in the other regions of the precursor polypeptide, numerous differences have been found. In view of the fact that the genome of *X. laevis* has apparently been duplicated about 30 million years ago [29], it is not surprising to find different mRNAs. For example, 2 groups of structurally different cDNAs coding for preproenkephalin [30] and preproopiomelanocortin [31] have been found, indicating that pairs of closely related genes are expressed in *X. laevis*. In case of the TRH-precursor genes, the structural data suggest the following interpretation: clones L4 [13] and the original clone 8/136 [10] are derived from alleles of the same gene as they differ by only about 1% in the nucleotide sequence determined for both clones. Clone C6 would be derived from the other pair of genes present due to the chromosome

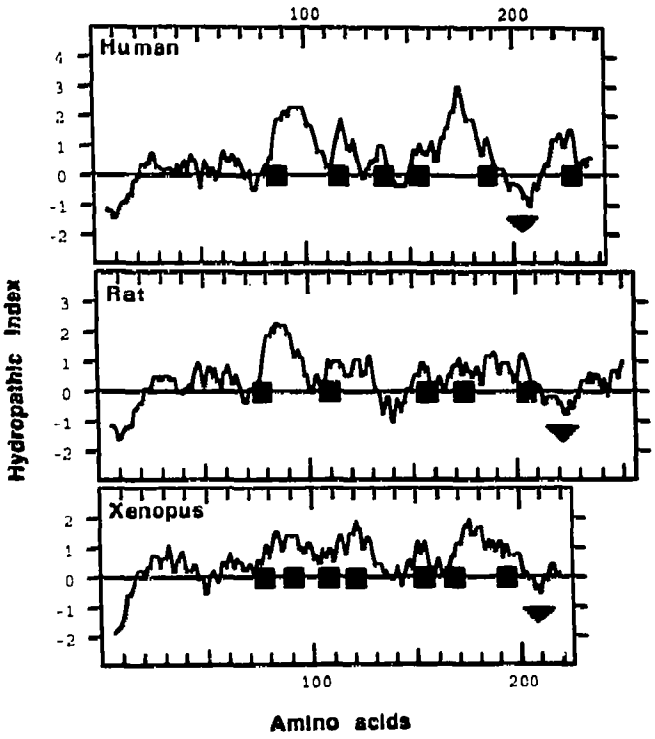

Fig. 4. Hydropathy profiles of the prepro-TRH from human, rat and *Xenopus laevis*. Hydropathy profiles were computed according to Hopp and Woods [33] using a window size of 10 residues. These give a quantitative estimate of hydrophilic (above the line) or hydrophobic (below the line) character of the sequences. Solid squares indicate the positions of the TRH progenitor sequences flanked by pairs of basic residues. Triangles indicate a peak of hydrophobicity present in both precursors. (Lower) Alignment of amino acid sequences of regions indicated by a triangle in the hydropathy profiles. Amino acid residues that are homologous are thrown into relief. Hyphens indicate gaps introduced to optimize homology. The sequences are from [11] and [12].

duplication. In addition, the sequence of the cDNA of the brain TRH-precursor presented in this communication may correspond to yet another gene generated by a gene duplication event that predates the tetraploidization of the genome. These assumptions could be tested by using *X. tropicalis*, a diploid relative of *X. laevis*.

Based on the deduced amino acid sequence of the *X. laevis* TRH prohormones, proteolytic processing of these gene-duplicated precursors is expected to produce 7 copies of TRH along with several other non-TRH peptides. These peptides should correspond to the leading and trailing pro-TRH peptide sequences and to connecting segments flanking the repeated TRH progenitor sequences. In addition, in the *X. laevis* skin, evidence was obtained for the presence of peptides which were extended on the NH_2-terminal or COOH-terminal side of TRH [32]. Unexpectedly, no similarity could be detected between the sequences of the connecting peptides in the TRH precursors from *X. laevis* and mammals,

respectively. Using hydropathy plots, one can see that the side chains of the amino acid sequences of the TRH polypeptides from human, rat and *Xenopus* show a strong hydrophilic character outside the portion corresponding to the predicted signal sequences (Fig. 4). However, in each of the precursors, a peak of hydrophobicity occurs in the COOH-terminal over a region of the order of 10 amino acids. In this region, the corresponding amino acid sequences of both precursors reveal a relatively high resemblance (Fig. 4). Whether these homologous sequences have a biological function is a matter of speculation, but they might participate in the intracellular routing of the prohormones.

The 5'-flanking region of *X. laevis* TRH gene, apart from the canonical TATA box, contains none of the actually known regulatory elements. Thus, a sequence, CAGGGTTTCC, located in the human [12] and rat [18] TRH genes, respectively at position −146 bp and −136 bp, was not found in the *X. laevis* counterpart. This sequence seems to be important for thyroid hormone gene regulation and the fact that it is not conserved suggests, as has already been shown at the pituitary level for the activity of TRH [19,20], that the amphibian TRH gene fulfills physiological roles different from those in mammals.

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