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Biosynthesis of thyrotropin releasing hormone in the skin of *Xenopus laevis*: partial sequence of the precursor deduced from cloned cDNA

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Skin of *Xenopus laevis* contains relatively large quantities of thyrotropin releasing hormone (TRH). Total mRNA isolated from skin was cloned in the plasmid pUC8. Among 1400 cDNA clones, one was found with an insert of 478 nucleotides coding for the amino-terminal part of prepro-TRH. This clone was detected using a mixture of two synthetic undecanucleotides for colony hybridization. The single open reading frame starts with a methionine residue and a stretch of hydrophobic amino acids, as is typical for signal peptides, and terminates at the poly(C) tail without a stop codon. The deduced polypeptide of 123 amino acids contains three copies of the sequences Lys-Arg-Gln-His-Pro-Gly-$^{Lys}_{Arg}$-Arg and a fourth incomplete copy at the carboxyl end. Typical prohormone processing at this sequence would yield pGlu-His-Pro.NH_2, i.e. TRH. It is concluded that the cloned part of the mRNA codes for prepro-TRH and that the TRH precursor from skin of *X. laevis* is a polyprotein containing at least four copies of the end product in its amino acid sequence.
Key words: cDNA cloning/frog skin peptides/precursor processing/prohormone/thyrotropin releasing hormone

Introduction

The isolation and structural analysis of thyrotropin releasing hormone (TRH) has been reviewed by Schally (1978) and by Guillemin (1978) in their Nobel lectures. From several hundred thousand ovine or porcine hypothalami, a few milligrams of purified peptide could be obtained for which the structure pGlu-His-Pro.NH_2 was determined. Several years later, Jackson and Reichlin (1977) made the important observation that TRH is present in the skin of the frog *Rana pipiens* at concentrations more than ten times higher than found in mammalian brain. This finding was corroborated for several other amphibian species including *Xenopus laevis*, where up to 15 μg TRH/g skin have been detected (Bennett *et al.*, 1981). Numerous studies by Erspamer and his colleagues have in fact shown that amphibian skin abounds in peptides also found in mammalian brain and gastrointestinal tract (Erspamer, 1971; Erspamer *et al.*, 1981; Erspamer and Melchiorri, 1983).

The biosynthesis of a simple tripeptide with a very high biological activity is of obvious interest. We have been operating under the assumption that TRH, like other, more complex peptide hormones, is derived from a larger precursor synthesized on ribosomes. In such a putative precursor, the sequence Gln-His-Pro-Gly should be present, where the glutamine residue cyclizes during processing and maturation to yield pyroglutamic acid and the extra glycine is required for the formation of the terminal amide (Suchanek and Kreil, 1977; Bradbury *et al.*, 1982; Eipper *et al.*, 1983). There has been one report that TRH is synthesized by soluble enzymes via a ribosome-independent mechanism (Mitnick and Reichlin, 1972) which could, however, not be confirmed in other laboratories (Bauer and Lipmann, 1976; Rupnow *et al.*, 1979). This possibility becomes even more unlikely since other small peptides like enkephalins (Noda *et al.*, 1982), vasopressin (Land *et al.*, 1982), and oxytocin (Land *et al.*, 1983) are all liberated from larger precursor polypeptides.

For an investigation of the biosynthesis of TRH, amphibian skin is the most promising starting material. We have previously prepared a cDNA library from total mRNA isolated from the skin of *X. laevis*. Using a synthetic decadeoxynucleotide as primer for the synthesis of radioactive cDNA, several clones could be found with inserts coding for precursors of caerulein, a skin peptide related to mammalian gastrin and cholecystokinin (Hoffmann *et al.*, 1983a). Moreover, the sequence of the precursor of a new peptide termed PYL[a] (Hoffmann *et al.*, 1983b) could also be deduced from the cDNA sequences of clones hybridizing with the radioactive cDNA obtained by primer extension.

Here we present the sequence of a cDNA clone from skin of *X. laevis* which codes for the NH_2-terminal 123 amino acids of a TRH precursor. This was the only clone out of 1400 which strongly hybridized with a mixture of two undecamers complementary to the codons for the tetrapeptide Gln-His-Pro-Gly (see Figure 1). Three complete and one incomplete copy of this peptide, flanked by pairs of basic residues, are present in this segment of the TRH precursor.

Results

Synthesis and labeling of oligodeoxynucleotides

A mixture of the two undecamers d(CC$^{C}_{T}$GGGTGTTG) was synthesized according to Wallace *et al.* (1981) with some modifications (see Materials and methods). These probes were originally used as primers for the synthesis of cDNA from total skin mRNA as described previously (Hoffmann *et al.*, 1983a). In DNA-RNA hybrids, dG-U and dT-rG base pairs are fairly stable (Ackermann *et al.*, 1979; Noyes *et al.*, 1979) and the two undecamers would thus be expected to hybridize with all the possible coding sequences for the tetrapeptide Gln-His-Pro-Gly, the predicted precursor form of TRH (see Figure 1).

The synthetic oligodeoxynucleotides were also end-labeled with T4 polynucleotide kinase to a specific activity of ~5 x 10^8 c.p.m./μg and then used for colony hybridization.

Cloning of frog skin mRNA and screening of the cDNA library

Total poly(A) RNA was prepared from skin of *X. laevis* and incubated with reverse transcriptase in the presence of oligo(dT) as primer. The mRNA-cDNA hybrids were directly inserted into the unique *Pst*I site of plasmid pUC8 (Vieira and

Messing, 1982) via poly(dC)-poly(dG) homopolymeric extensions. This approach yields a smaller cloning efficiency (Roninson and Ingram, 1981; Zain *et al.*, 1979), yet clones with larger inserts can be obtained and sequence artefacts encountered during preparation of double-stranded cDNA (Volckaert *et al.*, 1981) are less likely.

From more than 1500 ampicillin-resistant transformants, 1400 were selected, transferred onto 10 plates and filter replicas made on Whatman 541 paper (Gergen *et al.*, 1979). Such filters were originally hybridized with radioactive cDNA prepared from total skin mRNA using the two undecamers mentioned above as primers. More than 100 clones were found to hybridize with this cDNA. These positive clones were subdivided by hybridization with each of the seven strongest cDNAs isolated by fractionating the total cDNA by electrophoresis in 6% polyacrylamide gels (data not shown). From several clones from each of these groups as well as from the rest not hybridizing with any of the major cDNAs, minipreparations were made and plasmids digested with the restriction enzymes *Nci*I and *Bst*NI, which cleave at the sequences $CC^{C}_{G}GG$ and $CC^{T}_{A}GG$, respectively. One of these two enzymes must cleave at the codons of every Pro-Gly sequence and, consequently, the cloned mRNA of the TRH precursor should contain at least one cleavage site for either *Nci*I or *Bst*NI. Clones with inserts hydrolyzed by any of these restriction enzymes were partially sequenced in the region(s) covering this cleavage site(s). None of the sequences determined in this search coded for TRH.

A) pGLU-HIS-PRO.NH_2

B) ...GLN-HIS-PRO-GLY...

C) CA^{A}_{G} CA^{U}_{C} CCx GGx...3′

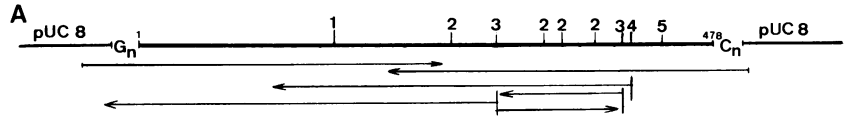
D) GTT GTG GG^{G}_{T} CC (5′)

Fig. 1. **(A)** Structure of TRH, **(B)** the postulated precursor form of TRH, **(C)** the corresponding segment of the mRNA, **(D)** the synthetic oligodeoxynucleotides used in this study.

Ultimately, the labeled undecamers were used directly for hybridization with the 1400 selected clones. Following the procedure described by Singer-Sam *et al.* (1983), the filters were hybridized for 20 h at 22°C and then washed at different temperatures (for details see Materials and methods). After each wash, filters were exposed to X-ray film overnight. At the end, after washing at 36°C, only clone number 8/136 still gave a strong signal on the film. A subsequent rescreening of the cDNA library with a labeled *Bgl*II fragment of clone 8/136 did not yield any additional positive clone.

Nucleotide sequence of clone 8/136 and partial amino acid sequence of the TRH precursor

The insert of clone 8/136 was found to contain four cleavage

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B    1
     AGCACAGAGCAGCACAAGGACACACTCTGCATATTGTGCTGCCGGACAAGGAGGTGACAGCCAGTCAGGCTGAGACAAAGGA
                                109
ACTTCCAGACCTCTGACAGCAGGAAAG ATG GTG TCT GTC TGG TGG TTG CTG CTT CTC GGT ACA ACC GTA TCT
                               Met-Val-Ser-Val-Trp-Trp-Leu-Leu-Leu-Leu-Gly-Thr-Thr-Val-Ser-
                                1                                                       15
CAC ATG GTG CAC ACA CAA GAG CAG CCT TTA CTG GAG GAG GAC ACA GCA CCA TTA GAT GAC TCG GAT
-His-Met-Val-His-Thr-Gln-Glu-Gln-Pro-Leu-Leu-Glu-Glu-Asp-Thr-Ala-Pro-Leu-Asp-Asp-Ser-Asp-

GTT CTT GAG AAA GCC AAA GGT ATC CTG ATC CGC AGT ATC CTG GAG GGA TTT CAA GAA GGG CAA CAA
-Val-Leu-Glu-Lys-Ala-Lys-Gly-Ile-Leu-Ile-Arg-Ser-Ile-Leu-Glu-Gly-Phe-Gln-Glu-Gly-Gln-Gln-

AAC AAT AGA GAT CTA CCA GAT GCA ATG GAA ATT ATA TCT AAG CGC CAG CAC CCA GGG AAA CGA TTC
-Asn-Asn-Arg-Asp-Leu-Pro-Asp-Ala-Met-Glu-Ile-Ile-Ser-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Phe-

CAG GAG GAG ATA GAA AAG AGA CAA CAC CCT GGA AAG AGG GAT CTG GAA GAT CTG AAT CTA GAG CTT
-Gln-Glu-Glu-Ile-Glu-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Asp-Leu-Glu-Asp-Leu-Asn-Leu-Glu-Leu-
                                                                                    478
TCC AAA AGG CAA CAC CCC GGA AGA AGA TTT GTG GAT GAT GTA GAG AAG AGG CAA CAT CCA CCCCC...
-Ser-Lys-Arg-Gln-His-Pro-Gly-Arg-Arg-Phe-Val-Asp-Asp-Val-Glu-Lys-Arg-Gln-His-Pro .....
                                                                                  123
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Fig. 2. Nucleotide sequence of the insert of clone 8/136. **(A)** Restriction endonuclease cleavage map and sequencing strategy. Arrows indicate direction of sequencing and length of sequenced region. Numbers refer to the following restriction endonucleases: (1) *Hgi*AI; (2) *Bst*NI; (3) *Bgl*II; (4) *Xba*I; (5) *Nci*I. **(B)** Nucleotide sequence of clone 8/136 and predicted amino acid sequence of the amino-terminal region of prepro-TRH. Only the coding strand is shown. Copies of the TRH sequence and the flanking basic and glycine residues are underlined.

sites for the restriction enzyme *Bst*NI and one for *Nci*I. The sequencing strategy and the location of these and some other restriction endonuclease sites present in the insert is shown in Figure 2A. The nucleotide sequence, comprised of 478 bp, contains only one open reading frame starting from an ATG codon 110 bp from the 5′ end (see Figure 2B), after an in-phase stop codon 39 bp earlier. The reading frame can be translated into a polypeptide of 123 amino acids which terminates without a stop codon. This incomplete sequence obviously represents the amino-terminal region of a larger polypeptide.

The predicted polypeptide (see Figure 2B) starts with a hydrophobic region typical for signal peptides, which probably encompasses the first 15 amino acids (von Heijne, 1983). Afterwards, the sequence Gln-His-Pro occurs four times, in each case preceded by the dipeptide Lys-Arg and followed, except for the last copy which terminates after the proline residue, by the sequence Gly-$^{Lys}_{Arg}$-Arg. The pairs of lysine/arginine residues are typical of processing sites found in many prohormones (Docherty and Steiner, 1982), while the glycine is required for the formation of terminal amides (Suchanek and Kreil, 1977; Bradbury *et al.*, 1982; Eipper *et al.*, 1983). On the basis of these considerations, we predict that the mRNA partly cloned in the insert of clone 8/136 codes for a secreted polypeptide which can be processed to yield four copies of the tripeptide pGlu-His-Pro.NH_2, i.e. of TRH.

The amino acid sequence of this fragment of prepro-TRH does not have any additional features which would suggest processing to other biologically active fragments. Interestingly, there is an internal homology discernible in the nucleotide sequence as well as in the polypeptide, which encompasses amino acids 61 – 92 and 93 – 123, respectively (see Figure 3). The four copies of TRH can thus be grouped in two pairs, the members of which are separated by the related hexapeptides Phe-Gln-Glu-Glu-Ile-Glu and Phe-Val-Asp-Asp-Val-Glu.

Discussion

Since the TRH present in skin of *X. laevis* constitutes only a few percent of the caerulein content, the original cDNA library, from which clones with inserts coding for caerulein precursors could be isolated (Hoffmann *et al.*, 1983a), was considered to be too small to search for the TRH precursor. A larger cDNA library was therefore constructed and we decided to directly clone the mRNA-cDNA hybrids hoping to obtain clones with larger inserts (Roninson and Ingram, 1981; Zain *et al.*, 1979). As the amount of poly(A) RNA which can be prepared from frog skin is not a limiting factor, the low cloning efficiency with this approach could be tolerated.

The screening method used to search for the caerulein precursor (Hoffmann *et al.*, 1983a) was not successful in the case of the TRH precursor. The labeled cDNA primed with the two synthetic undecamers hybridized to > 100 clones and, in spite of considerable effort, no insert containing a TRH sequence could be found. Direct screening with the end-labeled undecamers was therefore attempted, even though only two out of a possible 16 sequences were available (see Figure 1). A single clone, number 8/136, was still found to hybridize with the probe at 36°C and this one was found to contain an insert coding for part of the TRH precursor. It is noteworthy that this clone had not been picked up in the first approach, where we screened with oligonucleotide-primed cDNA.

The insert of clone 8/136 was found to be comprised of 478 bp excluding the GC tails. It contains a sequence complementary to one of the synthetic undecamers in the third TRH copy (see below) and two more with one mismatch each. The first ATG codon is preceded by a segment of 109 bp which has stop codons in all three reading frames. After this methionine codon, there is a single open reading frame which can be translated into a polypeptide containing 123 amino acids. This represents only the amino-terminal fragment of a larger protein as the last codon for a proline residue is followed by the poly(dC) tail. The sequence of the predicted polypeptide starts with a cluster of hydrophobic amino acids characteristic for signal peptides. Using the algorithm proposed by von Heijne (1983), it appears likely that this signal part ends at the serine in position 15. The following fragment of pro-TRH which we have cloned and sequenced, contains three copies of the sequence Lys-Arg-Gln-His-Pro-Gly-$^{Lys}_{Arg}$-Arg and a fourth at the carboxyl end which terminates at the proline residue. Processing at pairs of lysine/arginine residues and their subsequent cleavage by a carboxypeptidase B-like enzyme is the hallmark of numerous prohormone-hormone conversions. Invoking the same mechanism for the TRH precursor, the intermediate Gln-His-Pro-Gly would be generated. The terminal glycine has been shown to be essential for the formation of an amide bond (Bradbury *et al.*, 1982; Eipper *et al.*, 1983). Glycine residues following amino acids that are amidated in the end product have in fact been found in the precursors of a number of peptides including the hypothalamic hormones vasopressin and oxytocin (Land *et al.*, 1982, 1983), corticotropin releasing factor (Furutani *et al.*, 1983) and growth hormone releasing factor (Gubler *et al.*, 1983; Mayo *et al.*, 1983). Moreover, the conversion of pGlu-His-Pro-Gly to TRH has been observed *in vitro* in the presence of extracts from secretory granules of bovine pituitaries (Husain and Tate, 1983). The other reaction required to form TRH, i.e. the cyclization of the amino-terminal glutamine to yield pyroglutamic acid has, to our knowledge, not been investigated and it can thus not be decided whether this step is catalyzed by an enzyme or occurs spontaneously.

Besides the four TRH moieties and their flanking sequences, there are no noticeable features in the precursor fragment which would suggest the liberation of other hormones during processing. Also, no potential sites for N-glycosylation are present. It is noteworthy that the four TRH copies are not randomly distributed but rather arranged in two pairs each separated by a hexapeptide of similar structure. Homology is also apparent between the sequences preceding the first and third TRH moiety, which indicates that the size of this precursor has increased through duplica-

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 61
-Asn-Arg-Asp-Leu-Pro-Asp-Ala-Met-Glu-Ile-Ile-Ser-Lys-Arg-Gln-His-Pro-
-Lys-Arg-Asp-Leu-Glu-Asp-Leu-Asn-Leu-Glu-Leu-Ser-Lys-Arg-Gln-His-Pro-
 93

                                                              92
-Gly-Lys-Arg-Phe-Gln-Glu-Glu-Ile-Glu-Lys-Arg-Gln-His-Pro-Gly-
-Gly-Arg-Arg-Phe-Val-Asp-Asp-Val-Glu-Lys-Arg-Gln-His-Pro-.....
                                                             123
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Fig. 3. Internal homology regions in the TRH precursor. Only the amino acid sequence is shown and identical residues are underlined. In the corresponding nucleotide sequences, the homology between the two regions is 60%.

tion of a gene segment. We have previously shown (Hoffmann *et al.*, 1983a), that the caerulein precursor from skin of *X. laevis* also contains such an internal duplication. For the TRH precursor, the extent of this internal homology will only become clear when its entire sequence is known. In the arrangement depicted in Figure 3, the similarity is 65 and 60% between the amino acid and nucleotide sequences, respectively.

The TRH precursor joins the growing list of so-called polyproteins which can be processed to yield more than one molecule of end product. Several copies of the same peptide have previously been detected in the sequences of proenkephalin A (Noda *et al.*, 1982), proenkephalin B (Kakidani *et al.*, 1982) yeast α-mating factor (Kurjan and Herskowitz, 1982) and caerulein (Hoffmann *et al.*, 1983a). Similar situations exist for the precursors of glucagon (Lund *et al.*, 1982), epidermal growth factor (Scott *et al.*, 1983) and substance P (Nawa *et al.*, 1983), where peptides homologous to the respective end products are present. The internal duplications observed in the precursors of TRH and caerulein suggest one way by which such polyproteins with multiple identical or similar segments could have evolved and it will be interesting to check whether this arrangement also manifests itself in the exon-intron organization of the corresponding genes.

Materials and methods

Enzymes and reagents

Reverse transcriptase from avian myeloblastosis virus was obtained from Life Science Inc. (St. Petersburg, USA). Terminal deoxynucleotidyl transferase was purchased from P-L Biochemicals (St. Goar, FRG), restriction endonucleases and other enzymes from either New England Biolabs (Schwalbach, FRG) or from The Radiochemical Centre (Amersham, UK), which also supplied all the radiochemicals used in this study. Oligo(dT)-cellulose was obtained from Collaborative Research.

Synthesis of oligodeoxynucleotides

A mixture of the two undecamers d(CC$^{G}_{T}$GGGTGTTG) was synthesized by a modification of a published procedure (Wallace *et al.*, 1981). For the protection of the O-6 of deoxyguanosine during the synthesis, the nitrophenylethyl group (Trichtinger *et al.*, 1983) was incorporated into the fully protected mono- and dinucleotide deoxyguanosine phosphotriester derivatives (Chollet, personal communication). After de-protection, the probes were purified on 20% polyacrylamide/7 M urea gels followed by reverse-phase h.p.l.c. and sequenced by published methods (Jay *et al.*, 1974). The undecamers were labeled at the 5′ end with T4 polynucleotide kinase (Biolabs) and [γ-^{32}P]ATP as described by Boel *et al.* (1983) and then purified by chromatography over Sephadex G-25.

Preparation of poly(A) RNA

Total poly(A) RNA from dorsal and ventral skin of *X. laevis* (Herpetologisches Institut DeRover, The Netherlands) was prepared as described previously (Hoffmann *et al.*, 1983a).

Construction and cloning of recombinant plasmids

mRNA-cDNA hybrids were prepared by incubating 5 μg of poly(A) RNA with reverse transcriptase and oligo(dT) as described by Lehrach *et al.* (1979). After purification over a BioGel P60 column, the hybrids were lyophilized, redissolved and then precipitated with ethanol. The DNA-RNA hybrids were tailed with terminal deoxynucleotidyl transferase in the presence of 40 μM dCTP for 100 s at 30°C (Nelson and Brutlag, 1979). The tailed molecules were then annealed with the plasmid pUC8 (Vieira and Messing, 1982) which had previously been cleaved with *Pst*I and tailed with dGTP. The mixture of tailed plasmid and DNA-RNA hybrids was then used to transform *Escherichia coli* K12 RR1 (Bolivar *et al.*, 1977) following the procedure described by Dagert and Ehrlich (1979).

Colony screening

From >1500 transformants, 1400 were selected and transferred onto 10 plates. Filter replicas were made on Whatman 541 paper as described by Gergen *et al.* (1979) and filters were then hybridized with the labeled undecamers (Wallace *et al.*, 1981). A temperature of 22°C was chosen for the overnight hybridization in 6 x SET (1 x SET = 0.15 M NaCl, 1 mM EDTA, 0.015 M Tris/HCl, pH 7.5) following the strategy suggested by Singer-Sam *et al.* (1983). Filters were then washed 4 x 1 h in 6 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M Na-citrate, pH 7.2) at the following temperatures: 10, 22, 28, 32 and 36°C. After each eash, filters were exposed overnight to X-ray film.

Analysis of clones

Recombinant plasmids were isolated as described by Holmes and Quigley (1981) or, in the case of large-scale preparations, according to Birnboim and Doly (1979). After treatment with ribonuclease A and proteinase K, the DNA was extracted with phenol and purified by chromatography on BioGel A-15m. The isolated plasmids were cut with restriction enzymes, labeled at recessed ends with reverse transcriptase and one [α-^{32}P]deoxynucleoside triphosphate. Radioactive DNA fragments were then separated by electrophoresis in 0.8–2% agarose gels or by strand separation in non-denaturing acrylamide gels. Nucleotide sequences were determined by the chemical cleavage method (Maxam and Gilbert, 1980) with one modification for the A+G-reaction (Cooke *et al.*, 1981).

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