Expression in Brain of a Messenger RNA Encoding a Novel Neuropeptide Homologous to Calcitonin Gene–Related Peptide

Abstract. *As a consequence of alternative RNA processing events, a single rat gene can generate messenger RNA's (mRNA's) encoding either calcitonin or a neuropeptide referred to as alpha-type calcitonin gene–related peptide (α-CGRP). An mRNA product of a related gene has been identified in rat brain and thyroid encoding the protein precursor of a peptide differing from α-CGRP by only a single amino acid. The RNA encoding this peptide, which is referred to as β-CGRP, appears to be the only mature transcript of the β-CGRP gene. Hybridization histochemistry reveals a similar distribution of α- and β-CGRP mRNA's, but their relative levels of expression vary in different cranial nerve nuclei. Thus β-CGRP is a new member of a family of related genes with potential functions in regulating the transduction of sensory and motor information.*

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As a result of tissue-specific patterns of alternative RNA processing (*1–3*), one rat gene encodes both the precursor of the calcium-regulating hormone calcitonin and the precursor for a previously unknown 37–amino acid neuropeptide referred to as calcitonin gene–related peptide (α-CGRP). The neuropeptide, α-CGRP, is produced in both the central and peripheral nervous systems and in several endocrine tissues (*3–6*); its distribution in the nervous system and other tissues is consistent with functional roles in response to painful stimuli, ingestive behavior, and cardiovascular homeostasis, the latter effects being suggested by physiological studies (*7*). Alternative RNA processing events operate in the expression of many eukaryotic genes (*8–15*).

We have now identified a rat gene generating a messenger RNA (mRNA) related to α-CGRP and referred to as β-CGRP. We propose that proteolytic processing of the predicted protein precursor generates a 37–amino acid peptide product that is amidated at the COOH-terminal and differs by only a single amino acid from α-CGRP. β-CGRP RNA appears to be expressed in the brain, sensory ganglia, and the thyroid gland, in a pattern similar to that of α-CGRP mRNA; but the relative expression of each mRNA varies in different cranial nerve nuclei.

Polypeptide molecules serve critical functions as hormones and neuroactive regulators, and the importance of these peptides in normal brain development and function is suggested by their complex patterns of expression in specific subsets of neurons (*16*). The application of recombinant DNA technology accelerated the process of identifying previously unsuspected neuropeptides and revealed the existence of families of related genes encoding structurally similar, but not identical, peptide products [for review, see (*17*)]. The existence of such gene families accounts, in part, for the generation of diverse regulatory peptides in the brain (*17*).

We searched for genes related to the calcitonin–α-CGRP gene by screening a library of chimeric plasmids containing inserts complementary to mRNA's from rat medullary thyroid carcinomas. For this purpose, we used a clonal probe complementary (cDNA) to the α-CGRP coding region. Of the 35 positive colonies analyzed in detail, four clones exhibited a restriction enzyme map different from that characteristic of α-CGRP cDNA clones. Analysis of the DNA sequence of these clonal inserts revealed a novel mRNA (β-CGRP mRNA) related to α-CGRP mRNA (Fig. 1). The sequence of β-CGRP mRNA contains an open reading frame of 402 nucleotides. The first 258 nucleotides of the open reading frame encode an 86–amino acid NH_2-terminal region homologous to the NH_2-terminal portion common to the precursors of calcitonin and α-CGRP. This 258-nucleotide region of β-CGRP mRNA differs from the equivalent region of α-CGRP mRNA by 84 base substitutions, insertions, or deletions, altering 39 of 86 NH_2-terminal amino acids. Because there are two sets of paired basic amino acid residues in this predicted NH_2-terminal portion of the β-CGRP precursor, up to three polypeptides could potentially be generated from this region (Fig. 2), although Arg-Lys sites are not as readily cleaved as Lys-Arg sites, and hence may not be utilized. In contrast to the divergence of more than 30 percent in the 5′ portion of the coding sequence, β-CGRP mRNA differs from α-CGRP mRNA by only five base substitutions in the next 120 nucleotides. This region of the open reading frame predicts the excision of a 37–amino acid peptide containing a COOH-terminal phenylalanine-amide. This peptide differs by only a single amino acid (a Lys for Glu in position 35) from the primary sequence of α-CGRP. The β-CGRP protein precursor also predicts the cleavage of a COOH-terminal tetrapeptide. The 5′ and 3′ noncoding regions of β-CGRP and α-CGRP mRNA's diverge significantly, allowing the generation of α- and β-CGRP–specific hybridization probes (Fig. 2).

Analysis of restriction enzyme digests of rat liver genomic DNA with a series of clonal hybridization probes (Fig. 3A) provides evidence that the α- and β-CGRP genes are each single-copy. Thus, a 3′ noncoding probe derived from β-CGRP cDNA hybridized to a single 7.9-kilobase Bgl II restriction fragment (Fig. 3A). A probe encompassing the entire coding region of β-CGRP mRNA, which would be expected to hybridize to both α-CGRP and β-CGRP genes, and to any other gene with sequence homology to the coding region, hybridized to the predicted 7.3- and 3.4-kb Bgl II restriction fragments characteristic of the calcitonin–α-CGRP gene (*2, 4*) and to two fragments (3.8 and 1.2 kb) that appear to be derived from the β-CGRP gene (Fig. 3A). Although the 3.8- and 3.4-kb fragments are usually not resolved electrophoretically, independent identification of the 3.8-kb band was established by the observation that a 5′ noncoding clonal probe specific for β-CGRP cDNA hybridized to this band and to an additional 1.6-kb fragment. Therefore, the 3.8-kb band appears to encompass both the 5′ noncoding portion and the proximal 5′ coding portion of the β-CGRP gene,

while the 1.2-kb Bgl II fragment encompasses the remaining coding region of the β-CGRP gene. A probe corresponding to the 3′ portion of the α-CGRP coding region also hybridized to the 1.2-kb Bgl II fragment of the β-CGRP gene. This analysis of genomic DNA thus indicates the existence of two separate genes containing sequences hybridizing to CGRP cDNA probes. The β-CGRP gene appears to contain no sequences with close homology to the calcitonin-coding exon present in the calcitonin–α-CGRP gene because, even under relatively nonstringent conditions, a probe corresponding to the calcitonin-coding exon hybridizes only to the known 0.5-kb Bgl II fragment of the calcitonin–α-CGRP gene (Fig. 3A).

In the brain, α-CGRP mRNA is the exclusive or the predominant product of calcitonin–α-CGRP gene expression, and it is localized in specific neurons and pathways believed to mediate sensory, motor, and integrative functions. Analysis of RNA from various regions of the nervous system revealed that β-CGRP mRNA, like α-CGRP mRNA, is present in the trigeminal ganglia, lateral medulla, hypothalamus, and, to a lesser extent, midbrain (Fig. 3B). By this analysis, the relative distribution of β-CGRP mRNA is actually similar to that of α-CGRP mRNA, but in each region, the level of expression of β-CGRP mRNA is less than 20 percent that of α-CGRP mRNA. The size of the β-CGRP mRNA is 1100 to 1200 bases, compared to 1250 to 1350 for α-CGRP mRNA (Fig. 3C). With a polyadenylate [poly(A)] tract of approximately 200 bases and coding region of 402 bases, β-CGRP mRNA would be expected to contain 500 to 600 bases of noncoding information. As judged from the cDNA sequence, more than 340 nucleotides of the noncoding bases are represented in the 3′ noncoding sequence (Fig. 1). The 1150-nucleotide β-CGRP

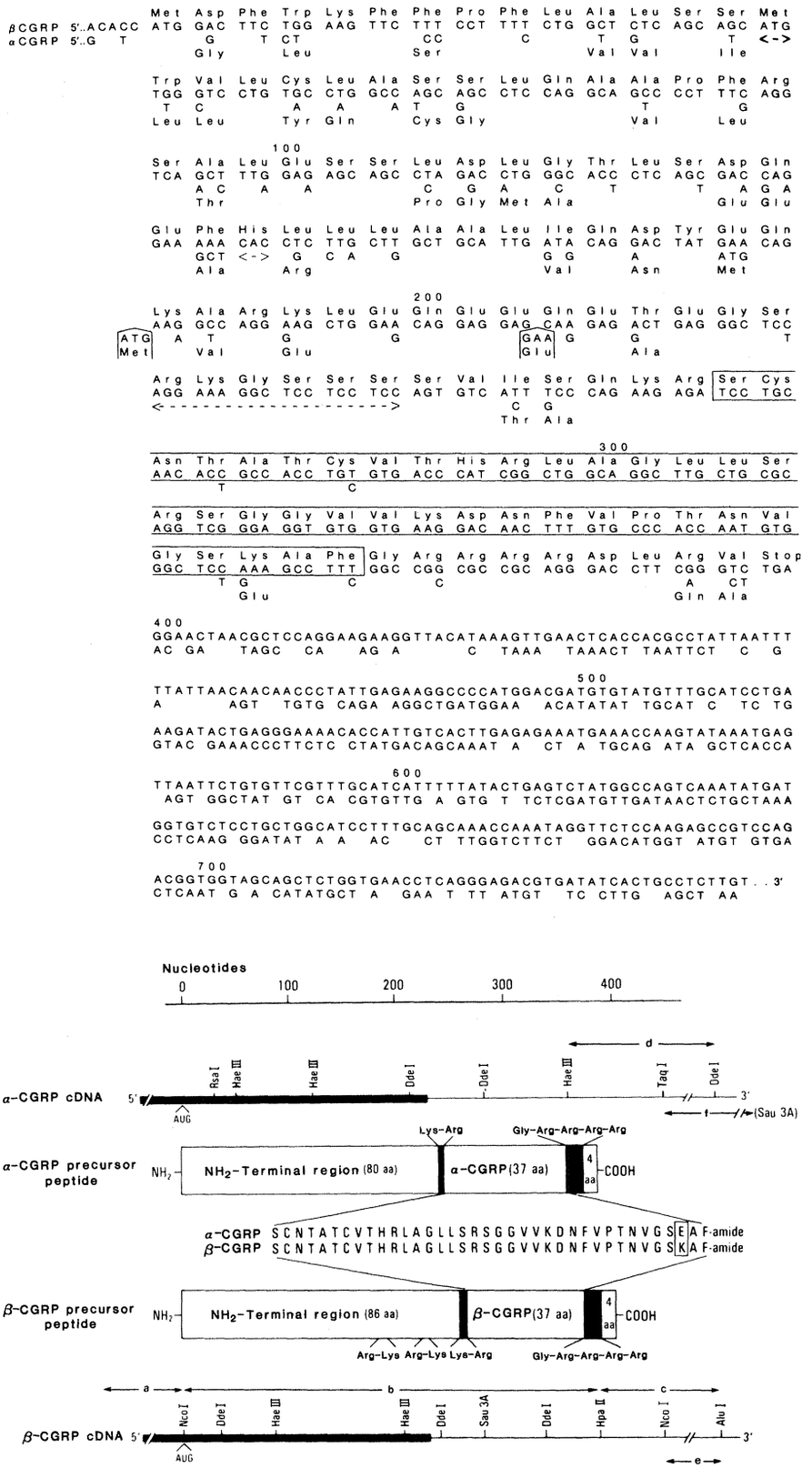
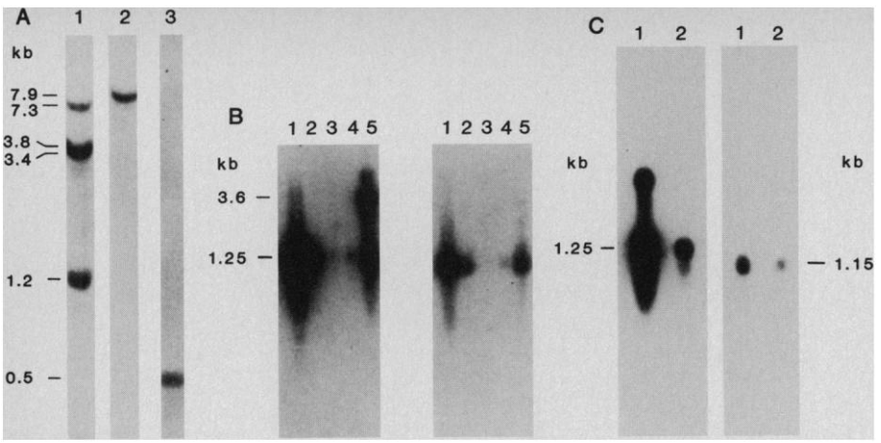


Fig. 1 (top). Sequence of rat β-CGRP cDNA. The sequence of two clones containing β-CGRP cDNA were determined; one (p-β-$CGRP_1$) encompasses the entire coding region and 46 bases of 3′ noncoding information. The sequence shown beyond the 46th 3′ noncoding nucleotide is derived from p-β-$CGRP_2$. The variations in sequence between β- and α-CGRP mRNA's and their encoded peptides are indicated; optimal alignment was achieved by leaving gaps to account for regions of additional β-CGRP sequence (indicated by bars demarcated with arrows) and by designating two amino acids of α-CGRP as insertions (shown as codons between bars). The predicted, excisable 37–amino acid β-CGRP peptide is boxed (*20*). Fig. 2 (bottom). Schematic diagram of α- and β-CGRP cDNA's and their encoded peptide products. Several of the restriction sites used for sequence analysis are indicated; the 5′ and 3′ noncoding regions are truncated in the diagram. The predicted peptides, with putative processing signals, are indicated for each precursor. The predicted α- and β-CGRP peptide sequences differ by only a single amino acid, as shown in the single-letter code, while flanking peptides exhibit considerable divergence (>35 percent). The one-letter symbols for the amino acids are A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan, Y, tyrosine; and V, valine. The heavy line in the 5′ portion of each cDNA corresponds to the NH_2-terminal region common to α-CGRP and calcitonin mRNA's. Clonal probes used in subsequent experiments are shown above and below the map of the cDNA's (designated a to f). Probe f is a 450-bp Taq I–Sau 3A genomic fragment containing 170 bp of a single-copy 3′-noncoding α-CGRP cDNA sequence.



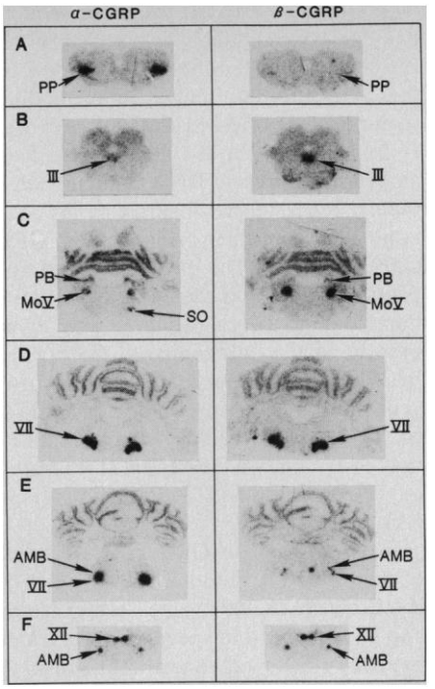


Fig. 3 (left). (A) Restriction enzyme analysis of rat α- and β-CGRP genomic DNA. Rat liver DNA was digested with Bgl II, and fragments were separated by electrophoresis, blotted onto nitrocellulose, and probed by the method of Southern (*23*). Each lane was subjected to hybridization with nick-translated probes representing the entire coding region of β-CGRP (lane 1), the unique 3′ noncoding region of β-CGRP (lane 2), and a calcitonin exon–specific probe (lane 3). Radioautographs were exposed for 72 hours (*24*). (B) Analysis of expression of β-CGRP mRNA in the brain and thyroid gland. Poly(A)-selected rat RNA from trigeminal ganglia (lane 1), lateral medulla (lane 2), midbrain (lane 3), hypothalamus (lane 4), and thyroid gland (lane 5) were fractionated under denaturing conditions and hybridized either to a clonal probe specific for α-CGRP (A) or to a clonal probe specific for β-CGRP (B). The radioautographs shown were exposed for 48 hours; additional exposure (130 hours) was required to visualize the hybridized band in midbrain (lane 3). A lower exposure confirmed the hybridization to the lateral medulla (lane 2). In additional experiments, RNA from lateral medulla was placed in lanes not adjacent to trigeminal ganglia RNA to unambiguously confirm β-CGRP expression; in each case β-CGRP mRNA was present in lateral medulla (*25*). (C) Analysis of expression of α-CGRP mRNA (left) and β-CGRP (right) with the specific probes described in (B). (lane 1) 1 μg of rat MTC tumor poly(A)-selected RNA; (lane 2) 5 μg of trigeminal ganglia poly(A)-selected RNA. Radioautographs were exposed for 8 hours (*25*). Fig. 4 (right). Localization of the α-CGRP and β-CGRP mRNA's by hybridization histochemistry. Adjacent frontal sections of rat brain were hybridized with asymmetric RNA probes specific for either α-CGRP (probe f in Fig. 2) or β-CGRP (probe e in Fig. 2). Arrows indicate regions of CGRP message expression; anatomical structures corresponding to these regions were identified by examination of adjacent Nissl-stained sections (not shown). (A) to (F) are brainstem sections, rostral to caudal: (A) junction of midbrain and diencephalon; (B) midbrain, (C) pons, (D to F) medulla. Abbreviations: PP, peripeduncular nucleus; III, oculomotor nucleus; PB, parabrachial nucleus; MoV, trigeminal motor nucleus; SO, superior olive; VII, nucleus of the facial nerve; AMB, nucleus ambiguus; and XII, nucleus of the hypoglossal nerve. Unidentified signals are irreproducible artifacts (*27*).

mRNA appears also to be expressed in the thyroid gland (Fig. 3B). The reactive RNA in thyroid hybridizes to both 5′ noncoding and 3′ noncoding probes specific for β-CGRP mRNA (data not shown). There is no evidence for a second mature transcript from the β-CGRP gene in brain or thyroid gland that would be analogous to the generation of alternative RNA products in the case of the calcitonin–α-CGRP gene expression. The level of β-CGRP in the thyroid gland is less than 20 percent that of α-CGRP mRNA (Fig. 3B) and less than 1 percent that of calcitonin mRNA.

The detailed pattern of differential expression of the α-CGRP and β-CGRP mRNA's was amenable to analysis by the technique of hybridization histochemistry because of the sequence divergence between the noncoding portions of these mRNA's. The pattern of expression of both mature transcripts in the brain (see Fig. 4) is consistent with the previously reported histochemical analysis of CGRP expression (*3*). Hybridization to probes specific for each mRNA is observed in the trigeminal ganglia; the motor nuclei of the third, fourth, fifth, seventh, tenth, and twelfth cranial nerves; in anterior horn cells (motoneurons); and in other nuclei—including the parabrachial and peripeduncular nuclei (*18*). Because the trigeminal ganglion contains more than ten times as much α-CGRP as β-CGRP, this tissue provided a criterion for the relative in situ hybridization signals for the two probes used. The α-CGRP probe gave a signal greater than ten times that of the β-CGRP probe in this tissue. Thus the two probes provide a relative, although not precise, quantitative indication of the ratio of the expression of the two mRNA's in each region of the brain. The hybridization signal for β-CGRP exceeds that for α-CGRP in the nuclei of the third (Fig. 4), fourth (not shown), and fifth (Fig. 4) cranial nerves and records at least equivalent signals in other areas (see Fig. 4). The similar distribution of the two mRNA's suggests that the calcitonin–α-CGRP and the β-CGRP genes are expressed in the same neurons but at variable levels (*30*).

The finding of a gene related to the calcitonin–α-CGRP gene exemplifies the biological potential of expression of families of genes encoding related neuropeptides generated as a consequence of gene duplication events. Whether α- and β-CGRP are expressed in the same or in different cells will be determined when antisera to the peptides encoded by their mRNA's become available. It will also be important to determine whether the two genes are independently regulated, because this would provide a potential functional advantage for the expression of two such related gene products. Finally, the discovery of β-CGRP augments the challenge of unraveling the physiological significance of multiple CGRP-related neuropeptides in rat and in man (*19*).

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24. Rat liver nuclear DNA, prepared by proteinase K, phenol, and chloroform extractions, was digested with Bgl II or other enzymes (Eco RI or Bam HI), and 20-μg aliquots were subjected to electrophoretic separation on an 0.8 percent agarose gel. DNA was transferred to nitrocellulose, baked at reduced pressure for 2 hours at 80°C, and hybridized to a series of clonal probes that were nick-translated (*26*) to a specific activity of 1×10^8 to 3×10^8 count/min per microgram of DNA, with [^{32}P]dCTP used as the radiolabeled nucleotide. Probes used were (i) a Hpa II–Nco I fragment from p-β-CGRP$_1$ representing unique 5′ noncoding information (designated a in Fig. 2), the Hpa II site is contributed by the vector; (ii) an Nco I–Hpa II fragment from p-β-CGRP$_1$ representing virtually the entire coding region of β-CGRP mRNA (designated b in Fig. 2); (iii) an Hpa II–Alu I fragment from p-β-CGRP$_2$ representing the unique 3′ noncoding region of β-CGRP (designated c in Fig. 2); and (iv) a 0.5-kb Bgl II fragment of the rat calcitonin–α-CGRP gene, representing the calcitonin coding exon (*3*). All probes, under conditions of hybridization used, are mRNA-specific except for the Nco I–Hpa II fragment of p-β-CGRP$_1$, which encompasses the entire coding region and which hybridizes to both α- and β-CGRP mRNA's. Hybridizations were performed at 40°C under conditions previously reported (*2*, *3*); washing was in 0.2× standard saline citrate (SSC) at 65°C, except in the case of the calcitonin probe, which was washed in 0.5× SSC at 50°C. Radioautographs were exposed for 72 hours. Calculation of RNA sizes was based on the migration of size standards provided by λ-Hind III markers.
25. Total cell poly(A)-rich RNA was prepared from normal rat thyroid, trigeminal ganglia, and a series of brain regions, as previously described (*21*). Aliquots (5 μg) were denatured and subjected to electrophoresis on 1.5 percent agarose-formaldehyde gels. RNA was transferred to nitrocellulose, washed in prehybridization buffer, and hybridized to clonal probes nick-translated to a specific activity of 1×10^8 to 3×10^8 cpm per microgram of DNA, with [α-^{32}P]dCTP as the labeled nucleotide. A p-α-CGRP$_1$ 3′ Hae III–Dde I fragment is specific for the noncoding region of α-CGRP mRNA (designated d in Fig. 2); a p-β-CGRP$_1$, Hpa II–Nco I fragment corresponding to 5′ noncoding information, and a Hpa II–Alu I fragment of p-β-CGRP$_2$ corresponding to the 3′ noncoding region of β-CGRP mRNA provided the β-CGRP mRNA-specific probes. The 5′- and 3′-noncoding β-CGRP probes gave identical results; the 5′ noncoding probe was used in the data shown in (B). Size standards were provided by migration of calcitonin and CGRP mRNA species from rat medullary thyroid tumors (*2*).
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27. Tissue was fixed by vascular perfusion and frozen sections 25 μm thick were cut on a sliding microtome (*28*). Sections were mounted on poly-L-lysine–coated slides and air-dried. Pretreatment, hybridizations, and washing conditions have been described for RNA probes (*29*). Briefly, sections were digested with proteinase K (10 μg/ml, 37°C, 30 minutes), acetylated, and dehydrated. After thorough drying, 50 μl of hybridization mix containing ^{32}P-labeled probe (10^8 cpm/ml) was spotted on each slide. Slides were incubated at 50°C for 16 hours. Slides were rinsed, digested with ribonuclease (RNase A, at 20 μg/ml 37°C, 30 minutes) and washed in 0.1× SSC for 30 minutes at 53°C. After dehydration, the sections were exposed to Cronex 4 film (DuPont) for 15 days at 4°C. Specific RNA probes were generated with in vitro transcription vectors. An α-CGRP vector was produced by cloning the 450-bp Taq I–Sau 3A α-CGRP genomic fragment (f in Fig. 2) into pSP64. This fragment contains 170 bp of single-copy 3′ noncoding sequence and 280 bp of single-copy 3′ flanking sequence. The β-CGRP vector contains a single-copy 220-bp Nco I–Alu I cDNA insert (probe e in Fig. 2) cloned into pGEM-1 (Promega). After the vectors were linearized, runoff transcripts were produced with SP6 polymerase (α probe) or T7 polymerase (β probe) in standard reactions in which only ^{32}P-labeled UTP (600 Ci/mmol was used. Specificity of the α-CGRP and β-CGRP probes was confirmed by Northern blot RNA analysis [see (*30*)].
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30. Computer sequence homology analysis identified several regions of local homology between the β-CGRP and α-CGRP specific probes, so some cross hybridization might occur under nonstringent conditions. If such cross hybridization did occur under our conditions, it represented less than 1 percent of specific hybridization based upon parallel RNA blot analysis of MTC RNA tumor standards. Thus it is formally possible that some or all of the very weak signals for α-CGRP and β-CGRP mRNA found in the oculomotor and peripeduncular nuclei, respectively, could be due to cross hybridization.
31. We thank P. Sawchenko and L. Stolarsky for their advice and discussion of data in manuscript and M. Richards for its preparation. The work was supported by grants from the American Cancer Society and the National Institutes of Health. S.G.A. was supported by a Schering-Plough Foundation Fellowship from the Life Sciences Research Foundation. R.M.E., L.S., M.G.R., and S.L. acknowledge support from the Matthew, Clayton, and McKnight Foundations and the American Cancer Society, respectively. J.L.A. is a predoctoral trainee in the Department of Biology, University of California, San Diego.

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Immunohistochemical Localization in the Rat Brain of the Precursor for Thyrotropin-Releasing Hormone

Abstract. *A rabbit antiserum to a peptide sequence present in the precursor for thyrotropin-releasing hormone (proTRH), deduced from cloned amphibian-skin complementary DNA, was raised by immunization with the synthetic decapeptide Cys-Lys-Arg-Gln-His-Pro-Gly-Lys-Arg-Cys (proTRH-SH). Immunohistochemical studies on rat brain tissue showed staining of neuronal perikarya in the parvicellular division of the paraventricular nucleus of the hypothalamus and the raphe complex of the medulla, identical to that already described for thyrotropin-releasing hormone (TRH). Immunostaining was abolished by preincubation with proTRH-SH (10^{-6}M) but not TRH (10^{-5}M). Both TRH precursor and TRH were located in neurons of the paraventricular nucleus. However, in contrast to the findings for TRH, no staining was observed in axon terminals of the median eminence. These results suggest that a TRH precursor analogous to that reported in frog skin is present in the rat brain and that TRH in the mammalian central nervous system is a product of ribosomal biosynthesis.*

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The long-standing controversy (*1*) concerning the mode of biosynthesis of thyrotropin-releasing hormone (TRH) has recently been resolved. It is now clear that TRH, like other hypothalamic releasing factors such as luteinizing hormone–releasing (LH-RH) (*2*), arises from the post-translational cleavage of a large precursor protein (*3*) and not by soluble nonribosomal enzymatic mechanisms such as those that produce the small neural peptide carnosine (*4*). On the basis of studies reporting large quantities of TRH in amphibian cutaneous tissue (*5*), Richter *et al.* (*6*) isolated messenger RNA from the skin of the frog *Xenopus laevis* and were able to obtain a complementary DNA (cDNA) clone with an insert of 478 nucleotides coding for a portion of the preprohormone precursor of TRH (preproTRH). The deduced TRH precursor of 123 amino acids contains three copies of the sequence Lys-