Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 1994–1998, April 1985
Biochemistry

Alternative RNA processing events in human calcitonin/calcitonin gene-related peptide gene expression

(neuroendocrine/pituitary/RNA processing/hormone precursor)

VIVIAN JONAS*, CHIJEN R. LIN*, ERIC KAWASHIMA†, DOMINIQUE SEMON†, LARRY W. SWANSON‡, JEAN-JACQUES MERMOD†, RONALD M. EVANS§, AND MICHAEL G. ROSENFELD*

*Eukaryotic Regulatory Biology Program, University of California, San Diego, School of Medicine, La Jolla, CA 92093; §Molecular Biology and Virology and ‡Developmental Neurobiology Laboratories, The Salk Institute, San Diego, CA 92138; and †Biogen SA, Geneva, Switzerland

Communicated by J. Edwin Seegmiller, November 30, 1984

ABSTRACT **Two mRNAs generated as a consequence of alternative RNA processing events in expression of the human calcitonin gene encode the protein precursors of either calcitonin or calcitonin gene-related peptide (CGRP). Both calcitonin and CGRP RNAs and their encoded peptide products are expressed in the human pituitary and in medullary thyroid tumors. On the basis of sequence comparison, it is suggested that both the calcitonin and CGRP exons arose from a common primordial sequence, suggesting that duplication and rearrangement events are responsible for the generation of this complex transcription unit.**

We have documented that the rat calcitonin gene can generate two mRNAs encoding discrete polypeptide products as a result of tissue-specific alternative RNA processing (1–4). The products of these RNAs are the precursors to the calcium-regulating hormone, calcitonin, and to two other peptides in the thyroidal "C" cell and are the precursors to a novel neuropeptide and hormone referred to as CGRP (calcitonin gene-related peptide) and two other peptides. Histochemical analysis with antisera against a synthetic CGRP fragment and nuclease S1 mapping have demonstrated CGRP expression and localization in the brain and other tissues (4). On the basis of its anatomical distribution and initial physiological studies with chemically synthesized peptide, CGRP is suggested to exert regulatory effects on nociception, ingestive behavior, and cardiovascular homeostasis (4, 5). In addition to its localization in neural tissue, CGRP also is produced in the endocrine system (including thyroid "C" cells, adrenal chromaffin cells, and bronchiolar cells) and is distributed widely in sensory nerves and in nerves supplying vasculature in many other organs (e.g., skin, genitourinary system). The molecular basis for the alternative RNA processing appears to be selective utilization of calcitonin- or CGRP-specific poly(A) sites present in the transcription unit associated with different patterns of exon splicing and ligation reactions (6). Similar RNA processing events appear to operate in expression of other eukaryotic genes (e.g., refs. 7–13) and in a number of animal DNA viruses (14–17). Alternative patterns of RNA splicing in association with the use of a single poly(A) site are also observed in expression of a number of genes, including the rat calcitonin/CGRP gene (6, 18–22).

In this manuscript we report that the human calcitonin/CGRP gene can generate two mRNAs encoding the precursors of calcitonin and CGRP as a result of alternative RNA processing events, documenting the operation of this type of post-transcriptional regulation in the human neuroendocrine system. The structure of the human CGRP precursor predicts the excision of a 37-amino acid peptide differing in 4 amino acids from the primary sequence of rat CGRP. The predicted peptide is identical with the structure of a peptide from human medullary thyroid tumors in novel fast-atom-bombardment mass spectometry (23). Both RNA and peptide products of the human calcitonin/CGRP gene appear to be expressed in the human pituitary gland.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Cloning Procedures and DNA Sequence Analysis. Poly(A)-rich RNA from human medullary thyroid carcinomas prepared as described (2, 3) was used as substrate for oligo(dT)-primed cDNA synthesis. Double-stranded DNA was digested with nuclease S1 and blunt-ended by using reverse transcriptase; *Eco*RI linkers were ligated, and the DNA was inserted into the *Eco*RI site of the bacterial expression plasmid pUC8 (2). Transformation of *Escherichia coli* DH-1 produced a library of 40,000 clonal isolates, of which 2000 were ordered and subjected to a detailed analysis. Screening by immunological and hybridization techniques with a calcitonin antiserum or a rat calcitonin-coding region-specific clonal probe revealed 78 positive clones and with a rat CGRP-coding region-specific probe revealed 7 clones. Plasmids were prepared from positive colonies (24) and subjected to DNA sequence analysis by the chemical procedures of Maxam and Gilbert (25). All of the common region and calcitonin- or CGRP-coding regions were present in a single insert ($pCal_{H1}$ and $pCGRP_{H1}$), and sequences were confirmed by analysis of two additional clonal inserts. Plasmids were digested with *Eco*RI, *Dde* I, or *Hin*fI prior to end labeling with [γ-^{32}P]ATP in conjunction with T4 polynucleotide kinase and were digested with a second restriction enzyme prior to electrophoresis procedures. All DNA sequence data were confirmed by multiple determinations of overlapping fragments and sequencing in both strands and were across all sites used for labeling.

Isolation and Sequence Analysis of the Human Calcitonin Genomic Clone. A library of human genomic DNA [partial *Alu* I/*Hae* III-digested DNA cloned into a phage λ Charon 4A (26) library; the generous gift of T. Maniatis] was screened by using calcitonin and CGRP cDNA clones by the method of Benton and Davis (27). Positive plaques were rescreened at low density until pure; reactive regions were identified by restriction enzyme mapping with DNA blot analysis. For sequencing of clone $\lambda hCal_1$, a series of oligonucleotides based upon human calcitonin and CGRP cDNA sequences were synthesized (28) and used as primers to sequence genomic fragments subcloned into phage M13 in both orientations by the chain termination method of Sanger *et al.* (29).

Abbreviation: CGRP, calcitonin gene-related peptide.

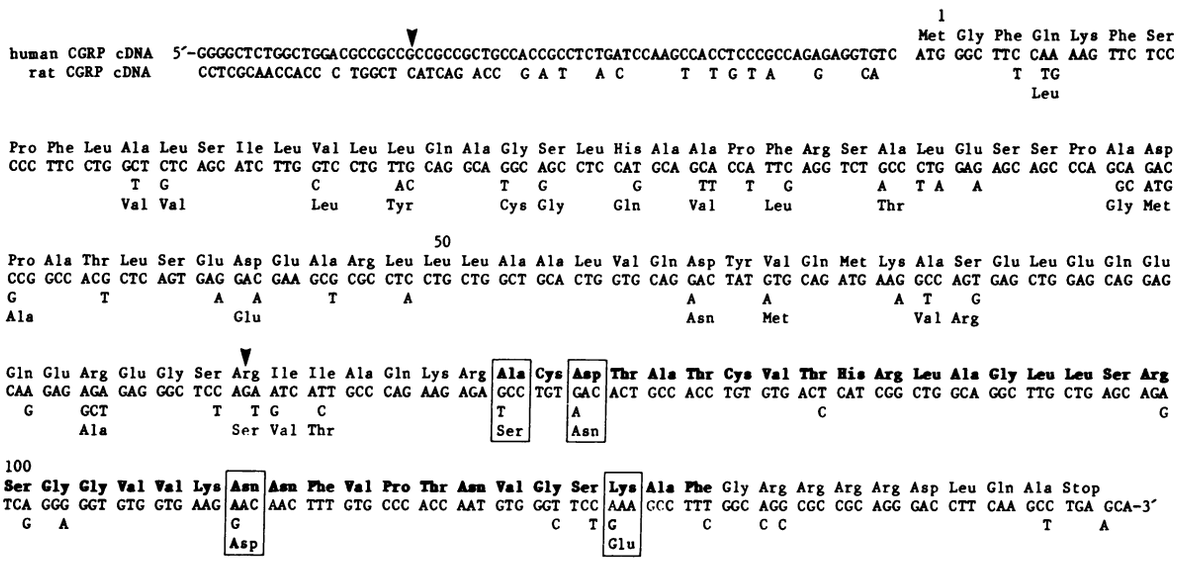

FIG. 1. Sequence of human CGRP and calcitonin cDNAs. The sequence of $pCal_{H1}$ exhibited complete identity with $pCGRP_{H1}$ over the region indicated by the arrowheads. The bold-faced sequence is the predicted CGRP-excised peptide; the boxed amino acids are those that differ from rat CGRP. Nucleotide divergences between rat and human CGRP cDNAs are listed below the human sequence.

RNA Analyses and Histochemical Procedures. RNA blot analysis was performed by using denaturing formaldehyde-agarose gels as described (2). Histochemical analyses with anti-CGRP and anti-calcitonin antisera were performed as described (4).

RESULTS

Sequence of Human CGRP and Calcitonin cDNAs. Several cDNA clones exhibiting calcitonin- and CGRP-specific reactivity were subjected to mapping and DNA sequence analysis. DNA sequence analysis confirmed that both calcitonin or CGRP mRNAs are expressed in a human medullary thyroid tumor (Fig. 1). Both mRNAs contain identical 5' noncoding and coding regions, with the sequence of the first 225 nucleotides of coding information being identical in both mRNAs, predicting a common 75-amino acid NH_2-terminal region between the peptides encoded by CGRP and calcitonin mRNAs. This region differs in 20-amino acid residues from the comparable rat calcitonin/CGRP "common region" sequence. A partial sequence of human calcitonin cDNA was first reported by Craig *et al.* (30), and the sequence reported here agrees entirely with that sequence and almost entirely with the additional sequence recently reported by Le Moullec *et al.* (31). After the region of sequence identity, the human calcitonin and CGRP mRNAs diverge entirely in sequence (Fig. 1), although both continue with open reading frames, and the encoded proteins exhibit comparable structural organization (Fig. 2). Thus, both calcitonin and CGRP sequences are flanked by paired basic amino acid residues signaling proteolytic excision from the primary translation product, and they contain a carboxyl-terminal glycine residue directing amidation of the excised peptide. The sequence of CGRP mRNA predicts a precursor peptide that will be proteolytically processed to generate a 37-amino acid COOH-terminally amidated peptide similar to rat CGRP except that the 1st, 3rd, 25th, and 35th amino acids (alanine, aspartic acid, asparagine, and lysine, respectively; Fig. 1) differ from the rat primary sequence. Therefore, the CGRP-coding region is better conserved between human and rat than is the "common region."

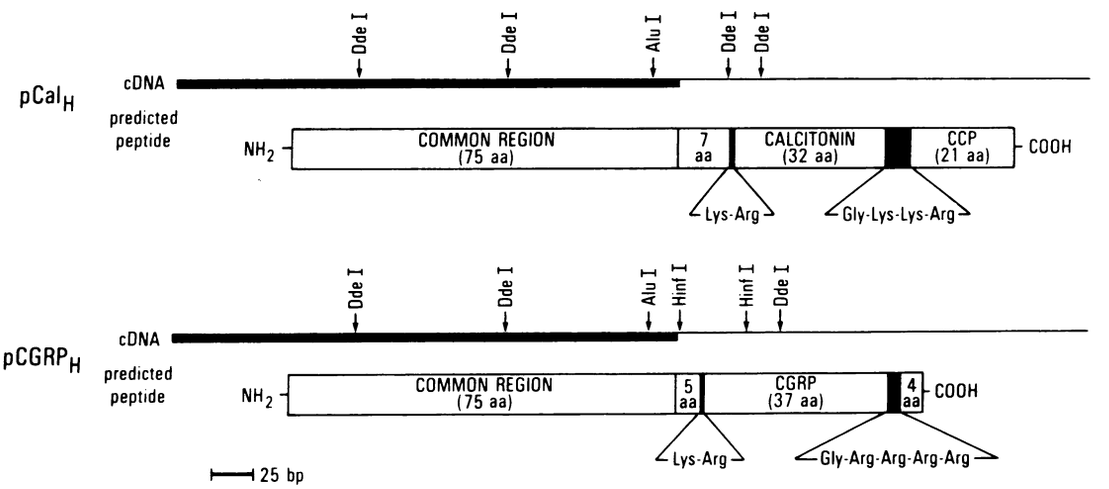

FIG. 2. Schematic representation of $pCal_{H1}$ and $pCGRP_{H1}$ cDNA cloned inserts and the structures of the protein precursors that they encode. Restriction sites used for DNA sequence analyses are those indicated, and regions of identical nucleotide sequence are thick black lines. The structures of the predicted protein precursors are represented below the cDNA, and the potential proteolytic processing sites and the resultant cleavage products are noted. aa, Amino acid(s); CCP, calcitonin C'-terminal peptide; bp, base pairs.

There Are Two Genes with Calcitonin- and CGRP-Related Sequences in the Human Genome. To determine the origin of the two mRNAs, we subjected human placental and fetal liver genomic DNA to restriction map analysis. In a series of restriction digests, two bands were visualized, each hybridizing to both calcitonin- and CGRP-specific probes (Fig. 3). On the basis of these analyses, it appears that there are at least two genomic loci containing sequences related to CGRP- and calcitonin-encoding exons. Two structurally distinct human calcitonin-related genes were isolated from a human genomic library (26), and a partial structural organization of each insert was established by restriction enzyme mapping and DNA sequence analysis. By using fragments prepared from rat and human calcitonin and CGRP cDNA clones as probes, it was established that both genomic clones exhibit a similar structural organization with regard to calcitonin- and CGRP-related sequences, with calcitonin-reactive sequences being located 5′ to CGRP-reactive sequences in both genomic clones. DNA sequence analysis

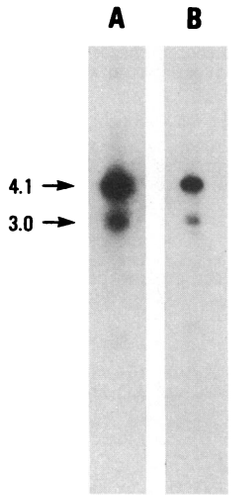

FIG. 3. Restriction enzyme analysis of calcitonin- and CGRP-reactive human genomic DNA. *Eco*RI/*Bam*HI-digested human placental DNA was fractionated by electrophoresis, transferred to nitrocellulose, and hybridized to a clonal rat calcitonin-specific (550 bp) (lane A) or CGRP-specific (220 bp) (lane B) probe as described (2). *Hin*dIII-digested phage λ DNA provided DNA size standards (shown in kilodaltons). A number of other restriction enzyme digests (*Bam*HI, *Eco*RI, and *Bgl* II) revealed two separable bands, each hybridizing both cDNA probes. Each of the two genomic clones ($\lambda hCal_1$ and $\lambda hCal_2$) generate *Eco*RI/*Bam*HI fragments, which comigrate with one of the two reactive bands observed in liver genomic DNA.

```
                                                    -100
---gatcctccaggttctggaagcatgagggtgacgcaacccaggggcaaaggaccctccgcc

                                          -50
cattggttgctgtgcatggcggaactttccgaccacagcggcgggaataagagcagtcgc

                    EXON 1
tggcgctgggagGCATCAGAGACATGCCCAGCCCAAGTGTCGCCGCCGCTTCCACAGGGG

50                                                          100
CTCTGGCTGGACGCCGCCGCCGCCGCTGCCACCGCCTCTGATCCAAGCCACCTCCCGCCA

Ggtgagccccgagattctggc---0.87 kb---atctcattcttcccttgcagAGAGGT

          EXON 2              1050
    MetGlyPheGlnLysPheSerProPheLeuAlaLeuSerIleLeuValLeuLeuGln
GTCATGGGCTTCCAAAAGTTCTCCCCCTTCCTGGCTCTCAGCATCTTGGTCCTGTTGCAG

AlaGlySerLeuHisAlaAlaProPheAr
GCAGGCAGCCTCCATGCAGCACCATTCAGgtaagacagcctgaagccag---0.65 kb-

                              EXON 3                        1800
                          gSerAlaLeuGluSerSerProAlaAspProAlaThrL
--agtttgcttcccctccacagGTCTGCCCTGGAGAGCAGCCCAGCAGACCCGGCCACGC

                                                    1850
euSerGluAspGluAlaArgLeuLeuLeuAlaAlaLeuValGlnAspTyrValGlnMetL
TCAGTGAGGACGAAGCGCGCCTCCTGCTGGCTGCACTGGTGCAGGACTATGTGCAGATGA

                                          1900
ysAlaSerGluLeuGluGlnGluGlnGluArgGluGlySerAr
AGGCCAGTGAGCTGGAGCAGGAGCAAGAGAGAGAGGGCTCCAGgtgaggctccccaagcg

                                    1950          EXON 4
                                        gLeuAspSerProArgSerLysAr
ctc---1.04 kb---ggtatgtgttttccctccagCCTGGACAGCCCCAGATCTAAGCG

                              2000
gCysGlyAsnLeuSerThrCysIleLeuGlyThrTyrThrGlnAspPheAsnLysPheHi
GTGCGGTAATCTGAGTACTTGCATCCTGGGCACATACACGCAGGACTTCAACAAGTTTCA

                         2050
sThrPheProGlnThrAlaIleGlyValGlyAlaProGlyLysLysArgAspMetSerSe
CACGTTCCCCCAAACTGCAATTGGGGTTGGAGCACCTGGAAAGAAAAGGGATATGTCCAG

          2100
rAspLeuGluArgAspHisArgProHisValSerMetProGlnAsnAlaAsnStop   2150
CGACTTGGAGAGAGACCATCGCCCTCATGTTAGCATGCCCCAGAATGCCAACTAAACTCC

2152                                                        2200
TCCCTTTCCTTCCTAATTTCCCTTCTTGCATCCTTCCTATAACTTGATGCATGTGGTTTG

                                          2250
GTTCCTCTCTGGTGGCTCTTTGGGCTGGTATTGGTGGCTTTCCTTGTGGCAGAGGATGTC

                              2300
TCAAACTTCAAGATGGGAGGAAAGAGAGCAGGACTCACAGGTTGGAAGAGAATCACCTGG

                         2350
GAAAATACCAGAAAATGAGGGCCGCTTTGAGTCCCCCAGAGATGTCATCAGCGCTCCTCT

          2400                                              2450
GTCCTGCTTCTGAATGTGCTGATCATTTGAGGAATAAAATTATTTTTCCCCAAAGATCCt

                                                    EXON 5
                                                gIleIleAlaGlnL
ctagagtcgacc---0.59 kb---tctccatccttgcaaatcagAATCATTGCCCAGA

          3200                                              3250
ysArgAlaLysAspThrAlaThrCysValThrHisArgLeuAlaGlyLeuLeuSerArgS
AGAGAGCCTGTGACACTGCCACCTGTGTGACTCATCGGCTGGCAGGCTTGCTGAGCAGAT

                                          3300
erGlyGlyValValLysAsnAsnPheValProThrAsnValGlySerLysAlaPheGlyA
CAGGGGGTGTGGTGAAGAACAACTTTGTGCCCACCAATGTGGGTTCCAAAGCCTTTGGCA

                                    3350
rgArgArgArgAspLeuGlnAlaStop
GGCGCCGCAGGGACCTTCAAGCCTGAGCAGCTGAATGACTCAAGAAGgtgactgcccttg

tatgatg---
```

FIG. 4. DNA sequence of $\lambda hCal_1$. The locations of the "TATA" box and the cap site are inferred from the striking homology with comparable rat genomic sequence data (2, 6); the genomic sequence corresponds precisely to the sequence of the two cDNA clones.

revealed that one genomic clone ($\lambda hCal_2$), although containing regions with homology to the "common region," calcitonin, and CGRP exons, differs in sequence from the comparable regions in the mature calcitonin or CGRP mRNAs. Therefore, this gene encodes neither the calcitonin nor CGRP mRNA. Analysis of a genomic clone ($\lambda hCal_1$) corresponding to the second gene detected by analysis of genomic DNA revealed that the genomic clone contains all of the primary sequence of both calcitonin and CGRP cDNAs shown in Fig. 1, and the point of divergence of calcitonin and CGRP mRNAs corresponds to intron/exon junctions (Fig. 4). The calcitonin- and CGRP-encoding exons are the fourth and fifth exons, respectively, while the second and third exons encode the NH_2-terminal sequences in common with the precursor of both polypeptides. Therefore, these data suggest that alternative RNA processing events do occur in expression of the human calcitonin/CGRP gene in a fashion analogous to events in rat calcitonin/CGRP gene expression and that a single genomic locus does generate both mature transcripts.

Calcitonin and CGRP mRNAs Are Expressed in the Human Pituitary Gland. The possible expression of the calcitonin gene in primate and human pituitary gland was analyzed in light of the previously ambiguous data in the rat. Although calcitonin immunoreactive material has been reported in the rat pituitary, the nature of the reactive material was uncertain (32–36), and calcitonin mRNA was not detected in the rat pituitary gland in RNA blotting analyses (4, 32). More sensitive analyses with nuclease S1-resistance procedures (3) did detect minimal amounts of CGRP mRNA in rat pituitaries (data not shown); however, the levels were so low that they could reflect either initial hypothalamic contamination of the collected pituitaries or the fact that the levels of calcitonin mRNA in pituitary are minimally above limits of detection by this method. Recently, calcitonin- and CGRP-like material have been reported by radioimmunoassay in the human pituitary (37, 38).

To investigate the possible identity of the reactive species as the products of calcitonin gene expression, we analyzed RNA from human autopsy pituitary gland specimens (24–48 hr postmortem). Although the poly(A)-rich RNAs prepared exhibited the expected considerable degree of degradation, calcitonin- and CGRP-reactive sequences were clearly and invariably demonstrable in each human pituitary gland examined. The major calcitonin cDNA-reactive mRNA migrated as a 1-kilobase RNA species, and the CGRP mRNA migrated as a 1.3-kilobase species; however, a smear of degraded RNA was observed below these bands. Quantitation by DNA-excess hybridization of immobilized RNA using a "nick-translated" clonal CGRP-coding region probe (2×10^8 cpm/μg) (39) revealed that the levels of CGRP mRNA in the pituitary glands were 2–4% of the extremely small amounts of CGRP mRNA present in the rat thyroid gland, while CGRP was not detected in human liver RNA [assay background, 10 μg of tRNA = 30 cpm; human liver RNA, 10 μg of poly(A)$^+$ RNA = 32 cpm; human pituitary RNA, 10 μg of poly(A)$^+$ RNA = 140 cpm; rat thyroid RNA, 10 μg of poly(A)$^+$ RNA = 2900 cpm]. The predicted expression of calcitonin- and CGRP-encoded peptides in pituitary is further supported by the observation of immunostaining of monkey pars intermedia cells by anti-CGRP (see Fig. 5) and anti-calcitonin antisera (data not shown). Coproduction of both peptides within individual cells has not yet been established. In conjunction with the extensive literature in this area (e.g., refs. 32–38), it would appear that the calcitonin/CGRP gene is expressed in specific cells within the pituitary gland.

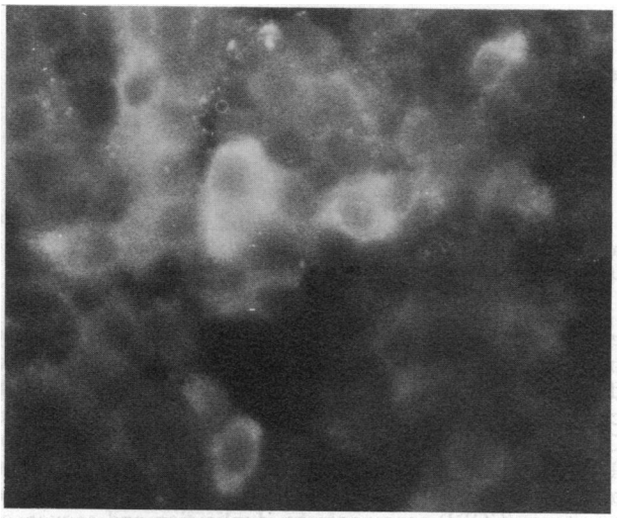

FIG. 5. Histochemical analysis of primate pituitary gland with anti-rat CGRP antisera: a section of monkey pituitary (pars intermedia) exhibiting staining with rat anti-CGRP antisera performed as described (4). Staining is specifically blocked by addition of excess CGRP. A similar pattern of staining was observed with anti-human calcitonin antiserum.

DISCUSSION

The data presented in this manuscript document several predictions concerning human neuroendocrine gene expression, based upon the precedent of alternative RNA processing events in rat calcitonin gene expression. The generation of discrete mRNAs encoding different hormonal products from a single transcription unit appears to occur in expression of the human and rat calcitonin gene. Therefore, the underlying mechanism is likely to involve factors that are evolutionarily highly conserved. Alternative RNA processing events appear to be important in expression of a wide variety of genomic loci (e.g., 2, 3, 7–22, 40, 41). The underlying molecular mechanisms include use of alternative transcriptional initiation sites (37, 38), the stochastic use of alternative splice donor sites (6, 18–22), and the use of alternative poly(A) sites (2, 9–13, 16). In the case of the human calcitonin/CGRP gene, the peptide products encoded by the alternative RNA species are quite similar to the structures of the rat mRNA products. Both RNA products of the human calcitonin/CGRP gene appear to be expressed in the pituitary gland and appear to generate the expected peptide products. These data are in concert with previous reports of immunoreactive calcitonin-like (32–35, 37, 38) and CGRP-like material (38) in rat and human pituitary. Because the levels of CGRP mRNA in the pituitary gland appear to represent only a fraction (<5%) of the low levels of CGRP mRNA present in thyroid gland, it is tempting to speculate that if pituitary CGRP and/or calcitonin exert any physiological functions, they might well occur within the pituitary gland itself. A wide biological variation in CGRP and calcitonin production in pituitary might account for the apparently discrepant data in the case of calcitonin/CGRP gene expression in rat pituitary (3, 31, 33).

To understand the evolutionary mechanisms that generate complex transcription units producing more than one mRNA product, we attempted to elucidate the genomic origins of the calcitonin and CGRP exons in the calcitonin/CGRP gene. Structural comparison of the human (or rat) CGRP and human calcitonin peptides reveals highly suggestive, but not statistically significant, homologies between the two peptides (see Fig. 6). A statistical analysis for evolutionary relatedness of peptides devised and performed by Doolittle

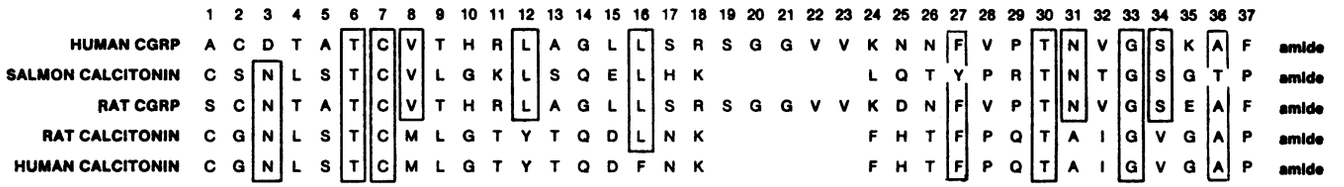

FIG. 6. Comparison of the primary sequences of human CGRP with human and salmon calcitonin by using computer-generated preferred alignment (39). Conserved amino acids between human CGRP and salmon calcitonin are boxed; human calcitonin and human CGRP also share homology at CGRP amino acids 27 and 36.

et al. (42) reveals potentially significant structural homology between salmon calcitonin and human CGRP (3.48 standard deviations from the mean by "jumble" analysis), albeit with caveats based on the small size of the peptide (Fig. 6). Rat CGRP is even more related to salmon calcitonin (Fig. 6). Therefore, it is suggested that calcitonin and CGRP exons are derived from a common primordial gene and that the calcitonin/CGRP gene arose by duplication and sequence-divergence events. The intriguing consequence of these evolutionary events was the generation of a gene with structural and/or sequence information conferring in rat and potentially in human a tissue-specific pattern of RNA-processing regulation; these data imply that there may be a biological advantage to production of CGRP, rather than calcitonin, in specific tissues.

The biological basis for the observation that salmon calcitonin is more potent in humans than is authentic human calcitonin (43) has not been established. Administration of salmon calcitonin has been reported to exert effects on eating behavior and pain perception (44–46). These effects appear, in a sense, to be paradoxical because in the rat it is CGRP and not calcitonin that is expressed in sensory ganglia and in the sensory, motor, and integrative areas subserving ingestive behavior (4). Detailed analysis of the tissue distribution and the biological actions of the CGRP molecule and proof of the predicted patterns of protein processing events are required to determine which, if any, of the potential physiological actions this novel peptide exerts in the human.

We thank Dr. Julia Polak and Dr. Bernard Roos for their efforts in generously providing tissues used in these studies, Dr. Russell Doolittle for comparing human CGRP and salmon calcitonin structures, and Dr. David Margulies for his comments concerning CGRP and calcitonin structural similarities. These studies were supported by grants from the National Institutes of Health and the American Cancer Society.

1. Rosenfeld, M. G., Amara, S. G., Roos, B. A. & Evans, R. M. (1981) *Nature (London)* **291,** 63–65.
2. Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. & Evans, R. M. (1982) *Nature (London)* **298,** 240–244.
3. Rosenfeld, M. G., Lin, C. R., Amara, S. G., Stolarsky, L. S., Ong, E. S. & Evans, R. M. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 1717–1721.
4. Rosenfeld, M. G., Mermod, J.-J., Amara, S. G., Swanson, L. W., Sawchenko, P. E., Rivier, J., Vale, W. W. & Evans, R. M. (1983) *Nature (London)* **304,** 129–135.
5. Fisher, L. A., Kikkawa, D. O., Rivier, J. E., Amara, S. G., Evans, R. M., Rosenfeld, M. G., Vale, W. W. & Brown, M. R. (1983) *Nature (London)* **305,** 534–536.
6. Amara, S. G., Evans, R. M. & Rosenfeld, M. G. (1984) *Mol. Cell. Biol.*, in press.
7. Rozek, C. E. & Davidson, N. (1984) *Cell* **32,** 23–34.
8. Heinkoff, S., Kelly, J. D. & Cohen, E. H. (1983) *Cell* **33,** 607–614.
9. Kitamura, N., Takagaki, Y., Furuto, S., Tanaka, T., Nawa, H. & Nakanishi, S. (1983) *Nature (London)* **305,** 545–549.
10. Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R. & Hood, L. (1980) *Cell* **20,** 313–319.
11. Alt, F. W., Bothwell, A. L., Kapp, M., Siden, E., Mather, G., Koshland, M. & Baltimore, D. (1980) *Cell* **20,** 293–301.
12. Rogers, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L. & Wall, R. (1980) *Cell* **20,** 313–319.
13. Maki, R., Roeder, W., Trauneker, A., Sedman, C., Wabe, M., Raschke, W. & Tonegawa, S. (1981) *Cell* **24,** 353–365.
14. Berget, S. M. & Sharp, P. A. (1979) *J. Mol. Biol.* **179,** 547–565.
15. Nevins, J. R. & Darnell, J. E., Jr. (1978) *Cell* **15,** 1477–1493.
16. Ziff, E. B. (1980) *Nature (London)* **287,** 491–499.
17. Chow, L. T. & Broker, T. R. (1978) *Cell* **15,** 497–510.
18. DeNoto, F. M., Moore, D. D. & Goodman, H. M. (1981) *Nucleic Acids Res.* **9,** 3719–3730.
19. Wallis, M. (1980) *Nature (London)* **284,** 512–516.
20. Crabtree, G. R. & Kant, J. (1982) *Cell* **31,** 159–166.
21. King, C. R. & Piatigorski, J. (1983) *Cell* **37,** 707–712.
22. Nawa, H., Hirose, T., Takashima, H., Inayama, S. & Nakanishi, S. (1983) *Nature (London)* **306,** 32–36.
23. Morris, H. R., Panico, M., Etienne, T., Tippins, J., Girgis, S. I. & MacIntyre, J. (1984) *Nature (London)* **308,** 746–749.
24. Clewell, D. B. & Helinski, D. R. (1969) *Proc. Natl. Acad. Sci. USA* **62,** 1159–1166.
25. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
26. Maniatis, T., Hankson, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. K. & Efstratiadis, A. (1978) *Cell* **15,** 678.
27. Benton, W. D. & Davis, R. W. (1977) *Science* **196,** 180–182.
28. Crea, R., Kraszewski, A., Hirose, T. & Itakura, K. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5765–5769.
29. Sanger, F., Coulson, A. R., Barell, B. G., Smith, A. J. A. & Roe, B. A. (1980) *J. Mol. Biol.* **143,** 161–178.
30. Craig, R. K., Hall, L., Edbrooke, M. R., Allison, J. & MacIntyre, I. (1982) *Nature (London)* **295,** 345–347.
31. Le Moullec, J. M., Jullienne, A., Chenais, J., Lasmoles, T., Guliana, J. M., Milhaud, G. & Moukhtar, M. S. (1984) *FEBS Lett.* **167,** 93–97.
32. Jacobs, J. W., Gottzman, D. & Habener, J. F. (1982) *Endocrinology* **111,** 2014–2019.
33. Deftos, L. J., Burton, D., Bone, H. G., Catherwood, B. D., Parthemore, J. G., Moore, R. Y., Minick, S. & Guillemin, R. (1978) *Life Sci.* **23,** 743–748.
34. Deftos, L. J., Burton, D., Catherwood, B. D., Bone, J. G., Parthemore, J. G., Guillemin, R., Watkins, W. B. & Moore, R. Y. (1978) *J. Clin. Endocrinol. Metab.* **47,** 457–459.
35. Cooper, C. W., Peng, T.-C., Obie, J. F. & Garner, S. C. (1980) *Endocrinology* **107,** 98–107.
36. Mendelsohn, G., D'Agostino, R., Eggleston, J. C. & Baylin, S. B. (1979) *J. Clin. Invest.* **63,** 1297–1306.
37. Fischer, J. A., Tobler, P. H., Kaufman, M., Born, W., Henke, H., Cooper, P. E., Sagar, S. M. & Martin, J. B. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7801–7805.
38. Tschopp, F. A., Tobler, P. H. & Fischer, J. A. (1984) *Mol. Cell. Endocrinol.* **36,** 53–57.
39. Potter, E., Nicolaisen, A. K., Ong, E. S., Evans, R. M. & Rosenfeld, M. G. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6662–6666.
40. Young, R. A., Hagenbuchle, O. & Schibler, U. (1981) *Cell* **23,** 451–458.
41. Nabeshima, Y., Fujii-Kuriyama, Y. F., Muramatsu, M. & Ogata, K. (1984) *Nature (London)* **22,** 333–338.
42. Doolittle, R., Feng, D.-F. & Johnson, M. F. (1984) *Nature (London)* **307,** 558–560.
43. Austin, L. A. & Heath, H., III (1981) *N. Engl. J. Med.* **304,** 269–278.
44. Braga, P., Terri, S., Santagostino, A., Olgiatio, V. R. & Pecile, A. (1978) *Life Sci.* **22,** 971–979.
45. Yamamoto, M., Tachukawa, W. & Maeno, H. (1981) *Neuropharmacology* **20,** 83–86.
46. Twery, M. J., Obie, J. F. & Cooper, C. W. (1982) *Peptides* **3,** 749–752.