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# Cloning and characterization of cDNAs encoding human gastrin-releasing peptide

(bombesin/mRNA/neuropeptide)

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**ABSTRACT** **We have prepared and cloned cDNAs derived from poly(A)$^+$ RNA from a human pulmonary carcinoid tumor rich in immunoreactivity to gastrin-releasing peptide, a peptide closely related in structure to amphibian bombesin. Mixtures of synthetic oligodeoxyribonucleotides corresponding to amphibian bombesin were used as hybridization probes to screen a cDNA library prepared from the tumor RNA. Sequencing of the recombinant plasmids shows that human gastrin-releasing peptide (hGRP) mRNA encodes a precursor of 148 amino acids containing a typical signal sequence, hGRP consisting of 27 or 28 amino acids, and a carboxyl-terminal extension peptide. hGRP is flanked at its carboxyl terminus by two basic amino acids, following a glycine used for amidation of the carboxyl-terminal methionine. RNA blot analyses of tumor RNA show a major mRNA of 900 bases and a minor mRNA of 850 bases. Blot hybridization analyses using human genomic DNA are consistent with a single hGRP-encoding gene. The presence of two mRNAs encoding the hGRP precursor protein in the face of a single hGRP gene raises the possibility of alternative processing of the single RNA transcript.**

Gastrin-releasing peptide (GRP) is a mammalian equivalent of the amphibian tetradecapeptide bombesin (1). GRP was originally isolated from porcine stomach by using the release of gastrin as a bioassay (2). Peptides related in structure have been isolated from avian proventriculus (3) and canine small intestine (4). Bombesin and the GRPs share similarities in their sequences and contain a common carboxyl-terminal heptapeptide.

GRP and bombesin have similar biological effects (5). Infusion of nanogram amounts of either peptide to dogs or humans increases plasma immunoreactive levels of gastrin, pancreatic polypeptide, glucagon, gastric inhibitory peptide, and insulin (5, 6). Bombesin is also a potent neuropeptide; intraventricular injections of 1 pM bombesin into rats elevates blood sugar (7) and produces hypothermia (8). These potent effects coupled with the wide distribution of bombesin-like peptides throughout the mammalian gastrointestinal tract (9) and nervous system (10) support the role of bombesin as an important neuroregulatory peptide.

Bombesin-like immunoreactivity is also detected in the neuroendocrine cells of the lung (11). In humans, the highest levels are found in the lung just after birth (12) and levels then decrease in parallel with the observed decrease in the number of pulmonary neuroendocrine cells (13). Bombesin immunoreactivity is also found in high levels in pulmonary carcinoid tumors as well as in many small cell carcinomas of the lung (14, 15). Hence, bombesin-like immunoreactivity is a potential chemical marker for these tumors.

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As is characteristic of many neuropeptides, mammalian bombesin-like peptides exist in multiple forms. Bombesin-related peptides of 10 and 27 amino acids have been isolated from porcine (16) and canine (4) tissues. Analyses by high-pressure liquid chromatography of fetal lung (17) and lung tumors (15, 18, 19) yield multiple peaks of immunoreactivity; one peak elutes near bombesin and one elutes near GRP. Multiple immunoreactive forms are also detected in gastrointestinal tissue (9, 20). In brain, immunohistochemistry reveals some distinct regions that react to bombesin antisera and other regions that react only with antisera to ranatensin (21), another amphibian peptide structurally related to bombesin.

To clarify these complexities of the bombesin-like family of peptides, we wish to analyze the gene(s) encoding members of this peptide family. As an initial step in these studies, we describe here the molecular cloning and characterization of cDNAs corresponding to mRNAs encoding the precursor of human GRP (hGRP; pre-proGRP).

## METHODS

Polyadenylylated RNA was obtained from a hepatic metastasis of a pulmonary carcinoid tumor rich in bombesin-like immunoreactivity (§), by using the guanidine thiocyanate procedure (23) and oligo(dT) chromatography (24). Double-stranded cDNA was prepared enzymatically as described by Crabtree and Kant (25). A recombinant cDNA library was prepared using homopolymeric dC·dG extensions and *Pst* I cut pBR322 (New England Nuclear). *Escherichia coli* MC1061 were transformed by the calcium-shock procedure (26).

Oligodeoxynucleotide probes were synthesized by the phosphate triester method (27). The probes (Fig. 1) corresponded to two different regions of amphibian bombesin and ranatensin to allow double screening and thus decrease the detection of false-positive recombinants. Duplicate high- (28) and low-density (29) colony screening was carried out with 5′-end-labeled (30) probes using hybridization conditions as described by Hanahan and Meselson (28). Cell-free translations using wheat germ extract and reticulocyte lysate, plasmid preparations, RNA hybridization, DNA hybridization, and DNA chemical sequence analyses were carried out by standard techniques (31–37).

RNA blots were hybridized for 18 hr at 42°C in 50% formamide/5× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate)/5× Denhardt's solution (1× Denhardt's solution = 0.02% Ficoll-400/0.02% bovine serum albumin/0.02% polyvinylpyrrolidone-40)/0.5% sodium dodecyl sulfate/sonicated denatured salmon sperm (100 μg/ml). Southern blots

Abbreviations: GRP, gastrin-releasing peptide; hGRP, human GRP; pre-proGRP, precursor of hGRP.

§Price, J., Nieuwenhuijsen-Kruseman, A. C., Clement-Jones, V. & Rees, L. H. (1983) Endocrine Society, 65th Annual Meeting, San Antonio, TX, June 8–10, 188 (abstr.).

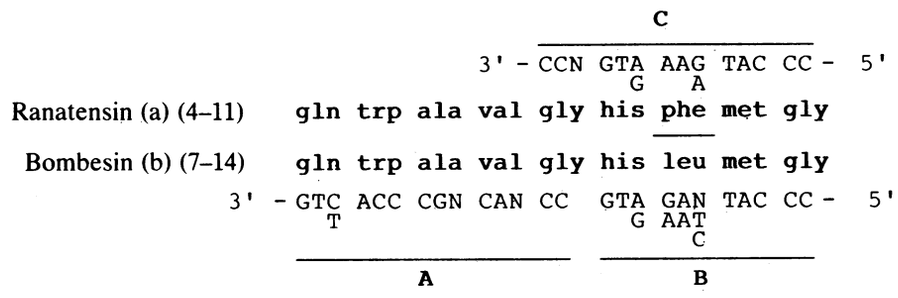


FIG. 1. Design of oligodeoxyribonucleotide probes. Probe A was prepared as two 16-fold mixed sets ($A_1$ and $A_2$); $A_1$ used the GTC and $A_2$ used the GTT complement to the glutamine codon. Probe B was synthesized as a 32-fold mixed set. Probe C was made to ranatensin-(8–11), which is closely related to bombesin-(11–14), but requires only a 16-fold mixed synthesis. N indicates the inclusion of all 4 bases. Hybridization temperatures for probes A, B, and C were 41°C, 28°C, and 37°C, respectively. Sequences are ranatensin-(4–11) (*a*) and bombesin-(7–14) (*b*).

were hybridized for 36 hr at 45°C in the same hybridization solution.

## RESULTS

From 3 g of tumor, 100 μg of poly(A)$^+$ RNA was prepared. Addition of 1 μg of RNA to cell-free translation systems resulted in 10- to 30-fold increases in protein synthesis, thus demonstrating the integrity of the tumor mRNA. By using 10 μg of poly(A)$^+$ RNA, 0.8 μg of double-stranded cDNA was synthesized as described by Crabtree and Kant (25). Transformation of *E. coli* with 100 ng of cDNA produced 20,000 colonies. High- followed by low-density screening with probes $A_2$ and C (Fig. 1) yielded 15 hybridizing colonies (probe C hybridized to essentially the same colonies as probe B but with less background). Sequence analyses of the recombinant plasmids obtained from these colonies (Fig. 2) yielded close to the entire sequences for the mRNA encoding pre-proGRP (Fig. 3). Both probes $A_2$ and C hybridized appropriately despite single base mismatches.

The deduced amino acid sequence shows that hGRP is part of a 148-amino acid polypeptide that is delimited by an AUG start codon and three successive stop codons. The amino-terminal portion of this precursor contains a cluster of hydrophobic amino acids consistent with the "pre" or signal, sequence (38). At the carboxyl terminus, hGRP is flanked by two lysines following a glycine, which likely provides the signal for amidation of the terminal methionine residue (39). Pre-proGRP does not code for any additional GRP-like or bombesin-like peptides, nor are there any other peptides set off by two consecutive basic amino acids characteristic of sites that are cleaved during the post-translational processing of prohormones. The protein-coding region is followed by 289 untranslated bases before the poly(A) tail commences; a typical A-A-A-T-A-A polyadenylylation signal (40) occurs 19 bases before the poly(A) tail.

Hybridization analysis (RNA blot) demonstrates the presence of two mRNAs: a predominant mRNA of 900 bases and a minor RNA of approximately 850 bases (Fig. 4). Hybridization analysis (Southern blot) of genomic DNA from human lymphocytes shows only a single band with *Bam*HI, *Hin*dIII, and *Eco*RI digests, consistent with a single gene coding for pre-proGRP.

## DISCUSSION

Cloning of cDNA prepared from a pulmonary carcinoid tumor demonstrates that hGRP is encoded in a precursor protein. There is a hydrophobic signal sequence (38), although the exact cleavage point of the "pre" sequence is not deter-

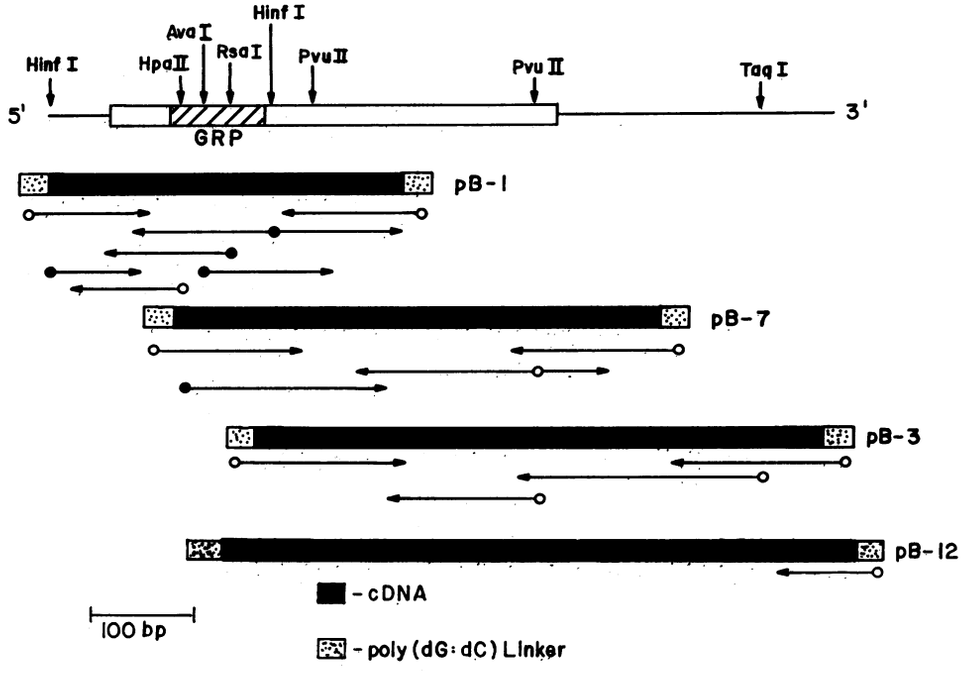


FIG. 2. DNA sequencing strategy for cDNAs encoding pre-proGRP. DNA fragments were produced by the indicated restriction enzymes and were either 5'- (●) or 3'- (○) end-labeled and sequenced by the method of Maxam and Gilbert (30). All DNA sequences were confirmed by sequencing on two different recombinant plasmids or on both strands of a single plasmid.

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                                                                    -23
                                                                    Met Arg Gly
5'....  AGTCTCTGCTCTTCCCAGCCTCTCCGGCGCGCTCCAAGGGCTTCCCGTCGGGACC ATG CGC GGC    64

                    Signal  Peptide          -5 ↓          -2  -1 ↓
Ser Glu Leu Pro Leu Val Leu Leu Ala Leu Val Leu Cys Leu Ala Pro Arg Gly Arg Ala   124
AGT GAG CTC CCG CTG GTC CTG CTG GCG CTG GTC CTC TGC CTA GCG CCC CGG GGG CGA GCG

+1   Gastrin-Releasing  Peptide  +13            +17
Val Pro Leu Pro Ala Gly Gly Gly Thr Val Leu Thr Lys Met Tyr Pro Arg Gly Asn His   184
GTC CCG CTG CCT GCG GGC GGA GGG ACC GTG CTG ACC AAG ATG TAC CCG CGC GGC AAC CAC

                    +27
Trp Ala Val Gly His Leu Met Gly Lys Lys Ser Thr Gly Glu Ser Ser Ser Val Ser Glu   244
TGG GCG GTG GGG CAC TTA ATG GGG AAA AAG AGC ACA GGG GAG TCT TCT TCT GTT TCT GAG

Arg Gly Ser Leu Lys Gln Gln Leu Arg Glu Tyr Ile Arg Trp Glu Glu Ala Ala Arg Asn   304
AGA GGG AGC CTG AAG CAG CAG CTG AGA GAG TAC ATC AGG TGG GAA GAA GCT GCA AGG AAT

Leu Leu Gly Leu Ile Glu Ala Lys Glu Asn Arg Asn His Gln Pro Pro Gln Pro Lys Ala   364
TTG CTG GGT CTC ATA GAA GCA AAG GAG AAC AGA AAC CAC CAG CCA CCT CAA CCC AAG GCC

Leu Gly Asn Gln Gln Pro Ser Trp Asp Ser Glu Asp Ser Ser Asn Phe Lys Asp Val Gly   424
TTG GGC AAT CAG CAG CCT TCG TGG GAT TCA GAG GAT AGC AGC AAC TTC AAA GAT GTA GGT

Ser Lys Gly Lys Val Gly Arg Leu Ser Ala Leu Gly Ser Gln Arg Glu Gly Arg Asn Pro   484
TCA AAA GGC AAA GTT GGT AGA CTC TCT GCT CTA GGT TCT CAA CGT GAA GGA AGG AAC CCC

Gln Leu Asn Gln Gln  Stop Stop Stop
CAG CTG AAC CAG CAA  TGA  TAA  TGA  TGGCCTCTCTCAAAAGAGAAAAACAAAACCCCTAAGAGACTGA   551

GTTCTGCAAGCATCAGTTCTACGGATCATCAACAAGATTTCCTTGTGCAAAATATTTGACTATTCTGTATCTTTCATCC   630

TTGACTAAATTCGTGATTTTCAAGCAGCATCTTCTGGTTTAAACTTGTTTGCTGTGAACAATTGTCGAAAAGAGTCTTC   709

CAATTAATGCTTTTTTATATCTAGGCTACCTGTTGGTTAGATTCAAGGCCCCGAGCTGTTACCATTCACAATAAAAGCT   788

TAAACACAT AAAAAAA .... Poly(A)                                          3'      797
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FIG. 3. Composite nucleotide and amino acid sequence of the mRNA encoding pre-proGRP deduced from sequencing the cDNAs shown in Fig. 2. The most probable signal peptide sequence is numbered −22 to −1; ↓ indicates possible signal peptide cleavage points. Box around the glycine at +28 indicates the source for amidation of the carboxyl-terminal methionine of hGRP. The cleavage site of two basic amino acids is marked by double lines. There are three consecutive stop codons at bases 500–508. The polyadenylylation signal at bases 779–784 is underlined.

mined and may occur after one of the short-chain hydrophilic amino acids indicated in Fig. 3. Studies on signal peptidase recognition sites (38) suggest the most likely site for cleavage would be after alanine (−1). In this case, hGRP would be a 27-amino acid peptide identical in length to previously isolated GRPs (Fig. 5). Alternatively, cleavage could occur after alanine (−5), thus necessitating removal of a tetrapeptide prosequence after arginine (−2). In this case, hGRP would be a 28-amino acid peptide. Such a cleavage at a single basic residue at the amino terminus is less common than cleavage at two basic residues, but it occurs in the precursors for somatostatin-28 (41), growth hormone releasing-hormone (42), and arginine vasopressin–neurophysin II (43). The carboxyl terminus of hGRP is flanked by two basic amino acids following the glycine, which signals the amidation of the terminal methionine residue.

GRP shares an identical 15-amino acid sequence at the carboxyl terminus with canine and porcine GRP; there is less sequence homology at the amino terminus (Fig. 5). The valine at position one makes hGRP more hydrophobic than porcine GRP, a finding consistent with reversed-phase high pressure liquid chromatography of GRP-like immunoreactivity in pulmonary carcinoid (18) or neonatal lung (44) extracts. The high pressure liquid chromatography data would also be consistent with the possibility that hGRP has an additional amino acid (alanine) at its amino terminus.

hGRP contains two potential internal tryptic cleavage sites that could generate hGRP-(14–27) or hGRP-(18–27). High-pressure liquid chromatography of GRP immunoreactivity demonstrates one species consistent with hGRP-(1–27) and a smaller form consistent with either hGRP-(14–27) (17) or hGRP-(18–27) (18, 44). The two forms of hGRP probably derive from alternative proteolytic processing of pre-proGRP into both GRP-(1–27) and a smaller GRP-like peptide, as occurs with somatostatin-28 and somatostatin-14 (41). Alterna-

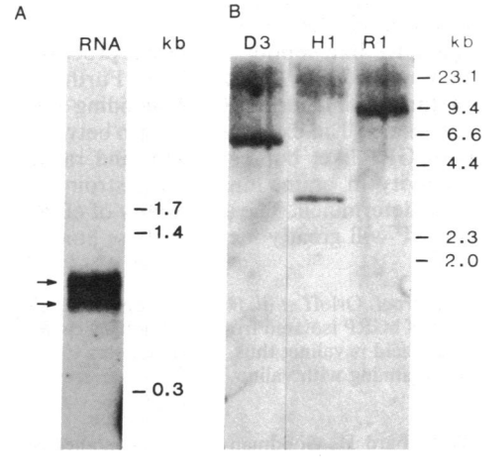


FIG. 4. RNA and DNA blot hybridization analyses. (*A*) Five micrograms of poly(A)$^+$ tumor RNA was fractionated on 1.5% agarose, transferred to Zetapor (AMF Cuno, Meriden, CT), and hybridized to nick-translated plasmid pB-1 (specific activity = $4 \times 10^8$ cpm/$\mu$g). Kodak XAR film was exposed 48 hr at −70°C without an intensifying screen. Size markers are pBR322 DNA digested with *Ava* II. (*B*) Human genomic DNA was digested to completion with *Hin*dIII (D3), *Bam*HI (H1), or *Eco*RI (R1), fractionated on 0.8% agarose, transferred to Zetapor membrane, and hybridized to nick-translated plasmid pB-1. Exposure was for 48 hr at −70°C with an intensifying screen. Size markers are bacteriophage λ DNA digested with *Hin*dIII.

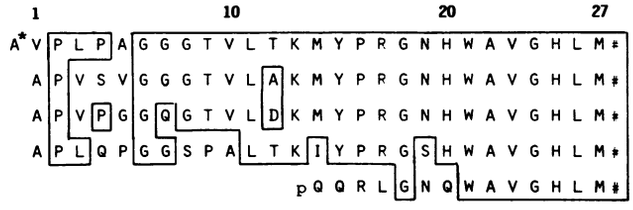


FIG. 5. Comparison of the amino acid sequence of hGRP with related peptides. Solid boxes enclose regions of homology with hGRP. M# indicates methioninamide. pQ indicates pyroglutamate. A* indicates that hGRP could (though probably does not) have an additional amino-terminal alanine. Amino acids are designated by standard one-letter abbreviations. Sequences of the following peptides are shown (from top to bottom): hGRP, porcine GRP, canine GRP, avian GRP, amphibian bombesin.

tively, the smaller GRP-like peptide could be encoded in a second mRNA. Conceivably, the smaller GRP-like immunoreactive forms could be artifacts of peptide extraction, but the consistency with which these forms are observed and their variation in concentration in different tissues makes this circumstance unlikely (15, 17–20, 44).

RNA blot analysis shows a major mRNA of ≈900 bases and a minor mRNA of ≈850 bases (Fig. 4). The size of the major mRNA is consistent with the 800-base cDNA sequence shown in Fig. 3. Additional cloned recombinant plasmids are being screened to determine whether the smaller mRNA encodes a second GRP-like peptide in possible analogy with the two mRNAs that encode substance P and the related peptide substance K (22).

Preliminary genomic Southern blot analysis shows only single bands in restriction enzyme digests with six-base recognition sites. This pattern is consistent with the existence of a single gene and suggests that the two GRP-encoding mRNAs may arise from alternative exon splicing of a single transcript, alternative sites of initiation of transcription, or alternative sites of transcription termination and polyadenylylation.

Inasmuch as GRP is produced by pulmonary carcinoid tumors and small cell carcinomas of the lung, it is a potentially important tumor marker. Because patterns of immunoreactivity observed after chromatography of tumor extracts reveal multiple GRP-like peptides, different processing of pre-proGRP may occur in different tissues. Further molecular analysis of tumor and normal GRP-encoding mRNAs will clarify these issues. The exact relationship between the various forms of GRP-like, bombesin-like, and ranatensin-like immunoreactivity in brain, lung, and gastrointestinal tract remains to be determined. The availability of cDNAs encoding pre-proGRP will greatly facilitate these studies.

**Note Added in Proof.** Orloff *et al.* (45) have reported a partial amino acid sequence of hGRP isolated from a pulmonary carcinoid tumor. The first amino acid is valine; thus, hGRP appears to be a 27 amino acid peptide beginning with valine.

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