Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 3548–3551, June 1985
Biochemistry

Coding sequences for vasoactive intestinal peptide and PHM-27 peptide are located on two adjacent exons in the human genome

(oligodeoxynucleotides/genomic library screening/restriction mapping)

MORDECHAI BODNER*†, MATI FRIDKIN†, AND ILLANA GOZES*‡

Departments of *Hormone Research and †Organic Chemistry, The Weizmann Institute of Science, Rehovot 76100, Israel

Communicated by Christian B. Anfinsen, January 7, 1985

ABSTRACT **The human precursor gene for vasoactive intestinal peptide (VIP) and PHM-27, a peptide that has an NH_2-terminal histidine and COOH-terminal methionine amide and is closely related in sequence and activity to VIP, was detected with synthetic oligodeoxynucleotide probes. These specific hybridization segments were constructed according to the neuroblastoma VIP cDNA sequence and contained up to 39 bases. The gene structure was partly deduced by hybridization to synthetic oligodeoxynucleotide probes and partly by direct chemical nucleotide sequencing. Four exons were discovered thus far; among them are two short exons separated by a 0.75-kilobase DNA stretch, one encoding PHM-27 and the second encoding VIP (exons 1 and 2). Each of these two exons encodes both the hormone amino acid residues as well as the post-translational processing signal sequences. The 3′ splice sites of the two exons contain an identical stretch of nine nucleotides. At the cDNA level, the 3′ splice sites contain the same stretch of six nucleotides, which are identically spliced. The occurrence of VIP and PHM-27 coding sequences on two separate exons of the human genome and the homology of their 3′ splice site may allow alternative RNA processing as discussed below.**

VIP is a 28-amino acid peptide originally isolated from porcine duodenum (1). This peptide is found in the central and peripheral nervous systems and in endocrine cells, where it exhibits both neurotransmitter and hormonal roles (2, 3). VIP-containing structures have been identified by light and electron microscopy techniques (3, 4) as being closely associated with blood vessels in the brain. In addition, VIP has induced relaxation of cerebral blood vessels (5), supporting the hypothesis that a VIP-like substance is the transmitter for vasodilation in the brain (3). VIP in the peripheral nervous system has been suggested recently as a neurotransmitter in penile erection (6, 7). As a hormone, VIP may be a candidate for the prolactin-releasing factor. However, immunohistochemical studies have revealed so far only single VIP-positive nerve fibers in the medial basal hypothalamus, from which hormones are released into the portal blood (8). VIP is closely related in its structure and function to other peptide hormones such as the recently discovered PHM-27, a peptide with an NH_2-terminal histidine and COOH-terminal methionine amide. This peptide is the human analog of the porcine PHI-27, which has a COOH-terminal isoleucine. PHI-27, in contrast to VIP, is present in the medial basal hypothalamus, forming a dense fiber network around the portal capillaries, and may be involved in the control of prolactin secretion (8). In human neuroblastoma and in other tumors that produce VIP, VIP and PHM-27 were shown to coexist on the same protein precursor (9, 10). As clearly outlined above, not all of the cells produce VIP and PHM-27 simultaneously (8, 9). Thus, we decided to investigate the structure of the VIP gene, which may shed light on its modes of expression. Short cDNA sequences (9) were chosen and chemically synthesized, which then allowed a facilitated gene library screening and characterization of the gene. We discovered that VIP and PHM-27 are encoded by two independent exons on the human genome, and the possibility of alternative RNA processing is discussed.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from New England Biolabs. Reverse transcriptase was obtained from Life Sciences (St. Petersburg, FL). Radiochemicals and additional enzymes were purchased from the Radiochemical Centre.

Methods. *Preparation of synthetic oligodeoxynucleotides.* Oligodeoxynucleotides (up to 21 bases long) were synthesized by the modified deoxynucleotide phosphoramidite approach with diisopropylphosphoramiditic nucleosides (11). Longer probes were produced by ligation as follows: oligodeoxynucleotides to be ligated were labeled with [γ-^{32}P]ATP in the reaction catalyzed by the enzyme T4 polynucleotide kinase (12); the labeled probes were hybridized to short synthetic linker sequences and ligated at 12°C using T4 DNA ligase by a published procedure (13). The synthetic probes obtained are outlined in Fig. 1.

Screening of a human genomic library for the VIP-encoding gene. The human gene library was a kind gift of R. Miskin and J. Axelrod. It was a collection of recombinant λ phage carrying human DNA fragments of 15–17 kilobases (kb) generated by partial digestion with *Eco*RI and joined to phage λ Charon 4A arms (14). Screening of the phage library was performed by hybridization with synthetic oligonucleotides end-labeled as above with [γ-^{32}P]ATP (5000 Ci/mmol; 1 Ci = 37 GBq).

Characterization of the VIP gene. λ phage was isolated by CsCl gradient centrifugation (13). Restriction endonuclease mapping and blot hybridization analysis were conducted as described (15). Hybridization temperatures for each probe were calculated by using the following formula: 69.3°C + [0.41°C multiplied by the percentage of G+C in the probe] − [650°C divided by the number of bases in the probe] (16). Subcloning of restricted gene fragments (*Eco*RI–*Pst* I) in bacterial hosts was conducted by using pBR322 vector lacking an *Eco*RI–*Pst* I fragment of 752 bases (ampicillin sensitive). Chimeric plasmids were used to infect *Escherichia coli* RR1 bacteria (13), and tetracycline-resistant transformants were screened by using the appropriate oligodeoxynucleotide probe. Cloned DNA fragments were further digested with the restriction enzyme *Hin*fI and labeled in the presence of reverse transcriptase and [α-^{32}P]dATP (3000 Ci/mmol) (17). DNA sequencing was performed basically as described by Maxam and Gilbert (18). The organization of *Hin*fI fragments in the cloned *Eco*RI–*Pst* I gene segments was deduced

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.

Abbreviations: VIP, vasoactive intestinal peptide; kb, kilobase(s).
‡To whom reprint requests should be addressed.

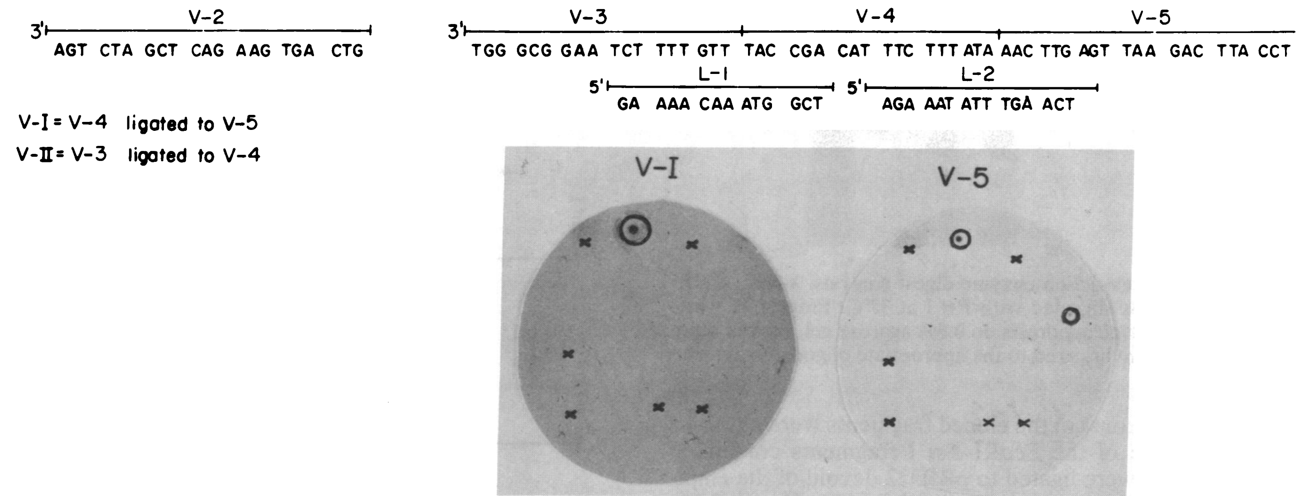

FIG. 1. Screening of human genomic library for VIP gene fragments. DNA hybridization was performed with synthetic oligodeoxynucleotides containing sequences as in the cloned VIP cDNA sequences. Each oligonucleotide (40 pmol) was labeled with [γ-^{32}P]ATP in the reaction catalyzed by T4 polynucleotide kinase (12) and was ligated by using an equimolar amount of synthetic linker (L-1; L-2) and T4 DNA ligase at 12°C for 17 hr (13). Hybridization was conducted at 50°C with the oligodeoxynucleotide probe denoted V-5 and at 66°C with probe V-I in 0.9 M NaCl/0.09 M sodium citrate, pH 7/0.1% polyvinylpyrrolidone/0.1% Ficoll/0.1% bovine serum albumin (13). Out of 300,000 plaques screened, 3 plaques gave positive hybridization to all the probes tested. An example of one such plaque is shown. A higher specificity was obtained with a longer oligodeoxynucleotide probe (V-I), as compared to one (encircled) nonspecific plaque obtained with V-5. Repeated subcloning steps enabled the isolation of a pure clone.

by labeling the *Eco*RI site with [α-^{32}P]dATP, followed by partial digestion with *Hin*fI and analysis of the products on a polyacrylamide gel.

RESULTS

Screening a Genomic Library. The human DNA library was screened for phage carrying the VIP precursor gene by hybridization *in situ* with four synthetic DNA probes corresponding to sequences 403–438 (probe V-I), 421–459 (probe V-II), 379–399 (probe V-2), and 439–459 (probe V-5) of the cloned cDNA (ref 9; see Fig. 1). The first two probes (V-I and V-II), which are of longer sequence and of higher (approximately doubled) specific activity as compared to the second two probes, hybridized identically to 3 plaques out of ≈300,000 recombinant phages screened. Probe V-2 hybridized to 10 plaques, 3 of which were identical to the former 3, with the rest probably representing nonrelated DNA segments. Similarly, probe V-5 hybridized to 9 additional plaques, among them 3 identical to the above 3 plaques hybridizing to all VIP-related probes.

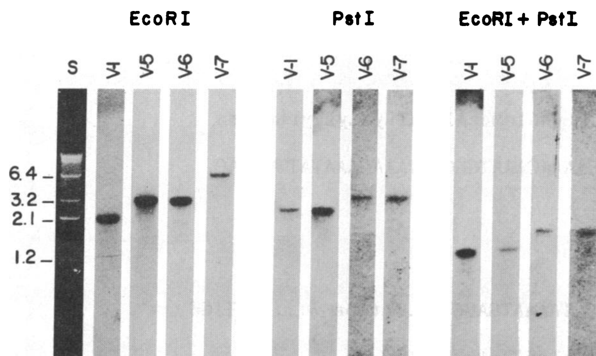

FIG. 2. Simplified restriction analysis of the human VIP gene. Cloned λ-VIP$_1$ DNA (10 μg) was cleaved with several restriction enzymes. The fragments were loaded on 0.8% agarose gel, electrophoresed, transferred to nitrocellulose, and hybridized to specific labeled oligonucleotide probes (15) shown at the top of each lane. Lane S shows *Eco*RI fragments (size in kb) stained with ethidium bromide. (See text and Fig. 1 for detailed description of the synthetic DNA probes.)

Restriction Mapping. The structure of one genomic clone (λ-VIP$_1$) was determined by *Eco*RI and *Pst* I digestions (Fig. 2).

Exons were identified by using synthetic probes complementary to the published neuroblastoma VIP mRNA sequences (9). The synthetic sequences are given in Table 1.

As shown in Fig. 2, probes denoted V-5 and V-6 hybridized to the same *Eco*RI fragment of 3.2 kb; probes V-1 and V-5 both hybridized to the same *Pst* I fragment of about 2.65 kb; probes V-6 and V-7 hybridized to the same *Pst* I fragment of ≈3.5 kb. To further characterize the *Pst* I fragments hybridizing to two remote probes, partial digests were conducted, and the two probes gave identical patterns of hybridization (Fig. 3). Detailed restriction maps for the mRNA cod-

Table 1. Sequences of synthetic probes

Probe*	Sequence
V-1 (244–264)	3′ C-G-A-C-T-A-C-C-T-C-A-A-A-A-G-T-G-G-T-C-A
V-2 (379–399)	3′ A-G-T-C-T-A-G-G-T-C-A-G-A-A-G-T-G-A-C-T-G
V-3 (403–420)	3′ T-G-G-G-C-G-G-A-A-T-C-T-T-T-T-G-T-T
V-4 (421–438)	3′ T-A-C-C-G-A-C-A-T-T-T-C-T-T-T-A-T-A
V-5 (439–459)	3′ A-A-C-T-T-G-A-G-T-T-A-A-G-A-C-T-T-A-C-C-T
V-6 (470–498)	3′ C-A-C-T-C-C-C-T-C-T-T-A-G-A-G-G-G-C-T-G
V-7 (1229–1248)	3′ C-A-C-T-T-C-A-C-T-T-A-C-T-T-T-G-T-G-A-G

*The locations of these sequences along the RNA are denoted in parentheses.

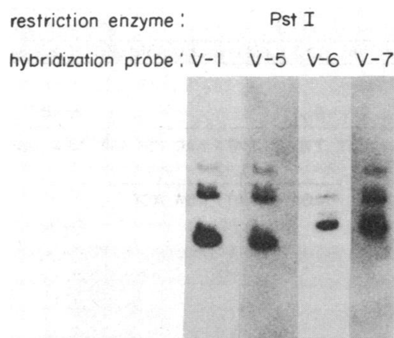

FIG. 3. Partial restriction enzyme digest analysis. λ-VIP$_1$ DNA (10 μg) was partially digested with *Pst* I at 37°C. Fragments were then subjected to electrophoresis on 0.8% agarose gel, blotted onto nitrocellulose, and hybridized to the appropriate oligodeoxynucleotide.

ing sequences (exons) in the cloned fragments were obtained as follows. Each of the *Eco*RI–*Pst* I fragments containing exon sequences were ligated to pBR322 devoid of the ampicillin-resistance site and were further cloned in *E. coli* RR1. The cloned gene fragments were then restricted with *Hin*fI, subjected to electrophoresis on 2.5% agarose gel, and analyzed by blot-hybridization (15). Using this approach, we established the location of exon sequences within the cloned gene fragment. The arrangement of the *Hin*fI fragments within the cloned gene segments was deduced from the partial *Hin*fI digest of the *Eco*RI end-labeled fragment compared with known lengths of *Hin*fI fragments, obtained after complete digestion (Fig. 4).

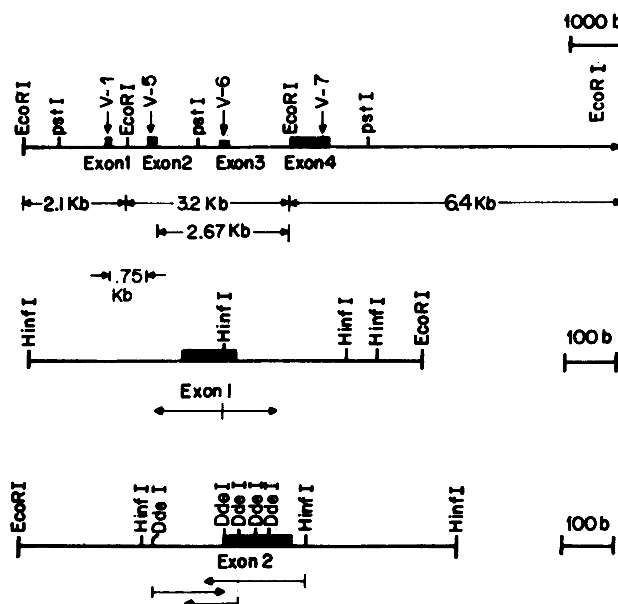

FIG. 4. Restriction mapping of cloned human genomic DNA containing the prepro-VIP gene and sequencing strategy. (*Top*) A part of the cloned human DNA containing four exons. (*Middle*) PHM-27-encoding sequences (exon 1). (*Bottom*) VIP-encoding sequences (exon 2).

Nucleotide Sequence Analysis. The *Hin*fI fragments containing exon sequences and their surrounding regions were subjected to chemical nucleotide sequence analysis by the

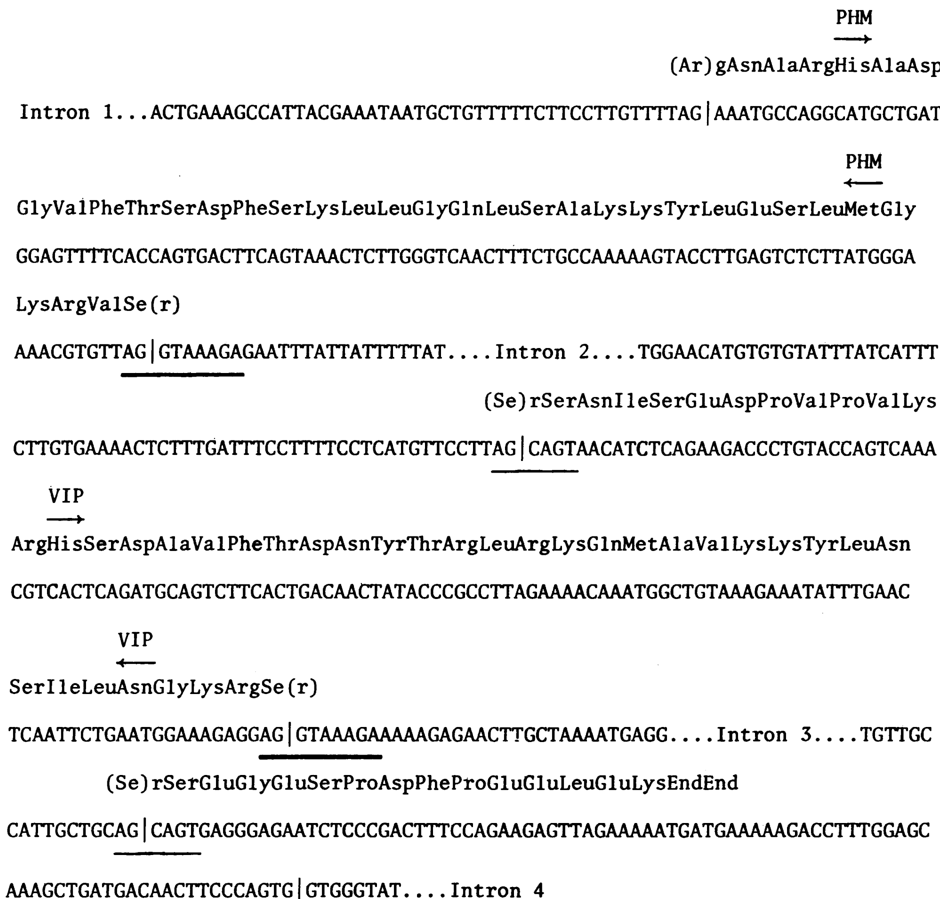

FIG. 5. A part of human genomic DNA encoding VIP and PHM sequences. Exons 1, 2, and 3 are denoted together with their flanking intronic sequences. The two pairs of nine-base-long (heavy line) and six-base-long (thin line) identical sequences are underlined at the splice junctions.

procedure of Maxam and Gilbert (18) according to the strategy indicated in Fig. 4. In the two peptide (VIP and PHM-27)-encoding exons sequenced, we have found a complete correlation to the published cDNA sequence. These exons are short, terminating a few bases (two or five) after the posttranslational amidation signal at the COOH terminus of the peptide (19). At the cDNA level, their 3′ splice sites both contain an identical stretch of six nucleotides (A-G-C-A-G-T) that also are spliced identically (9). Indeed, the same codon is spliced (AG/C of serine). At the gene level, the 3′ splice sites of the two exons and their adjoining introns contain an identical, repetitive, stretch of nine bases (Fig. 5). Moreover, their subsequent downstream introns contain an identical stretch of six nucleotides at their 3′ splice site (Fig. 5).

DISCUSSION

Molecular cloning and DNA sequence analysis have established the primary structure of a part of the VIP gene. Synthetic oligodeoxynucleotides were successfully used in the screening and sequencing of the VIP gene. The discontinuity of the genetic segments encoding mature RNAs and the complexity of RNA processing pathways suggest that alternative splicing events could be one of the mechanisms for increasing the flexibility of gene expression in the neuroendocrine system, as was found for the calcitonin gene (20). Indeed, our finding of two separate exons encoding either VIP or PHM-27 and the existence of specific cells expressing either VIP or PHI-27 (8) supports the possibility of alternative RNA processing. Blot-hybridization analysis with either the labeled gene fragments or oligodeoxynucleotides as hybridization probes for RNA detection revealed a high molecular weight RNA (≈7000 bases long) in all VIP-producing tissues examined thus far (unpublished data). A smaller possible precursor (≈4000 bases) of the mature VIP mRNA was identified in human neuroblastoma (21). One mature processed product of the VIP gene is a mRNA of ≈1600 bases described by us in a human buccal tumor (22, 23) and by others in a human neuroblastoma (21). In the neuroblastoma, this mRNA was shown to contain both VIP- and PHM-27-encoding sequences (21). In an islet cell tumor, a similar VIP mRNA encoding both VIP and PHM-27 was described (24). Future studies are aimed at screening more cell types for VIP and PHM-27 mRNA(s) production. However, in normal nontumorigenic cells, the low amounts of the specific mRNA may be below the level of detection, and investigations are now being designed to overcome these limitations.

We specially acknowledge the excellent participation of Einat Bril in the sequencing project. We also thank Yael Shani for invaluable technical assistance and Rona Levin for the typing and editing of this manuscript. Special thanks are due our collaborators from San Diego, Profs. F. E. Bloom and R. J. Milner. We are also grateful to Interpharm, Inc. for supporting M.F. This work was supported by grants (to I.G.) from National Institute of Neurological and Communicative Disorders and Stroke (1R1 NS 19860-01 A1), the United States–Israel Binational Science Foundation, the Fund for Basic Research administered by the Israeli Academy of Sciences and Humanities, the Jewish Agency, and the Leo and Julia Forchheimer Center for Molecular Genetics and by a Bergmann Memorial Prize to I.G., who is the incumbent of the Samuel O. Freedman Career Development Chair in Cancer Research in perpetuity, established by the Montreal Chapter, Canadian Society for the Weizmann Institute of Science.

1. Mutt, V. & Said, S. I. (1974) *Eur. J. Biochem.* **42**, 581–589.
2. Said, S. I. (1984) *Peptides* **5**, 143–150.
3. Said, S. I., ed. (1982) *Vasoactive Intestinal Peptide* (Raven, New York).
4. Eckenstein, F. & Baughman, R. W. (1984) *Nature (London)* **309**, 153–155.
5. Lee, T. J.-F., Saito, A. & Berezin, I. (1984) *Science* **224**, 898–901.
6. Ottesen, B., Wagner, G., Virag, R. & Fahrenkrug, J. (1984) *Br. Med. J.* **288**, 9–11.
7. Dixson, A. F., Kendrich, K. M., Blank, M. A. & Bloom, S. R. (1984) *J. Endocrinol.* **100**, 249–252.
8. Hökfelt T., Fahrenkrug, J., Tatemoto, K., Mutt, V., Werner, N., Hulting, A.-L. Terenius, L. & Chang, K. J. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 895–898.
9. Itoh, N., Obata, K.-i., Yanaihara, N. & Okamoto, H. (1983) *Nature (London)* **304**, 547–549.
10. Bloom, S. R., Cristofides, N. D., Delamarter, J., Buell, G., Kawachima, E. & Polak, J. M. (1983) *Lancet* **ii**, 1163–1165.
11. Beaucage, S. L. & Caruthers, M. H. (1981) *Tetrahedron Lett.* **22**, 1859–1962.
12. Richardson, C. C. (1965) *Proc. Natl. Acad. Sci. USA* **54**, 158–165.
13. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
14. Lawn, R. M., Fritsch, E. F., Parker, R. C, Blake, G. & Maniatis, T. (1978) *Cell* **15**, 1157–1174.
15. Delbadie-McFarland, G., Cohen, L. W., Riggs, A. D., Morin, C., Itakura, K. & Richards, J. H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6409–6413.
16. Wetmer, R. & Davidson, N. (1968) *J. Mol. Biol.* **31**, 349–370.
17. Verma, I. M. (1977) *Biochim. Biophys. Acta* **473**, 1–38.
18. Maxam, A. & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 560–564.
19. Mains, R. E., Eipper, B. A., Glembotsky, C. C. & Dores, R. M. (1983) *Trends Neurosci.* **6**, 229–235.
20. 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.
21. Hayakawa, Y., Obata, K.-i., Itoh, N., Yanaihara, N. & Okamoto, H. (1984) *J. Biol. Chem.* **259**, 9207–9211.
22. Gozes, I., Bodner, M., Shwartz, H., Shani, Y. & Fridkin, M. (1984) *Peptide* **5**, 161–166.
23. Gozes, I., Bodner, M., Shani, Y. & Fridkin, M. (1984) *J. Cell Biochem.* **26**, 147–156.
24. Horovitch, S. J., Habener, J. F. & Goodman, R. H. (1984) in *Seventh International Congress of Endocrinology* (Elsevier, Amsterdam), abstr. 644.