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Detection of mRNAs Containing Regulatory Peptide Coding Sequences Using Synthetic Oligodeoxynucleotides

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To understand the regulation of the production of peptide hormones, it is vital to elucidate their biosynthetic pathways. We chose to study a major regulatory peptide, vasoactive intestinal peptide (VIP), a peptide possessing both neurotransmitter and neurohormone actions. To identify the specific peptide mRNA we are using, as hybridization probes, radiolabeled synthetic oligodeoxynucleotides with sequence complementary to the predicted peptide mRNA sequence. Employing this approach, we identified and partially purified a ~1600-base long mRNA containing VIP related sequences which can be translated *in vitro* into VIP-immunoreactive polypeptides. Such mRNA was detected in normal VIP producing tissue (rat brain), as well as in a tumor producing VIP (human buccal tumor). This mRNA differs in size from a known VIP-mRNA identified in human neuroblastoma cells, suggesting the possibility of different VIP-mRNAs in different cell types.

Key words: VIP, oligodeoxynucleotides, mRNAs

Regulatory peptides exhibiting multiple functions may be controlled both at their production site and their function loci, ie, receptor site. We chose to study one such molecule, vasoactive intestinal peptide (VIP), a peptide with a broad biological activity, including induction of prolactin release through the hypothalamus-pituitary axis [1]. This peptide is widely distributed throughout the body in nerve cells, as well as in endocrine cells [2].

Hormonal activity of VIP is mediated through cyclic AMP [1]. Evidence supporting the neurotransmitter function attributed to VIP, in addition to its presence in neurons, include its localization in synaptosomal preparation [3] and its potassium induced release from those preparations [3,4]. VIP may also act as a neuromodulator. Indeed, the peptide and acetylcholine are simultaneously released from the parasympathetic nerve that innervates the submandibular salivary gland [5]. Acetylcholine is known to act via muscarinic receptors in activating salivary secretion and VIP potentiates this action. Recent experiments by Lundberg and colleagues [6] have

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shown that the affinity of muscarinic receptors for acetylcholine is increased by 10^5-fold in the presence of VIP. This may be the basis for the potentiating effect of VIP on cholinergic response. VIP is also involved in systemic vasodilation, smooth muscle relaxation [7], and glycogenolysis in the periphery [8] and in the brain [9].

In view of the variety and the multitude bioactivities of the peptide, it is plausable that several controlling mechanisms exist for its generation. To understand the regulation of VIP production, it is essential to elucidate its biosynthetic pathways. Peptide hormones are usually produced in minute quantities and the identification of their respective mRNAs might thus be thought as an unattainable task. However, current methodology enables the chemical synthesis of specific oligodeoxynucleotides probes, with sequences deduced from the peptide amino sequence, which can thereafter be used to identify and characterize the appropriate mRNA. The nucleic acid probes can be of unique sequences, following the predictions and rules derived by Agarwal et al [10]. Alternatively, owing to the extensive redundancy of the genetic code, it might be advantageous to use all possible sequence combinations, in order to decrease mismatches in hybridization [11]. The recent developments in the production of synthetic oligodeoxynucleotides [10–12], along with the possibility of obtaining very high specific radioactively labeled probes [13] and the progress in RNA-DNA hybridization methodology [14], have greatly increased the feasibility of studying biosynthesis of regulatory peptides.

Using the above approach, we were able to detect and partially characterize a mRNA containing VIP-related sequence in a human buccal tumor [15]. We have now extended these studies, and by the use of a battery of oligodeoxynucleotide probes specific for VIP, we have identified this mRNA in rat brain as well. Thus, the same mRNA containing VIP sequence is expressed in normal, as well as in a tumorogenic tissue of different species.

MATERIALS AND METHODS

Materials

Protected nucleosides: 5'Dimethoxy trityl thymidine, 5'dimethoxy trityl N-benzoyl adenosine, 5'dimethoxy trityl N-benzoyl cytidine, and 5'dimethoxy trityl N-isobutyryl guanosine were purchased from Vega Biochemicals (Tucson, AZ). Rats used were of the inbred Wistar strain obtained from an in house colony. Oligo(dT)-cellulose was purchased from Collaborative Research (Waltham, MA). Radiochemicals and enzymes were obtained from Amersham Radiochemical Center (Amersham International plc., England).

Synthesis of Oligodeoxynucleotides

Methyl phosphorochloroamidite was reacted with each of the protected nucleosides essentially as described [12]. The chemically reactive nucleoside dimethylamino phosphoramidites obtained were used as the building blocks in the oligodeoxynucleotide synthesis. The synthesis was conducted in a stepwise manner on a modified silica as a solid support [12]. The sequence of the synthetic chain was confirmed by the Maxam and Gilbert approach [16]. For radioactive ^{32}P labeling, the procedure described by Richardson [13] was employed. In short, 20 pmol of oligodeoxynucleotide were labeled with 20 pmol γ-labeled ^{32}P-ATP (7,000 Ci/mmole) in the presence of 8 U T_4 polynucleotide kinase. The probes were separated from the free ^{32}P-ATP by gel filtration on a Sephadex G-50 column.

Preparation of Poly(A)-Rich mRNA

Brain RNA was prepared from mature rat brains (Wistar rats, 4–8 weeks old) according to the procedure described before [17]. Briefly, frozen tissues (1 volume) were homogenized in the presence of 9 volumes phenol, m-cresol (8/1; vol/vol) and 9 volumes of 4% Na-para-amino salicylic acid in H_2O and RNA was extracted into the aqueous phase. Tumor RNA was prepared from HEp 3 (a neoplasm derived from a lymph node containing metastatic epidermoid cancer, primary in the buccal mucosa [18], which can be passed in nude mice). The tumor was kindly provided as frozen tissue by Professor Ruth Miskin and Mr. Jonathan Axelrod. For tumor mRNA preparation, the urea-LiCl [19] method was adapted. RNA precipitated in 6 M urea containing 3 M LiCl was then extracted with phenol-chloroform mixtures [19]. Poly(A)-rich RNA was obtained by oligo(dT)cellulose affinity chromatography [20].

mRNA Blot Analysis

Poly(A)-rich mRNA was subjected to electrophoresis on 1.5% agarose slab gels in the presence of 3.25% formaldehyde and transferred to nitrocellulose filters [21,22]. Hybridization to ^{32}P-labeled oligodeoxynucleotide probes was performed as described [15].

Separation of RNA on Sucrose Gradients

RNA was separated on sucrose gradients essentially as described previously [15]. For VIP mRNA localization, samples were spotted onto nitrocellulose filters which were presoaked in 10 × SSC (which is 10 times concentrated SSC and 1× SSC = 0.15M NaCl, 0.015 M Na citrate) and then processed for hybridization as above [15].

Translation of VIP Putative mRNAs In Vitro

Reticulocyte lysate was purchased from Amersham Radiochemical Center, and mRNA was translated according to the protocol provided by the company. ^{3}H-leucine was used to label the newly synthesized proteins. The translation reaction was carried out in the presence of human placental RNAse inhibitor (1 U/μl), purchased from Amersham. The putative peptide precursors were immunoprecipitated by the addition of rabbit anti-VIP antiserum (Antibody No. 5600-9, [23], a gift from Dr. J. Fahrenkrug, Clinical Chemistry Department, Frederiksborg Amts Centrabyglehus, 3400 Hillerod Sweden). Immunoprecipitation was performed according to Obata et al [24]. $NaDodSO_4$-polyacrylamide gel electrophoresis and autoradiography were performed as described previously [17,25].

RESULTS

A mRNA Containing VIP Sequences in Rat Brain

In this paper, we show that a peptide-related mRNA can be identified using mixtures of synthetic oligodeoxynucleotides, of lengths ≥ 17 bases, with sequences predicted from the peptide's amino acid sequence. This technique may be generally applicable to the study of the biosynthesis of regulatory peptides. Our preliminary studies with a short 11-base long VIP oligodeoxynucleotide probe did not give a specific hybridization to a single mRNA band (I. Gozes, R.J. Milner, R.A. Lerner, and F.E. Bloom, unpublished results). The DNA probe contained the unique nucleo-

tide sequence: 3′...TTT.GTT.TAC.CG...5′, corresponding to the VIP sequence, starting from amino acid 15 as follows: .lys.glu.met.ala...[2]; this probe was custom synthesized by Collaborative Research (Waltham, MA). We concluded that, in the case of VIP, a probe with a unique sequence of 11 bases may be too short for specific hybridization and may recognize irrelevant mRNAs. In addition, the specificity of hybridization is not determined only by the length of the probe. Thus, when longer oligodeoxynucleotides of mixed sequences are used, an increased number of sequences present can obscure any specific hybridization. For example, a mixture of 17-base long probes for VIP prepared for us by Biologicals (Toronto, Canada) through McNeil Pharmaceuticals (Spring House, PA) (containing 64 different sequences that are actually a mixture of probes 1-4 described below) showed hybridization to quite a few RNA bands (I. Gozes, R.J. Milner, R.A. Lerner, and F.E. Bloom, unpublished results). However, the possibility of multiple mRNA species containing VIP sequences cannot be ruled out.

For further studies and precise characterization of mRNAs containing regulatory peptide sequences, we are using an efficient system for facilitated synthesis of oligodeoxynucleotides and we use these compounds to identify and partially characterize the specific mRNAs.

We synthesized eight different oligodeoxynucleotides (four specific for the N-terminal region of the VIP molecule and four specific for the C-terminal region of the VIP molecule) as hybridization probes for the identification of VIP-mRNA, as outlined below.

The VIP sequences is as follows [2]:

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          5         10        15        20        25
HisSerAspAlaValPheThrAspAsnTyrThrArgLeuArgLysGlnMetAlaValLysLysTyrLeuAsnSerIleLeuAsnNH2
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We chose two stretches of amino acids of limited codon redundacy, N-terminal-related probes—amino acids 6–11 and C-terminal related probes—amino acids 15–21.

N-terminal probes

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                6
Peptide:    . . .Phe.Thr.Asp.Asn.Tyr.Thr. . .

mRNA:       5′..UU(U/C).ACN.GA(U/C).AA(U/C).UA(U/C).AC. . . . .3′

Probes: 1   3′. .AA(A/G).TGT.CT(A/G).TT(A/G).AT(A/G).TG. .5′
        2              —A—
        3              —C—
        4              —G—
```

(Each of these probes is a mixture of 16 different unique sequence probes).

C-terminal Probes

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                15
Peptide:    . . .Lys.Gln.Met.Ala.Val.Lys.Lys. . .

mRNA:       5′.AA(A/G).CA(A/G).AUG.GCN.GUN.AA(A/G).AA. . 3′
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Probes: 5 $3'.TT^{T}_{C}.GT^{T}_{C}.TAC.CGT.CAN.TT^{T}_{C}.TT.\ .\ ^{5'}$
6 —A—
7 —C—
8 —G—

(N denotes any nucleotide: each of the above probes is a mixture of 32 unique sequence probes).

The oligodeoxynucleotides described above were hybridized to mRNA from a known VIP-containing tissue, ie, rat brain. The probes were first end-labeled with ^{32}P, as described in Materials and Methods, and thereafter hybridized to the mRNA which had been resolved on denaturing agarose gels. All eight probes were tested for hybridization at various temperatures (21°C–40°C), according to Dalbadie-Mc-Farland [11]. Probe 1 showed specific hybridization to a 1,600–1,700-base mRNA, which was maintained up to 40°C (Fig. 1).

Probe No. 4 also showed hybridization to the same region, but in addition it hybridized to other mRNAs as well (one mRNA of about 1,300 bases and one of about 2,100 bases; the latter could possibly be 18S ribosomal RNA). At elevated temperatures, the hybridization to the additional (1,300 and 2,100 bases) mRNAs decreased relative to the hybridization to the 1,600–1,700 base band (at 34°C the hybridization to this band amounted to 45% of the total, while at 30°C it amounted to 28%) (Fig. 1). Thus, a single base change (which is the difference between probe 1 and 4), in this case, still allowed hybridization to a 1,600–1,700-base RNA band; however, this hybridization decreased at 40°C in contrast with the hybridization to probe 1 (please see above). Similarly, but less definitive, hybridization to both the 1,600–1,700-base mRNA and the ~1300-base mRNA was obtained with probe 2; the latter is in agreement with the known sequence of VIP complementary DNA from human neuroblastoma [26].

Of the C-terminal probes, the most substantial hybridization to the 1,600–1,700-base band was obtained with probe 6 as depicted in Figure 2. However, hybridization to additional bands was observed as well. Several washes with 2× SSC at increasing temperatures (up to 40°C) were performed in order to differentiate the specific hybridization to the various bands, resulting in no observable change in their relative intensity. This result could possibly be explained by the multiplicity of sequences in probe 6 (32 sequences) as compared to probe 1 (16 sequences) and the possible multiplicity of mRNAs' containing VIP related sequences. Indeed, probe 6 can recognize the ~1300-base VIP-related mRNA as well (Fig. 2), as was found with probe 4 (Fig. 1). A similar mRNA (12–13S) coding for VIP was characterized before [26]. We have thus identified an additional, as yet unknown, putative VIP mRNA of 1,600-1,700 bases that hybridizes to both C-terminal and N-terminal VIP-specific oligodeoxynucleotide probes.

A Similar mRNA Containing VIP Sequences in Normal and Tumor Tissues

The N-terminal probe 1 was used to screen different tissues for VIP gene expression. Using this approach, we discovered the existence of elvated amounts of VIP-mRNA produced by a human buccal Tumor (Fig. 3). Since this tumor, which can be readily passaged in nude mice, provides a very rich source of total mRNA (yields at least ten times more material than brain per g tissue) and, as we have recently discovered contains elevated amounts of VIP [15], we chose to use this source of material for further studies on VIP gene expression.

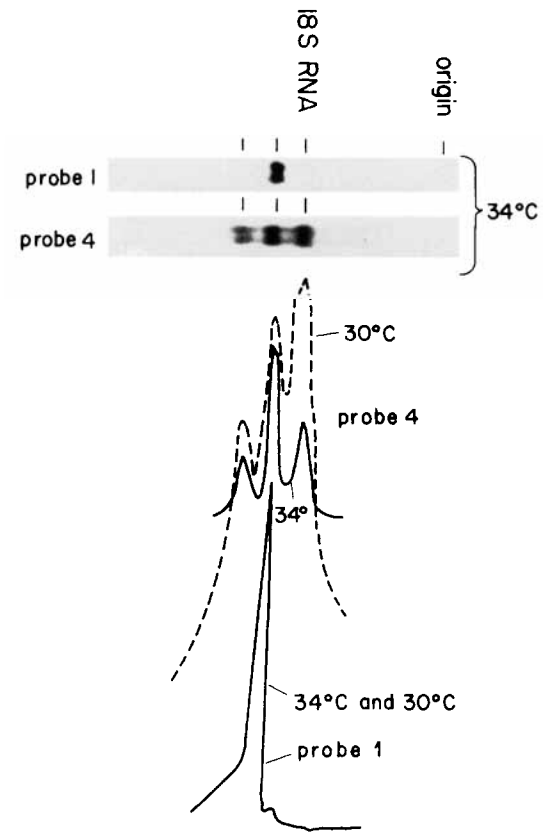

Fig. 1. Hybridization of synthetic oligodeoxynucleotides corresponding to the N-terminal region of VIP to brain mRNA. Brain mRNA prepared from mature rats [17] was resolved on agarose-formaldehyde gels and then transferred onto nitrocellulose filters [21,22]. Synthetic VIP specific oligodeoxynucleotides were end labeled with ^{32}P [13] and hybridized to this mRNA according to Stetler et al [14]. Following hybridization, the filters were exposed to autoradiography and the autoradiogram was subjected to densitometric scanning at 500 nm. The densitometric trances are depicted on the graph. It is worthwhile repeating here that one base alteration can at certain instances still allow hybridization. As depicted in the figure, oligodeoxynucleotide 4 (which has one base difference from oligodeoxynucleotides 1) also hybridized to the 1,600–1,700 bases mRNA, which is the only mRNA recognized by oligodeoxynucleotide 1.

We partially purified the putative VIP-mRNA from human buccal tumor (Fig. 3) using sucrose density gradient centrifugation (see Materials and Methods). The VIP-like mRNA fraction was thereafter identified using ^{32}P-labeled probe 1 as a VIP-specific hybridization probe [15]. The fractions giving the highest hybridization (binding the highest amount of radioactivity in dot hybridization) represented about 1,600-base long mRNA.

The final identification of the VIP-like mRNA species was achieved by its efficient translation into VIP-immunoreactive protein in a reticulocyte lysate cell-free system (Fig. 4). Further characterization of the translation products was obtained by immunoprecipitation, using specific anti-VIP antibodies. followed by gel electrophoresis and autoradiography. Various gradient fractions were processed in that way and

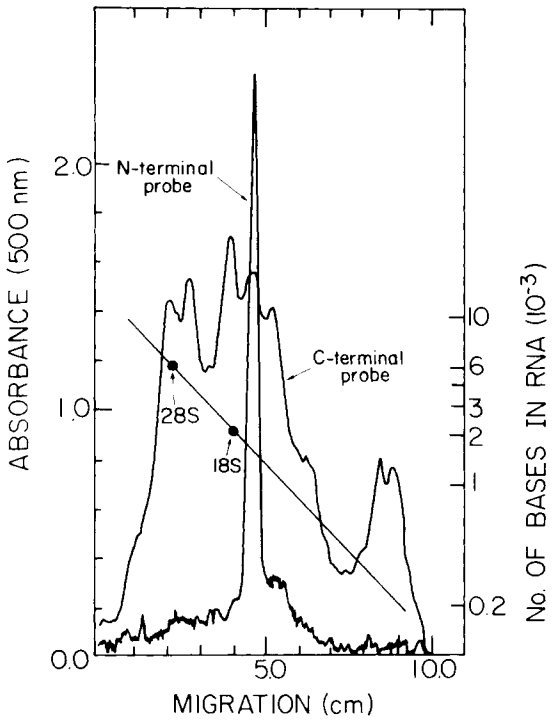

Fig. 2. Hybridization of synthetic oligodeoxynucleotide probes containing VIP coding sequences to brain mRNA. Experiments were performed as described in the legend to Figure 1. Oligodeoxynucleotide 1 (as a N-terminal hybridization probe) hybridized to a single mRNA band of 1,600–1,700 bases. Oligodeoxynucleotide 6 (as a C-terminal hybridization probe) hybridized specifically to the above RNA. Although this oligodeoxynucleotide hybridized to additional RNA bands as well, such hybridization could arise due to the multiplicity of sequences in probe 6 as compared to probe 1.

the ~1,600-base fraction gave distinct VIP-immunoreactive proteins, while other fractions did not.

An ~11,000-dalton protein band was identified using the antibody 5600-9. This is in close agreement with our previous observations using a different antiserum [15]. This protein may very well contain the entire VIP sequence since its binding to anti-VIP antiserum was totally inhibited by synthetic VIP [15]. It is particularly interesting to note that a protein having a similar molecular weight was proposed as the VIP precursor by pulse-labeling experiments in human neuroblastoma cells [27]. The 15,000-dalton VIP-related protein (Fig. 4) may contain VIP sequences as well. However, the major VIP-related protein, coded for by the mRNA containing VIP sequences, is an 11,000-dalton protein.

DISCUSSION

In this paper, we describe the use of synthetic oligodeoxynucleotides for the identification of mRNA species containing the VIP sequences. The possibility of multiple mRNAs coding for one peptide or for very similar peptides is demonstrated.

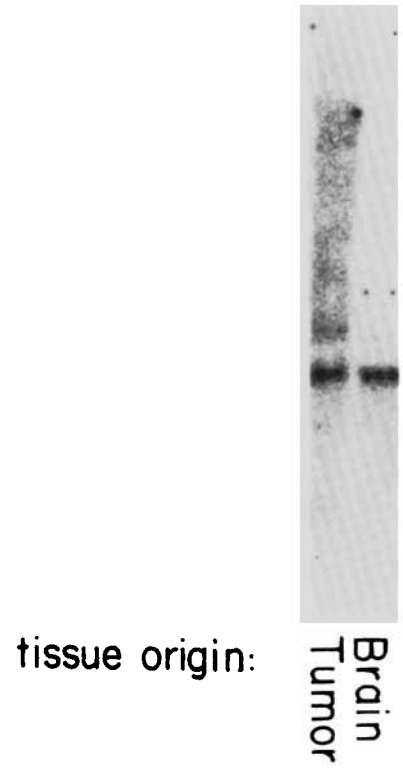

Fig. 3. Hybridization of VIP-related oligodeoxynucleotide No. 1 to tumor mRNA. Human buccal tumor mRNA and brain mRNA (prepared as described in Materials and Methods) were electrophoretically resolved and hybridized to probe 1 as in Figures 1 and 2. The picture shows the autoradiography. (Ribosomal RNA was used for molecular weight determination, see Fig. 2.)

Indeed, human neurobastoma prepro-VIP mRNA seems to be shorter than the 1,600–1,700-base mRNA that we have described (Figs. 1–3) and corresponds to ~1,300-base mRNA [26]. In addition, several high molecular weight proteins containing VIP-like sequences have been described, some of which are too high in molecular weight to be coded for by a 1,600–1,700-base mRNA [28–30]. Moreover, we identify here a noval source for VIP production namely a human buccal tumor [15]. This tumor also produces molecules similar to gonadotropin-releasing hormone (GnRH) and, thus, VIP and GnRH sequences may actually be found in the same cell (data not shown). The mRNA containing VIP-related sequences in brain, in the human buccal tumor and, as recently described, in a pancreatic tumor [31] are similar in size but differ from the one described for human neuroblastoma [26]. To further elucidate the VIP genomic organization, we used synthetic oligodeoxynucleotides specific for the peptide sequence and identified a recombinant phage inserted with human genomic DNA that is positive for the hormone sequence; elucidation of the gene structure is in progress (in preparation).

In conclusion, the resolving power of specific oligodeoxynucleotide hybridization may allow precise elucidation of regulatory-peptide biosynthesis and provide a

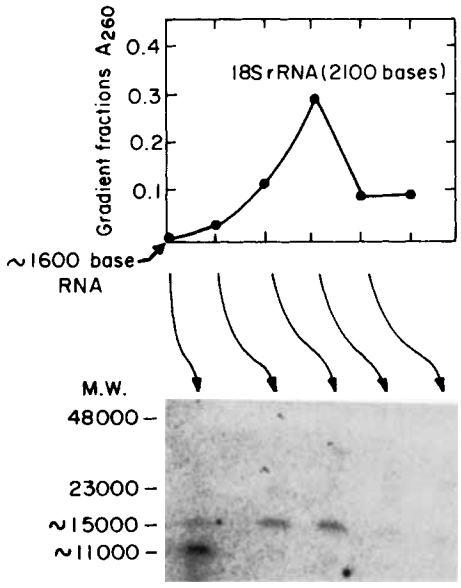

Fig. 4. Isolation of a mRNA containing VIP sequences on sucrose gradients and its in vitro translation. We isolated human buccal tumor mRNA and resolved it on sucrose gradients (see Materials and Methods). Samples were collected, spotted on nitrocellulose paper, and hybridized to ^{32}P-labeled oligodeoxynucleotide 1. The various samples were autoradiographed. The samples shown are of a size < 18S RNA, (see upper panel of RNA absorption profile at 260 nm). Samples > 18S did not show significant hybridization (Figs. 1-3). The gradient fractions were translated in a reticulocyte lysate cell-free translation system. The ^{3}H-leucine labeled newly synthesized proteins were immunoprecipitated with anti-VIP antibodies and subjected to $NaDodSO_4$ (15%) polyacrylamide gel electrophoresis (as described in Materials and Methods). The resulting autoradiograms of the gel are shown.

better understanding of multicellular interactions. Molecular cloning and rapid sequence analysis of the genes corresponding to the detected mRNA bands should yield information on sequences controlling gene expression and, in the long run, may shed light on the mechanism of cell differentiation and cell-cell communication.

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REFERENCES

1. Nicosia S, Oliva D, Giannathasio G, Spada A: J Endocrinol Invest 6:235, 1983.
2. Said SI: "Vasoactive Intestinal Peptide," Advances in Peptide Hormone Research. New York: Raven Press, 1982.
3. Giacchetti A, Said SI, Reynolds RC, Koniges FC: Proc Natl Acad Sci USA 74:3424, 1977.
4. Jessel TM, Iverson LL: Brain Res 143:174, 1978.
5. Lundberg JM: Acta Physiol Scand (Suppl 496): 112:1, 1981.
6. Lundberg JM, Hedlund B, Bartfai T: Nature 295:147, 1982.
7. Piper PJ, Said SI, Vane JR: Nature 225:1144, 1970.
8. Kerins C, Said SI, Proc Soc Exp Biol Med 142:1014, 1973.
9. Magistretti PJ, Morrison JH, Shoemaker WJ, Sapin V, Bloom FE: Proc Natl Acad Sci USA 78:6535, 1981.
10. Agarwal KL, Brunstedt J, Noyes BE: J Biol Chem 256:1023, 1981.
11. Dalbadie-McFarland G, Cohen LW, Riggs AD, Morin C, Itakura K, Richards JH: Proc Natl Acad Sci USA 79:6409, 1982.
12. Beaucage SL, Caruthers MH: Tetrahedron Lett 22:1859, 1981.
13. Richardson CC: Proc Natl Acad Sci USA 54:158, 1965.
14. Stetler D, Das H, Nunberg JH, Saiki R, Sheng-Dong R, Mullis KB, Weissman SM, Erlich HA: Proc Natl Acad Sci USA 79:5966, 1982.
15. Gozes I, Bodner M, Schwartz H, Shani Y, Fridkin M: Peptides 5:161, 1984.
16. Maxam A, Gilbert W: Proc Natl Acad Sci USA 74:560, 1977.
17. Gozes I, de Baetselier A, Littauer UZ: Eur J Biochem 103:13, 1980.
18. Moore AE, Sabachewsky L, Toolan HW: Cancer Res 15:598, 1955.
19. Auffray C, Nageotte R, Chambraud B, Rougeon F: Nucleic Acid Res 8:1231, 1980.
20. Aviv H, Leder P: Proc Natl Acad Sci USA 69:1408, 1972.
21. Nevins JR, Wilson MC: Nature 290:113, 1981.
22. Derman E, Krauter K, Walling L, Weinberger L, Ray M, Darnell JE Jr: Cell 23:731, 1981.
23. Fahrenkrug J, Schaffalitzky de Muckadell OB: J Lab Clin Med 89:1379, 1977.
24. Obata K-i, Itoh N, Okamato H, Yanaihara C, Yanaihara N, Suzuki T: FEBS Lett 136:123, 1981.
25. Gozes I, Schmitt H, Littauer UZ: Proc Natl Acad Sci USA 73:701, 1975.
26. Itoh N, Obatas K-i, Yanaihara N, Okamoto H: Nature 304:547, 1983.
27. Hioki Y, Hoshino M, Yanaihara C, Ogino K, Sato H, Yanaihara N: In Sakakibara S (ed): "Peptides Chemistry." Osaka: Protein Research Foundation, 1983, pp 263–266.
28. Gozes I, O'Connor DT, Bloom FE: Regul Peptides 6:111, 1983.
29. Gozes I, Milner RJ, Liu F-T, Johnson E, Battenberg ELF, Katz D, Bloom FE: J Neurochem 41:549, 1983.
30. Gozes I: In Zomzely-Neurath C, Walker WA (eds): "Gene Expression in Brain." John Wiley and Sons, Inc., (in press).
31. Delamarter J, Buell G, Kawashima E, Polak J, Bloom S: Regul Pept 7:283, 1983.