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Cloning, characterization, and DNA sequence of a human cDNA encoding neuropeptide tyrosine

(recombinant DNA/oligonucleotide/preproneuropeptide Y)

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ABSTRACT **_In vitro_ translation of the RNA isolated from a human pheochromocytoma demonstrated that this tumor contained a mRNA encoding a 10.5-kDa protein, which was immunoprecipitated with antiserum raised against porcine neuropeptide Y. Double-stranded cDNA was synthesized from total RNA and inserted into the *Pst* I site of pUC8. Transformants containing the neuropeptide Y cDNA were identified using the mixed hybridization probe d[A-(A,G)-(A,G)-T-T-(A,G,T)-A-T-(A,G)-T-A-(A,G)-T-G]. The probe sequences were based on the known amino acid sequence, His-Tyr-Ile-Asn-Leu, found in porcine neuropeptide Y. The nucleotide sequence of the cDNA was determined and contained 86 and 174 bases in the 5′- and 3′-untranslated regions, respectively. The coding sequence consisted of 291 bases, suggesting a precursor to neuropeptide Y that was 97 amino acids long (10,839 Da). The deduced amino acid sequence of the precursor suggested that there were at least two sites of proteolytic processing, which would generate three peptides having 28 (signal peptide), 36 (human neuropeptide Y), and 30 (COOH-terminal peptide) amino acid residues. A partial NH_2-terminal sequence obtained by Edman degradation of the immunoprecipitated *in vitro* translation product identified the positions of methionine and leucine in the first 30 residues of the prepropeptide. A highly sensitive single-stranded complementary mRNA hybridization probe specific for neuropeptide Y mRNA was prepared using the bacteriophage SP6 promoter. This probe was used to identify a mRNA corresponding to neuropeptide Y of ≈800 bases.**

Neuropeptide Y (NPY) is one of the most abundant and widespread peptides in the mammalian nervous system (1). This 36 amino acid peptide was originally isolated from porcine brain by Tatemoto *et al.* (2) using a chemical assay that detected the presence of a COOH-terminal amide in proteins. NPY exhibits sequence homology with two other regulatory peptides localized in endocrine cells: pancreatic polypeptide and peptide YY. The biological properties of these three peptides have recently been reviewed by Tatemoto (3).

To date, NPY has been identified exclusively in tissue of neuronal origin (4). The extensive distribution of NPY immunoreactivity in the brain suggests that this peptide might have a role as a neurotransmitter or a neuromodulator (1). In the peripheral nervous system, NPY immunoreactivity is found in nerve fibers of the human heart associated with nodal tissues, coronary vessels, and cardiac muscle fibers (5). In addition, NPY immunoreactivity has been observed in the adrenal medulla (6) and in tumors arising from this tissue [pheochromocytomas (7)], as well as in noradrenergic neurons (8).

This report describes the cloning, characterization, and nucleotide sequence of a neuronal-specific gene product, the human mRNA, which encodes a precursor to NPY.

EXPERIMENTAL PROCEDURES

Materials. Avian myeloblastosis virus reverse transcriptase was provided by J. Beard (Life Sciences, St. Petersburg, FL). The mixed tetradecamer was synthesized by P-L Biochemicals. SP6 RNA polymerase was purchased from New England Nuclear and RNasin was purchased from Promega. The *Staphylococcus aureus* bacterial membranes were obtained from Calbiochem. γ-Labeled nucleotide triphosphates were purchased from ICN and α-labeled nucleotide triphosphates were purchased from Amersham. The plasmid pSP62-PL, containing the bacteriophage SP6 promoter, was obtained from D. Melton (Department of Biochemistry and Molecular Biology, Harvard University).

Isolation of Total RNA and Construction of cDNA Clones. Total RNA from a human pheochromocytoma was purified by the guanidinium isothiocyanate method of Chirgwin *et al.* (9). Total RNA served as a template for double-stranded cDNA synthesis as described (10), with the following modifications. The double-stranded cDNA was base-digested by adjusting the solution to 0.3 M NaOH and incubating for 20 min at 46°C (11). The pH was adjusted to neutrality by adding 1 M HCl, and the entire reaction mixture was passed over a Sephadex G-100 column (1 × 10 cm) equilibrated in 10 mM triethylammonium carbonate (pH 7.4). The S1 nuclease-digested, dC-tailed double-stranded cDNA was annealed to *Pst* I-cleaved pUC8, which had been tailed with ≈15 dG-residues. The annealed material was used to transform *Escherichia coli*, strain JM83, as described (10).

Identification of cDNA Complementary to the mRNA Encoding NPY. A total of 3200 transformants were screened by the method of Grunstein and Hogness (12), using the ^{32}P-labeled tetradecamers as the hybridization probes. The probe was 5′-end-labeled to a specific activity of 9×10^7 cpm/μg. The filters were prehybridized 4 hr at 37°C in 5× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate)/10× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/*E. coli* DNA (500 μg/ml). They were hybridized for 30 hr at room temperature in the above buffer including 300 μg of Torula yeast RNA per ml and 2×10^5 cpm of probe per ml. The filters were washed twice at 0°C for 15 min in 4× NaCl/Cit/1× Denhardt's solution, once at room temperature for 15 min in 5× NaCl/Cit/0.2% $NaDodSO_4$, and twice at 30°C for 15 min in 5× NaCl/Cit/0.2% $NaDodSO_4$.

Characterization and Sequence Analysis. Plasmids from the positive clones were analyzed in more detail by several restriction enzymes and were found to be subsets of one another. Both strands of the largest clone, pNPY3-75, were sequenced using the chemical degradation method of Maxam and Gilbert (13) and the Sanger dideoxy-sequencing method (14).

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Abbreviations: NPY, neuropeptide Y; NaCl/Cit, standard saline citrate.

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RNA Blot Analysis. Five micrograms of total RNA was electrophoresed in a 1.5% agarose gel containing 5 mM methylmercuric hydroxide (15). The gel was blotted onto nitrocellulose according to the procedure of Southern (16). The nitrocellulose was prehybridized, hybridized, and washed according to Thomas (17), except all procedures were carried out at 60°C. The complementary mRNA probe was generated from the phage SP6 promoter (5×10^6 cpm per filter; specific activity, 1×10^8 cpm/μg).

Synthesis of Complementary mRNA to Human NPY. The plasmid used as a template for synthesis of probes complementary to the coding strand of NPY was constructed by inserting the *Bam*HI/*Hin*dIII insert of pNPY3-75 into the polylinker region of pSP62-PL (18). This recombinant plasmid, pSP6-NPY, was linearized with *Bam*HI, phenol-extracted, extracted with chloroform/isoamyl alcohol, and precipitated prior to transcription. Transcription of the linearized plasmid with phage SP6 polymerase was carried out as described (19).

Cell-Free Translation and Immunoprecipitation. Ten micrograms of total pheochromocytoma RNA was translated in a wheat germ cell-free lysate (20) and the products were immunoprecipitated as described (21).

RESULTS

Cloning and Selection of cDNA Coding for Human NPY. Approximately 25 μg of total RNA was used to construct the human pheochromocytoma cDNA library. The frequency of transformation was 2×10^4 transformants per μg of dG-tailed pUC8 vector. Two thousand transformants were initially screened with the 5′ ^{32}P-labeled probes. The mixture of probes, d[A-(A,G)-(A,G)-T-T-(A,G,T)-A-T-(A,G)-T-A-(A,G)-T-G], was a combination of 48 codon possibilities based on the amino acid sequence, His-Tyr-Ile-Asn-Leu, which is present in porcine NPY. Three positive colonies were obtained from the initial screening. The largest clone, pNPY3-75, was nick-translated and used to screen an additional 1200 transformants. The collective screenings produced 9 positive clones, all of which were mapped with restriction enzymes and shown to be subsets of pNPY3-75.

Characterization and Sequence Analysis. The restriction map of pNPY3-75 is shown in Fig. 1. Both strands of the DNA corresponding to the insert were sequenced, and the nucleotide sequence of the two strands were complementary. The inserted cDNA is 591 nucleotides long and includes a stretch of 40 adenosyl residues at the 3′ terminus. It contains 174 bases in the 3′-untranslated region, 291 bases coding for the preprohormone and 86 bases in the 5′ untranslated region. In addition, the poly(A) recognition site, A-A-T-A-A-A, precedes the site of poly(A) addition by 13 bases (Fig. 2).

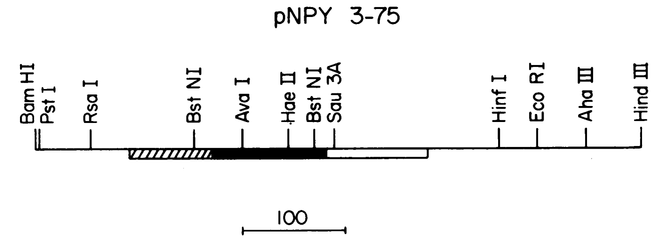

FIG. 1. The restriction map of pNPY3-75. The boxed area represents the prepro-NPY. The hatched area represents the suggested signal peptide, the blackened area denotes the mature neuropeptide (NPY), and the open area represents the COOH-terminal peptide. The restriction sites for *Bam*HI, *Pst* I, and *Hin*dIII are located within pUC8.

Analysis of Prepro-NPY. The sequence of the mRNA encoding prepro-NPY contains only one open reading frame. Fig. 2 shows the deduced amino acid sequence of human prepro-NPY, which contains 97 amino acids and has a predicted molecular size of 10,839 Da. The 5′-untranslated RNA region contains no additional AUG codons that might serve as translation start sites. There is also an in-phase stop codon 36 bases 5′ to the suggested initiator methionine. The NH_2-terminal sequence of the precursor is composed of numerous hydrophobic amino acids, a feature common to signal peptides (22). Cleavage of the signal peptide between alanine-28 and tyrosine-29 should result in formation of the propeptide, with NPY being located at the NH_2 terminus. An additional proteolytic cleavage and post-translational modification are required to form NPY. Proteolytic cleavage of pro-NPY on the carboxyl side of the Lys-Arg sequence (residues 66 and 67) would result in an intermediate form of human NPY. Removal of the Gly-Lys-Arg COOH-terminal extension and amidation of tyrosine would generate human

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                                                                                        MetLeuGlyAsnLysArgLeuGlyLeuSerGlyLeu
ACCCCATCCGCTGGCTCTCACCCCTCGGAGACGCTCGCCCGACAGCATAGTACTTGCCGCCCAGCCACGCCCGCGCGCCAGCCACCATGCTAGGTAACAAGCGACTGGGGCTGTCCGGACTG
                       26                      50                      74                      98                     122

                      20                                                                      40
ThrLeuAlaLeuSerLeuLeuValCysLeuGlyAlaLeuAlaGluAlaTyrProSerLysProAspAsnProGlyGluAspAlaProAlaGluAspMetAlaArgTyrTyrSerAlaLeu
ACCCTCGCCCTGTCCCTGCTCGTGTGCCTGGGTGCGCTGGCCGAGGCGTACCCCTCCAAGCCGGACAACCCGGGCGAGGACGCACCAGCGGAGGACATGGCCAGATACTACTCGGCGCTG
                      146                     170                     194                     218                     242

                      60                                                                      80
ArgHisTyrIleAsnLeuIleThrArgGlnArgTyrGlyLysArgSerSerProGluThrLeuIleSerAspLeuLeuMetArgGluSerThrGluAsnValProArgThrArgLeuGlu
CGACACTACATCAACCTCATCACCAGGCAGAGATATGGAAAACGATCCAGCCCAGAGACACTGATTTCAGACCTCTTGATGAGAGAAAGCACAGAAAATGTTCCCAGAACTCGGCTTGAA
                      266                     290                     314                     338                     362

              97
AspProAlaMetTrp***
GACCCTGCAATGTGGTGATGGGAAATGAGACTTGCTCTCTGGCCTTTTCCTATTTTCAGCCCATATTTCATCGTGTAAAACGAGAATCCACCCATCCTACCAATGCATGCAGCCACTGTG
                      386                     410                     434                     458                     482


CTGAATTCTGCAATGTTTTCCTTTGTCATCATTGTATATATGTGTGTTTAAATAAAGTATCATGCATTC(A)41
                      506                     530
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FIG. 2. The nucleotide sequence encoding the precursor to human NPY. The amino acid sequence of prepro-NPY is numbered 1 through 97. The termination codon is denoted by asterisks. The mature hormone is underlined. The putative signal for poly(A) addition is underlined with a broken line.

NPY. In the absence of further proteolysis, a peptide of 30 residues corresponding to the COOH-terminal sequence of the precursor would also be formed.

Single-Stranded Complementary mRNA Hybridization Probes. To obtain a sensitive, single-stranded hybridization probe for mRNA encoding NPY, the *Bam*HI/*Hin*dIII insert of pNPY3-75 has been cloned into a plasmid containing the promoter of the bacteriophage SP6 (Fig. 3*A*). The construction of this plasmid, pSP6-NPY, was carried out as described above. *In vitro* transcription of the *Bam*HI-cleaved pSP6-NPY with phage SP6 polymerase resulted in a single-stranded RNA that was complementary to the mRNA encoding NPY (i.e., complementary mRNA). This probe was used to analyze RNA isolated from the pheochromocytoma by RNA blot analysis. RNA blot analysis showed an intense band of ≈800 nucleotides that hybridized with the complementary mRNA (Fig. 3*B*).

Cell-Free Translation. Total RNA from the human pheochromocytoma was used to direct the *in vitro* synthesis of [^{35}S]methionine-labeled proteins in a wheat germ cell-free extract. Translation products were fractionated on a $NaDodSO_4$/polyacrylamide gel and detected by fluorography (Fig. 4). The translation product corresponding to prepro-NPY was identified by immunoprecipitating the reaction mixture with antiserum raised against porcine NPY. Fig. 4 (lane D) shows that a polypeptide was recovered from the cell-free translation mixture using this antiserum and that this protein has an estimated mass of 10.5 kDa. When 5 μg of porcine NPY was included in the immunoprecipitation mixture, it inhibited the precipitation of the 10.5-kDa protein. In addition, preimmune antiserum did not precipitate this or any other protein from the reaction mixture (Fig. 4).

NH_2-Terminal Sequence Analysis of Prepro-NPY. Cell-free translation mixtures were labeled with either [^{35}S]methionine or [^{3}H]leucine and were immunoprecipitated with antiserum directed against porcine NPY. The immunoprecipitation product was subjected to automated sequential Edman degradation. The results of the NH_2-terminal sequence analyses are presented in Fig. 5. Radioactivity corresponding to [^{35}S]methionine was found only in cycle 1, while peaks of [^{3}H]leucine were present in cycles 2, 7, 9, 12, 14, 16, 18, 19, 22, and 25. The positions of [^{35}S]methionine and [^{3}H]leucine were identical to the locations of these residues in the amino acid sequence deduced from the cDNA sequence analysis.

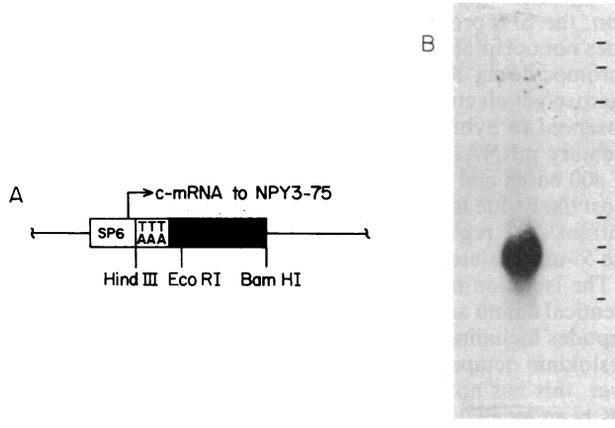

FIG. 3. (*A*) *In vitro* transcription of pSP6-NPY. The location of the phage SP6 transcription initiation site and direction of transcription are schematically illustrated. The portion of the subclone containing NPY sequences is denoted by a blackened box. The position of the poly(A) is indicated. The plasmid was linearized for transcription using the *Bam*HI site present in the linker region of pUC8. (*B*) RNA blot analysis of total pheochromocytoma RNA. Five micrograms of total RNA was subjected to electrophoresis on a 1.5% agarose gel containing 5 mM methylmercuric hydroxide, transferred to nitrocellulose, and hybridized with 5×10^6 cpm of the complementary mRNA generated by *in vitro* transcription of pSP6-NPY. The autoradiograph was exposed for 48 hr using Fuji film with an intensifying screen at −80°C. The molecular size markers from top to bottom are 23, 9.5, 6.67, 1.35, 1.08, 0.87, and 0.6 kilobases.

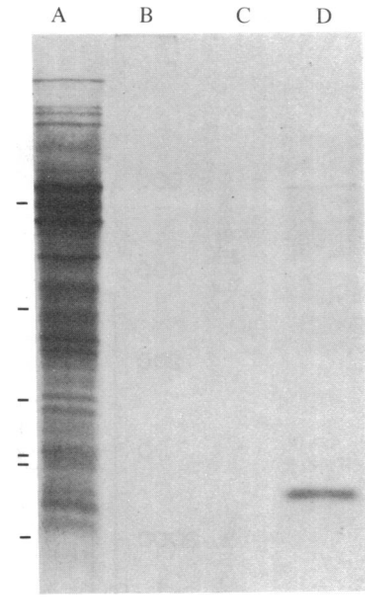

FIG. 4. Immunoprecipitation of translation products directed by human pheochromocytoma RNA. Translations were carried out in a wheat germ cell-free translation system and then analyzed on $NaDodSO_4$/15% polyacrylamide gels. Lanes: A, total translation products (one-tenth of reaction); B, precipitation with preimmune serum; C, precipitation with anti-NPY serum (YN-12) in the presence of 5 μg of porcine NPY; D, precipitation with anti-NPY (YN-12). Protein standards (in Da) from top to bottom are ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), lysozyme (14,300), cytochrome *c* (12,300), and bovine trypsin inhibitor (6200).

DISCUSSION

This report describes the cloning and characterization of a mRNA isolated from a human pheochromocytoma that encodes prepro-NPY. The sequence of the precursor is 97 amino acids long and has a calculated size of 10.8 kDa. The cell-free translation product that immunoprecipitated using antiserum to porcine NPY has an estimated size of 10.5 kDa. The two molecular size determinations are in good agreement. The NH_2-terminal sequence of the precursor to NPY deduced from the cDNA sequence was confirmed by Edman degradation of the immunoprecipitated cell-free translation product. The positions of methionine and leucine in the first 30 cycles corresponded exactly to those predicted by the cDNA sequence.

Tatemoto *et al.* (2) have reported the only known sequence of NPY, a 36 amino acid peptide that was isolated from porcine brain using a chemical method to detect the presence of a COOH-terminal amide. When the sequence of the human prepro-NPY is compared with the 36 amino acid residues of porcine NPY, a single amino acid substitution is noted. Methionine at position 45 in human NPY has been substituted for leucine in porcine NPY. This is consistent with the observation that human and porcine NPY elute from reversed-phase chromatography columns with different retention times (23). In addition, human and porcine NPY appear to have similar molecular weights by gel filtration chromatography (7), suggesting that the processing of human prepro-NPY most likely results in formation of a ma-

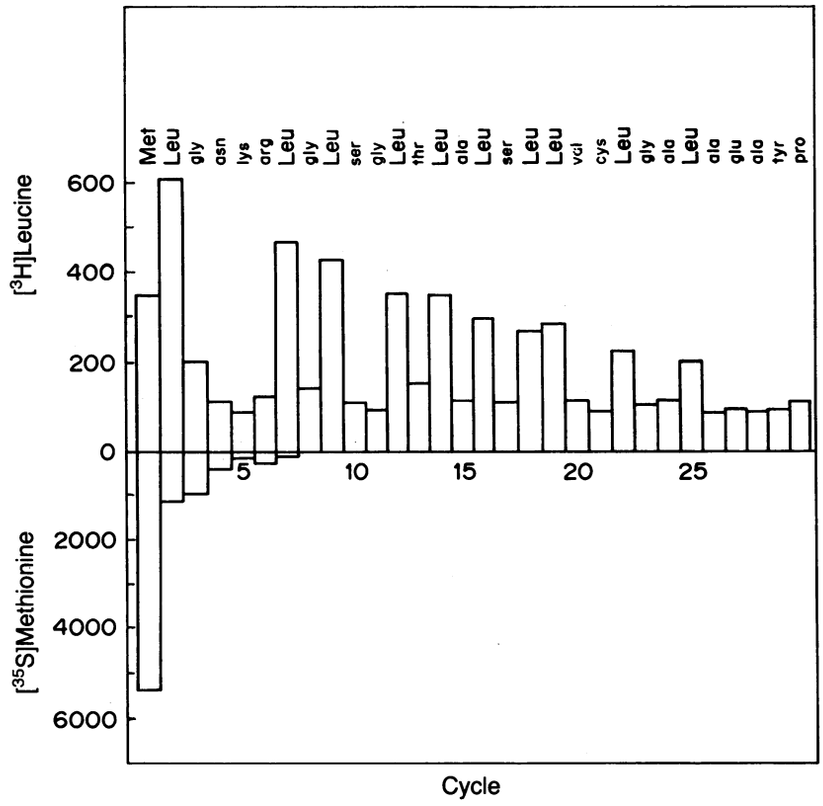

FIG. 5. The NH_2-terminal sequence analysis of prepro-NPY. Sequential Edman degradation was carried out on the *in vitro* cell-free translation product immunoprecipitated with antiserum directed against porcine NPY. The radioactivity corresponding to [^{35}S]methionine and [^{3}H]leucine was determined for 30 cycles. The positions of the radiolabeled amino acids in the immunoprecipitated translation product are shown along with the amino acid sequence determined from nucleotide sequence analysis. The positions of Leu and Met are capitalized.

ture peptide having structural homology to the porcine peptide. These observations suggest that the signal peptide is cleaved after residue 28, resulting in pro-NPY. Proteolysis of pro-NPY most likely occurs after the single Lys-Arg sequence found in the precursor. Two adjacent basic residues are often the site where post-translational proteolytic processing of prohormones occurs (24). To form the amidated tyrosine residue, the COOH-terminal glycine must be removed. The presence of glycine following a COOH-terminal amidation site has been observed in several prohormones and appears to be a general feature associated with amidation (3).

No larger precursor to porcine NPY or to the gut peptide YY has been isolated or extensively characterized. However, a precursor to canine pancreatic polypeptide, the other member of this peptide family, has been isolated and sequenced by Schwartz and Tager (25). The precursor to pancreatic polypeptide has a COOH-terminal extension of ≈20 residues. The amino acid sequence of the precursor to NPY suggested that it has a COOH-terminal extension of 30 residues. The amino acid sequences of human NPY and canine pancreatic polypeptide are ≈50% conserved. However, when the COOH-terminal extensions of canine pancreatic polypeptide and human NPY are aligned, only 3 of 20 amino acids show identity. This is a striking change in the degree of amino acid homology found in different regions of the amino acid sequence. Since a high degree of conservation of amino acid sequence between peptides has often been cited as evidence for conservation of biological function (26), the divergence of this region may suggest a lack of function. However, speculations on the biological importance of these observations must await additional structural data from other members of this peptide family.

A specific probe complementary to NPY mRNA (complementary mRNA) was prepared using the *Salmonella* bacteriophage SP6 promoter and cloned NPY cDNA. The single-stranded probe generated from this plasmid is specific and cannot undergo intermolecular reannealing. It has a low background when used with nitrocellulose filters. In addition, the SP6 promoter is highly efficient and transcription does not occur at other sites within the plasmid. When pheochromocytoma RNA was analyzed on nitrocellulose after agarose gel electrophoresis, a single band of 800 bases was observed to hybridize to the pSP6 NPY-generated complementary mRNA. The difference between the apparent size of 800 bases and the 591 bases of the insert of pNPY3-75 is most likely due to the presence of poly(A) attached to the 3′-untranslated region and an additional sequence present in the 5′-untranslated region.

The isolation from both gut and brain of peptides having identical amino acid sequences has been reported for several peptides including substance P (27), neurotensin (28), cholecystokinin octapeptide (29), and somatostatin-28 (30). However, this has not been observed in the case of NPY. NPY has been localized exclusively to the central and peripheral nervous system (1) and in a subpopulation of the chromaffin cells of the mammalian adrenal medulla (6). The tissue-specific expression of this peptide emphasizes the importance of understanding the factors that govern differential gene expression.

Because NPY was isolated using a chemical assay method, no specific biological function was originally assigned to the peptide. Recently, several biological activities of NPY have been reported. For example, NPY caused inhibition of electrically induced contractions of the vas deferens, while it seemed to have a stimulatory effect on vascular smooth muscle (8, 31). In studies of isolated perfused rabbit heart, NPY decreased the flow of perfusate through the coronary

vasculature (32). Since NPY is present in cardiac nerves, this may have important physiological significance pertinent to the control of blood pressure. NPY is also present in large amounts in the brain. The abundance and wide distribution of this neuropeptide in the central and peripheral nervous systems certainly underscores the importance of understanding its function and regulation.

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