Structure and expression of the gene encoding the vasoactive intestinal peptide precursor

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ABSTRACT The gene encoding the human vasoactive intestinal peptide (VIP) and the histidine-methionine amide (PHM-27) peptide hormone was isolated from a phage library. The human gene was found to be composed of seven exons spanning ~9 kilobase pairs. The first exon codes for an untransladed leader sequence, and the second exon codes for a putative signal peptide. DNA sequences coding for the VIP and PHM-27 hormones are located in two different exons. Southern blot analysis with genomic DNA suggested that a single copy of the VIP/PHM-27 gene is present in the human haploid genome. The expression of VIP/PHM-27 precursor mRNA in various tissues in the rat was analyzed by RNA gel blot hybridization. In the organs examined, expression was only detected in the brain and duodenum. RNA isolated from various regions of the rat brain—including the cortex, hypothalamus, and hippocampus—hybridized to both VIP- and PHM-27-specific probes. The same pattern of hybridization was found when VIP- and PHM-27-specific probes were used, suggesting that possible differences in the localization of VIP and PHM-27 peptides between different brain regions cannot be accounted for by differential RNA processing.

The growing number of isolated peptide hormones can be grouped into families of structurally related peptides. Examples of such families are the glucagon–secretin group, the gastrin–cholecystokinin group, and the tachykinins. Of these, the glucagon–secretin family consists, at present, of six peptides: glucagon, secretin, vasoactive intestinal peptide (VIP), glucose-dependent insulinotropic peptide, peptide hormone with N-terminal histidine and C-terminal isoleucine amide (PHI-27), and growth hormone releasing factor (1). Itoh et al. (2) have shown that VIP and PHI-27 are synthesized as parts of the same precursor polyprotein in human neuroblastoma cells. The human counterpart of PHI-27 has a C-terminal methionine and is, therefore, designated PHM-27. VIP and PHM-27 were originally isolated by Mutt and co-workers (3, 4) who purified their porcine counterparts from extracts of intestinal mucosa. VIP is a 28-amino acid peptide that is not only present in intestinal tissue but also in neural tissues. Several biological activities have been ascribed to VIP. These include vasodilation, release of prolactin, a role in penile erection, and effects on pancreatic and intestinal secretion (for a review, see refs. 5 and 6). Nerves immunoreactive to VIP are widely distributed. They have been demonstrated to be present in conjunction with smooth muscle in the intestinal tract and in the wall of cerebral arteries (7). Subsequent histochemical studies have shown a widespread distribution of VIP-positive cell bodies and fiber networks (see refs. 6 and 8). PHI-27 was isolated on the basis of its C-terminal amide structure (9) and shown to have several biological activities. These include the release of insulin, glucagon, and prolactin (for a review, see ref. 10).

We report the structure of the VIP/PHM-27 precursor gene, which consists of seven exons. Sequences coding for the two hormones were found to be located in two exons, and sequences for the signal peptide were in a third exon. Since VIP and PHM-27 are members of a family of related peptides, we searched for nucleic acid sequences coding for such peptides by hybridization at low stringency. We have also examined the expression of the precursor gene in different organs of the rat. In particular, we have addressed whether RNA isolated from different regions of the brain predominantly contains VIP or PHM-27 sequences.

MATERIALS AND METHODS

Preparation of Oligonucleotide Probes. Two partially complementary 18-mer oligonucleotides corresponding to the VIP cDNA sequence at nucleotides 373–401 were synthesized (KabiGen AB, Stockholm, Sweden). The oligonucleotides were mixed in stoichiometric amounts and extended in the 3' direction using DNA polymerase (Klenow fragment) with [32P]dCTP and unlabeled dATP, dTTP, and dGTP. The resulting 28-mer was used as a probe for the screening of phage libraries. For the mapping of exon sequences, 15-mer oligonucleotides were 5' labeled using polynucleotide kinase and [γ-32P]ATP and were hybridized to Southern blots of cloned phage or plasmid DNA.

Isolation and Characterization of the VIP/PHM-27 Precursor Gene. Two different λ phage libraries of human genomic DNA were screened. The VIP-4 phage was isolated from the Charon 4A library of Lawn et al. (11). An EMBL3 λ phage library of human lymphocyte DNA partially digested with Mbo I and ligated to BamHI-cleaved EMBL3 phage DNA (12) was supplied by Hans Lehrach (EMBL Molecular Biology Laboratory, Heidelberg, F.R.G.). The libraries were screened following the procedure of Benton and Davies (13). Filters were hybridized to the labeled 28-mer probe at 45°C in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 3× Denhardt's solution (1× Denhardt’s solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), 0.5% NaDodSO₄, and denatured salmon sperm DNA (100 μg/ml). Areas from the bacterial lawn containing phages that hybridized with the probe were isolated, and positive phage isolates were purified by several rounds of plaque purification. λ phage particles were purified by polyethylene glycol precipitation followed by glycerol gradient centrifugation (14). Phage DNA was analyzed by restriction enzyme digestion and agarose gel electrophoresis. From

Abbreviations: kb, kilobase pair(s); VIP, vasoactive intestinal peptide; bp, base pair(s); PHI-27, peptide hormone with an N-terminal histidine and a C-terminal isoleucine amide; PHM-27, peptide hormone with an N-terminal histidine and a C-terminal methionine amide.

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this analysis, phage VIP-D and VIP-4 were chosen for further studies. EcoRI fragments from phage VIP-D were subcloned into the pUC18 vector (15) using standard procedures. Plasmid DNA was prepared from 50-ml unamplified cultures using a slight modification of the alkaline extraction/LiCl precipitation technique (16). A restriction map of EcoRI, Pst I, and HindIII sites was constructed by hybridizing subcloned DNA to blots of phage DNA cleaved with these enzymes. DNA was sequenced by the dyeoxy method (17), either after subcloning into M13mp18 or mp19 vectors or by sequencing supercoiled plasmid DNA directly following the protocol of Chen and Seeberg (18). Primers for sequencing were purchased from New England Biolabs or were synthesized directly to be complementary to the established cDNA sequence (2). Southern blotting (19) of nucleic acids to filters was performed using the alkaline procedure of Chomczynski and Qasba (20). Probes were labeled by nick-translating the protocol of Meinkoth and Wahl (21). Hybridizations were done in 5X SSC/0.5% NaDodSO4/1× Denhardt’s solution/0.1% salmon pyrophosphate/denatured salmon sperm DNA (100 μg/ml)/10% (wt/vol) dextran sulphate.

Analysis of RNA Expression. Brain regions were dissected from decapitated adult Sprague–Dawley rats as described by Whittemore et al. (22). The hypothalamic was dissected to include the paraventricular nucleus. RNA was isolated by the guanidine isothiocyanate procedure (23) and was chromatographed on oligo(dT)-cellulose columns (24). RNA was separated on formaldehyde-containing gels (25) and blotted onto nitrocellulose filters. Hybridizations to nick-translated probes were conducted in 4X SSC/1× Denhardt’s solution/denatured salmon sperm DNA (100 μg/ml)/40% (vol/vol) formamide/10% (wt/vol) dextran sulphate at 43°C for 16–18 hr. Filters were washed in 0.2X SSC and 0.1% NaDodSO4 at 45°C.

RESULTS

Isolation of the Human VIP and PHM-27 Precursor Gene. A synthetic oligonucleotide complementary to the cDNA sequence of the human VIP/PHM-27 precursor mRNA (2) was used as a probe in the isolation of the human gene from phage DNA libraries. Initially, the human genomic DNA library of Lawn et al. (11) was screened. Several positive plaques were identified, isolated, and partially characterized. All recombinant phages that were isolated contained an identical DNA insert corresponding to the 5′ portion of the VIP/PHM-27 gene. An EMBL3 human genomic library was subsequently screened, and one phage (VIP-D) was recovered that spanned the 5′ region of the gene.

Restriction enzyme analysis showed that the insert of the phage VIP-D contained six EcoRI fragments whereas VIP-4 contained four. Three of these fragments were shared between the phages (Fig. 1). Both phages were found to harbor VIP and PHM-27 sequences and together covered 18.5 kilobase pairs (kb) of human DNA.

The Human VIP/PHM-Gene Consists of Seven Exons. To localize coding sequences, several oligonucleotide probes complementary to the precursor cDNA sequence were synthesized. The oligonucleotides were labeled and hybridized to blots of phage DNA cleaved with different restriction endonucleases. Seven different regions of the phage inserts hybridized to the oligonucleotides.

The first six exons of the gene were sequenced by the dyeoxy method (Fig. 2). The first exon represents an untranslated leader sequence of 164 nucleotides. Primer extension of human neuroblastoma mRNA yielded several cDNA products, the most abundant corresponding to a 160-base-pair (bp) leader sequence (data not shown). A "TATAAA" motif was present 29 bp upstream from the presumed cap site. Our data, then, point to a final size of the transcribed mRNA, excluding the poly(A) tail, of 1460 nucleotides. The observed size of the polyadenylated mRNA of about 1600 bases in human VIP-producing tumors (26, 27) is in good agreement with our results.

The second exon consisted of mRNA nucleotides 165–281. This sequence codes for a putative signal peptide of 21 amino acids and for 15 additional amino acids. The third exon contains 123 nucleotides. A spacer protein sequence of unknown function is encoded by this sequence. The PHM-27 encoding exon consists of nucleotides 405–509, and the VIP encoding exon has nucleotides 510–631. The sixth exon dictates the synthesis of the remainder of the protein coding sequence. All exons are flanked by consensus splice junction sequences (G-T donor and A-G acceptor sequences). As noted (28), the 3′-splice junctions flanking the VIP and PHM exons contain an identical run of nine nucleotides, AGGTAAAGA. The entire VIP/PHM-27 precursor gene spanned ~9 kb of DNA.

A region containing repeated DNA sequences was found upstream from exon 1 as shown by hybridization to labeled human genomic DNA. A probe containing an Alu sequence did not hybridize to the repeated sequence (data not shown). Hybridization to labeled repetitive DNA from the upstream region of the VIP/PHM-27 gene to human genomic DNA cleaved with EcoRI or HindIII resulted in bands corresponding to fragments of 2.8 kb and 3.7 kb, respectively (data not shown), with an intensity suggesting that they were moderately repeated in the human genome.

Southern blots of human DNA were hybridized to VIP and PHM-27 DNA probes and washed under low stringency conditions (Fig. 3). These analyses demonstrated that the structure of the cloned DNA reflected the genomic structure. In particular, HindIII and Bgl II fragments of identical
CATCCCATGCGCTATCTGACCTTCTTCCAGAGGCTTTGAGGTGATGTGAGCTTCTCCTCTTATAGAGTTCCTAGTGGGTTTGTAGGACAGAGAGTTGCTGTG
GACGAGGAGGGCGAGCGGCTAGACGCGCTCCCTATCGGATTGCGGAGGTCTTCCTCTCTCCCGACCTCCACTAAGTAATAGCTCTAATTTAGGCTTTTGAATTT

THM-27 in molecular weight from prepared and BH VIP PHM

FIG. 2. Sequence analysis of the VIP/PHM-27 gene. Splice junctions inferred from the VIP cDNA sequence (2) are shown by arrowheads. Underlined in the 5′ part of the gene are putative "TATA" and cap elements. PHM-27 and VIP hormone sequences are enclosed within arrows.

molecular weight were observed after hybridization with VIP- and PHM-specific probes, showing linkage of VIP and PHM-27 in genomic DNA (Fig. 3). Furthermore, probes prepared from the 5′ portion of the gene hybridized to fragments of identical molecular weights in cloned and genomic DNA (data not shown).

Expression of VIP/PHM mRNA in Different Tissues of the Rat. The expression of the VIP/PHM-27 precursor in different rat organs was studied by RNA gel blot hybridization. Among RNAs from the organs examined, only poly(A)+ RNA extracted from the duodenum and the brain hybridized to a VIP-specific probe (Fig. 4 Left). The observed transcript appeared to be 1800 nucleotides long. Expression of a VIP-encoding mRNA was observed in several areas of the rat brain including the hippocampus, cortex, colliculum, olfactory bulb, thalamus, and hypothalamus (Fig. 4 Middle and Right). Weak hybridization was also observed with mRNA from the septum, while no detectable hybridization occurred with material from pons, medulla, and striatum. A PHM-27-specific probe also hybridized to an 1800-nucleotide transcript from the cortex and hypothalamus (Fig. 4, Right). Furthermore, the PHM-27 probe hybridized to a similar RNA species present in the hippocampus, colliculum, olfactory bulb, and thalamus in a manner analogous to the VIP probe (data not shown). Thus, our data suggest that both VIP and PHM-27 sequences were present in the mRNA expressed in the different brain regions.

DISCUSSION

The gene coding for the precursor of the human VIP and PHM-27 hormones was found to be 9 kb long and to consist of seven exons. Of these, exon 2 contains the coding
information for a potential signal peptide, while the fourth and fifth exons code for the PHM-27 and VIP hormones, respectively. The third exon codes for a spacer protein sequence (amino acids 37–77). The function of spacer protein sequences in polypeptide precursors is unclear, but they may be important for a proper folding of the polypeptide that allows processing. Parts of the processed precursor may also be used as carrier for the storage of the hormones in secretory vesicles, as has been described for vasopressin/oxytocin (29). Provided that the spacer peptide does have a distinct function, four of seven exons constitute functional domains of the polypeptide. The structure of the VIP/PHM-27 gene may thus support the suggestion that exons are building blocks of functional domains of their corresponding proteins (30).

The gene of the VIP/PHM-27 precursor was found to direct the synthesis of a 5'-terminal untranslated exon of 164 bp. Only 20 bp of this leader was represented in the cDNA clone isolated by Itoh et al. (2). A similar structure with introns interrupting the 5'-noncoding region have been found in a number of other peptide hormone precursor genes, including preproinsulin, gastrin, preproparathyroid, preproenkephalin, preproopiocalcinor, and prolactin (for a review, see ref. 31). The significance, if any, of the occurrence of 5'-noncoding exons in genes coding for peptide hormone precursors is unknown.

VIP and PHM-27 are both members of the glucagon–secretin family of peptide hormones. Glucagon, secretin, VIP, and PHM-27 all share an N-terminal His-1, a Phe-6, and a Thr-7. The structural relatedness may possibly be explained by gene duplication events. The homology between sequences coding for VIP and PHM-27, as well as adjacent flanking sequences, was examined using a computer alignment program. Only small regions of homology were found, and the overall homology over a 520-bp region was only 37%. The homology between the sequences coding for VIP and PHM-27 was 42 of 81 nucleotides (52%). Nine nucleotides at the splice junctions on the 3' side of VIP and PHM-27 coding sequences were identical (AGGTAAAGA), and some further homology was found downstream from the splice junctions. One of the aims of the present study was to use VIP/PHM-27-derived DNA probes to search for other members of the glucagon–secretin family of hormones. We were, however, unable to detect hybridization to other sequences in the human genome even under conditions of low stringency of hybridization. Our findings suggest that the members of the glucagon–secretin family have diverged extensively after presumptive ancient gene duplication events.

The structure of the human VIP/PHM-27 gene shows similarities to that of the human preproglucagon gene (32). The preproglucagon gene has possibly evolved through a tandem duplication of an exon encoding a glucagon-like peptide resulting in three hormone coding sequences (glucagon, glucagon-like peptides 1 and 2). Similarly, VIP and PHM-27 sequences may have diverged after a duplication of an ancient hormone coding sequence. Such a hypothetical ancestral hormone sequence may also have duplicated several times to give rise to other members of the secretin–glucagon gene family and possibly to part of the gene coding for prealbumin. The low level of conservation between VIP and PHM-27 coding sequences (52%) and the fact that flanking sequences lack homologies suggest that the duplication occurred early during evolution. Genomic DNA sequences in the rat and calf but not chicken or Xenopus laevis genomes were observed to hybridize to VIP and PHM-27 probes (unpublished data). Hybridization of the human probes with rat DNA was relatively weak, however, considering the reported 85–89% sequence homology between human and rat VIP and PHM-27 sequences (33). The weak hybridization suggests that the sequences constituting most of the probe, representing intron sequences, do not hybridize between the species and have diverged strongly.

Bodner et al. (28) and Tsukada et al. (42) reported the isolation of recombinant λ phages similar to those described here. Our mapping and characterization of the VIP and PHM-27 exons agrees with their findings. Bodner et al. (28) suggested that the VIP/PHM mRNA may be differentially spliced to generate expression of VIP in the brain cortex and PHM-27 in the hypothalamus. The finding that the 3' ends of VIP and PHM exons border splice junctions with the identical sequence AGGTAAAGA was interpreted as support of this suggestion. In the present study, we have examined the expression of the VIP/PHM-27 gene in various organs of the rat. The rat was chosen as an experimental model due to the ease with which material can be obtained, and since rat and human VIP/PHM-27 gene sequences cross-hybridize (unpublished information). We were able to detect low levels of expression of transcripts hybridizing to VIP and PHM-27 probes in various regions of the rat brain in agreement with the wide distribution of VIP-containing neuronal cell bodies in the rat brain (6, 8, 35, 36). These transcripts were not observed to vary in size between different brain regions. Furthermore, all brain regions exam-
ined that showed an mRNA hybridizing to the VIP probe also contained an mRNA encoding PHM-27. Our data, then, suggest that tissue-specific processing of the precursor mRNA cannot explain observed regional differences in the distribution of VIP and PHM-27 peptide hormones (37, 38). In accord with our data is the previous isolation of a CDNA clone containing both VIP and PHM-27 coding sequences from the rat brain cortex (33).

It has been suggested that the preferential demonstration of PHI-like immunoreactivity in the paraventricular nucleus with immunocytochemistry (37, 38) represents an artificial cross-reaction of some PHI-antiseras with the C-terminal isoleucine of corticotropin releasing hormone (39). This has been confirmed for the antiserum used by Hökfelt et al. (37, 38, and T.H., unpublished results). However, the existence of a small population of VIP-positive neurons in the parvocellular part of the paraventricular nucleus is demonstrable in colchicine-treated, lactating rats (40). Both VIP- and PHI-immunoreactive cells, in approximately similar numbers, can be demonstrated in this nucleus also in colchicine-treated rats after severe stress and during lactation. Some of these cells contain corticotropin releasing factor-like immunoreactivity (T.H., unpublished data). These histochemical results suggest the presence of approximately similar numbers of VIP- and PHI-positive hypothalamic cell bodies, in agreement with the demonstration of VIP and PHI in a 1:1 molar ratio in the portal blood (40).

Further studies are required to establish whether VIP and PHI/PHM hormones necessarily are coexpressed at the level of the single neuron. Our findings do not rule out that differential mRNA processing may take place in a subset of neurons. Alternatively, specific expression of VIP and PHM-27 hormones may be accomplished by differences in the processing of the precursor polypeptide in different neurons (41).

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