Nucleotide sequence divergence and functional constraint in VIP precursor mRNA evolution between human and rat

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The nucleotide sequence analysis of cloned cDNA for VIP precursor from rat cerebral cortex reveals that the precursor contains both rat VIP and PHI-27. The deduced primary structure of rat VIP is identical with human VIP. The amino acid sequence of rat PHI-27 differs by 4 amino acids from human PHM-27. When each VIP precursor is divided functionally into 6 domains, the amino acid sequence homology between rat and human precursors ranges from 69 to 100%. In contrast, any domain exhibits an essentially equal degree of nucleotide sequence homology.

Rat VIP *Rat PHI-27* *VIP precursor* *VIP precursor mRNA* *Human PHM-27* *Sequence homology*

1. INTRODUCTION

Vasoactive intestinal polypeptide (VIP), a 28 amino acid peptide originally isolated from porcine duodenum [1], is present not only in gastrointestinal tissues but also neural tissues, possibly as a neurotransmitter [2,3]. VIP is structurally similar to members of the glucagon-secretin family, especially to porcine PHI-27 [4]. We found that the primary structure of the human VIP precursor, deduced from the DNA sequence complementary to the mRNA coding for human VIP, contains the sequences of both VIP and a novel PHI-27-like peptide, PHM-27 [5,6]. We reported that the transcription rate of the VIP precursor mRNA in human neuroblastoma cells is regulated by cyclic AMP [7].

Abbreviations: VIP, vasoactive intestinal polypeptide; PHI-27, peptide having amino-terminal histidine, carboxy-terminal isoleucine amide and 27 amino acid residues; PHM-27, peptide having amino-terminal histidine, carboxy-terminal methionine amide and 27 amino acid residues; bp, base pairs

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Here, we have determined the DNA sequence complementary to the mRNA coding for rat VIP precursor. The comparative analysis of nucleotide and amino acid sequence homologies of rat and human VIP precursors implies that selection pressures during evolution have eliminated base substitutions which might result in amino acid replacements.

2. MATERIALS AND METHODS

2.1. *Materials*

Guanidinium thiocyanate was purchased from Fluka; oligo(dT)-cellulose from Pharmacia P-L Biochemicals; restriction endonucleases from Nippon Gene (Toyama, Japan) and Takara Shuzo (Kyoto); avian myelomatosis virus reverse transcriptase from Seikagaku Kogyo (Tokyo); calf thymus terminal deoxynucleotidyl transferase from Takara Shuzo; *E. coli* RNase H from Wako (Osaka); *E. coli* DNA ligase from Pharmacia P-L Biochemicals; *E. coli* DNA polymerase I from New England BioLabs; membrane filters for colony and blot hybridizations and DPT paper from Schleicher & Schuell; [α-^{32}P]dCTP, dGTP, and TTP (spec. act. 3000 Ci/mmol) and an M13 clon-

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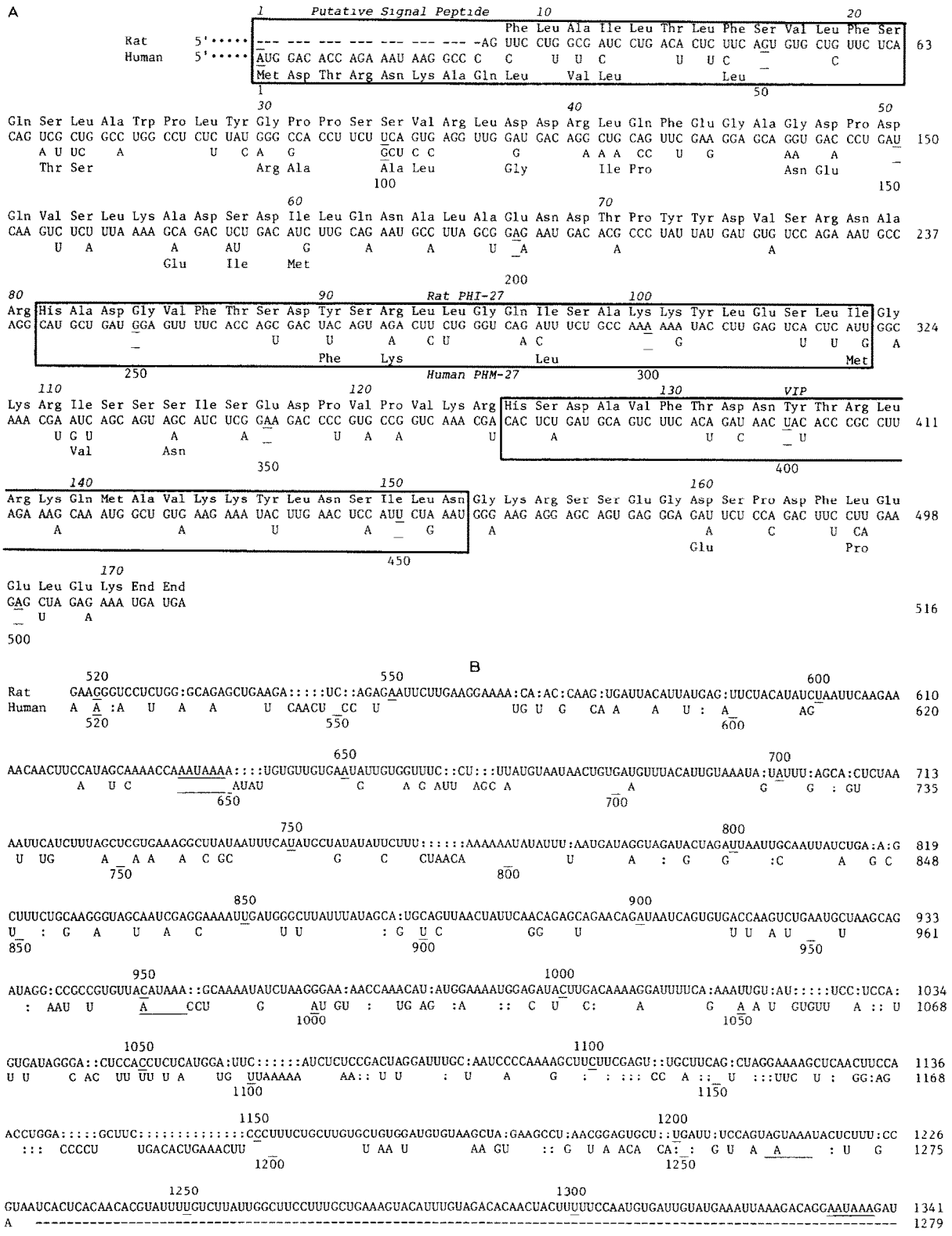
A
Putative Signal Peptide
Rat PHI-27
Human PHM-27
VIP
B

ing kit from Amersham; an M13 sequencing kit from Takara Shuzo.

2.2. *Probe DNA for colony and blot hybridizations*

The 409-bp *Sau*3AI-*Eco*RI restriction fragment (nucleotide residues 148–556 in fig.1), which contains human VIP and PHM-27 cDNA sequences, was derived from human prepro-VIP/PHM-27 cDNA clone pVIP-1 [5,6]. This fragment was labelled by nick-translation [8] with [α-^{32}P]dCTP and used as a hybridization probe to screen for recombinant plasmids carrying rat VIP precursor cDNA sequences.

2.3. *Purification of RNA containing rat VIP precursor mRNA and cDNA cloning*

Total RNA was extracted from cerebral cortex of male Wistar rats as described previously [9]. About 400 μg of poly(A)$^+$ RNA isolated by oligo(dT)-cellulose chromatography was applied to a linear 5–25% sucrose gradient, centrifuged at 26000 rpm at 20°C for 20 h in a Beckman SW27.1 rotor, and fractionated [10]. Glyoxalated RNA from each fraction was electrophoresed on a 1.5% agarose gel and transferred to DPT paper [11]. The paper was hybridized with the nick-translated *Sau*3AI-*Eco*RI restriction fragment from human prepro-VIP/PHM-27 cDNA clone pVIP-1 and subjected to autoradiography [7]. RNA (about 9 μg) was ethanol-precipitated from the hybridization-positive fraction. With this partially purified mRNA, cDNA cloning was performed according to the method of Okayama and Berg [12].

2.4. *Transformation of* E. coli *strain DHl and screening*

Transformation of *E. coli* strain DHl was carried out according to the method of Shigesada [13]. Ampicillin-resistant transformants were screened by colony hybridization [14] with the nick-translated *Sau*3AI-*Eco*RI restriction fragment from human prepro-VIP/PHM-27 cDNA clone pVIP-1. Sizes of cDNA inserts of candidate clones were estimated by Southern blot analysis [11].

2.5. *DNA sequence analysis*

The largest cDNA insert was cleaved with various restriction endonucleases and the cleaved fragments were subcloned in M13 vectors mp10 and mp11 [15]. The sequence of each restriction fragment was determined by the dideoxy chain-termination method [15].

3. RESULTS

The partially purified mRNA from rat cerebral cortex was 3-fold enriched over poly(A)$^+$ RNA for rat VIP precursor mRNA by fractionation after passage through the sucrose density gradient. The content of rat VIP precursor mRNA was estimated at 0.005% by northern blot hybridization assays using the human VIP precursor cDNA (pVIP-1), and the rat VIP precursor mRNA was found to be about 1600 nucleotides long (unpublished). With the partially purified mRNA, cDNA cloning was performed according to the method of Okayama and Berg [12]. In the first screening, 11 hybridization-positive clones were isolated from approx. 800000 transformants by colony

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Fig.1. Nucleotide and deduced amino acid sequences of rat VIP precursor mRNA cloned into pVIPrat-B, compared with human sequences. (A) The coding regions and (B) the 3′-nontranslated regions. Nucleotide residues are numbered in the 5′ to 3′ direction, beginning with the first residue of AUG triplet encoding the initiative methionine of the human VIP precursor mRNA. Amino acid residues are numbered beginning with the initiative methionine of the human VIP precursor [5,6]. The upper nucleotide sequence shows the rat VIP precursor mRNA and the deduced amino acid sequence is given above this. The nucleotide and amino acid differences found in the human sequence are displayed beneath the rat sequence. Nucleotide sequences are aligned to give maximum homology with the introduction of minimal deletions. Deletions are indicated by colons on a one-to-one basis. The hyphens indicate sequences that have not been determined. A possible site for cleavage of a signal peptide is after amino acid residue 21 and possible glycosylation sites are at amino acid residues 68–70 and 133–135 [5]. The polyadenylation signals (AAUAAA) present downstream of the translational termination site are underlined.

hybridization with the nick-translated *Sau*3AI-*Eco*RI restriction fragment from human prepro-VIP/PHM-27 cDNA clone pVIP-1. We re-screened these 11 clones twice, and 3 independent clones varrying rat VIP precursor cDNA sequences were isolated. One of the clones, pVIPrat-B, which carried the largest cDNA insert (about 1500 bp long), was subjected to nucleotide sequence analysis by using M13 cloning and sequencing methods [15].

The nucleotide sequence of the mRNA coding for rat VIP precursor protein is shown in fig.1. The amino acid sequence (residues 125–152) deduced from the nucleotide residues 373–456 coincides precisely to the amino acid sequence of human VIP [5]. The primary structure of rat VIP, deduced from the cDNA sequence of rat cerebral cortex in this study, is in agreement with that of rat VIP recently isolated from rat intestine [16].

The deduced amino acid sequence at residues 81–107 has histidine as amino-terminus and isoleucine as carboxy-terminus and consists of 27 amino acid residues. This amino acid sequence differs by 1, 2, and 4 amino acid(s) from bovine PHI-27, porcine PHI-27, and human PHM-27, respectively [4,5,17]. Although rat PHI-27-like peptide has not been isolated, we estimate that the sequence of nucleotide residues 241–321 corresponds to that of 'rat PHI-27'.

Thus, as shown in fig.1A, the rat VIP precursor contains both rat VIP and rat PHI-27, and has post-translational processing sites for generation of these two peptides. A pair of basic amino acid residues (Lys-Arg) precede the amino-terminus of rat VIP and only one basic amino acid (Arg) precedes the amino-terminus of rat PHI-27. A glycine and a pair of basic amino acids (Gly-Lys-Arg), a sequence which is known to be a carboxy-terminal amidation signal [18], lie adjacent to the carboxy-terminus of both rat VIP and rat PHI-27. These suggest that both rat VIP and rat PHI-27 are liberated from their precursor by proteolytic processing, and that the peptides are carboxy-terminally amidated.

Two translation termination codons, UGA (nucleotide residues 511–516), are present at a site 55 nucleotides downstream from the carboxy-terminus of rat VIP, as is observed in the nucleotide sequence of human VIP precursor mRNA [5,6], but the presumed initiation codon is not found. As shown in fig.1A, our cDNA clone pVIPrat-B can encode 162 amino acids and human VIP precursor is 170 amino acids long. Thus, our cDNA clone pVIPrat-B may be missing 22 nucleotides of the translated sequence in addition to the 5′-nontranslated sequence.

As shown in fig.1B, the 3′-nontranslated regio of the rat VIP precursor mRNA is unusually long and has two polyadenylation sites, AAUAAA (nucleotide residues 633–638 and 1333–1338). The rat 3′-nontranslated region is 75% homologous to the human counterpart, when gaps are counted as one substitution regardless of their length.

4. DISCUSSION

In the preceding paper, we have shown the primary structure of human VIP precursor [5,6]. Here, nucleotide sequence analysis of the cloned cDNA pVIPrat-B has disclosed the primary structure of rat VIP precursor. Comparison of rat and human VIP precursors has revealed that the positioning of the processing sites for generation of VIP and PHI-27 in the rat VIP precursor is the same as that in the human precursor (fig.1A). As shown in fig.2, each VIP precursor protein is func-

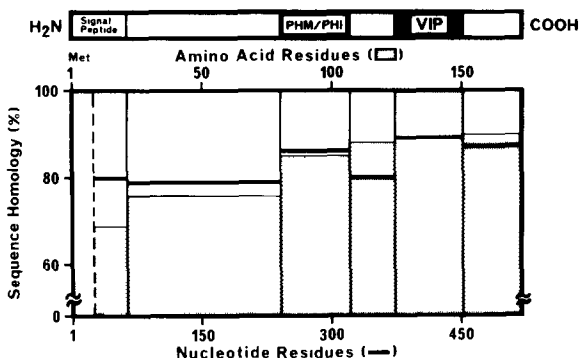

Fig.2. Sequence homology of each functionally divided domain in rat and human VIP precursors and corresponding mRNAs. The structure of the VIP precursor is presented schematically in the upper part of the figure. Numbering of the amino acid and nucleotide residues is as in fig.1. Each VIP precursor is functionally divided into 6 domains by the post-translational processing sites, and percentage of homology is calculated in each domain. Amino acid sequence homology is shown by shaded boxes and nucleotide sequence homology is solid lines.

tionally divided into 6 domains by post-translational processing sites (after amino acid residues 21, 80, 107, 124, and 152). The amino acid sequence for VIP is completely conserved in rat and human VIP precursors, while the degree of amino acid sequence homology of other domains varies from 69 to 90%. In contrast, at the nucleotide level, the degree of homology is 89% in the VIP domain and any domain exhibits an essentially equal degree of homology (79–89%). Although these findings suggest that rat and human VIP precursor genes have evolved from a common ancestral gene, the comparative analysis of nucleotide and amino acid sequence homologies of each domain in rat and human VIP precursors implies that selection pressures have eliminated base substitutions which might result in amino acid replacements. In other words, the degree of amino acid sequence conservation of the peptide during evolution might strongly contribute to its essential biological functions. In fact, VIP is conserved in various mammalian species [1,5,6,19,20] and exhibits important biological actions [21], but the physiological significance of PHI-27 and PHM-27 is still nuclear.

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