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A NOVEL PEPTIDE WHICH STIMULATES ADENYLATE CYCLASE: MOLECULAR CLONING AND CHARACTERIZATION OF THE OVINE AND HUMAN cDNAs

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SUMMARY : A novel neuropeptide which remarkably stimulates adenylate cyclase in rat anterior pituitary cell cultures has been recently isolated from ovine hypothalami by A. Arimura and his collaborators (*Biochem.Biophys.Res.Commun.*164, 567-574(1989)). This peptide was designated as PACAP38(*P*ituitary *A*denylate *C*yclase *A*ctivating *P*olypeptide with *38* residues). In an attempt to investigate physiological implications of PACAP38, we have succeeded in cloning the cDNAs encoding the precursor of PACAP38 from ovine hypothalamus and human testis. An ovine cDNA encodes a protein of 176 amino acids in which PACAP38 is proceeded by a putative signal peptide and a "pro"-region (107 amino acids), and followed by a Gly-Arg-Arg sequence for proteolytic processing and amidation. Deduced amino-acid sequence of human PACAP38 was completely identical to that of the ovine isolated peptide. Cloning of PACAP38 cDNAs confirms the expression of the corresponding mRNAs and the presence of this neuropeptide in ovine hypothalamus and also in human testis.

A novel neuropeptide which remarkably stimulates adenylate cyclase in rat anterior pituitary cell cultures has been recently isolated from ovine hypothalami by A. Arimura and his collaborators (1). This peptide was named PACAP38(*P*ituitary *A*denylate *C*yclase *A*ctivating *P*olypeptide with *38* residues) (1). The amino-acid sequence of PACAP38 was determined to be : His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-

ABBREVIATIONS: PACAP38, pituitary adenylate cyclase activating polypeptide with 38 residues; AMP, adenosine 3',5'-monophosphate; VIP, vasoactive intestinal polypeptide; GHRH, growth hormone releasing hormone.

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Tyr-Leu-Ala-Ala-Val-Leu-Gly-Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-NH_2. Although the N-terminal portion (1-28) exhibits 68% homology to vasoactive intestinal polypeptide (VIP), adenylate cyclase stimulating activity of PACAP38 was at least 1000 times greater than that of VIP in cultured rat pituitary cells(1). More recently an extraordinary large increase in cyclic AMP accumulation has been demonstrated in cultured rat astrocytes after addition of PACAP38 (10^{-9}M), whereas VIP at the same concentration failed to increase intracellular cyclic AMP levels in rat pituitary cells, neurons and astrocytes (2). Though distribution of PACAP38 in the brain remains to be clarified, it is possible that various central actions which have been attributed to VIP may actually be controlled by PACAP38. All of the so far isolated hypothalamic hypophysiotropic hormones stimulate adenylate cyclase activity of the anterior pituitary cells (3-6), although the extent of stimulation varies and this stimulation may not be necessarily linked with the secretion of the respective pituitary hormone. PACAP38 did not, however, induce a significant increase in the release of any known pituitary hormones in static rat pituitary cell cultures (1). These observations imply that PACAP38 stimulates the release of some as yet unknown pituitary hormones, or only their biosynthesis. It is also possible that PACAP38 may be a modulator of other hypophysiotropic hormone and/or a novel nerve-surviving factor. Meanwhile our preliminary study indicated that the immunoreactivity specific for PACAP38 was detected not only in rat hypothalamus but also in the brain cortex and testis (A. Arimura *et al.* unpublished observation), suggesting that this peptide is expressed in several tissues in mammals. These situations have prompted us to study the physiological implications of PACAP38.

In the present study, we have succeeded in cloning cDNAs encoding the precursor of PACAP38 from an ovine hypothalamus cDNA library. Furthermore, in order to deduce and elucidate the structure of human PACAP38 as a first step in investigating physiological functions of PACAP38 in human, we have also cloned a cDNA encoding human PACAP38 from a testis cDNA library. An ovine cDNA encodes a protein of 176 amino acids in which PACAP38 is proceeded by a putative signal peptide and a "pro"-region (107 amino acids), and followed by a Gly-Arg-Arg sequence for proteolytic processing and amidation. Deduced amino-acid sequence of human PACAP38 was completely identical to that of the ovine isolated peptide. Cloning of PACAP38 cDNAs confirms the expression of the corresponding mRNAs and the presence of this neuropeptide in ovine hypothalamus and also in human testis.

MATERIALS AND METHODS

Cloning and sequence analysis of ovine cDNA encoding the precursor of PACAP38

An ovine hypothalamus cDNA library constructed in λgt11 (7) was screened with a synthetic oligonucleotide probe corresponding to amino acid residues 1-27 of PACAP38. Oligonucleotides 5'-CACTCTGATGGAATCTTCACAGATAGCTA-

CAGCCGCTATAGAAAGCAAATG-3' and 3'-TCGGCGATATCTTTCGTTTAC-CGACACTTCTTTATGAACCGGCGGCAAGAT-5' were chemically synthesized with a DNA synthesizer, model 380B (Applied Biosystems Inc.). These two oligonucleotides were annealed to each other and labeled to a specific activity of 1.2 x 10^9 c.p.m./μg DNA by the action of Klenow fragment of *E. coli* DNA polymerase I (Takara Shuzo, Kyoto, Japan) in the presence of [α-^{32}P]dCTP (110 TBq/mmol, Amersham). Approximately 2 x 10^6 plaques were screened by plaque hybridization which was performed at 60°C for 16 h in slightly modified hybridization buffer-S(8)(2x SSC, 10x Denhardt's solution, 150μg/ml of sonicated and denatured salmon sperm DNA and 670μg/ml of yeast RNA), and followed by washing in 1x SSC at 50°C. The filters were then dried and autoradiographed onto Kodak X-AR film at -80°C with intensifying screens. Five hybridization-positive clones were obtained, and their cDNA inserts were subcloned into plasmid pUC118. The plasmids were rescued as single-stranded DNA and sequenced from both strands by the dideoxynucleotide chain-termination method with Klenow fragment or Sequenase (United States Biochemical Corporation, Cleaveland, Ohio). All other recombinant DNA manipulations were carried out principally as described (9).

Southern blot analysis of ovine genomic DNA

Ovine genomic DNA (10 μg each, Clontech, Palo Alto, CA) was digested in completion by restriction endonucleases, *Eco* RI, *Hin* dIII, *Bgl* II and *Bam* HI, respectively, electrophoresed in 0.7% agarose gel and transferred to nylon filters (BIODYNE A, PALL) as the procedures recommended by the manufacturer. The *Pst* I to *Acc* I cDNA fragment (240 bp) which fully encodes the mature PACAP38 (indicated in Fig. 1*a*) was labeled by the action of Klenow fragment in the presence of random sequence hexanucleotide primers (Amersham) and [α-^{32}P]dCTP(110 TBq/mmol, Amersham) to a specific activity of 4.5 x 10^8 c.p.m./μg DNA, and employed as a probe. Hybridization was carried out at 32°C in 50% formamide, 5x Denhardt's solution, 5x SSPE(43.5g NaCl, 6.9g $NaH_2PO_4.H_2O$ and 1.9g EDTA in 200 ml), 0.1% SDS and 100μg/ml sonicated and denatured salmon sperm DNA. Filters were then washed in 2x SSC, 0.1% SDS at 45°C and autoradiographed onto Kodak X-AR film with a intensifying screen at -80°C for 7 days.

Cloning and sequence analysis of cDNA encoding PACAP38 from human testis

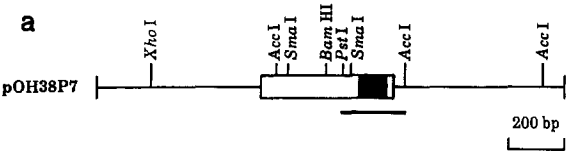
A human testis λgt11 cDNA library (Clontech) was screened with a synthetic oligonucleotide probe identical to the ovine PACAP38 cDNA encoding amino acid residue 1-27 of ovine PACAP38. Oligonucleotides 5'-CACTCGGACGGCATCT-TCACTGACAGCTACAGCCGCTACCGGAAGCAAATG-3' and 3'-TCGGCGA-TGGCCTTCGTTTACCGACAATTCTTTATGAACCGCCGACAGGAT-5' were chemically synthesized, annealed to each other and labeled to a specific activity of 6.8 x 10^8 c.p.m./μg DNA as described above. Approximately 1.8 x 10^6 plaques were screened by hybridization at 50°C in slightly modified hybridization buffer-S as mentioned above. The nitrocellulose filters were finally washed in 2x SSC at 48°C, then dried and autoradiographed. Three identical clones were obtained. The insert (2.1kbp) was subcloned into the *Eco* RI site of plasmid pUC118, and sequenced as described above.

RESULTS AND DISCUSSION

Cloning and characterization of cDNA encoding the precursor of PACAP38 from ovine hypothalamus

The probe used was chemically synthesized oligonucleotide coding 1-27 of PACAP38. The nucleotide and deduced animo acid sequences of a representative ovine cDNA clone are shown in Fig. 1. The nucleotide sequence is different from that of prepro-VIP(10), although the amino-acid sequence of the N-terminal 28 residues of PACAP38 is similar to that of VIP. The largest open reading frame

(nucleotide 577-1104) specifies the coding region for the precursor of ovine PACAP38. One of the cloned cDNAs (pOH38P7 in Fig. 1) has *ca.*570 nucleotides of 5' untranslated sequence. Analysis of ovine other clones has revealed that the



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1     CTGCTAACTGCCCAGATAAATAGGAGCAGAGGGCTGGTCACCTCTGTAATAACCACCGGCAGCAGTAGAAGAAAC   75

76    CGCAGCTTCAGAAGCAGCCAGAGAGACTTCTGAGCAGCGAAGGCGCTGCCTGCTCGAGCTGCCTGGCCGGGCGGCTGCC   154

155   CCAGACGCCGACTTCGCCGAGGCCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTGCTTCTTTC   233

234   CTTATCACTCCTTTCTTCTCAGTGGACTTCAGGCCACTTTGTCTCCCACCCCCACTCAGCTCGTCGCCTCCTCCGTCTT   312

313   CCTTCTCCATCTCTCCTCTCGCCCCCCTTCTCTCAGTGTCACGCTCCGTCCTAGTTCCGAGCGTCGTCAAACTTTTGAA   391

392   CAGAATAACAGGACTCAGCAAACAAGTCCTCCAGCTCCTCCCGCGGCTCCGGCTCGTTCCTGCGGCTCCTGCTCAGACA   470

471   CTAACGCCAGACGGCGATGCCTCTTGGGTTGTGACTACAGCGCACAAACTTGGAGAAGCTCTTTGCCCGCCGTCCTACT   549

550   TGGCAGCAAATCCTCTCCTGGCAGCGA ATG ACC ATG TGT AGC GGA GCG AGG CTG GCC CTG CTC GTT   615
1                                 Met Thr Met Cys Ser Gly Ala Arg Leu Ala Leu Leu Val   13

616   TAC GGG ATA CTG ATG CAC AGC AGC GTC TAC GGC TCA CCT GCC GCC TCC GGA CTC CGG TTC   675
14    Tyr Gly Ile Leu Met His Ser Ser Val Tyr Gly Ser Pro Ala Ala Ser Gly Leu Arg Phe   33

676   CCG GGG ATC AGG CCG GAG AAC GAG GCG TAC GAC GAG GAC GGA AAC CCG CAG GAC TTC   735
34    Pro Gly Ile Arg Pro Glu Asn Glu Ala Tyr Asp Glu Asp Gly Asn Pro Gln Gln Asp Phe   53

736   TAC GAC TCG GAG CCG CCA GGC GTG GGG AGC CCC GCC TCC GCG CTG CGC GAT GCC TAC GCG   795
54    Tyr Asp Ser Glu Pro Pro Gly Val Gly Ser Pro Ala Ser Ala Leu Arg Asp Ala Tyr Ala   73

796   CTC TAC TAC CCG GCG GAG GAA AGA GAT GTC GCC CAC GGG ATC CTT GAT AAG GCC TAC CGC   855
74    Leu Tyr Tyr Pro Ala Glu Glu Arg Asp Val Ala His Gly Ile Leu Asp Lys Ala Tyr Arg   93

856   AAA GTG CTG GAC CAG CTG TCC GCC AGG AGA TAC CTG CAG ACG CTC ATG GCC AAG GGC TTG   915
94    Lys Val Leu Asp Gln Leu Ser Ala Arg Arg Tyr Leu Gln Thr Leu Met Ala Lys Gly Leu   113

916   GGT GGG ACC CCG GGC GGC GGC GCG GAC GAC GAC TCG GAG CCG CTC TCC AAG CGC CAC TCG   975
114   Gly Gly Thr Pro Gly Gly Gly Ala Asp Asp Asp Ser Glu Pro Leu Ser Lys Arg His Ser   133

976   GAC GGC ATC TTC ACT GAC AGC TAC AGC CGC TAC CGG AAG CAA ATG GCT GTT AAG AAA TAC   1035
134   Asp Gly Ile Phe Thr Asp Ser Tyr Ser Arg Tyr Arg Lys Gln Met Ala Val Lys Lys Tyr   153

1036  TTG GCG GCT GTC CTA GGG AAA AGG TAT AAA CAA AGG GTT AAG AAC AAA GGA CGG CGA ATA   1095
154   Leu Ala Ala Val Leu Gly Lys Arg Tyr Lys Gln Arg Val Lys Asn Lys Gly Arg Arg Ile   173

1096  CCG TAC TTG TAG CGACGAGTTACCAGCTATCCTGTGTATACAGCCCTGACACAATGAGAAGTCGTTTTTCCCAAC   1170
174   Pro Tyr Leu ***                                                                  177

1171  TGACTGAACTGTCATCGCTGCTGTGTTCTGTCCCACATGTATTTATGTATGAAGTCAAGCCATTAAATGAATATTTTGA   1249

1250  TAATAATATTGTTTTTCTTTTTACGAAGCACTGGAGAATGCACAGATATACTTTGTGGACCAATTATTGATATTGACAT   1328

1329  ATATATTACGAATATATAAAGAGTATATATATATATATATAAGTATAATAGAGAGCCGTTCATACAGTGTGCACAAGGA   1407

1408  CTGAAGATTCGCCTGAGCTGTTTGTTTTTATATAAAATAAATAGAAAAATAGACAATCATTGTTTTGAATATTACTCCT   1486

1487  ATTTTTGTAAACTGGAATTAAAAGGATAGTATTTTTATCCACAATAGGCCTGAAGATATTAATCCTGACCATTTGCTAC   1565

1566  TGTACATAAACAGTGATGCCCTGCTCCAGGGAGACTTTGAGGTAATGATTTGGGAGGATTGCTGAAGGTCTCTCTTTCC   1644

1645  CAGGGAGTCTCTGGGGCAGGCTGCTTCAATCCCAGCTGAACTCGACTGAGGCTCTGTCTACCCCTTGCTGGGTGGCAAT   1723

1724  GCCAATACTTCCGCTTTCTTTGATTCTATTTTTATGTGTA                                          1763
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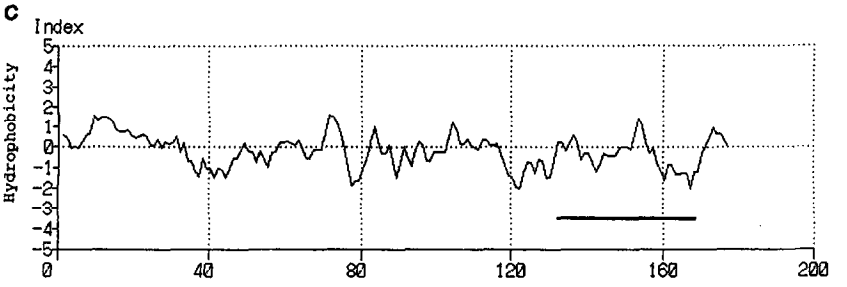
c



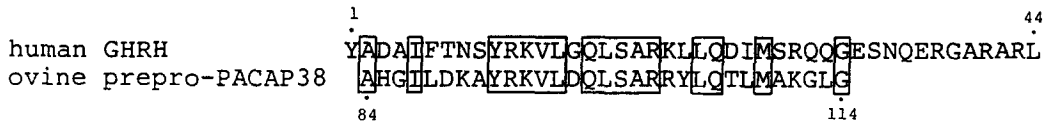


Fig. 2 Sequence comparison between GHRH and the precursor of PACAP38. The one-letter amino acid notation is used. Amino acid residues 1-44 of human GHRH(19)(top) are aligned with residues 84-114 of ovine prepro-PACAP38 (bottom). Sets of identical residues are enclosed by solid lines.

coding region is followed by *ca.*1400 nucleotides of a 3' untranslated region containing an AGTAAA polyadenylation signal (11) shortly upstream of the poly(A) tail (data not shown). The first ATG initiation codon (position 577) is followed by an in-frame ATG two codons downstream. This second ATG, but not the first, is within the consensus sequence analysed for eukaryotic translation initiation site (12). The possibility that the second ATG is the translation initiation site can not be excluded. The coding region for PACAP38 is located between nucleotides 970 and 1083, followed by the code for only seven amino acids(Fig. 1*b*). The possible cleavage site of the putative signal peptide is located between Gly^{24} and Ser^{25} based on a weight-matrix approach (13), which is well visualized on the hydrophobicity profile of the precursor protein (Fig. 1*c*). The deduced amino acid sequence of the precursor for PACAP38 consists of 176 amino acid residues with molecular weight of 19,458. The predicted primary structure of ovine PACAP38 corresponds precisely to the amino acid sequence of the peptide isolated from ovine hypothalami (1). The amino-terminus of ovine PACAP38 is preceeded by a pair of basic amino acid residues (Lys^{130}-Arg^{131}) which is known to be frequent sites for post-translational proteolytic processing of various peptide hormone precursors (14). A glycine residue and a paired basic amino acids (Arg^{171}-Arg^{172}) are found adjacent to the carboxy-terminal Lys^{169} residue of PACAP38. The glycine residue is thought to serve as an amino donor to the carboxy-terminus of this peptide, as with other polypeptide precursors (15-17). PACAP38 contains another signal sequence for proteolytic processing and amidation (Gly^{159}-Lys^{160}-Arg^{161}), which is consistent with the experimental evidence that PACAP27-NH_2 was also isolated from ovine hypothalamic tissues

Fig. 1 *a*, Schematic representation of the structures of an ovine PACAP38 precursor cDNA clone with its restriction map. Lines and a rectangle denote the noncoding and coding region, respectively. The position corresponding to mature PACAP38 is indicated by a closed box. The probe employed in ovine genomic Southern blot analysis is indicated by a solid line. *b*, Nucleotide sequence of the PACAP38 precursor cDNA and deduced amino-acid sequence. The overlined ATG denotes an alternative translational initiation codon. The arrow indicates the possible cleavage site of the signal peptide. The code and the mature PACAP38 are boxed. *c*, Hydrophobicity index along the amino-acid sequence of ovine PACAP38 precursor. The hydrophobicity values were calculated with the hexapeptide averaging method (20). The position of PACAP38 sequence is underlined.

(18). Whether PACAP27-NH_2 is generated from PACAP38 or these two peptides are directly cleaved from the same precursor respectively remains to be studied. In any case, it is noteworthy and extremely rare that two peptides with amidated C-terminus are cleaved from the same precursor.

In the remainder of the PACAP38 precursor, there is a region which is flanked by paired basic residues (Tyr^{104}-Ser^{129}) and is presumably co-liberated by post-translational processing. Whether this peptide has biological functions or not is presently unknown. The precursor does not contain any sites for *N*-glycosylation (Asn-X-Thr/Ser). Interestingly, not only the N-terminal sequence of PACAP38 itself(1), but also the upstream region (Ala^{84}-Gly^{114}) of the mature peptide have a sequence similarity to that of growth hormone releasing hormone (GHRH)(19) (Fig. 2). These observations imply evolutional relations among these precursor proteins, which is concievable in view of the fact that GHRH, PACAP38 and, to a lesser extent, VIP have adenylate cyclase stimulating activity.

Southern blot analysis of ovine genomic DNA probed with the ovine PACAP38 precursor cDNA (240 bp *Pst* I-*Acc* I fragment which fully encodes the mature PACAP38) was performed in an effort to identify homologous genes. Even in low-stringency conditions, only single band appeared with each restriction endonuclease examined (Fig. 3), indicating that the PACAP38 gene is probably

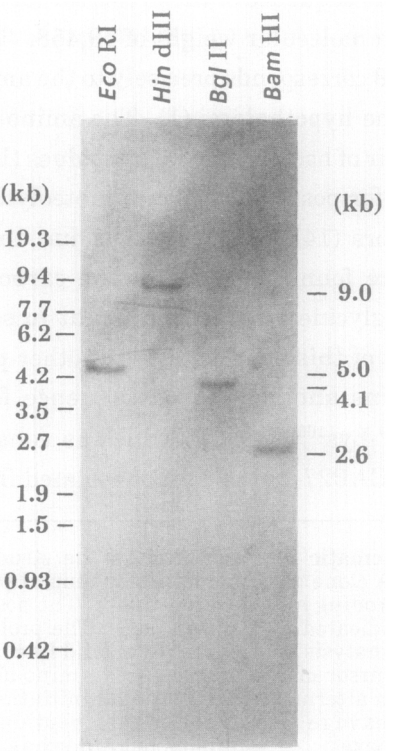
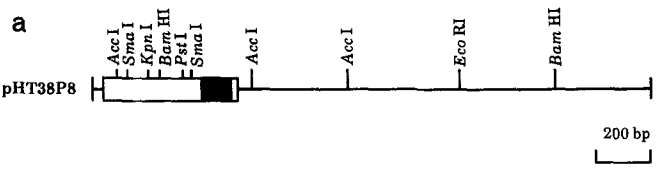


Fig. 3 Southern blot analysis of ovine genomic DNA probed with the ovine PACAP38 cDNA.



b

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Ovine   Gly Gly Thr Pro Gly Gly Gly Ala Asp Asp Asp Ser Glu Pro Leu
        GGT GGG ACC CCG GGC GGC GGC GCG GAC GAC GAC TCG GAG CCG CTC
        *** *** * * *   *** *** *** *** *   *** ***  **  *** *** ***
Human   GGT GGG AGC CTC GGC GGC GGC GCG GGG GAC GAC GCG GAG CCG CTC
        Gly Gly Ser Leu Gly Gly Gly Ala Gly Asp Asp Ala Glu Pro Leu

        Ser Lys Arg His Ser Asp Gly Ile Phe Thr Asp Ser Tyr Ser Arg Tyr
        TCC AAG CGC CAC TCG GAC GGC ATC TTC ACT GAC AGC TAC AGC CGC TAC
        *** *** *** *** *** *** **  *** *** **  *** *** *** *** *** ***
        TCC AAG CGC CAC TCG GAC GGG ATC TTC ACG GAC AGC TAC AGC CGC TAC
        Ser Lys Arg His Ser Asp Gly Ile Phe Thr Asp Ser Tyr Ser Arg Tyr

        Arg Lys Gln Met Ala Val Lys Lys Tyr Leu Ala Ala Val Leu Gly Lys
        CGG AAG CAA ATG GCT GTT AAG AAA TAC TTG GCG GCT GTC CTA GGG AAA
        *** **  *** *** *** **  *** **  *** *** *** **  *** *** *** **
        CGG AAA CAA ATG GCT GTC AAG AAG TAC TTG GCG GCC GTC CTA GGG AAG
        Arg Lys Gln Met Ala Val Lys Lys Tyr Leu Ala Ala Val Leu Gly Lys

        Arg Tyr Lys Gln Arg Val Lys Asn Lys Gly Arg Arg Ile Pro Tyr Leu
        AGG TAT AAA CAA AGG GTT AAG AAC AAA GGA CGG CGA ATA CCG TAC TTG
        *** *** *** *** *** *** **  *** *** *** **  *** ***  *  **  ***
        AGG TAT AAA CAA AGG GTT AAA AAC AAA GGA CGC CGA ATA GCT TAT TTG
        Arg Tyr Lys Gln Arg Val Lys Asn Lys Gly Arg Arg Ile Ala Tyr Leu

        End
        TAG CGA CGA GTT ACC AGC TAT CCT GTG TAT
        *** ***  *  *** *** *** **  *** *** ***
        TAG CGA TGG GTT ACC AGC TAC CCT GTG TAT
        End
```

Fig. 4 *a*, Structure of a human PACAP38 precursor cDNA clone with its restriction map. Lines and a rectangle indicate the noncoding and coding regions, respectively. The sequence encoding mature PACAP38 is denoted by a closed box. *b*, Partial nucleotide sequence of the cDNA encoding a human PACAP38 precursor and deduced amino acid sequence (bottom) aligned with the corresponding ovine sequences (top). The regions of the mature PACAP38 are boxed.

unique in sheep. More direct evidence was provided by obtaining ovine genomic clones (EMBL-3) probed with a PACAP38 cDNA fragment. The restriction maps of the ovine genomic isolates were fully consistent with the above results from the ovine genomic Southern blots (data not shown). Furthermore, partial sequence analysis showed that an intervening sequence, intron (*ca.* 1000 bp), was present between nucleotide 911 and 912 in Fig. 1*b*, supporting the possibility that the cloned cDNA is derived from a spliced mature mRNA.

Cloning and characterization of cDNA encoding PACAP38 from human testis

The availability of a cDNA clone for ovine PACAP38 has allowed us to isolate and characterize the human PACAP38 cDNA and gene. Radioimmunoassay indicated that immunoreactive PACAP38 was demonstrated in rat testis in high

concentrations (A. Arimura *et al.* unpublished observation). Indeed, we have succeeded in cloning of the cDNA encoding PACAP38 from human testis λgt11 cDNA library (Clontech). Partial nucleotide sequence of the human cDNA and deduced amino-acid sequence are presented in Fig. 4 together with the ovine corresponding sequences aligned. Sequence analysis has revealed that the amino acid sequence of human mature PACAP38 is completely identical to that of the ovine peptide, though some amino acid substitutions were observed in the "pro-" region (manuscript in preparation). However, the restriction map of the region encoding around the precursor of human PACAP38 resembles the map of the cDNA coding the ovine PACAP38 precursor protein (Fig. 1*a* and Fig. 4*a*), indicating that the structure of human PACAP38 precursor is analogous to that of the ovine precursor.

In this report we have described molecular cloning and structural characteristics of the precursor of ovine and human PACAP38. Identity of ovine and human PACAP38 evokes conservation of the PACAP38 gene and functions of this peptide. These cloned cDNAs will in turn become powerful probes for understanding the mechanism of biosynthesis and secretion of the mature peptide. Cloning of the PACAP38 cDNAs confirms the expression of the corresponding mRNAs and this neuropeptide in ovine hypothalamus, as demonstrated by Arimura and his colleagues (1), and also in human testis. Further unveiling of biological functions and physiological significance of this peptide in the brain, as well as in the testis, is urgently expected.

ACKNOWLEDGMENTS

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