

Expression of the vasopressin and oxytocin genes in human hypothalami

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Poly(A)⁺ RNA isolated from post-mortem human hypothalami has been used to characterize the poly-protein precursors to vasopressin and oxytocin. Translation in a cell-free system and subsequent immunoprecipitation with antibodies raised against either vasopressin or neurophysin identified a product of M_r 19 000 (prepro-vasopressin). A second less intense product of M_r 16 500 was tentatively identified as prepro-oxytocin. A cDNA library derived from the human hypothalamic poly(A)⁺ RNA was screened for vasopressin and oxytocin-encoding cDNA using heterologous probes; clones encoding the two precursors were identified and found to be organized as their rat and bovine counterparts. Northern blot analysis shows that the mRNAs for the two prepro-hormones consist of ~840 (AVP) and ~700 (OT) nucleotides.

<i>cDNA cloning</i>	<i>Prepro-vasopressin</i>	<i>Prepro-oxytocin</i>	<i>(Human hypothalamus)</i>
	<i>mRNA</i>	<i>Cell-free translation</i>	

1. INTRODUCTION

Vasopressin and oxytocin are structurally related peptide hormones which regulate respectively the water balance in the distal kidney tubuli and uterine contraction during birth. The major site of their biosynthesis is the hypothalamus. From nucleotide sequence analysis of cDNA clones from rat and bovine hypothalami it is known that both hormones are synthesized as composite precursors, additionally containing their respective carrier proteins, the neurophysins. Only the vasopressin precursor contains a third unit, a glycopeptide [1–3].

Analysis of the vasopressin and oxytocin genes from rat and cow has shown that both are composed of 3 distinct exons, each encoding a functional domain of the precursor [4–6]. The hormones are located on exons A together with their signal peptides and the variable N-termini of the neurophysins; the major part of the neurophysins

are on exons B while exons C contain the variable C-termini of the neurophysins as well as the glycopeptide in the case of the vasopressin gene. Only a single gene for each hormone has been found in the 2 species tested.

No such data were hitherto available for the human nonapeptide hormone precursors mostly due to the scarcity of suitable hypothalamic tissue. Cell-free translation studies using human hypothalamic poly(A)⁺ RNA, however, indicated that the size of the prepro-vasopressin precursor was similar to that reported from rat and cow [7]. This was in agreement with protein data which suggested that high-molecular-mass material with vasopressin- and neurophysin-like structure was present in extracts from human pituitary [8] and lung carcinoma [9]. We present here sequence data on the structure of the human vasopressin and oxytocin precursors.

2. MATERIALS AND METHODS

Poly(A)⁺ RNA was prepared by the

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guanidinium isothiocyanate method [10]. Restriction and other enzymes were purchased from Boehringer, Mannheim or from New England Biolabs, Schwalbach, FRG.

2.1. Screening and sequencing

cDNAs prepared from human hypothalamic poly(A)⁺ RNA were inserted into the *Eco*RI site of the phage λ gt10 [11]. The bank (complexity 130.000) was screened for vasopressin-specific plaques by filter hybridization at 65°C, using a rat vasopressin cDNA fragment (*Hind*III-*Dra*I; Schmale, unpublished) nick-translated with [³²P]dCTP to a specific activity of 10⁸ cpm/ μ g.

The oxytocin-specific clones were identified by screening the same library sequentially using the *Mn*II fragment (230 bp) and the complete *Eco*RI cDNA insert of the human vasopressin clone (see restriction map); the *Mn*II fragment specifically encodes the 3'-end of the vasopressin precursor. The oxytocin cDNA should cross-react with the *Eco*RI cDNA but not with the *Mn*II fragment. The identified clones were subcloned into plasmid pUC9 and sequenced according to Maxam and Gilbert [12].

2.2. Northern blot analysis

Total hypothalamic RNA prepared by the guanidinium isothiocyanate method was subjected to methyl mercuric hydroxide-agarose gel electrophoresis [13] and transferred to nylon membranes (Amersham Hybond N) [14].

Northern blot analysis was carried out according to Maniatis et al. [15] with the following modifications. Filters were prehybridized in 50% formamide, 5 \times SSPE, 5 \times Denhardt's solution, 0.5% SDS, 100 μ g/ml denatured herring sperm DNA and 100 μ g/ml poly(A) at 42°C, for at least 6 h. Hybridization was performed in the same solution to which ³²P-labelled nick-translated probes (spec. act. >4 \times 10⁸ cpm/ μ g) were added (10⁷ cpm/ml).

Hybridization was carried out at 42°C for 16 h. Filters were washed at 55°C, with 2 changes of 2 \times SSC, 0.1% SDS, and once each with 1 \times SSC, 0.1% SDS and 0.2 \times SSC, 0.1% SDS. They were subsequently exposed to Kodak X-Omat AR film at -70°C, using 2 intensifying screens.

2.3. Cell-free translation

Poly(A)⁺ RNA was translated in a rabbit reticulocyte lysate in vitro system, specific translation products were immunoprecipitated and isolated as described [7].

3. RESULTS AND DISCUSSION

3.1. Cell-free translation products

When mRNA from human hypothalami was translated in a cell-free reticulocyte lysate system a precursor of 19 kDa could be precipitated with antibodies raised against either vasopressin or neurophysin (fig.1). The presumed oxytocin precursor migrated with a slightly smaller molecular mass of 16.5 kDa (fig.1, arrowhead). The low intensity of the oxytocin precursor band probably reflects the ratio of the 2 nonapeptide mRNAs in the human hypothalamus.

3.2. Sequence and organization of the hormone precursors

Since cell-free translation showed that the poly(A)⁺ RNA from post-mortem human hypothalami was of sufficiently good quality to direct the in vitro synthesis of the 2 nonapeptide

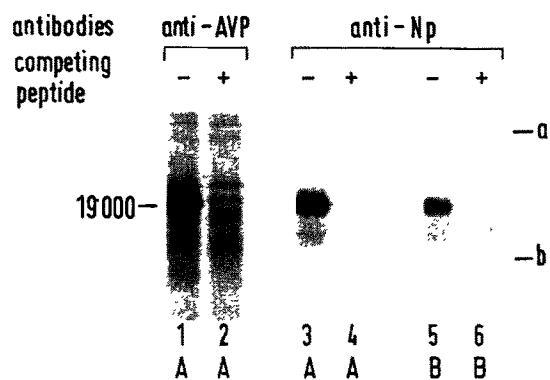


Fig.1. Cell-free translation of hypothalamic RNA; A and B, RNA prepared from 2 different autopsies. Poly(A)⁺ RNA was translated in a reticulocyte lysate system in the presence of [³⁵S]cysteine and immunoprecipitated using antibodies against bovine vasopressin (AVP) or mixed neurophysin I and II (Np). Where indicated 10–25 μ g/ml of the competing antigens was added prior to immunoprecipitation. Translation products were separated on SDS-polyacrylamide gels and subjected to fluorography [7]. a, b, molecular mass markers (30 and 14 kDa, respectively).

nucleotide sequence for the neurophysin is highly conserved, cross-hybridization was expected with the human cDNA clone. Of the positively hybridizing recombinant clones 2 were subjected to

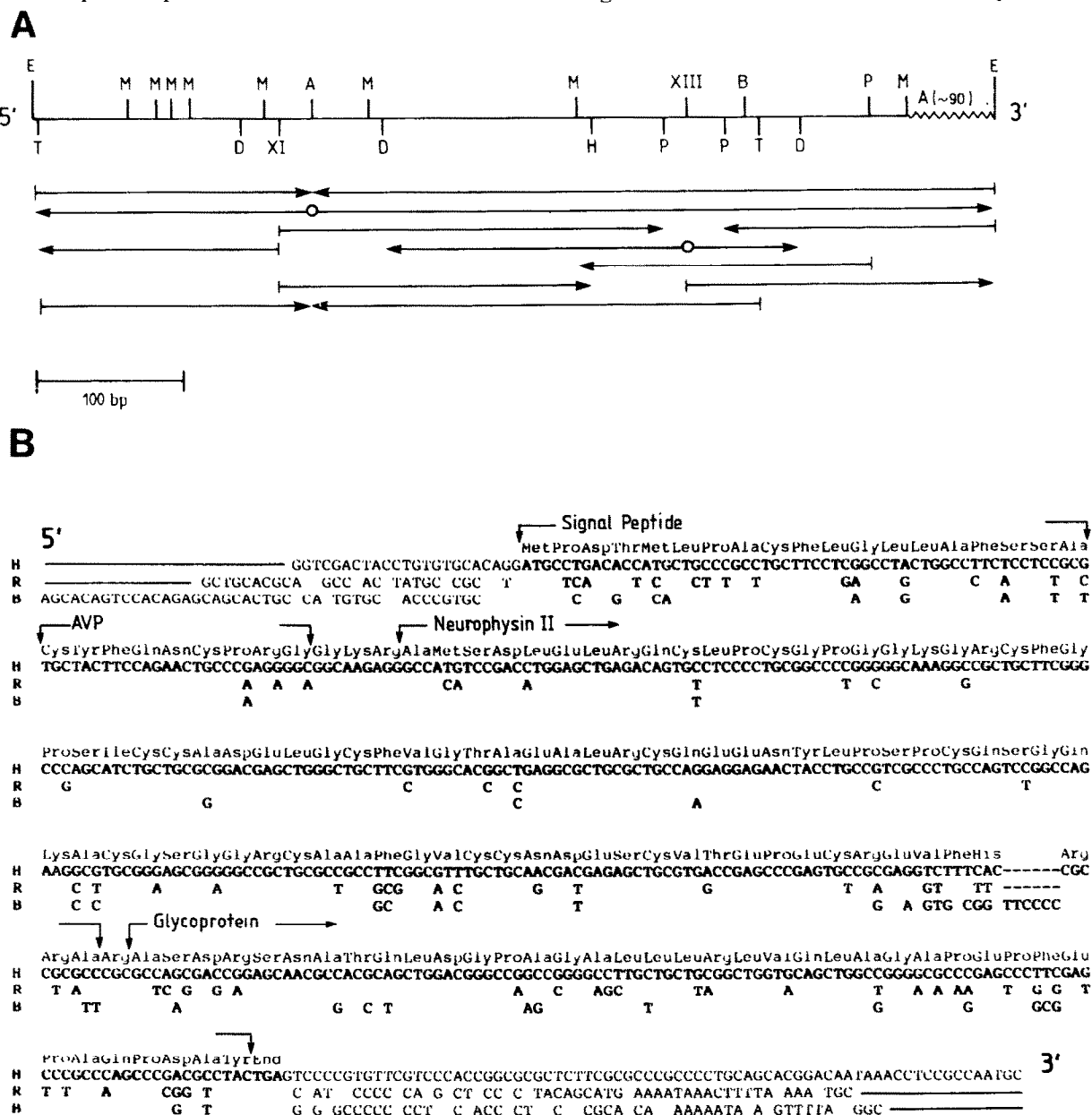


Fig. 2. (A) Restriction map and sequencing strategy of the human vasopressin precursor cDNA. A, *Apa*I; B, *Ban*I; D, *Dde*I; E, *Eco*RI; H, *Hph*I; M, *Mn*II; P, *Pst*I; T, *Taq*I; XI, *Xma*I; XIII, *Xma*III. Direction of sequencing is indicated by arrows. (B) Nucleotide sequence of the human (H) vasopressin precursor cDNA and its comparison to the rat (R) and bovine (B) counterpart. Nucleotides different to the human vasopressin sequence are indicated. The dashed lines show the position where the human and rat sequences are shorter by 6 nucleotide residues compared to the bovine sequence. Solid lines were drawn where corresponding nucleotides were either absent or not sequenced.

nucleotide sequencing (pHV-1, pHV-2), the strategy for which is illustrated in fig.2A. The nucleotide sequence of the human vasopressin precursor compared to that from cow and rat is presented in fig.2B.

According to the nucleotide sequence analysis the human prepro-vasopressin consists of 164 amino acid residues with a molecular mass of 17423 Da. The precursor starts with a putative signal peptide consisting of 19 amino acid residues and is separated from the pro-hormone by a small amino acid, alanine.

As in the rat and bovine vasopressin precursors the human counterpart includes the nonapeptide hormone immediately adjacent to the signal peptide. The hormone is separated from the respective neurophysin by the triplet Gly-Lys-Arg; the third moiety is a glycopeptide, C-terminally located, and separated from the neurophysin by a single arginine residue. The glycosylation site is predicted by the sequence Asn-Ala-Thr.

In general the deduced amino acid sequences agree with those obtained by conventional Edman degradation with one exception [16]. The cDNA sequence predicts that the human neurophysin of the vasopressin precursor contains a valine instead of a glycine residue in position 88; this may reflect a microheterogeneity or a polymorphism as has been reported for the cow [17], where at position 89, 70% of neurophysin molecules include an isoleucine, the remainder valine.

The nucleotide sequence for the human vasopressin precursor stops short a few nucleotides upstream of the first AUG codon. Attempts to identify the residual nucleotides by S_1 mapping have been hampered by the difficulty of obtaining adequate quantities of post-mortem human hypothalamic mRNA. At the 3'-end of the sequence a typical polyadenylation site AATAAA is indicated. Inspection and comparison of the

nucleotide sequence from man, rat and cow show a remarkably high degree of sequence homology, which is particularly strong in the region encoding the conserved part of the neurophysin (fig.2B). Also striking is the homology found in the 5'- and 3'-untranslated regions where in the latter the sequences around the polyadenylation sites are highly conserved.

Rescreening of the phage λ gt10 library with the 3'-vasopressin specific *MnII* DNA fragment of clone pHV-1 indicated that less than 0.01% of the recombinant clones encoded the vasopressin precursor. Subsequent screening of the library with the total cDNA now including the DNA sequence encoding the constant neurophysin region revealed additional positive signals, attributable to the oxytocin gene product. The ratio of vasopressin to oxytocin-specific clones was approx. 15:1. This uneven ratio reflects the proportions of the magnocellular neurones immunostaining for either oxytocin or vasopressin [18] as well as the relative peptide levels in extracts of different human brain regions [19]. It would also explain the relatively very low intensity of the putative oxytocin precursor visualized in the cell-free translation experiment (fig.1).

Sequencing of 2 selected oxytocin-specific clones (pHO-1, pHO-2) confirmed their identification (fig.3), and showed that the human oxytocin precursor is organized just as in other species, there being no C-terminal glycopeptide moiety as in the vasopressin precursors of all mammal species studied to date. As in the rat and calf the C-terminus of the neurophysin includes an extra basic amino acid, arginine, which has presumably to be removed by a post-translational cleavage process.

3.3. mRNA analysis

To determine the length of the vasopressin- and

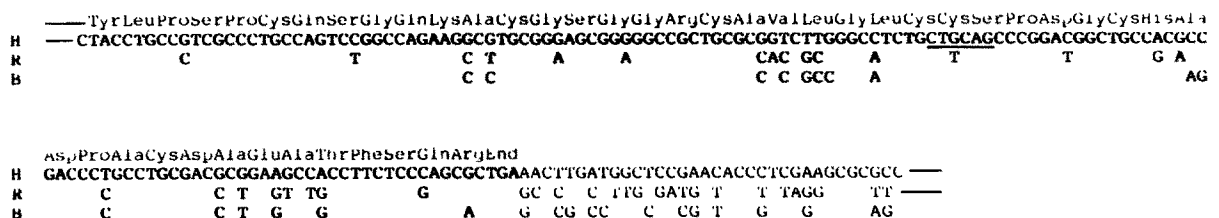


Fig.3. Partial nucleotide sequence analysis of the human oxytocin precursor.

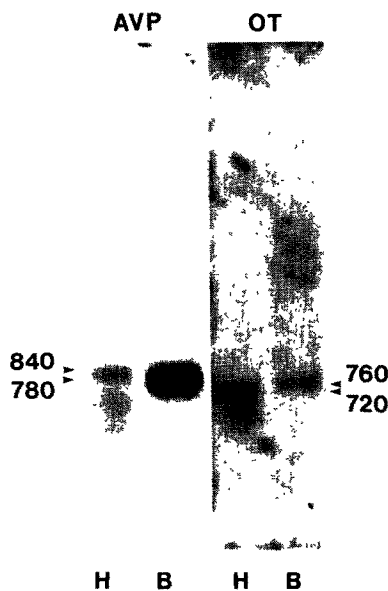


Fig.4. Northern blot analysis. 20 μ g human (H) and 40 μ g bovine (B) hypothalamic total RNA were separated on 1.2% agarose gels as described in section 2. Hybridization was carried out with 32 P-labelled nick-translated probes of the complete human vasopressin cDNA (hVP-1) or with a *Pst*I/*Eco*RI fragment of the human oxytocin cDNA (see fig.3).

oxytocin-encoding mRNAs, poly(A)⁺ RNA from post-mortem human hypothalami was separated on agarose gel, transferred to nylon filters and hybridized to 32 P-labelled cDNAs encoding either vasopressin (pHV-1) or oxytocin (pHO-1).

The length of the human vasopressin-encoding mRNA was determined to be ~840 nucleotides which is slightly larger than the corresponding bovine one (~780). Since the size predicted from the cDNA sequence is ~600 nucleotides, the poly(A) tail must comprise in excess of 200 A residues. The human oxytocin-encoding mRNA migrated at ~700 nucleotides, being smaller than the bovine oxytocin mRNA (~720). The smear seen in the lower range of the gel presumably results from degradation of the post-mortem isolated mRNA.

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