

# The Human Vasopressin Gene Is Linked to the Oxytocin Gene and Is Selectively Expressed in a Cultured Lung Cancer Cell Line\*

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The human genes for prepro-arginine-vasopressin-neurophysin II (prepro-AVP-NPII) and prepro-oxytocin-neurophysin I (prepro-OT-NPI) were cloned from a human genomic library and the nucleotide sequence of both genes was determined. The two genes are similar in their intron-exon structure, linked together with 12 kilobases intervening, and transcribed from opposite DNA strands. A human small cell lung cancer cell line, H378, produces significant quantities of prepro-AVP-NPII mRNA using a transcription unit predicted from the genomic DNA sequence. Despite the proximity of the actively transcribed prepro-AVP-NPII gene, transcription of prepro-OT-NPI is not detected in this cell line.

tide hormones, including AVP, pro-opiomelanocortin, calcitonin, and bombesin/gastrin-releasing peptide (Gazdar and Carney, 1984). As a prelude to understanding the basis for selective hormone gene expression by certain SCLC tumors and cell lines we have cloned the human prepro-AVP-NPII gene from a genomic library. The same screening procedure also allowed us to characterize the human prepro-OT-NPI gene. We report here the structure of the human prepro-AVP-NPII and prepro-OT-NPI genes and show that they are physically linked within 12 kb in the human genome with an inverted arrangement of their coding strands. In addition, a SCLC-derived cell line transcribes this prepro-AVP-NPII gene in a way predicted by the gene structure. In contrast, transcription of prepro-OT-NPI in this cell line is not observed.

The conventionally accepted activities of the posterior pituitary hormones AVP<sup>1</sup> and OT are in the regulation of water excretion, parturition, and lactation. In addition, AVP receptors and AVP-like immunoreactive material exist in central and autonomic neurons (Hanley *et al.*, 1984; Buijs *et al.*, 1983). AVP is derived from a larger precursor, prepro-AVP-NPII, whose primary nucleotide structure was first elucidated in a bovine cDNA clone by Land *et al.* (1982). The precursor consists of a single polypeptide containing a secretory signal sequence (Blobel and Dobberstein, 1975), the nonapeptide AVP (preceded by a signal peptide), the hormone carrier protein neurophysin II, and from the carboxyl terminus another peptide of unknown function but resembling a previously detected pituitary glycoprotein. Bovine prepro-OT-NPI has an analogous structure (Land *et al.*, 1983; Ruppert *et al.*, 1984) except that OT is present in place of AVP and neurophysin I is present in place of neurophysin II. There is no peptide in prepro-OT-NPI analogous to the carboxyl-terminal glycoprotein present in prepro-AVP-NPII. Although the primary sequence of prepro-AVP-NPII and prepro-OT-NPI has been characterized in cow and rat, it has not been known whether these two structurally similar genes are linked in the genome.

Human small cell lung cancer (SCLC) is distinguished from other types of lung cancer by selective elaboration of polypep-

## MATERIALS AND METHODS<sup>2</sup>

## RESULTS AND DISCUSSION

**Structure of the Human Prepro-AVP-NPII Gene**—A probe from the neurophysin portion of bovine prepro-AVP-NPII was obtained by preparing a 0.3-kb *SmaI-PvuII* fragment from a cDNA clone (pVNpII-1) of Land *et al.* (1982). This probe (designated cc-bov) has homology to both neurophysins I and II, due to their extensive nucleic acid sequence similarity (Land *et al.*, 1983; Schmale *et al.*, 1983). In addition, a 0.2-kb *PstI-SmaI* fragment from the AVP-specific 5' region of bovine prepro-AVP-NPII, designated 5' bov was also used. Of 10<sup>6</sup> plaques from a human genomic library derived from *MboI*-cut placental DNA cloned into Charon 28A, eight positive clones were obtained and characterized.

The library clones fell into two classes represented by hAVP3 and hAVP4, whose structure is outlined in the top portion of Fig. 1. Homology to both 5' bov and cc-bov exists on the 15-kb insert of hAVP4. Restriction mapping of hAVP4 reveals that homology to cc-bov is found on both 2.5- and 5.0-kb *BamHI-BamHI* fragments. Homology to the 5' bov probe in hAVP4 exists only on the 2.5-kb fragment. The fact that homology with cc-bov in hAVP3 is linked to phage arms when the phage is digested with *BamHI* shows that the 2.5-kb *BamHI-BamHI* fragment in hAVP4 is bounded by an artificial *BamHI* site on its 5' border that is created from an *MboI*

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<sup>1</sup> The abbreviations used are: AVP, 8-arginine-vasopressin; OT, oxytocin; prepro-AVP-NPII, prepro-AVP-neurophysin II; prepro-OT-NPI, prepro-OT-neurophysin I; kb, kilobase, SCLC, small cell lung cancer; hAVP, human 8-arginine-vasopressin.

<sup>2</sup> Portions of this paper (including "Materials and Methods," part of "Results and Discussion," Figs. 4-6, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M3411, cite the authors, and include a check or money order for \$3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

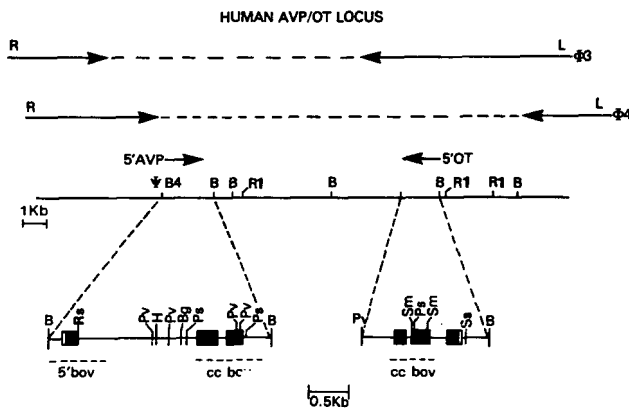


FIG. 1. Schematic organization of human prepro-AVP-NPII and prepro-OT-NPI genes. The top portion indicates the relative extent of overlap of library phage clones hAVP3 and hAVP4 (dashed lines) with respect to the human chromosomal segment in the middle portion of the figure. The bottom indicates the detailed restriction endonuclease map for the genomic region. The transcriptional orientation of prepro-AVP-NPII (5' AVP) as well as prepro-OT-NPI (5' OT) is indicated. Solid bars indicate exons; white areas adjacent to solid bars indicate 5' untranslated regions of exons. The horizontal dashed lines show regions with homology to the *PstI-SmaI* (5' bov) and *SmaI-PvuII* (cc-bov) fragments of the bovine cDNA clone (Land *et al.*, 1982). B, *BamHI*; RI, *EcoRI*; R1, *RsaI*; Pv, *PvuII*; H, *HindIII*; Bg, *BglII*; Ps, *PstI*; Sm, *SmaI*; Ss, *SstI*.  $\Psi$ B4 indicates the *BamHI* site present in hAVP4 that is created by ligation of an *MboI* site into Charon 28A, and is not present in genomic DNA.

site in genomic DNA by ligation into the *BamHI* site of the cloning vector.

The 2.5-kb *BamHI-BamHI* fragment from hAVP4 was subcloned and sequenced, as shown in Fig. 2. A Goldberg-Hogness consensus site for initiation of transcription (Breathnach and Chambon, 1981) is identified at position 146. The next adjacent ATG triplet 79 base pairs downstream at position 225, begins an open reading frame which contains the AVP sequence (position 283–308). The peptide, as in cow and rat (Land *et al.*, 1982; Ivell and Richter, 1984), appears to be derived from a larger polypeptide precursor. Preceding AVP in the prepro-AVP-NPII sequence is a series of largely hydrophobic or uncharged amino acids encoded by nucleotides 225–281. This leader peptide could function as a signal sequence for further processing, as has been described for other secreted proteins (Blobel and Dobberstein, 1975). Following AVP, there is a spacer tripeptide (Gly-Lys-Arg). This arrangement has been seen in bovine prepro-AVP-NPII (Land *et al.*, 1982), and may serve as a signal for preteolytic cleavage and processing as has been described for other hormone precursors (see Amara *et al.*, 1980). Following the spacer (starting at nucleotide 318) there is a translation product identical with human neurophysin II, whose protein sequence was described by Chauvet *et al.* (1983). This moreover aligns precisely with the bovine cDNA sequence (Land *et al.*, 1982) until a break in homology with the bovine sequence occurs at position 344, preceding a consensus 5' splice donor site (Breathnach and Chambon, 1981), thus marking the end of the first exon of the human prepro-AVP-NPII gene. An intron of 1374 nucleotides follows, with resumption of homology with the bovine cDNA at position 1718. The second exon is comprised entirely of sequences encoding neurophysin II, consistent with the protein sequence (Chauvet *et al.*, 1983).

Another break in homology with the bovine cDNA sequence is found at position 1921, defining a second intervening sequence that is only 165 base pairs in length. Homology with the bovine cDNA resumes at position 2087 and defines a third

exon which contains the remaining neurophysin II sequences. Following these sequences, a 40 amino acid human peptide is predicted which is considerably less homologous to the putative prepro-AVP-NPII associated glycopeptide predicted by the bovine cDNA sequence (Land *et al.*, 1982) and rat genomic sequence (Schmale *et al.*, 1983) than is the hormone or neurophysin portion of the precursor. From the sequence in Fig. 2 a potential *N*-glycosylation site is also predicted in the human case to exist at the amino acids encoded by nucleotides 2155–2163 (Pless and Lennarz, 1977). A termination codon exists in-phase with this reading frame at position 2258. The 3' untranslated portion of this gene continues through a polyadenylation signal (Proudfoot and Brownlee, 1976) at position 2309, and an approximate 3' border of the third exon is indicated at about position 2345.

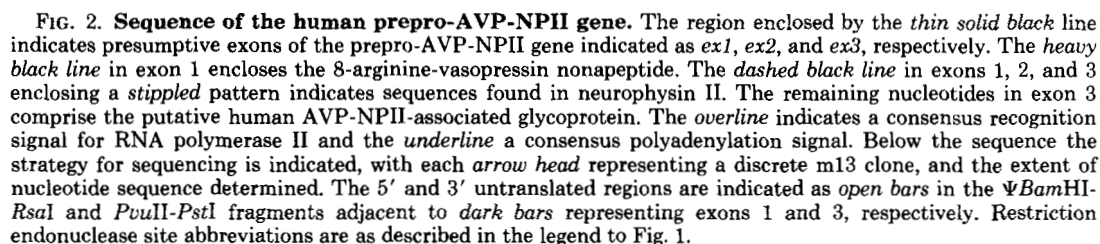
Therefore, the human prepro-AVP-NPII gene has a three-exon genomic structure in common with cow and rat (Land *et al.*, 1982; Ivell and Richter, 1984). The human AVP peptide is derived from a protein precursor comprised of a signal peptide, AVP, neurophysin II, and another peptide in the 3' portion of the gene of as yet unknown function. Comparison of the nucleic acid sequence obtained here for neurophysin II with the observed protein sequence (Chauvet *et al.*, 1983) shows that the differences between the human protein sequence and the cow and rat proteins are precisely predicted by the human nucleic acid sequence for neurophysin II presented here.

**Structure of the Human Prepro-OT-NPI Gene**—Although the entire sequence of human prepro-AVP-NPII is localized to the 2.5-kb *BamHI-BamHI* fragment of hAVP4, homology to the cc-bov fragment derived from a bovine cDNA clone can also be found on the 5-kb *BamHI-BamHI* fragment present in hAVP4. The AVP-specific 5' bov cDNA probe was limited in its homology to the 2.5-kb *BamHI-BamHI* fragment from hAVP4 (Fig. 1). To reconcile these observations, a 1.4-kb region of hAVP4 containing the additional homology region was sequenced.

The sequence shown in Fig. 3 contains a recognition site for mRNA initiation (Breathnach and Chambon, 1981) at position 355. The first ATG thereafter defines an open reading frame containing a 9 amino acid peptide segment expected for human oxytocin (nucleotides 475–501). By analogy to the bovine prepro-OT-NPI gene (Land *et al.*, 1983), the human oxytocin sequence (residues 475–501) is preceded by a largely hydrophobic peptide from residues 418–474, and followed by a tripeptide (Gly-Lys-Arg) before commencing a putative translation product with a sequence expected for human neurophysin I (Chauvet *et al.*, 1983) at position 511. A break in homology with the expected translation product occurs at position 538, with the characteristics of a splice donor site (Breathnach and Chambon, 1981), defining the first exon of human prepro-OT-NPI.

A 299-bp intervening sequence separates the first and second exon, and homology with human neurophysin I resumes (position 838). The second exon is comprised solely of neurophysin I-related sequences, extending to position 1036. A short intervening sequence (83 bp) separates the second and third exons, with the remaining sequences of neurophysin I encoded in the third exon. As in the cow and rat (Ruppert *et al.*, 1984; Ivell and Richter, 1984), a termination codon follows directly after the neurophysin I coding sequences with no apparent analog of the 3' putative glycopeptide segment found in prepro-AVP-NPII. A double consensus polyadenylation signal (Proudfoot and Brownlee, 1976) is found at position 1249, indicating the end of the transcription unit.

The only difference observed between our predicted se-



**Orientation and Comparison of AVP and OT Genes**—The nucleotide sequence of prepro-AVP-NPII and prepro-OT-NPI predicts that the two genes exist in opposite transcriptional orientations. This was confirmed by hybridizing OT-specific and AVP-specific probes to *Hind*III-*Sst*I digests of hAVP4 blotted to nitrocellulose. An 11.7-kb *Hind*III-*Sst*I fragment hybridized to exons 2 and 3 of both prepro-AVP-

Nucleotide sequences encoding the nonapeptide AVP and OT hormones are highly homologous. In addition, dot matrix analysis (Maizel and Lenk (1981) reveals (data not shown) that the sequence of a portion of the first intron and exon 2 of prepro-AVP-NPII (nucleotides 1512-1920) is highly homologous to part of the first intron and exon 2 of prepro-OT-NPI (nucleotides 620-1036). Analogous features of the bovine genes for prepro-AVP-NPII and prepro-OT-NPI had previously been noted (Ruppert *et al.*, 1984). The results of our

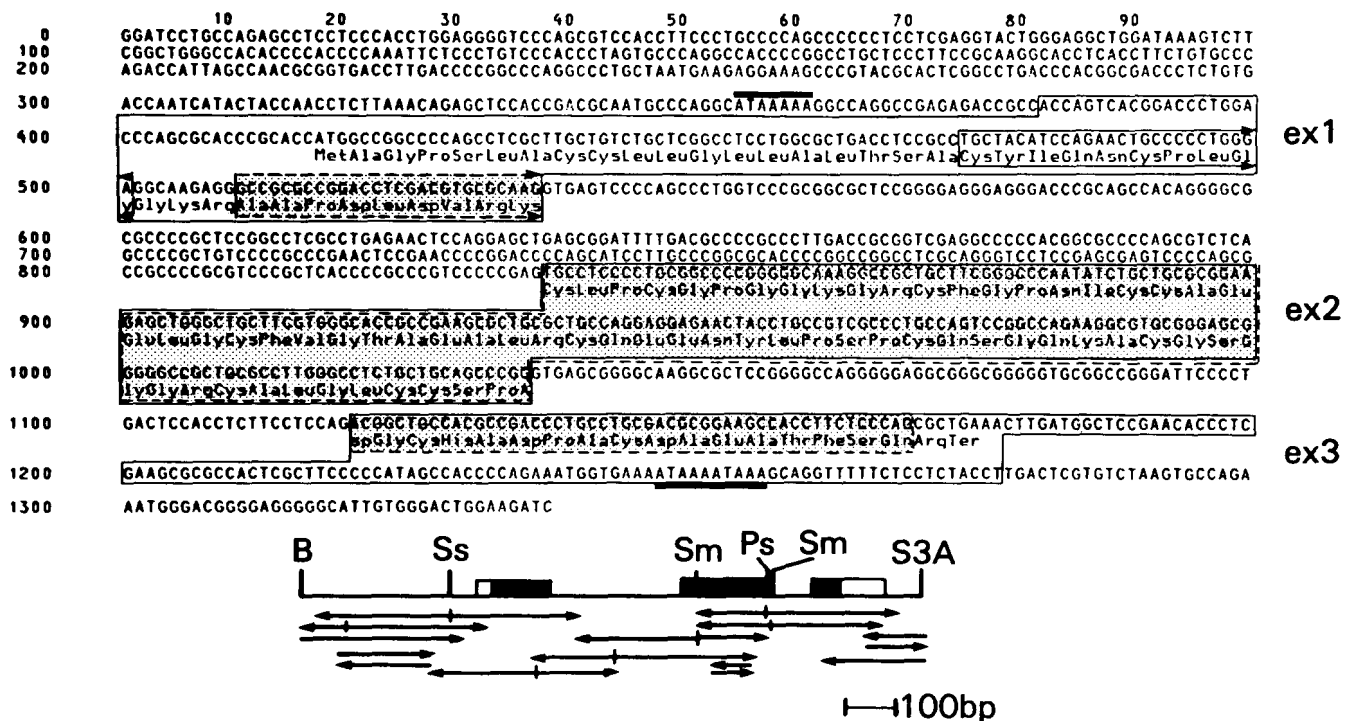


FIG. 3. Sequence of prepro-OT-NPI gene. Symbols are as designated in Fig. 2, except that oxytocin is the nonapeptide found in place of vasopressin in exon 1 enclosed in the heavy black line, and neurophysin I is present within the stippled area in place of neurophysin II. Note the absence of a 3' exon-associated peptide after neurophysin I.

sequence analysis suggest that to explain the evolution of these two structurally similar genes from a common ancestor, both gene duplication and an inversion are required. Apparent duplication and inversion has also been described in the structurally related egg-laying hormone-related merge of *Aplysia* (Scheller *et al.*, 1983), although its significance in this system is unclear. Boorstein *et al.* (1982) have noted a duplicated and inverted arrangement of genes potentially encoding the B-subunits of human chorionic gonadotropin. Consequences of an inverted arrangement of related genes could include the establishment of differences in transcriptional regulation, chromatin protein binding properties, and decreased recombination frequency.

Extensive interspecies homology is found when the bovine and rat 5' flanking sequences of prepro-AVP-NPII are compared (Ruppert *et al.*, 1984). This is also true for 5' flanking sequences of cow and rat prepro-OT-NPI. The human genes for these two hormones also share this interspecies homology of 5' flanking sequences, although there is negligible homology in 5' flanking sequences when the two human genes are compared to each other (data not shown). Since it has recently been shown that expression of a polypeptide hormone gene can be influenced in a tissue specific fashion by the adjacent 5' flanking sequences (Supowit *et al.*, 1984) it is possible that these conserved sequences are responsible for the differential expression of AVP-NPII and OT-NPI in tissues which specifically produce either hormone. *In vitro* alteration of these flanking sequences followed by DNA transfection and expression studies in appropriate cell hosts may allow sequences important in differential hormone gene expression to be defined.

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Additional references are found on p. 10241.

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## Supplementary Material to

The Human Vasopressin Gene is Linked to the Oxytocin Gene and is Selectively Expressed in an Cultured Lung Cancer Cell Line

by Edward Sausville, Desmond Carney, and James Battey

## Materials and Methods

## Cloning

A recombinant Charon 28A (Blattner et al., 1977) library of  $1 \times 10^6$  clones was screened as described with a nucleic acid probe derived from pNpII-1 of Land et al., (1982), which was a gift to J. Battey from I. Hennighausen. Plasmid subclones were in all cases constructed in pBR327, a derivative of pBR327 with the polylinker region of mp 11 DNA substituted for the small EcoRI-HindIII fragment. Plasmid growth in general was in *E. Coli* LE392 under NIH P1 containment conditions.

## DNA Sequencing

M13 mp 10 and mp 11 subclones were derived and sequencing performed essentially as described by the Bethesda Research Laboratory Manual (1981). Where favorable restriction sites did not exist, size-selected exonuclease Bal31 digests of plasmids were cloned into the SmaI site of M13 phages. Approximately 90% of the prepro-AVP-NP11 gene and >90% of the prepro-OTNP1 gene was sequenced from both strands.

## Cell Lines

Fresh specimens from patients with SCLC were minced and grown in HITES medium (RPMI -1640 (GIBCO) plus  $10^{-8}$  M each of estradiol and hydrocortisone,  $3 \times 10^{-8}$  M sodium selenite, 5 mcg/ml insulin and 10 mcg/ml transferrin) or in RPMI-10% fetal bovine serum. H378, a cell line derived from a patient with SCLC who was originally suspected to have the syndrome of inappropriate AVP secretion, was grown in HITES and was found by radioimmunoassay to have >10,000 pg/mg protein of immunoreactive AVP (A. Gazdar, unpublished results).

## Genomic Organization and Expression

Total cellular DNA was isolated from cell lines as described by Hieter et al., 1981. Total cellular RNA was prepared as described by Chirgwin et al., (1979), and poly A(+) RNA by selection with dt-cellulose (Aviv and Leder, 1972). DNA samples were transferred to nitrocellulose as described by Southern (1975) after electrophoresis. RNA samples were electrophoresed (2 mcg of poly A+ or 10mcg of total cell RNA/ lane) through agarose gels containing 0.22 M formaldehyde (Lehrach et al., 1977) and transferred to nitrocellulose. In all hybridizations to DNA or RNA immobilized on nitrocellulose, indicated fragments were prepared free of plasmid vector by preparative electrophoresis and then labelled to high specific activity with [ $^{32}$ P]dCTP as described by Rigby et al., 1977. Hybridization to nitrocellulose blots of transferred RNA and DNA in 10% dextran sulfate was conducted as described by Wahl et al., (1979) and blots were washed at  $52^\circ$  in 15mM NaCl/1.5mM sodium citrate pH 7.0/0.1% SDS. S1 nuclease protection experiments were as described by Battey et al., (1983). Single stranded probes complementary to transcribed sequences were synthesized by primer extension of an m13 subclone in the presence of [ $^{32}$ P]dATP.

## RESULTS AND DISCUSSION

## Organization of AVP and OT Genes

The structure of the prepro-OT-NP1 gene in hAVP4 derived from the fetal liver library predicts that in genomic DNA the gene should occur on a 4.9 kb BamHI fragment, and both prepro-AVP-NP11 and prepro-OT-NP1 should be contained on a single SstI fragment. Figure 4 demonstrates that DNA derived from H378, a SCLC cell line which produces substantial quantities of immunoreactive AVP and DNA from H209, a SCLC cell line not producing AVP have a similar pattern of hybridization to both AVP and OT -specific probes. An AVP exon 1-specific probe hybridizes to a 9.6 kb BamHI fragment and a 19 kb SstI fragment. An OT exon 3-specific probe hybridizes to the same SstI fragment as well as the expected 4.9 kb BamHI fragment. A probe derived from the neurophysin domain of prepro-AVP-NP11 hybridizes to both the 4.9 kb and 9.6 kb BamHI fragments as well as the 19 kb SstI fragment. This experiment verifies the physical linkage of prepro-AVP-NP11 and prepro-OT-NP1 predicted from the structure and sequence of the cloned genes. The pattern of hybridization shown in Fig. 4 was identical to that seen in DNA from normal lymphocytes (data not shown), and analogous results with DNA from six other SCLC cell lines suggests no frequent amplification or rearrangement of the prepro-AVP-NP11 gene in SCLC.

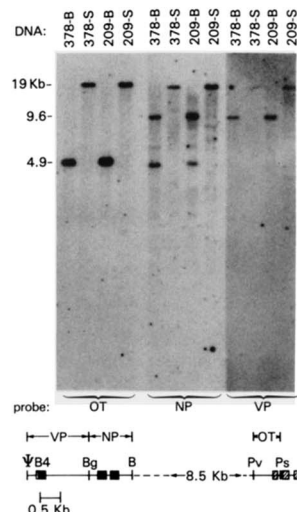


Figure 4. Linkage of Prepro-AVP-NP11 and Prepro-OT-NP1. Genomic DNA from SCLC lines H378 or H209 were digested with BamHI (B) or SstI(S), electrophoresed, and transferred to nitrocellulose. Results of hybridizations with AVP, OT, and neurophysin(NP)-specific probes derived from the locus as indicated in the diagram are shown.

## Expression of Prepro-AVP-NP11 in SCLC

To validate the predicted transcription unit of prepro-AVP-NP11 in human DNA, the expression of this gene was studied in two SCLC cell lines. H378 produces substantial quantities of AVP, whereas H209 does not.

Fig. 5, panel 1 shows that a 1.5 KB Bam-BglII fragment (probe 1) encoding only the first exon of prepro-AVP-NP11 detects a 0.7 kb species in polyA+ RNA from H378 and is not found in RNA from H209. In addition, a 2.5 kb species is also detected by probe 1 in polyA+ RNA only from H378. This material is clearly separated from the observed position of the 18S ribosomal subunit. Probe 1 also apparently hybridizes nonspecifically to the 28S rRNA in non-polyA+ RNA. Figure 5, panel 2 shows results with probe 2, deriving from the first intervening sequence Rsa-HindIII segment of prepro-AVP-NP11. This probe hybridizes only to the 2.5 kb species, but not to the small 0.7 kb species. We conclude that the 0.7 kb species is the mature spliced mRNA for prepro-AVP-NP11. This is consistent with the size predicted from the sequence. We consider that the 2.5 kb species is a polyadenylated precursor which retains intervening sequences. Its size is what would be expected for the primary prepro-AVP-NP11 transcript. Figure 5, panel 3 reveals that a probe from the BglII-BamHI segment of the prepro-AVP-NP11 gene hybridizes to both the 0.7 kb and 2.5 kb species in RNA from H378 but not H209. Since this probe contains neurophysin sequences, it would not be expected to distinguish between AVP and OT transcripts. However, Fig. 5, panel 4 reveals that the OT-specific exon 3 PstI-PvuII probe 4 does not specifically hybridize to any polyA+ RNA from H378 or H209.

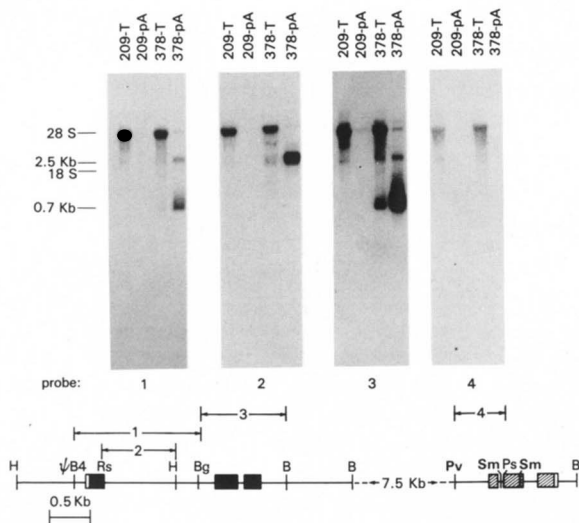


Figure 5. Transcription of Prepro-AVP-NP/OT-NPII in SCLC Cell Lines. Total(T) or polyA+(pA) RNA from either H209 or H378 were electrophoresed and transferred to nitrocellulose. Hybridization was carried out with probes 1-4 prepared from the indicated regions of the AVP-NP/OT-NPII locus.

An implication of the results presented in Fig. 5 is that there is selective expression of the prepro-AVP-NP/OT-NPII gene and not the prepro-OT-NPII gene, despite their close proximity. To examine this point directly, utilization of the promoter regions predicted in the sequence analysis in Figs. 2 and 3 was assessed by S1 nuclease analysis after hybridization of RNA to AVP and OT-specific probes. If the ATAAA box identified in the sequence (Fig. 2, positions 146-152) is an active signal for transcription, an approximately 170 base fragment complementary to sequences in the first exon of AVP should be protected from S1 nuclease digestion when a 398 base probe containing the first exon of AVP is hybridized to RNA from H378. Fig. 6 shows that there is protection of a major species of approximately 180 nucleotides. Other similarly sized minor species may also be protected, but the 180 base species predominates in both non-selected and polyA+ RNA. SCLC cell lines H209 and H434, not known to produce immunoreactive AVP, do not specifically protect any portion of the probe from S1 digestion, even with prolonged autoradiographic exposures.

Analogous S1 nuclease protection experiments using an OT-specific exon 1 BamHI-SmaI probe (nucleotides 1-855, Fig. 3) and an overlapping TaqI-TaqI probe(nucleotides 71-523, Fig. 3) showed no S1-protected fragments after hybridization to polyA+ RNA from H378 (data not shown), confirming the relative transcriptional inactivity of prepro-OT-NPII suggested by the nitrocellulose filter hybridization experiments of Fig. 5.

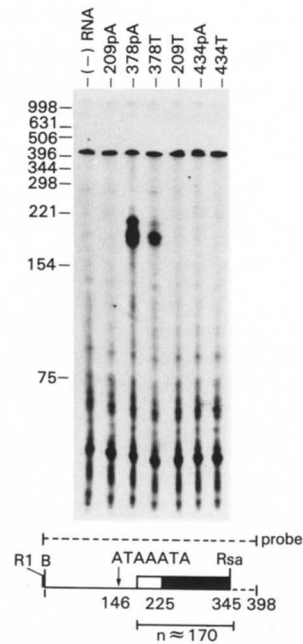


Figure 6. S1-nuclease Protection Assay by RNA from Human SCLC Cell Lines. A single-stranded DNA probe was prepared by primer extension using as template an M13 subclone of the prepro-AVP-NP/OT-NPII gene containing the 5' untranslated portion and the entire first exon. The probe was hybridized to either total cellular (T) or polyA+ (pA) RNA from the indicated SCLC cell lines. Following hybridization, non-annealed probe was largely digested with S1 nuclease, and the products of digestion separated on an 8M urea - 5% polyacrylamide gel after denaturation. Residual undigested probe (approximately 1% of input probe) is present in all samples at 396 nucleotides.

Further experiments must focus on whether the differential activation of the prepro-AVP-NP/OT-NPII and prepro-OT-NPII genes in this cell line reflects specific recognition of their differing 5' flanking regions. An alternative is that this pattern of transcriptional activation reflects a program of neuroendocrine differentiation independent of specific sequence elements.

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