

Sequence analysis of the promoter region of the rat vasopressin gene

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The vasopressin gene is highly transcribed in magnocellular neurons of the supraoptic (SON) and paraventricular nucleus (PVN) in the rat hypothalamus. In order to identify *cis*-acting elements involved in the expression of the vasopressin gene, approximately 1 kb upstream of the transcription start site has been sequenced. Several putative regulatory elements have been detected, including a glucocorticoid response element (GRE), a cAMP response element (CRE), and four AP2 binding sites. In gel shift assays performed with a labelled DNA fragment corresponding to nucleotide residues –214 to –36 and nuclear proteins extracted from SON-derived tissue enriched in magnocellular neurons, three specific protein-DNA complexes have been detected. Complex formation is effectively competed by addition of an excess of unlabelled fragment.

Vasopressin gene expression; Glucocorticoid response element; Binding site, AP2; cyclic AMP response element; Gel shift assay; Nuclear protein fraction

1. INTRODUCTION

To date little is known about the molecular events governing the expression of the gene encoding the precursor for the peptide hormone vasopressin. In the rat and human genome, the vasopressin gene is linked to the closely related oxytocin gene [1,2]. Yet the two genes are transcribed in different subtypes of magnocellular neurons of the supraoptic (SON) and paraventricular nucleus (PVN) of the hypothalamus [3]. Although both hormones control rather different physiological functions in the organism, e.g. water retention in the kidney (vasopressin) and smooth muscle contraction in the uterus (oxytocin), there are several reports that demonstrate a concomitant upregulation in the expression of the vasopressin and oxytocin genes under various physiological conditions. For instance prolonged osmotic stimulation of rats results not only in a 2–3-fold increase of vasopressin message [4], but also of oxytocin-encoding mRNA in magnocellular neurons [5]. A similar rise in vasopressin- and oxytocin-encoding transcripts has also been shown to occur during late gestation and lactation [6]. On the other hand, after systemic administration of vasopressin, a reduction in the two mRNA species has been observed in rat hypothalami [7].

To gain an initial insight into the mechanisms controlling the expression of the vasopressin gene, a DNA

segment upstream of the transcriptional start site of the vasopressin gene has been sequenced and analyzed for regulatory elements.

2. MATERIALS AND METHODS

2.1. Sequence analysis and preparation of nuclear extracts

Nucleotide sequence was determined as reported [3]. Where specified male Wistar rats were treated with 2% saline for seven days. SON were dissected from about 1.5 mm thick brain slices and collected in ice-cold PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.2). Tissue pools from 5 animals were frozen in liquid nitrogen after removal of PBS. Nuclei from SON tissue or from cerebellum were prepared as published [8]. Nuclear proteins were extracted [9] and their protein concentration determined [10]. Aliquots of nuclear protein were frozen in liquid nitrogen and stored at –80°C.

2.2. Mobility shift assay

Binding reactions contained 3.5 µg nuclear protein, 3 µg poly (dJdC), 20 ng sonicated *E. coli* DNA in 10 mM Hepes, pH 7.9, 10% glycerol, 50 mM KCl, 0.25 mM PMSF, 5 mM DTT, 1 mM EDTA. Prior to the addition of labelled DNA fragment (2.5 fmol/reaction), binding reactions were preincubated for 15 min at 4°C. Labelled DNA and if included, unlabelled competitor DNAs (see legend to fig.3) were added simultaneously. The mixture was incubated at 4°C for 30 min and immediately loaded on 4% prerun acrylamide gels (acrylamide:bisacrylamide 80:1). Electrophoresis was performed for 2 h (20 mA) at room temperature in 6.7 mM Tris-HCl, pH 7.5, 3.3 mM sodium acetate, 1 mM EDTA, with constant buffer circulation. Gels were dried and exposed to X-ray film at –80°C for 15–24 h.

2.3. Labelling of DNA fragments

377 bp (–751 to –374) and 338 bp (–374 to –36) *Pst*I DNA fragments derived from the 5' region of the rat vasopressin gene were subcloned individually into the *Pst*I site of pUC9. DNA fragments were labelled at the unique *Sal*I- or *Hind*III-site of the vector by filling in the 5' cohesive ends with [α -³²P]dCTP (sp. act. 3000 Ci/mmol) and Klenow polymerase according to standard procedures [11]. Subsequently, DNAs were recut with *Aha*II (–561) and *Bal*I (–214) re-

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The nucleotide sequence(s) presented here has (have) been submitted to the EMBL/GenBank database under the accession number no. Y0531

spectively. The fragments were electrophoretically eluted from native 8% polyacrylamide gels [12]. The specific activity of the labelled fragments ranged between 4 and 8×10^7 cpm/ μ g.

3. RESULTS AND DISCUSSION

The rat vasopressin gene was previously subcloned as a 3.2 kb DNA fragment (pRAVP 3.2) excised from a 18 kb genomic clone harbouring both the vasopressin and oxytocin gene [1]. In addition to the complete coding region, clone pRAVP 3.2 contains roughly 1 kb of 5' untranscribed DNA (fig.1A). Several putative *cis*-acting elements located proximal and distal to the transcriptional start site were identified on the basis of their homology with known *trans*-acting factor binding sites (table 1).

At position -247 a CCAAT motif is found, the presence of such an element being known to increase the constitutive rate of transcription in many genes [13]. Further downstream at position -227, a region resembling a cAMP response element (CRE) is present. The putative CRE differs from the classical octamer sequence 5' TGACGTCA 3' in 2 positions yielding a non-palindromic sequence. Nevertheless, it is well documented that CRE-like sequences found in promoter regions of other genes show deviations from the classical sequence but are still cAMP-responsive [14].

Four elements, fitting the consensus sequence for transcription factor AP2 binding sites [15] were found at position -345, -146, -95, and -84. AP2 has been shown to bind to the SV40 enhancer as well as to the promoter regions of various cellular genes, and to stimulate the rate of transcription by protein kinase A and C dependent pathways [15]. A sequence similar to a glucocorticoid response element (GRE) is present more distally at nucleotide position -622. Detailed analysis of functional GREs present in several genes has led to the conclusion that the last six nucleotide residues 5TG-TYCT3' within the GRE motif are strongly conserved, while there is a greater degree of variability intrinsic in the nucleotides at the 5' end of the element [16].

In order to identify nuclear proteins that bind to the putative promoter regions of the AVP gene protein-DNA binding experiments were performed using four different DNA fragments (schematically outlined in fig.1B) and nuclear extracts prepared either from SON tissue or from cerebellum of rats treated with 2% saline. In vitro, three complexes I, II, and III are formed when a SON-derived nuclear protein fraction was added to the most proximal promoter fragment 1 (position -214 to -36), which harbours three putative AP2 binding sites (fig.2, lane 2), but none of the other *cis*-acting elements mentioned above. No complex formation was observed with DNA fragments 2-4 (fig.2, lanes 4,6,8) although at least DNA fragment 2 contains, in addition to one putative AP2 binding site, a CCAAT motif and a CRE-like sequence. The failure to detect a protein/DNA complex could be due to the low amount of

Table 1
Putative regulatory elements in the 5' region of the rat vasopressin gene

CRE	5'					3'
consensus		T G A C G T C A				
- 227		T C C C G T C A				
						- 220
AP2 binding site	5'					3'
consensus		T C C C C A / C	N	G / C	C / G	G / C
- 336		T C C C C A	G	G	T	C - 345
- 146		T C C C C A	G	A	T	G - 137
- 86		T C C C C A	G	G	A	G - 95
- 75		T C C C C A	G	T	G	G - 84
GRE	5'					3'
consensus		G G T A C A N N N T G T Y C T				
- 622		T G T C A C A A C T G T C C T				
						- 608

AP2, activator protein 2; CRE, cAMP response element; GRE, glucocorticoid response element. The numbering indicates the position of the elements upstream of the transcriptional start site (1). The bold letters are in agreement with published sequence motifs

protein used in these experiments. Unfortunately, the small number of vasopressinergic neurons per rat brain (about 5000 cells [17]) precludes the preparation of nuclear protein in higher amounts. The AP2-like elements may, on the other hand, represent low affinity binding sites so that stable complexes cannot be formed under the experimental conditions used. Alternatively, respective proteins may act to modulate the transcriptional rate of the vasopressin gene only during certain developmental stages, physiological conditions, or in non-neuronal tissues.

To decide whether complexes I to III result from interactions between DNA fragment 1 and proteins restricted to magnocellular neurons, mobility shift experiments similar to those described above were carried out with a nuclear protein fraction extracted from rat cerebellum tissue. One complex exhibiting an electrophoretic mobility very similar or identical to complex I was formed upon incubation with DNA fragment 1 (fig.2, lane 1); however once again no shifted bands were present after addition of fragments 2, 3 or 4 to the binding reactions (fig.2, lanes 3,5,7). The binding of nuclear proteins from SON, as well as from cerebellum, to DNA fragment 1 seems to be specific, because an excess of unlabelled fragment 1 effectively competed for binding to the labelled DNA (fig.3, lanes 2,6). In contrast, the same molar excess of unlabelled fragment 2, or of a 183 bp DNA fragment excised from pUC9 by restriction enzymes *Pvu*II and *Hind*III, was not able to inhibit complex formation (fig.3, lanes 3,4,7,8). None of these complexes appears to arise from interaction of AP2-like proteins with DNA fragment 1. Addition of synthetic double-stranded oligonucleotides (30-mers) centrally harbouring the individual putative AP2 binding sites as competitors, did not abolish the formation of complexes I, II, or III respectively (data not shown). This finding is in agreement with a failure of unlabelled

A

GATCAAGAGTTCAATGCCAGCTTTCTGCTATGTAGTAAGGTCAAGGTCAGCCTGGACTAAACGACTGCCTTAGAAACAACAAATGACTTACCGTCTA -901
CTAGTTCTCAAGTTACGGTCGAAAGACGATACATCATTCCAGTTCAGTCGGACCTGATTGTGTCGCGGAATCTTTGTTGTTTACTGAATGGCAGAT

AAGTCAGGAACACTACTGCTTTCTCAGACTGTGTCTGTCTGTCTGGGGCTCCTCCCATTTCTCTCTTAACAACATCCACTTCCACTCCTGCCCTAGAT -801
TTCAGTCCCTGATGTGAACGAAAGAGTCTGACACAGACAGACAGACCCCGAGGAGGGTAAAGGAGAGGATTGTTGTAGGTGAAGGTGAGGACGGAATCTA

CTGAGATAGTACCAGCCTCAGGGCATGGGGTCTCCCATAGCTTTCTCTCTGCAGTACTGTGGGCTCACCTAGGACTGTTTCTGAACATATATCCTACCCT -701
GACTCTATCATGGTCGGAGTCCCGTACCCAGAGGGGTATCGAAAAGGAGACGTATGACACCCGAGTGGATCCTGACAAAGACTTGATATAGGATGGGA

AGCTCTCTACCCTAGAAGGCCTGAAACTCACAGAAATTCCTCGCTCTGCTTTTCCAATGGCTGGGGTTAAAGCATGTGTCACTGTCTTTTATT -601
TCGAGAGATGGGATCTTCGGACTTTGAGTGTCTTTAAGAGGACGGAGACGAAAGTTACCGACCCCAATTTTCGTACACAGTGTGACAGGAAAAATAA

CTTTTAATATCGAGACAGGGTCTCACCAAGTTGCCCAAGACGCCAGCCACCTGGGACAGGGCCTTTGGCTCTATGTTCACTGTTGACTCCATG -501
GAAAATTATAGCTCTGTCCAGAGTGGTTCAACGGGGTCTCGGGTCGGTGTGGACCTGTCCCGTCCGAAACCGAGATACAAGTCAGAACTGAGGTAC

ACTGTGGCCGCTAGCCCATGAGGCTGCGCGTGGGAATTTCTTCTGAAAGCTACCTGGTATCGATGCTTCTCTTATCTACACCACAACAAAC -401
TGACACCGGCGATCGGGTACTCCGACGCGCACCTTAAAGGAAGACTTTCGAGTGGACCATAGCTACGAAGGAGAATAGGATGTGGTGTGATTGTTTGG

TGCCCCACCTCCTGGTCTGACCTGTCTGACAGCTGTAGTCTTGGTGAATGAGACCTGGGGACCCCTCTAGTCTGTTGAGAGCTGTGAAATGCTCA -301
ACGGGGTGGAGGACAGGACTGGGACGACGTCTGGACGATCAGGAACCACTTACTCTGGACCCCTGGGGAGATCAGACAACCTCTGACGACTTTACGAGT

ACTATGATTTCCAGGTGACCTCAAGTCGGCTCACCTCCCTGATTGCACAGCACCAATCACTGTGGCGGTGGCTCCCGTCAACGGTGGCCAGTGACAGC -201
TGATATAAAGGTCCACTGGGAGTTTCAGCCGAGTGGAGGGGACTAACGTGTCTGTTAGTGACACCGCCACCGAGGGCAGTGTGCCACCGGTCACTGTCTG

CTGATGGCTGGCTCCCTCTCCACCACTCTGCATTGACAGGCCACGTGTGTCCTCCAGATGCCGTAATCACTGCTGACAGCTTGGGACCTGTCTAGCT -101
GACTACCGACCGAGGGGAGGAGTGGTGGGAGACGTAAGTGTCCGGTGCACACAGGGGTCTACGGACTTAGTGACGACTGTGCAACCTGGACAGTCTGA

GTGGGCTCCTGGGGAGCCACTGGGGAGGGGTTAGCAGCCACGCTGTGCGCTCTAGCCAACACCTGCAGACATAAATAGACAGCCAGCCGCTCAGGC -1
CACCGAGGAGCCCTCGGTGACCCCTCCCCAATCGTCGGTGCACAGCGGAGGATCGGTTGTGGACGTCTGTATTTATCTGTGGGTTCGGGCGAGTCCG

* MET
AGCAGAGCAGAGCTGCACGCAGTGCACCACTATG +34
TCGTCCTGCTCGACGTGCGTCAACGGGTGGATAC

B

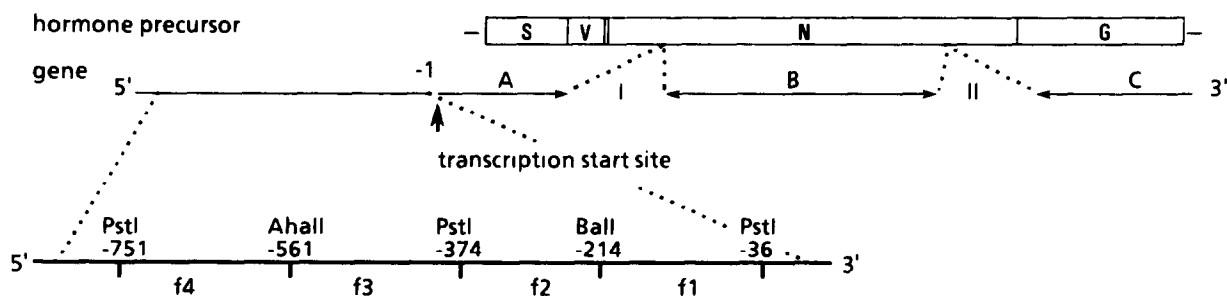


Fig.1. (A) Nucleotide sequence of the 5' region of the rat vasopressin gene. The modified TATA box is underlined. Putative binding sites for glucocorticoid receptor (GR), CCAAT-box binding proteins, cAMP response element binding protein (CREB) and AP2 are indicated by arrows. The protein coding regions and the intron sequences of the vasopressin gene are not shown. The asterisk marks the transcriptional start point. (B) Schematic representation of the rat vasopressin gene, the deduced hormone precursor structure and the derived DNA fragments (f1 to f4) used in mobility shift assays. The numbers indicate the position relative to the transcriptional start point (+1). S, signal peptide; V, vasopressin; N, neurophysin; G, glycopeptide; A, B, C, indicate the three exons of the vasopressin gene, I and II, the two introns.

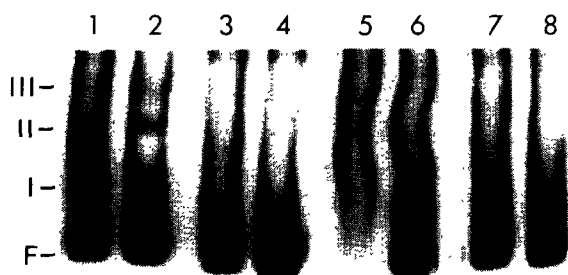


Fig.2. Mobility shift assay. Nuclear protein fraction extracted from rat cerebellum (lanes 1,3,5,7) or SON tissue (lanes 2,4,6,8) was incubated with 2.5 fmol each of 32 P-labelled DNA derived from the 5' region of the rat vasopressin gene. Lanes 1,2: fragment 1 (-214 to -36); lanes 3,4: fragment 2 (-374 to -214); lanes 5,6: fragment 3 (-561 to -374); lanes 7,8: fragment 4 (-751 to -561). F, unbound probe; I, II, III, bound probe.

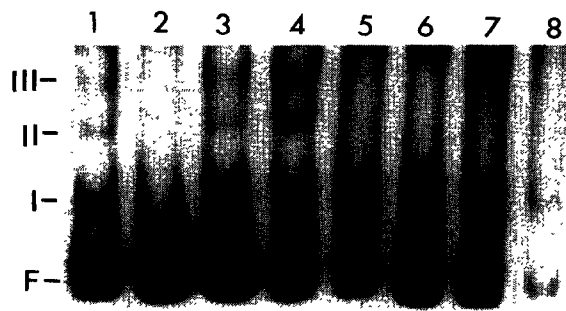


Fig.3. Mobility shift assay. Nuclear protein fraction extracted from rat SON tissue (lanes 1-4) or cerebellum (lanes 5-8) was incubated with 2.5 fmol of 32 P-labelled DNA fragment 1 (-214 to -36) in the absence (lanes 1,5) or presence of various unlabelled competitor DNAs, added in a ~30-fold molar excess. Lanes 2,6: unlabelled fragment 1; lanes 3,7: unlabelled fragment 2; lanes 4,8: unlabelled pUC9 DNA. F, unbound probe; I, II, III, bound probe.

DNA fragment 2 to inhibit or reduce the formation of any complex in the competition experiments described above, as well as the lack of complex formation between DNA fragment 2 and SON nuclear protein. The data may indicate that at least complexes II and III represent cell type-specific protein factor(s) bound to presently unknown enhancer and/or promoter elements within DNA fragment 1. Complex I, on the other hand, seems to contain a protein, which is not restricted to magnocellular neurons, and which might therefore function as a more general transcription factor.

The gel shift data suggest the existence of proteins in magnocellular neurons of the SON that can specifically bind to a DNA fragment, which spans the 5' region from nucleotide -214 to -36. One of the putative *trans*-acting factors is probably also present in cerebellum nuclei, thus representing a more ubiquitous protein. A precise characterization of protein-DNA contact sites by footprint analysis is rendered technically extremely difficult due to the small number of vasopressinergic neurons present in the hypothalamus (approximately 5000 cells per rat brain [17]). Thus linkage of the putative *cis*-acting elements of the promoter region of the AVP gene to a heterologous promoter should help to clarify whether these elements would be responsive to externally added stimuli.

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REFERENCES

- [1] Mohr, E., Schmitz, E. and Richter, D. (1988) *Biochimie* 70, 649-654.
- [2] Sausville, E., Carney, D. and Battey, J. (1985) *J. Biol. Chem.* 260, 10236-10241.
- [3] Mohr, E., Bahnsen, U., Kiessling, C. and Richter, D. (1988) *FEBS Lett.* 242, 144-148.
- [3] Burbach, J.P., De Hoop, M.J., Schmale, H., Richter, D., De Kloet, E.R., Ten Haaf, J.A. and De Wied, D. (1984) *Neuroendocrinol.* 39, 582-584.
- [5] Van Tol, H.H.M., Voorhuis, D.T.A.M. and Burbach, J.P.H. (1987) *Endocrinology* 120, 71-76.
- [6] Zingg, H.H. and Lefebvre, D.L. (1988) *Mol. Brain Res.* 4, 1-6.
- [7] Rehbein, M., Hillers, M., Mohr, E., Ivell, R., Morley, S., Schmale, H. and Richter, D. (1986) *Biol. Chem. Hoppe-Seyler* 367, 695-704.
- [8] Schibler, M., Hagenbüchle, O., Wellauer, P.K. and Pittet, A.C. (1983) *Cell* 33, 501-508.
- [9] Dignam, J.D., Lebovitz, R.H. and Roeder, R.G. (1983) *Nucleic Acids Res.* 11, 1475-1489.
- [10] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248-254.
- [11] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular cloning. A Laboratory Manual*, Cold Spring Harbor, NY.
- [12] Maxam, A.M. and Gilbert, W. (1980) *Methods Enzymol.* 65, 499-559.
- [13] Maniatis, T., Goodbourn, S. and Fischer, J.A. (1987) *Science* 236, 1237-1245.
- [14] Comb, M., Hyman, S.E. and Goodman, H.M. (1987) *Trends Neurol. Sci.* 10, 473-478.
- [15] Imagawa, M., Chiu, R. and Karin, M. (1987) *Cell* 51, 251-260.
- [16] Beato, M. (1987) in: *DNA Protein Interactions and Gene Regulation* (Thompson, E.B. and Papaconstantinou, J. eds) Austin, 269-279.
- [17] Swaab, D.F., Nijveldt, F. and Pool, C.W. (1975) *J. Endocrinol.* 67, 461-462.