Volume 242, number 1, 144–148 FEB 06608 December 1988

Expression of the vasopressin and oxytocin genes in rats occurs in mutually exclusive sets of hypothalamic neurons

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Received 20 October 1988

The genes for the hypothalamic hormones vasopressin and oxytocin are located in close proximity to each other within the rat genome. They are separated by only approx. 11 kbp of DNA sequence and oriented in such a way that their transcription occurs on opposite DNA strands. Although the two genes are structurally very similar including common potential regulatory elements in their putative promotor regions, they are expressed in discrete populations of magnocellular neurons of the hypothalamus. In rats placed under osmotic stress, the vasopressin gene is upregulated; concomitantly transcription of the oxytocin gene is also stimulated. To address the question of whether this coordinated rise in oxytocin-encoding mRNA is the result of switching on oxytocin gene transcription in vasopressinergic neurons, in situ hybridization with double labelled cRNA probes was carried out. Biotinylated and [α-^{35}S]CTP labelled antisense cRNA probes specific for either vasopressin or oxytocin mRNA were constructed and hybridized to hypothalamic sections from salt-loaded rats. The results demonstrate that upregulation of oxytocin gene transcription is restricted solely to oxytocinergic cells; no oxytocin gene transcripts can be detected in vasopressinergic neurons.

In situ hybridization; Double labeling; Oxytocin gene; Promoter region; Consensus sequence; Vasopressin gene regulation; Osmotic stress

1. INTRODUCTION

To date little information is available concerning transcriptional regulation of the genes encoding the hypothalamic hormones vasopressin and oxytocin. Rats placed under osmotic stress by drinking saline accumulate not only vasopressin but also oxytocin transcripts in the hypothalamus (reviewed in [1]). Conversely the two genes appear to be downregulated when rats are exposed to excess of serum vasopressin [2]. Another regulatory mechanism has been observed in certain cell types of the hypothalamo-pituitary axis where expression of the vasopressin gene is negatively controlled by steroids [3–6]. Upregulation of the oxytocin gene has been observed in female rats during pregnancy and lactation [7,8] and in analogy to the osmotic stress situation the vasopressin gene is concomitantly upregulated [8].

In man [9] and rat [10] both genes are closely linked on the same chromosome [11] and transcribed from opposite DNA strands, yet immunological studies suggest that vasopressin and oxytocin are produced in different sets of hypothalamic neurons [12]. This implies that in vasopressinergic hypothalamic cells the oxytocin gene is maximally suppressed, while in oxytocinergic cells the vasopressin gene appears to be dormant. Stress conditions such as thirst could either cause release of suppression of the oxytocin gene in vasopressinergic neurons or alternatively, could activate oxytocinergic cells, implying limited specificity of response. To test these possibilities, hypothalamic sections obtained from osmotically stressed rats were analyzed by in situ hybridization using double labelled cRNA probes specific either for vasopressin or oxytocin mRNA. The data demonstrate that even when the two genes are

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

upregulated the oxytocin gene is not transcribed in vasopressinergic cells.

2. MATERIALS AND METHODS

For constructing the oxytocin-specific oligonucleotide probe, a 190 bp *AvaI/BalI* fragment of the pOλEcoRI genomic subclone [13] was first cloned into the vector pUC8. Subsequently, using the multiple cloning sites of the vector the fragment was excised by *Eco*RI and *Hin*dIII restriction and cloned into the vector pSP64. This clone is referred to as pSP10. The vasopressin-specific probe (230 bp *PstI/DraI* fragment; [13]) was cloned into the *SmaI/PstI* site of pSP65 and designated pSP2. pSP10 and pSP2 both yield cRNA probes representing the anti-sense strand of their respective mRNAs.

2.1. *Preparation of ^{35}S-labelled cRNA*

Synthesis of labelled cRNA (spec. act. 5×10^8–1×10^9 cpm/μg) was carried out according to the SP6 polymerase protocol of Promega Biotec (Madison, WI, USA) using 117 μCi of [α-^{35}S]CTP (spec. act. 37 TBq/mmol) and pSP10 or pSP2.

2.2. *Synthesis and biotinylation of cRNA*

cRNA was synthesized from pSP2 or pSP10 DNA in the SP6 RNA polymerase reaction using 5′-(3-amino)allyluridine 5′-triphosphate (allylamine-UTP; Bethesda Research Laboratories, Neu Isenburg, FRG) as described in the protocol by BRL. For RNA quantitation, [α-^{35}S]CTP (spec. act. 37 TBq/mmol) was included in the reaction mixture. The biotinylated probes were analyzed by spotting various amounts of RNA on a nitrocellulose filter followed by colorimetric assay using streptavidin and biotin-conjugated alkaline phosphatase of BRL DNA detection system. A positive signal was observed at 50 pg of the respective cRNA.

2.3. *In situ hybridization*

Preparation and fixation of the tissue was carried out as reported in [14]. Hybridization was performed in the presence of 4 ng of ^{35}S-labelled and/or 40 ng of biotinylated cRNA probes as described [14], except that the final dehydration steps through ascending ethanol concentrations were omitted.

2.4. *Development of the sections*

2.4.1. Biotinylated probe

Biotinylated signals were detected using a modification of the BRL DNA detection protocol. Unspecific binding was reduced by treating the sections with 1 ml buffer A (0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100, 3% bovine serum albumin) at room temperature for 1 h in a moist chamber. Following a 5 min wash with buffer A, sections were incubated with 100 μl of streptavidin (BRL) in buffer A (2 μg/ml) for 20 min in a moist chamber. The slides were washed 4 × 5 min in buffer A. Each section was covered with 100 μl of biotinylated alkaline phosphatase (1 μg/ml) in buffer A for 20 min. The slides were washed 4 × 5 min with buffer A, then 4 × 5 min with buffer B (0.1 M Tris-HCl, pH 9.2; 50 mM $MgCl_2$). The colorimetric reaction was carried out using variamin blue as dye. A freshly prepared solution of 50 ml buffer B containing 25 mg α-naphthylphosphate and 50 mg of variamin blue was filtered and used to incubate the sections at 4°C in the dark for 15 min. The staining reaction was stopped by washing the slides with distilled water.

2.4.2. Radioactively labelled probe

Dried slides were dipped into Kodak NTB-3 nuclear track emulsion diluted 1:1 by 600 mM ammonium acetate [15] at 42°C, dried and exposed up to two weeks. Autoradiograms were developed with Kodak chemicals and cover slipped (Kodak protocol).

3. RESULTS AND DISCUSSION

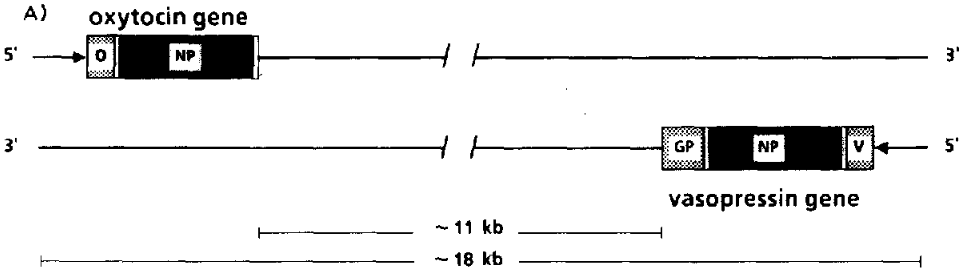
Fig.1A shows schematically the structural organization of the two hypothalamic genes for oxytocin and vasopressin. The overall sequence of the 18 kbp DNA segment (Schmitz, E., unpublished) indicates the existence of homologous putative promoter regions at the 5′-flanking regions of the two genes. For instance the promoter region of the oxytocin gene (fig.1B) contains several potential glucocorticoid (GRE)-[16] and estrogen-responsive elements (ERE) [17] upstream of the transcription start site, suggesting that this gene is under steroid control (table 1). In this region the oxytocin gene also shares homology with a negative regulatory sequence motif (AGTGCATGACTGGGCAGC-CAGCCAGTGGCAG) of the human polyoma-virus BK enhancer which binds a HeLa cell-specific potential repressor [18]. This sequence element overlaps partially with an ERE of the oxytocin gene −91 to −72) suggesting that positively and negatively acting protein factors may be involved in the expression of this gene. Similar potential regulatory sequence elements can be found in the

Table 1

The putative promoter region of the rat oxytocin gene contains several potential glucocorticoid (GRE)- and estrogen-responsive elements (ERE) upstream of the transcription start site

GRE	5′ 3′	
consensus	**G G T A C A N N N T G T T C T**	
−2392	C G G T C T C C T T G T T C T	−2406
−2256	C T A A G C C C G T G T T C T	−2270
ERE	5′ 3′	
consensus	**G G T C A N N N T G A C C**	
−170	**G G T G A C C T T G A C C**	−158
−112	**G A A C A G T T T G A C C**	−100
−92	**C C T G G C T G T G A C C**	−80

The numbering indicates the position of the sequence starting upstream of the transcription start site (−1). The bold letters are in agreement with published GRE or ERE sequence motifs



B)

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                  GATCTGTGATTCAGGGTCAGAAGAGAATGTGCTTTGCCCCACTGGCCCCAGCTCCTTGAGGAGAAGGCTGATCATAAGAACA  -2401
AGGAGACCGGAGCTTTTCCATCCTCTCTAGGGGCTGAGACTGCATGTAGTGAGTGGGTCTCGGGGGCACTGGCTGTTACTTCTTCCTGGATGTCGCAGGT  -2301
CACTTCTTAAGAGAGCCTGAGATGGAGTTTAGAACACGGGCTTAGGCCCCACCTCTATGGAAGAGAAGGGGTGGGCAACTAGAACCAGTATGCAGACCTC  -2201
AGCTAACCCAAAGCCCAAAGCTGACATGCAGTTGGGGTGCCCAGCATTCGTACCAAAGTGGACCAACCATTATATGTGGACACCTGGGAAGGCCAGGTG  -2101
GCTTTCTGCAGCTAAGACCACACCCAGCCACTGATGGCAAGTGTCTTCTTAAACAGGGGCCTAGGTACCTGGCACGGTGGTACACCCAGCACTTTGGGAG  -2001
GCAGGGGGATCGCTGTGAGTTCAAGCCCAGCCTAGTCTACACAGACAGTCCCAGGTCAACTAAGAGTCTATCTCAAAACAAAATAAAACGACTCTGGATA  -1901
ATGCTTCTCCTTTCTTTTAAATGTTTTAGGTCTATTTATTTTATTTTATGTGTCTGAACGTTCTGCCTGCAAGTGTGTAATGCACTATTTGGGTACTGG  -1801
CTCCACTGGAACTGGGATAAGTGATGTGAGCCACCCTGTGGGTGCTGGGAATCAAACCTAGGACTTCTGCAAGAGCAACTCAACCACTGAGCCAGTTCTC  -1701
CAGCCCTGAGATGGTAAGATATTAAGATGGGGCCCAGTGGAAGATCTTCCAGTCACTGAGGGCAAGCCTTGAAGGGAGCAGTGAGACCTTGGTCTCTCCT  -1601
GGTGATCTGTTTTCGATTTCTCAGCCGTCAAATGAAGAGGTTGAGGCTCCCATGTCTTCCTGCCATGGTTTGTCTGTGTCTTTATTTTGTTTTGGTTTTG  -1501
CTTTTGTTTTTTTGTGTAGCCCAGGCTGGCCTGGAACTAGGTCTGTAGACCAGGCTGGCCTGGAACTCACAGACATTCTCCTGCCTCTGCCTCCTGAGTT  -1401
CTGGGATTAAAAGTTTGCATCACTGTCACCGTGGCTTAATGTGCATTTCATTTTAAACTGTATTCAAATGCAAAATCAAGGAGCTGGGGAGATGGCTTAA  -1301
TGGTTAAGTCTGCACACTGCTCTTGCAGAATTCTGAATTTTGTTCCCAGCACCCACATAGGCCTACTCATGATCCCCTTTAATGACAGCTCCTGGGGATA  -1201
CAACATCCTCTTCTGGTTGCCAAGGAAACCTGTGTACACAAACATACACACAAATAAAAGTTAAAATTTAAAGCAAAAACTGTTCCCAAAATGTCTTGCT  -1101
ACCACCATGCTGGGGACTGGACCCAAGGTCTCTCACACACTAGGCAGGCAGTCTGCTACTGAGCTGCATGTAGCATTCTTCCCATGCAGACCTATGCAGG  -1001
CCTGCAGCCCAACCCTGAAATGCAACACGAGATGCCTAGTTGTATGTACTAAAATGGGCCACCATTACATGTGGCCACCAGGAAGGGTATGGCCTTAGGA  - 901
CAAATGGCTCTCTGTAGCTGAGGCCGCCTGTTGGCCACGCTCAAGCAGCTGATGGCCTGTGTTTTCTCAAACGGGAGTGTAGGTACCTAGCATTTGGGAG  - 801
GCAGAAGCAAATAGATCACTGTGAGTTCAAGGCCAGCCTGGTCCTCATAGCAAGTTCTAATACAGAGTTTTAAAGAGTTTTAAAGTTATTTATTATATAT  - 701
AAGCACATTGTAGCTCTCTTCAGACACACCAGAAGAGGGCATCAGATCCCATTACAGATGGTTGTGAGCCACCATATGGTTGCTGGGATTTGAACTCAGG  - 601
GCCTTTGGTAGAGCAGTCAGTGCTCCTAACCACTGAGCCATCTCTCCAGCCCTAATACAGAGTTGTAAGTGCAAGTGTGATTTTTCCTTTTATTGTGATT  - 501
TTTCTTTCTTTTTACTTTATTTGGTCTGTTTGTTGTGTTTTTGGGTTTGATTTCTACTGATATTTTGTTTTACGGGCTAGTTAGAAGAAGGGCTGAGCT  - 401
GCACTCAGAGATTGAACAAGATCCTTTTCCCCATCTAAGCTTTAGATCCACGTAAGGGCTTCCTCACCCACTTGCGCTATCCAACCTCATTCTGAGGTAT  - 301
TGGATTTCTATGAAAAAACAGCTCTTGGCTAGGCTGCACCTCTACCCCCTCCAAGTCTCTTTATCCTCTTGTAGCTTAGGCCTCCCCTTCTAGGCTGTGT  - 201
CCCTTTGAGCTCAGGTCATTAGCTGAGGCGGTGACCTTGACCCCAGCCCAGACCCTGCAAATGAAGGGCCTGCTTCTAAACAGTGTGGAACAGTTTGAC  - 101
GCAAGAGACCTGGCTGTGACCAGTCATGCAGTCACCCTCTTAGACTGGCCCCACCATGGCAGTGGACAAGGCATAAAAAGGTCGGTCTGGGCTGGAGAA  -   1
ACCATCACCGACGGTGGATCTCGGACTGAACACCAACGCCATGGCCTGCCCCAGTCTCGCTTGCTGCCTGCTTGGCCTACTGGCTCTGACCTCCGCCTGC  + 100
                                        M  A  C  P  S  L  A  C  C  L  L  G  L  L  A  L  T  S  A  C
TACATCCAGAACTGCCCCCTGGGCGGCAAGAGGGCTGCGCTAGACCTGGATATGCGCAAGGTGAGTCTCCCCGACGCCATCCCGTCCCGTTCTGGCAAGG  + 200
Y  I  Q  N  C  P  L  G  G  K  R  A  A  L  D  L  D  M  R  K
CTAAGGACCAGAGATGCTCTCCCACCTTCAGAGAGCATCCCCTCACACTTGCCAGCCCTACGCGGCCTCGCATGGGAACCCGGAGCTCCCTCTTGACGCC  + 300
GTGAAGGCACGCTGTCCCTGCCCCACCACAGTCCCGATATGGATTGTGCACAGCGCCCATCCGCGTCTTTCCCCCCGCAGTGTCTTCCCTGCGGACCCGG  + 400
                                                                                 C  L  P  C  G  P  G
CGGCAAAGGGCGCTGCTTCGGGCCGAGCATCTGCTGCGCGGACGAGCTGGGCTGCTTCGTGGGCACCGCCGAGGCGCTGCGCTGCCAGGAGGAGAACTAC  + 500
 G  K  G  R  C  F  G  P  S  I  C  C  A  D  E  L  G  C  F  V  G  T  A  E  A  L  R  C  Q  E  E  N  Y
CTGCCCTCGCCCTGCCAGTCTGGCCAGAAGCCTTGCGGAAGCGGAGGCCGCTGCGCCACCGCGGGCATCTGCTGTAGCCCGGGTGAGCAGGAGGGGGCCT  + 600
L  P  S  P  C  Q  S  G  G  K  P  C  G  S  G  G  R  C  A  T  A  G  I  C  C  S  P  D
AGCAGGACCGACCGGCAGGGAGCCGTCGGGTTTGCTGCTCAGGCCACTGACCCATTTCTCTTGCAGATGGCTGCCGCACCGACCCCGCCTGCGACCCTGA  + 700
                                                                    G  C  R  T  D  P  A  C  D  P  E
GTCTGCCTTCTCCGAGCGCTGAGCCCTGCTTTGTGATGATACCTTTAGGGCGCTTCCTTCGTTCCCCATGGCCACTACCAGAAAAAAATTAAAAAAAAAA  + 800
 S  A  F  S  E  R
CATAAATAAATAAATAAAGCAGATTTCCTTTTC                                                                     + 833
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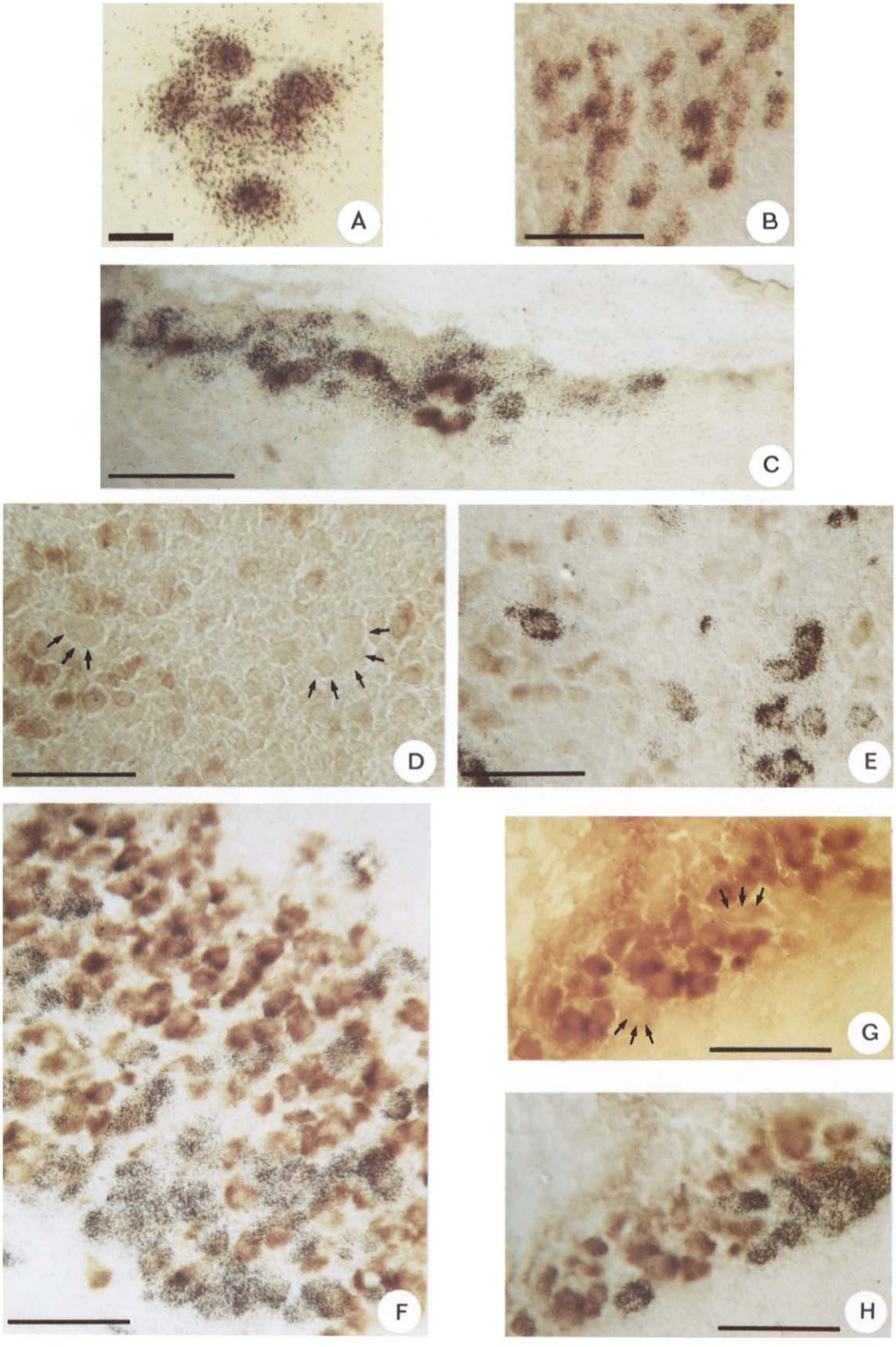
Fig.1. (A) Schematic representation of the organization of the rat oxytocin and vasopressin gene. O, oxytocin; NP, neurophysin; GP, glycopeptide; V, vasopressin. (B) Sequence of the rat oxytocin gene with its putative promoter region (sequence has been submitted to the EMBL data library under the accession no. X12792). For sequencing [19,20], genomic DNA restriction fragments were subcloned into M13 and Bluescript plasmids; sequence was determined completely on both strands. GRE, ERE and the modified TATA sequences are boxed. A putative interferon-responsive enhancer sequence (−1593 to −1579) is underlined [21]. There are multiple octanucleotide enhancer sequences (ATGCAAAT/C) [22] at positions −1381 to −1388, −1333 to −1325, −145 to −136.

Fig.2. In situ hybridization of hypothalamic tissue sections through supraoptic (C,F,G,H), paraventricular (B,D,E) or suprachiasmatic nucleus (A) from normal rats (A–E) or rats loaded with 2% saline for seven days (F–H) to vasopressin- and oxytocin-specific cRNA probes labelled with biotin or [α-^{35}S]CTP. In A, B, C and F, sections are double labelled: biotinylated and ^{35}S-labelled V-specific cRNAs (A), biotinylated and ^{35}S-labelled O-specific cRNAs (B); biotinylated V-specific and ^{35}S-labelled O-specific cRNAs (C); biotinylated V-specific and ^{35}S-labelled O-specific cRNAs (F). (D) Biotinylated O-specific and ^{35}S-labelled V-specific cRNAs, this section was photographed before the slide was dipped into emulsion; (E) same section as in D labelled with ^{35}S-specific V-cRNA. (G) Biotinylated V-specific and ^{35}S-labelled O-specific cRNAs, the section was photographed before the slide was dipped into emulsion; (H) same section as in G showing the ^{35}S-labelled O-cRNA. Thick bar, 20 μm; thin bar, 100 μm. The arrows point to examples of single cells showing signals with the ^{35}S-labelled probe but not with the biotinylated cRNA. V, vasopressin; O, oxytocin.


A
B
C
D
E
F
G
H

promoter region of the vasopressin gene (Mohr, E., unpublished) which agrees with the finding that upregulation of one gene concomitantly affects expression of the other [1–8].

To address the question of the cellular specificity of oxytocin and vasopressin gene transcription and their upregulation in osmotically stressed rats, we designed double labelled cRNA probes labelled either with biotin or [α-^{35}S]CTP, these being specific respectively for vasopressin and oxytocin-encoding transcripts. In situ hybridization of sections through the supraoptic nucleus of saline-exposed rats compared to control rats is shown in fig.2 (A–H). In neither case was colocalization of the two transcripts observed in one and the same neuronal cell, supporting the earlier immunological data which suggested that the two peptides were present in mutually exclusive sets of neurons [12]. Identical results were obtained with sections through the suprachiasmatic and paraventricular nucleus (not shown). These data imply that the osmotic stimulus causing the upregulation of the two genes cannot trigger activation of the suppressed oxytocin gene in vasopressinergic cells but in contrast does stimulate oxytocin gene transcription in oxytocinergic cells. It would be of interest to see which factor(s) and regulatory sequence elements of the two genes are involved in their suppression and stimulation.

Acknowledgements: This work was supported by Deutsche Forschungsgemeinschaft. The double labelling in situ hybridization data are part of the thesis by C.K.

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