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Neuron-specific expression and physiological regulation of bovine vasopressin transgenes in mice

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We have used transgenic mice to analyse the regulation of the bovine vasopressin (BVP) gene. We find that the restriction of BVP gene expression to anatomically and functionally distinct hypothalamic neuronal groups is achieved, in part, by selective repression. The expression of a 1.25 kb BVP proximal promoter, which on its own confers general expression of a reporter to most peripheral and brain tissues, was limited by sequences in the BVP structural gene to neural cells in the adrenal medulla and brain. Transgene expression in the hypothalamus was shown to be regulated by the physiological stimulus of dehydration in parallel with the endogenous gene. The expression of a larger 13.4 kb BVP transgene, containing 9 kb of 5′ upstream sequence, the VP structural gene and 1.5 kb 3′ of the transcription unit, was even more restricted and resembles that of the endogenous mouse gene. Hypothalamic expression of the 13.4 kb BVP transgene was regulated appropriately in response to an osmotic challenge.

Key words: hypothalamic neurons/neuronal gene regulation/physiological regulation/transgenic mice/vasopressin

Introduction

A major function of the mammalian hypothalamus is to integrate the physiological systems that maintain the constancy of the conditions in the internal environment. Information from peripheral receptor systems is received and processed by the hypothalamus, which responds to perturbations by modulating the synthesis and release of peptide neurohormones. A neuropeptide may, however, be involved in many different integrative processes: one of the best examples of such functional plasticity is the hypothalamic vasopressin (VP) system. VP is synthesized in anatomically defined groups (nuclei) of hypothalamic neurons each of which is involved in the maintenance of distinct physiological systems and is subject to functionally appropriate regulatory cues.

The best known role of VP is as a central component of the systems that regulate water balance. Two groups of magnocellular neurons in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) are involved in osmoregulation. Within the cell bodies of these neurons, VP is synthesized as a prepropeptide, a precursor which is subject to cleavage and other modifications as it is transported down the axon to terminals located in the posterior (neural) portion of the pituitary gland (Brownstein *et al.*, 1980; Rehbein *et al.*, 1986; Ivell, 1987; Richter, 1988). The mature peptide products—the nonapeptide hormone VP, a putative carrier molecule termed neurophysin II and a 39 amino acid glycopeptide of unknown function—are stored in the axon terminals until a physiological stimulus elicits their release. The physiological challenge of dehydration results in a rise in plasma osmolality which is detected by an undefined osmoreceptor mechanism. Subsequent excitation of the magnocellular hypothalamic neurons leads to a release of VP into the general circulation from posterior pituitary stores. VP travels through the bloodstream to its targets which exhibit specific receptors. Particularly, through an interaction with V2-type receptors located in the kidney, VP increases the permeability of the collecting ducts to water. Thus, under conditions of reduced water intake, and hence increased plasma osmolality, the hypothalamus (through VP) promotes water conservation by decreasing the amount of water lost in the urine.

Different functions have been ascribed to the anatomically distinct groups of parvocellular neurons which synthesize VP. Vasopressinergic neurons in the PVN project to the median eminence from where VP released into the portal blood travels to the anterior pituitary gland (Zimmerman and Silverman, 1983; Horn *et al.*, 1985). By stimulating the secretion of adrenocorticotrophic hormone (Gillies *et al.*, 1982; Rivier and Vale, 1982) it is involved in the regulation of the hypothalamic-pituitary-adrenal 'stress axis'. VP is also synthesized in the parvocellular neurons of the dorsomedial region of the rat suprachiasmatic nucleus (SCN). The central circadian generator of the mammalian brain is thought to reside in this part of the hypothalamus (van den Pol 1980; Moore, 1983). Vasopressinergic SCN neurons project to defined target areas of the brain (Watts and Swanson, 1987) and there is some evidence that VP transmits circadian signals (Sodersten *et al.*, 1985; Buijs *et al.*, 1990).

Studies on VP gene expression have benefited from the exploitation of well established paradigms for the modulation of the activity of vasopressinergic neurons. These experiments have revealed differential regulation of VP gene expression in different groups of VP neurons in response to different physiological stimuli. For example, we and others (Carter and Murphy, 1989a, 1991a; Murphy and Carter, 1990; references contained therein) have shown that VP gene expression in the SON responds both transcriptionally and post-transcriptionally to osmotic challenges, such as dehydration. Osmotic challenge stimulates VP release, which engenders an increase in plasma VP levels and a depletion of pituitary stores. Functional demand leads to an increase in VP gene transcription (Murphy and Carter, 1990; Herman *et al.*, 1991), VP RNA abundance (Burbach *et al.*, 1984; Zingg *et al.*, 1986; Lightman and Young, 1987; Sherman *et al.*, 1988), and a dramatic increase in the VP RNA poly(A) tail length (Carrazana *et al.*, 1988; Zingg *et al.*, 1988; Carter and Murphy, 1989a, 1991a). However, the pattern of VP gene expression in the parvocellular

neurons of the SCN changes in a different way in response to osmotic stimuli. Although the poly(A) tail length of the SCN VP RNA increases following dehydration (Carter and Murphy, 1990), there is no change in message abundance (Carter and Murphy, 1990). Similarly, SON magnocellular neurons do not change their pattern of VP gene expression in response to stimuli that affect the parvocellular neurons of the PVN and SCN. For example, in contrast to the SON, VP gene expression in the parvocellular PVN is dependent upon circulating glucocorticoid levels (Wolfson *et al.*, 1985; Young *et al.*, 1986). Similarly, in the SCN, the level of transcription of the VP gene (Carter and Murphy, 1991b), the abundance of VP RNA (Uhl and Reppert, 1986; Carter and Murphy, 1991b) and the length of the VP mRNA's poly(A) tail (Robinson *et al.*, 1988; Carter and Murphy, 1989b, 1991b) are all regulated by circadian cues. However, VP gene expression is not regulated by time of day in the SON (Carter and Murphy, 1991b).

The hypothalamic VP system not only epitomizes neuroendocrine regulation, but, being regulated in different ways in different groups of neurons, also represents a unique and accessible example of the plasticity of gene expression in mature neurons. Thus, the molecular mechanisms underlying both the cell-specific expression and the physiological regulation of the VP gene are currently the subject of much interest and speculation (Ivell and Burbach, 1991). However, the description of these processes has been hampered by the lack of an appropriate (i.e. VP-synthesizing) cell line (Ivell and Burbach, 1991). Further, it is not possible to study an integrated homeostatic mechanism outside of the intact organism. We have therefore chosen to study the regulation of the VP gene *in situ* using transgenic mice.

Results

Generation of the transgenic mouse lines carrying bovine vasopressin transgenes

In order to define regions in the bovine vasopressin (BVP) gene responsible for appropriate tissue-specific expression, three constructs were used to generate transgenic mouse lines. Construct VP-A is a fusion of the 1.25 kb of the BVP proximal promoter linked to the chloramphenicol acetyl transferase (CAT) reporter gene (Figure 1A). Three independent lines were generated from this construct. They were designated VP-A1 to 3. Construct VP-B contains the same 1.25 kb BVP proximal promoter region linked to its own structural gene and ~200 bp of downstream sequence (Figure 1A). Three lines were generated from this construct, designated VP-B1 to 3. Construct VP-C is a 13.4 kb genomic fragment comprising 9 kb of 5′ and 1.5 kb of 3′ sequences flanking the structural gene (Figure 1A). Four independent lines were generated from this construct and were designated VP-C1 to 4.

Following the positive identification of a transgenic founder, lines were generated through crosses with wild-type F1 (CBA/J×C57Bl/10) mates. At each generation, individual obligate heterozygote transgenic mice were identified by Southern hybridization analysis. Each line was then analysed for transgene expression as described below. The expression pattern of the VP gene in the wild-type mouse has not been studied in great detail before. Here we confirm expression in the hypothalamus (Figure 1B; Hara *et al.*, 1991; Murphy *et al.*, 1992a) and the presence of VP RNA (of unknown origin; Murphy *et al.*, 1993) in the neuro-

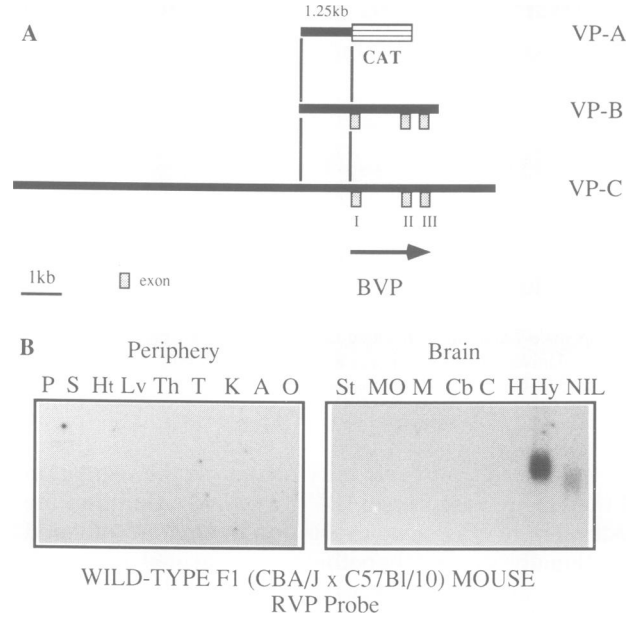


Fig. 1. **(A)** Structure of the constructs used to generate transgenic mice. **The VP-A construct**: This construct is derived from the plasmid pbAVPCAT1.25 (Murphy *et al.*, 1987), which contains a 1.25 kb BVP promoter region, defined by *SalI* sites, linked to the bacterial CAT reporter gene. The 1.6 kb CAT coding region contains its own translation start site, the SV40 small t-antigen splice and the SV40 early region poly(A) signal and transcription termination sequences. **The VP-B construct**: This construct contains the same 1.25 kb BVP proximal promoter linked to its own coding sequence consisting of three exons and 0.2 kb 3′ of the native poly(A) addition site. **The VP-C construct**: This is a 13.4 kb *SalI* fragment containing 9 kb of 5′ sequence, the BVP structural gene and 1.5 kb of 3′ sequence (Ruppert *et al.*, 1984). **(B)** Expression of the endogenous VP RNA in wild-type mice. Total RNA was extracted from tissues of F1 (CBA/J×C57BL/10) non-transgenic mice, fractionated on a 1% (w/v) formaldehyde−agarose gel, transferred to a solid matrix and probed with a radiolabelled oligonucleotide specific for RVP. Peripheral tissues examined: P, pancreas; S, spleen; Ht, heart; Lv, liver; Th, thymus; T, testis; K, kidney; A, adrenal gland; O, ovary. Brain regions examined: St, striatum; MO, medulla oblongata and pons; M, midbrain; Cb, cerebellum; C, cortex; H, hippocampus; Hy, hypothalamus; NIL, neurointermediate lobe of the pituitary. 50 μg total cellular RNA was loaded into each lane except for NIL (5 μg) and Hy (5 μg). Filters were reprobed for the internal control (α-tubulin) to confirm that the RNAs were loaded in equivalent amounts and were not degraded (data not shown).

intermediate lobe of the pituitary (Figure 1B; Murphy *et al.*, 1989; Levy *et al.*, 1990; Mohr *et al.*, 1991). Expression could not be detected in any other mouse tissue (Figure 1B). A detailed description of the expression of the endogenous vasopressin gene in the mouse hypothalamic neurons is presented below, and compared with the expression of the bovine transgene. Knowledge of the expression pattern of the vasopressin gene in cattle is limited. Expression has been described in hypothalamus (Ivell and Richter, 1984; Murphy *et al.*, 1992a) and VP RNA is present in the neurointermediate lobe of the pituitary (Murphy *et al.*, 1992a). Aberrant VP gene derived transcripts have been described in the bovine ovary (Morley and Ivell, 1987).

Expression of the VP-A transgene

CAT RNA expression was detected in all VP-A1 brain and peripheral tissues examined (Figure 2). Similarly, CAT RNA was detected in all brain regions and most peripheral

tissues of VP-A2 (not shown). No CAT RNA could be detected in any VP-A3 tissue (not shown). CAT assays were conducted on tissue extracts from all three lines and, except for low levels of CAT activity in the liver in VP-A1, no enzyme activity was detectable. Transfection of the VP-A construct into monkey kidney (CV 1) cells resulted in high CAT activity, confirming that this construct is a functional transcription and translation unit (Pardy *et al.*, 1992).

Expression of the VP-B transgene

The three VP-B lines shared very similar transgene expression patterns (Figure 2). Peripheral expression of the transgene was restricted to the adrenal gland of VP-B1 and VP-B2. Most brain regions expressed the transgene, but the relative level of expression differed between lines. Such quantitative, rather than qualitative, differences in expression patterns have been attributed to chromosome position effects (Wuenschell *et al.*, 1990; Mercer *et al.*, 1991; McKnight *et al.*, 1992).

In situ hybridization histochemistry (ISHH) was then used to identify precisely some of the cell types expressing the VP-B transgene. For example, ISSH of VP-B1 brain sections showed that in the hypothalamus, there was general expression of the BVP RNA with no apparent clustering in particular neurons. This contrasted with the VP-B2 line, in which high levels of the BVP transcript were localized in the PVN, SON and SCN (Figure 3A, right panel), accessory magnocellular cells and cells of the retrochiasmatic area (Figure 3B). BVP transcript levels were also low in the lateral regions of the SCN and midbrain. Thus the transgene expression pattern in the VP-B2 hypothalamus resembles that of the endogenous VP gene (Figure 3B and C). In the VP-B1 brain, transgene RNA was also detected in the CA1–3 regions of the hippocampus (Figure 3E and F).

Since the adrenal gland contains cell types that develop from different embryological lineages, the adrenal cortical layers having arisen from the fetal mesoderm and the adrenal medulla from the primitive cells of the neural crest (Goldfien, 1986), we used ISHH to identify the cell types that express the BVP transgene (Figure 3D). Expression of BVP transcripts in line VP-B1 was restricted to the corticomedullary junction and small groups of medullary cells that were sometimes organized into short anastomosing cords (Leeson *et al.*, 1988) with a consequent cord-like pattern of transgene expression when viewed under dark-field microscopy. A similar pattern of expression was noted in the adrenal gland of VP-B2 (data not shown).

Expression of the VP-C transgene

Three of the four transgenic lines demonstrated transgene transcripts in the hypothalamus. In the VP-C3 (Figure 2) and VP-C4 (not shown) lines the hypothalamus was the only brain region expressing the transgene. Only VP-C1 showed prominent ectopic expression in brain cells outside of the hypothalamus, in cortex and hippocampus (Figure 2). Outside of the brain, transgene transcripts were detected in the ovary of all of the three lines examined (VP-C1 and VP-C3, Figure 2; VP-C2), the kidney in one line (VP-C2, not shown) and the anterior pituitary gland in two lines (VP-C2 and VP-C4, not shown).

ISHH of brain sections from VP-C4 revealed localization of BVP transcripts to neurons of the SON, PVN, accessory magnocellular neurons and cells of the retrochiasmatic area. However, more cells within the SON and PVN expressed the endogenous VP RNA than expressed the transgene transcript (Figures 3C and 4). SON and PVN cells also displayed a marked heterogeneity in the level of expression of the transgene. No BVP transcripts were detected in the SCN (Figure 4), whereas a probe specific for the rodent VP (RVP) message identified endogenous gene expression in this group of hypothalamic neurons (Figures 3C and 4). Endogenous VP transcript levels in the SCN were considerably lower than those in the PVN and the SON. Hypothalamic sections of VP-C3 brains showed transgene transcripts in neuronal populations of the SON including its retrochiasmatic division and the PVN (data not shown).

Regulation of the bovine vasopressin transgenes by physiological stimuli

Transgenic mice from the VP-B1 and VP-B2 lines were subjected to the osmotic challenge of 7 days of salt-loading. This treatment involves the replacement of the tap water diet of the mice with 2% (w/v) NaCl, and results in a consistent increase in plasma osmolality and a well defined upregulation in hypothalamic VP gene expression (Murphy *et al.*, 1989; Murphy and Carter, 1990). In both lines, the level of the hypothalamic BVP RNA did not significantly change when assayed by Northern blotting (Figure 5A; Table I). However, it is possible that cell-specific increases in transgene expression in response to salt-loading would not be detected using this method of analysis due to their being masked by the general BVP expression in the hypothalamus. This proved to be the case. ISHH of brains from control and salt-loaded VP-B1 and VP-B2 transgenic males revealed that both lines upregulated BVP expression specifically in PVN and SON in response to the osmotic stimulus (Figure 6). A similar effect of salt-loading on the hypothalamic expression of the VP-C transgene was seen in both VP-C3 and VP-C4. Transgene expression increased in parallel with the endogenous gene and could be detected clearly by Northern blotting (Figure 5B, Table I). ISHH of hypothalamic sections from salt-loaded VP-C4 males showed increased transgene expression in the accessory magnocellular neurons (data not shown), the SON and the PVN (Figure 7). It was evident that, following salt-loading, transcript levels per cell were increased and that more cells expressed detectable transgene message (Figure 7). Transgene expression levels in SON neurons continued to display heterogeneity following salt-loading. Similar results were obtained with the VP-C3 line (data not shown).

Discussion

Using transgenic mice, we have identified genomic regions that are sufficient for the appropriate tissue-specific expression and physiological regulation of the BVP gene. Whilst the 1.25 kb proximal BVP promoter confers general expression of a reporter element in both peripheral and brain tissues (VP-A transgene), expression of this same promoter is limited to cells derived from neuronal progenitors by elements contained within the BVP structural gene (VP-B transgene). The expression of a 13.4 kb BVP genomic fragment containing 9 kb of 5′ upstream sequence, the BVP coding sequence and 1.5 kb of sequence 3′ of the structural gene (VP-C transgene) was further restricted to discrete groups of hypothalamic neurons in three lines. In one

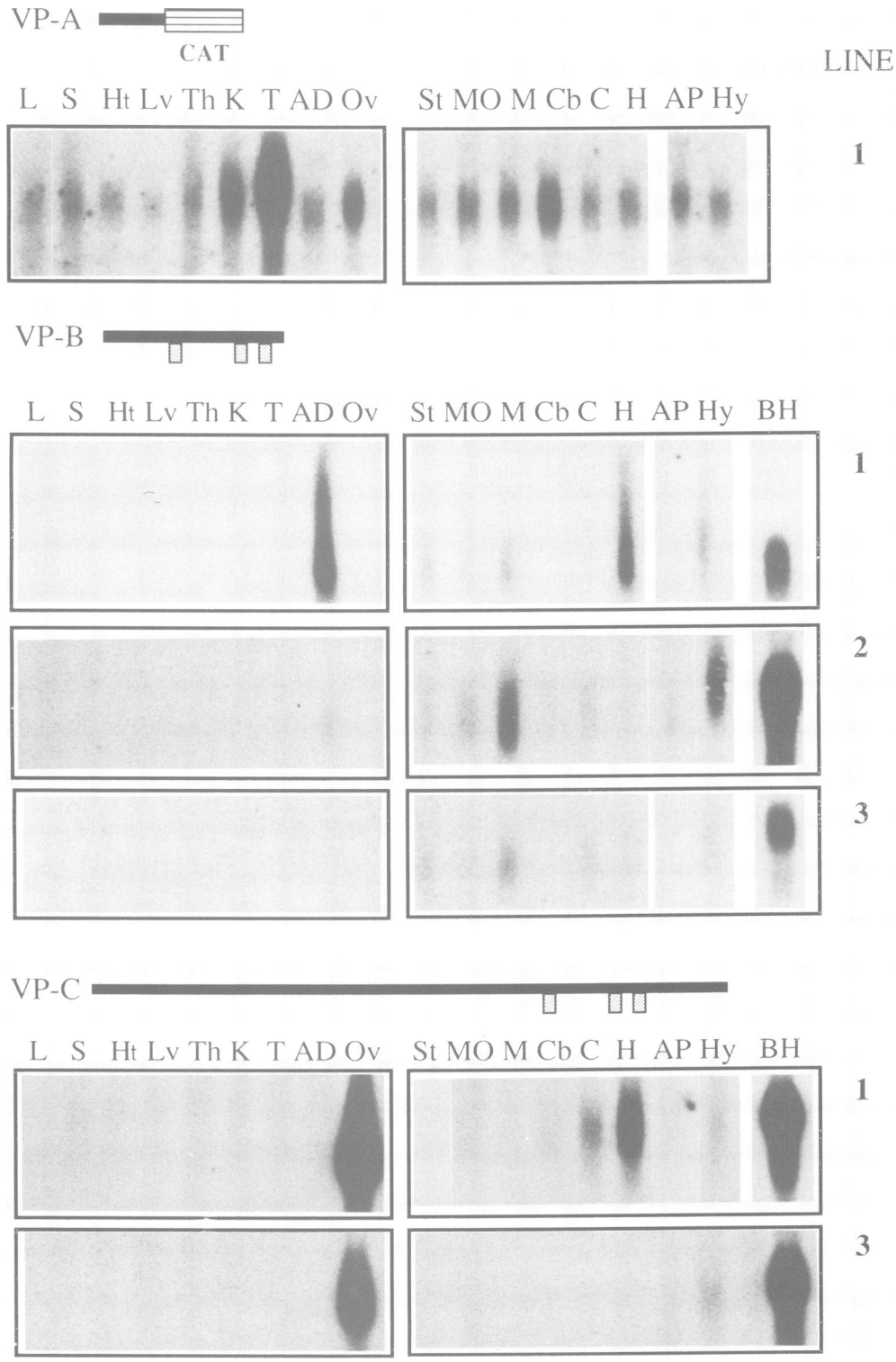


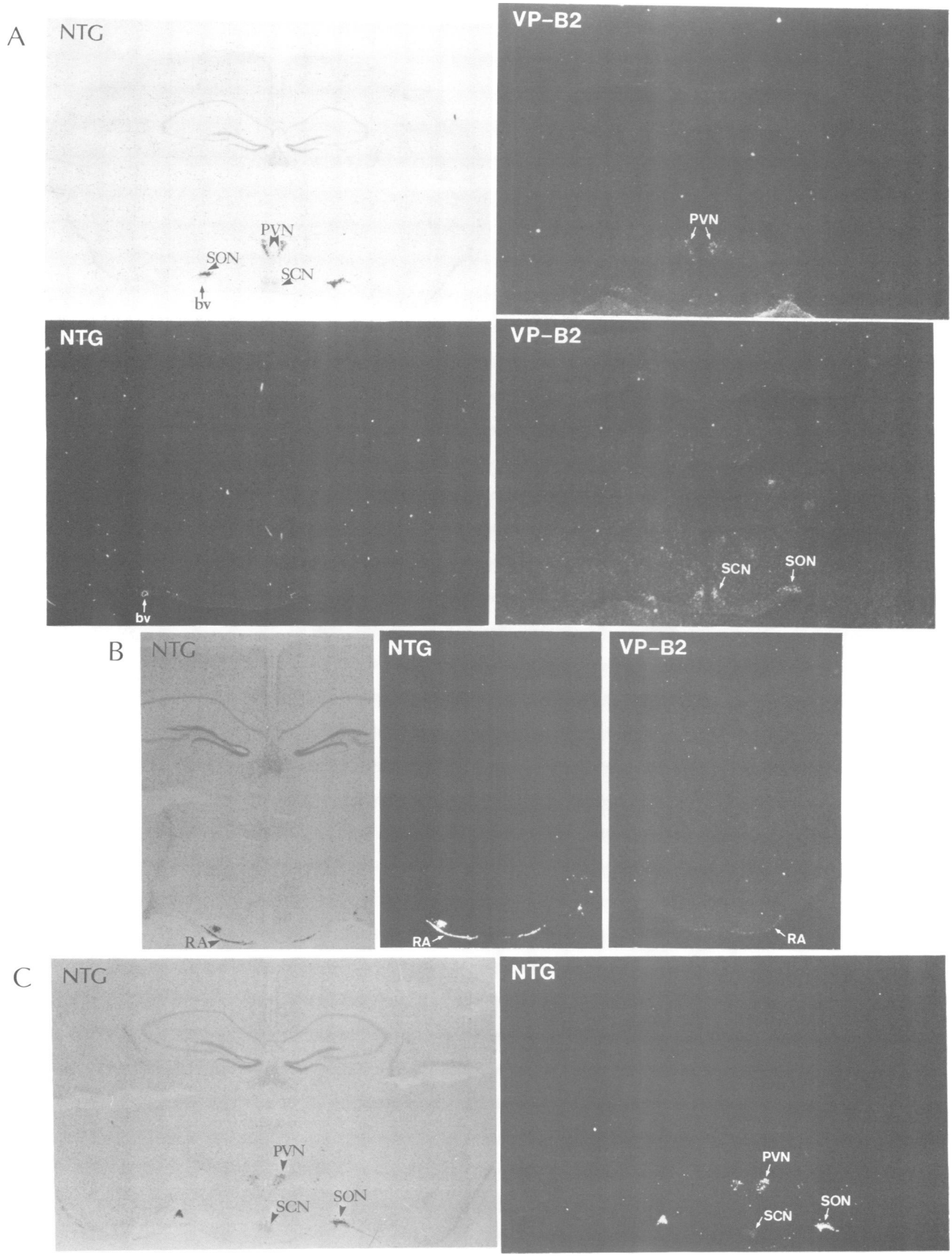
Fig. 2. Expression of transgene RNA in peripheral and brain tissues of VP-A, VP-B and VP-C lines. Northern analysis of total cellular RNA extracted from the following tissues: lung (L), spleen (S), heart (Ht), liver (Lv), thymus (Th), testes (T), kidney (K), adrenals (AD), ovary (Ov), striatum (St), medulla oblongata (MO), midbrain (M), cerebellum (Cb), cortex (C), hippocampus (H), anterior pituitary (AP) or whole pituitary, hypothalamus (Hy) and the positive control, bovine hypothalamus (BH). Unless otherwise indicated, 50 μg of RNA from each tissue was loaded. 3–5 μg of BH total RNA was used. VP-A1: AD and Ov (20 μg each). 15 μg of RNA from each brain region was loaded. AP (15 μg). VP-B2: AD (40 μg), AP (22 μg). VP-C1: AD (37 μg), AP (14 μg). VP-C3: The pituitary was not dissected into the anterior and neuro-intermediate lobes (24 μg). The VP-A RNA was probed with a radiolabelled double stranded CAT probe. The VP-B and VP-C RNAs were hybridized to an oligonucleotide specific for BVP.

additional line, ectopic expression in hippocampus and cortex was also seen. The VP-C transgene recapitulates the appropriate physiological regulation, such that salt-loading results in a significant increase in BVP transcript levels which parallels the change in activity of the endogenous gene. However, sufficient regulatory information is also contained

within the limits of the smaller VP-B transgene (containing the 1.25 kb proximal promoter and the BVP structural gene) to confer an appropriate response to osmotic challenge within the confines of the PVN and SON.

Neuron-specific expression of BVP in the mouse hypothalamus

Our results suggest that repressor elements may play an important role in achieving appropriate tissue-specific



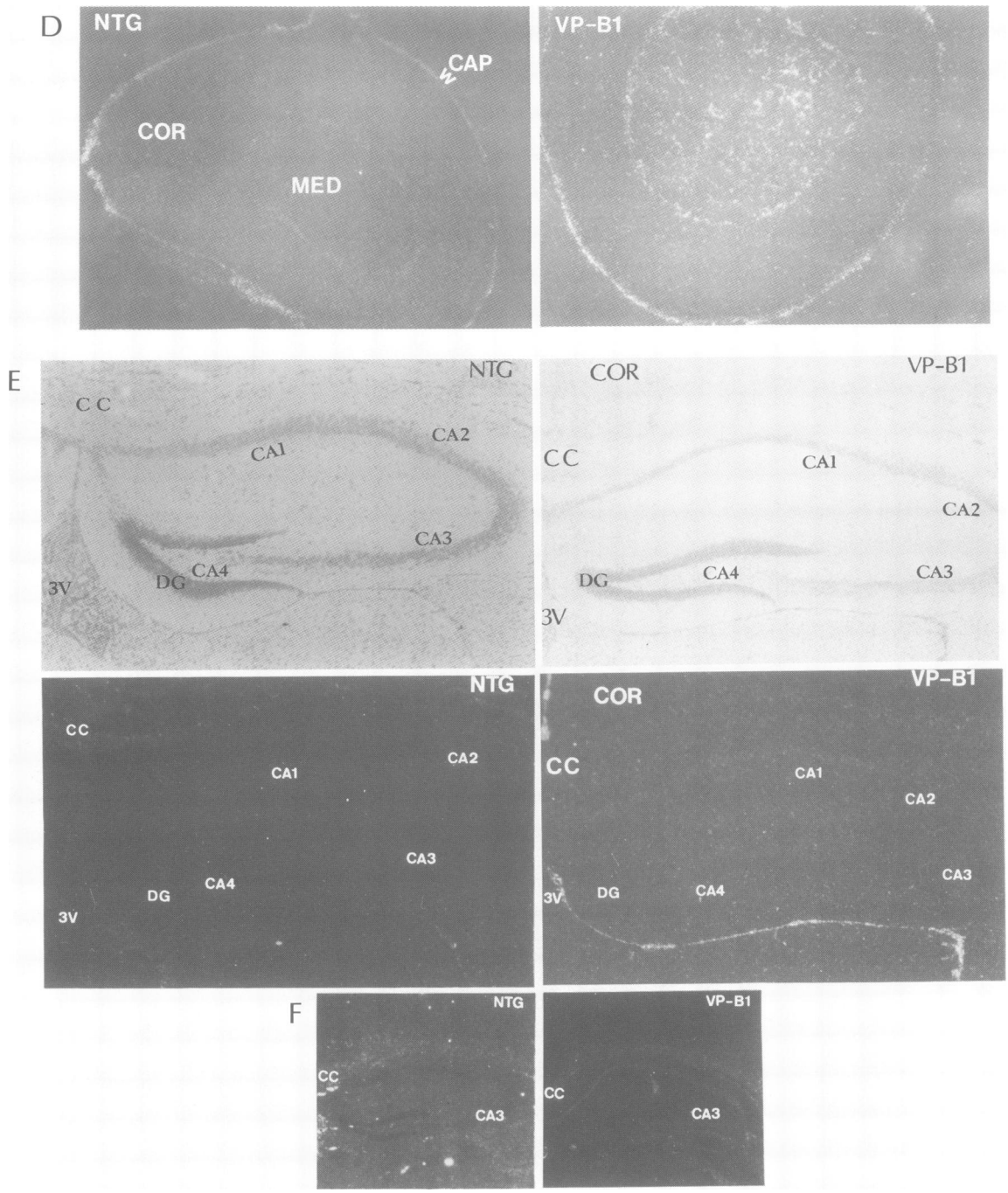


Fig. 3. ISHH of adrenal gland and brain sections from non-transgenic (NTG) and VP-B mice. (**A**) Dark-field microscopy of coronal sections of NTG and VP-B2 brains probed for BVP transcripts. The bright-field image (top, left panel) of a NTG brain, exhibiting high levels of endogenous VP expression, shows the topographical localization of the PVN, SON and SCN within the hypothalamus in a coronal section. High levels of BVP transcripts are detected in the PVN (top right panel), SON and SCN (bottom right panel) of the VP-B2 brain. Low levels are detected in the midbrain. The signal below the base of the brain is due to non-specific association of this probe with the microscope slide. The NTG brain section did not give any specific signal with the bovine probe (bottom left panel). Some non-specific signal is observed in the blood vessels (bv). (**B**) Bright-field and dark-field microscopy of hypothalamic sections of NTG and VP-B2 brains showing VP expression in cells of the retrochiasmatic area. NTG and VP-B2 sections are probed for endogenous VP (RVP) and BVP transcripts respectively. The VP-B2 hypothalamus exhibits BVP expression in the cells of the retrochiasmatic area (RA). This pattern of expression is similar to that of the endogenous VP gene in the NTG brain. (**C**) Bright-field and dark-field microscopy of a coronal section of NTG brain probed for endogenous VP (RVP) transcripts. RVP expression is detected in the SON, PVN and the SCN. (**D**) Dark-field microscopy of adrenal sections from NTG and VP-B1 mice probed for BVP transcripts. BVP expression is restricted to groups of cells in the medulla (MED) of the transgenic adrenal gland. No BVP transcripts are detected in the cortex (COR). Cells in the medulla are organized in small clusters or in short anastomosing cords. Expression of the transgene in these groups of cells consequently takes on a cord-like pattern under dark-field microscopy. NTG adrenal sections do not give any specific signal with the bovine probe. Some non-specific signal can be detected around the capsule (CAP) of NTG and VP-B1 sections. (**E** and **F**) Bright-field and dark-field microscopy of NTG and VP-B1 hippocampal sections probed for BVP transcripts. BVP expression is detected in the CA1–3 cells of the transgenic hippocampus only. Some non-specific signal is detected in the corpus callosum and the third ventricle of the NTG and VP-B1 brains. The following abbreviations are used: cortex (COR), corpus callosum (CC), third ventricle (3V) and dentate gyrus (DG).

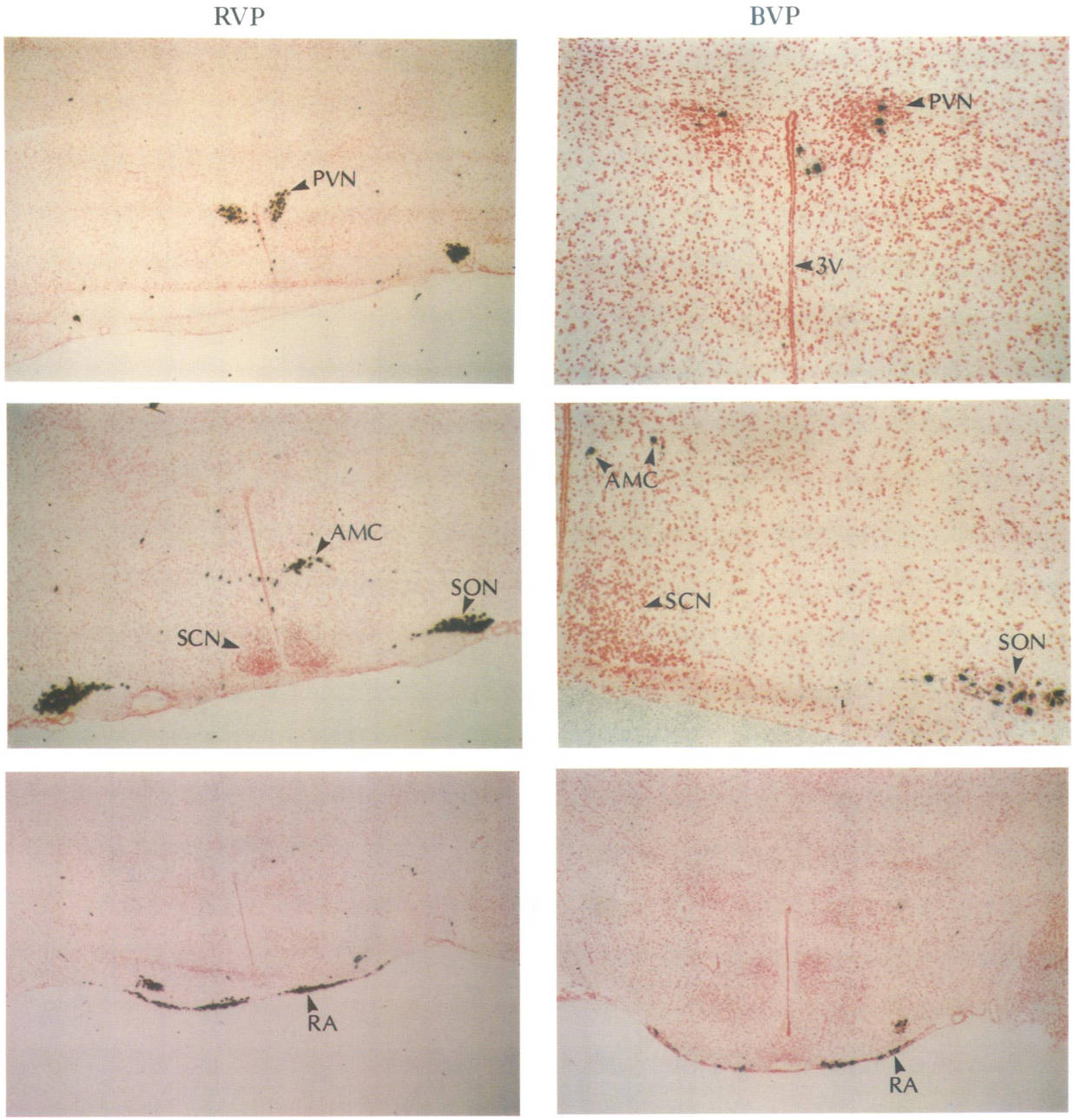


Fig. 4. ISHH of brain sections from VP-C4 mice demonstrating hypothalamic expression patterns of the endogenous VP gene (RVP) and the VP-C transgene (BVP). Bright-field microscopy of hypothalamic sections of VP-C4 brain probed for RVP (left panels) and BVP (right panels) transcripts. RVP transcripts are abundant in the supraoptic nucleus (SON) and its retrochiasmatic division (RA), accessory magnocellular neurons (AMC), the para-ventricular nuclei (PVN) and suprachiasmatic nuclei (SCN). Except for the SCN, BVP transcripts are detected in a subset of neurons in the same hypothalamic regions. The sections have been counter-stained with 1.2% neutral red.

expression of the BVP gene. It would appear that the proximal BVP promoter contains little or no tissue-specific information, and is profoundly influenced by neighbouring elements. When linked to the CAT reporter, in the case of the VP-A transgene, the BVP proximal promoter is almost ubiquitously expressed. An almost identical segment of the human VP promoter directed the expression of a human growth hormone reporter to many brain regions, including the SON, PVN, SCN, hippocampus (CA3, pyramidal cells and dentate gyrus), the neocortex (layers II and IV) and the nucleus of the lateral olfactory tract and piriform cortex (Russo *et al.*, 1988). No peripheral expression was described in this study. Activity of the BVP promoter was limited to the somatotrophs in the anterior pituitary gland (Stefaneanu *et al.*, 1992), to the endocrine pancreas and to the ovary by linkage to the SV40 early region (Murphy *et al.*, 1987, 1992a). Here we show that the activity of the proximal BVP promoter is selectively repressed in non-neuronal peripheral tissues and neuronal extrahypothalamic brain regions by factors interacting with at least two silencer elements located in the homologous BVP structural gene and flanking regions. The first silencer element(s) were defined by the restriction of the activity of the 1.25 kb BVP proximal promoter to the brain and the adrenal gland by downstream, structural gene sequences (the VP-B transgene). Analysis of VP-B transgenic mouse adrenal glands proved to be particularly instructive

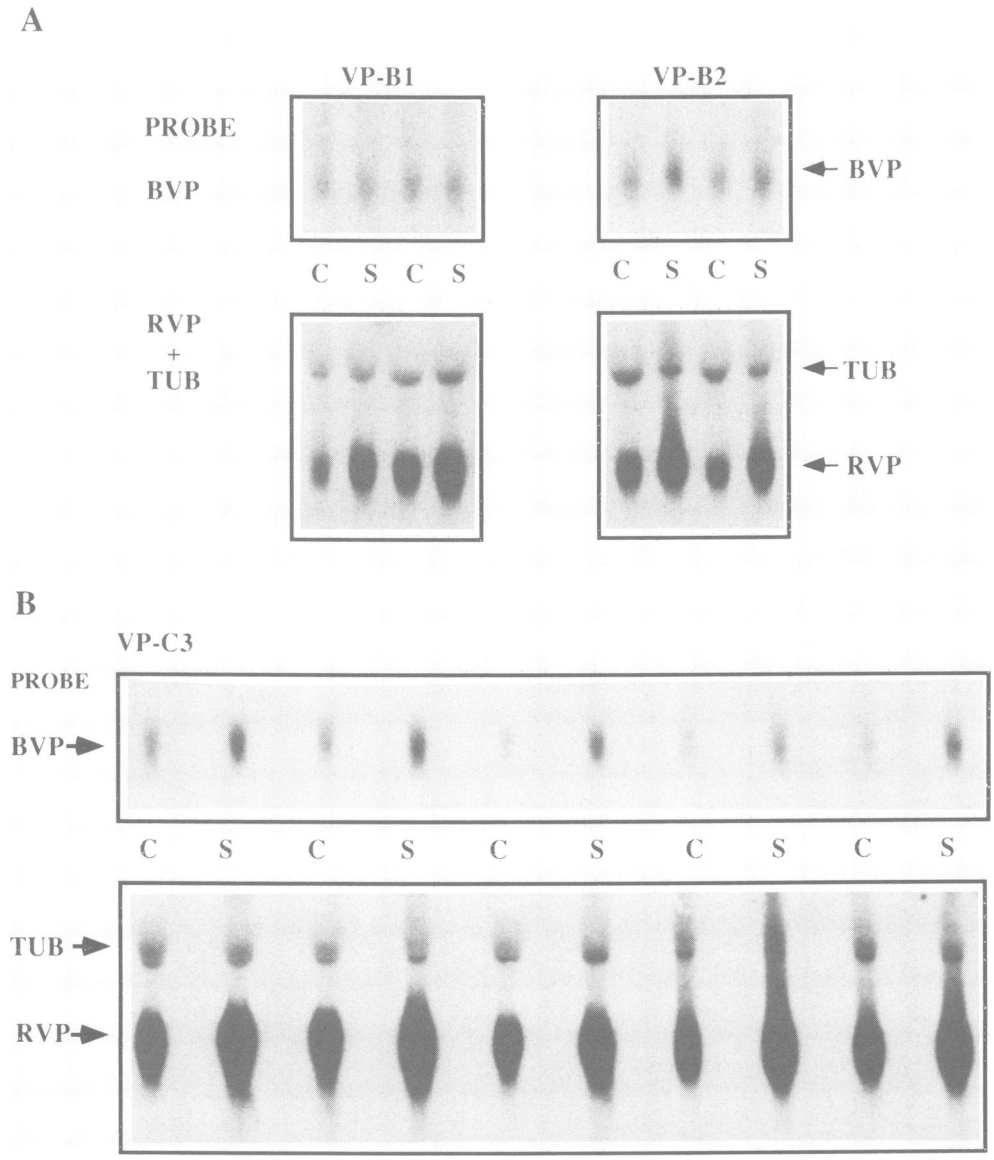


Fig. 5. Effect of salt-loading on RVP and BVP expression in the hypothalami of the (A) VP-B1 and VP-B2 and (B) VP-C3 transgenic mouse lines. Northern analysis of total cellular RNA from the hypothalami of control (C) and salt-loaded (S) transgenic males. Each track represents a separate experimental group. In each experiment, the α-tubulin message (TUB) served as the internal standard. (**A**) Representative Northern filter showing transgene (BVP) and endogenous (RVP) vasopressin transcript levels in control and salt-loaded VP-B1 and VP-B2 males. Three and four experiments were conducted for the VP-B1 and VP-B2 lines respectively. Each experiment comprised two groups (one group of controls, one group of salt-loaded) of four animals per group. The data from two independent experiments for each line are presented here. 50 μg total hypothalamic RNA was loaded into each lane. (**B**) Expression of endogenous (RVP) and transgene (BVP) vasopressin transcripts in control (C) and salt-loaded (S) VP-C3 males. Five independent experiments are shown. Each experiment carried two groups (one control, one salt-loaded) of five animals per group. 50 μg total hypothalamic RNA was loaded into each lane.

in this regard. In VP-B1 adrenal gland, transgene RNA was found to be restricted to cells in the medulla, indicating expression in neural cells. These data suggest that putative silencer element(s) contained within the BVP structural gene thus confine expression to neural cells. Neuron-specific expression is further restricted with the addition of more 5′ and 3′ flanking sequences. With the exception of the VP-C1 transgene, which is also expressed ectopically in the hippocampus and cortex, the VP-C transgene is expressed in specific hypothalamic cell groups, suggesting the involvement of additional silencer element(s). These data are consistent with the hypothesis that selective repression is, in part, responsible for the cell-specific expression of the BVP gene. Silencer elements may act in a tissue-specific manner such that expression in vasopressinergic cells in the hypothalamus remains unrepressed, whilst repressor function(s) are active in non-vasopressinergic neurons and in non-neuronal tissues.

A similar selective repression mechanism has been described for the neural-specific gene, SCG 10. Using transgenic models, Wuenschell *et al.* (1990) showed that SCG 10 has a 0.5 kb promoter proximal region with a constitutive enhancer-like element that elicited expression in the brain and a wide variety of non-neuronal tissues. A silencer located in a 3.5 kb fragment upstream of this promoter–enhancer region restricted transgene expression specifically to the brain and the adrenal glands. These authors proposed that the deregulated expression conferred by the

Table I. Mean percentage values (± SEM) of the levels of transgene BVP RNA and endogenous RVP RNA expression in response to osmotic stimuli in VP-B1, VP-B2, VP-C3 and VP-C4 transgenic mice

Line	*n*	Transcript	Control/salt-loaded (C/S)	Mean % (± SEM) (densitometric values)
VP-B1	3	RVP	C	100 ± 12
	3	RVP	S	196 ±36
	3	BVP	C	100 ± 19
	3	BVP	S	128 ± 20
VP-B2	4	RVP	C	100 ± 21
	4	RVP	S	189 ± 5*
	4	BVP	C	100 ± 31
	4	BVP	S	117 ± 8
VP-C3	5	RVP	C	100 ± 9
	5	RVP	S	141 ± 22
	5	BVP	C	100 ± 20
	5	BVP	S	241 ± 51*
VP-C4	5	RVP	C	100 ± 17
	5	RVP	S	223 ± 15*
	5	BVP	C	100 ± 18
	5	BVP	S	639 ± 172*

The values were calculated from densitometric readings of autoradiographic signals of Northern analyses. The levels of BVP or RVP RNA were determined relative to the level of an α-tubulin internal control and are expressed as a percentage of the mean of the control groups (mean ± SE). * $P < 0.05$ compared with controls (Mann–Whitney U-test). *n* represents the number of independent experiments carried out for the different lines.

proximal promoter region is restricted in a neural-specific way with the added tissue-specific repressor function mediated by the distal domain.

Transgene expression in the ovary is mediated by a strong enhancer element that attenuates repressor function

In the VP-C transgenic mouse lines we observed very abundant BVP RNA expression in the ovaries of mature females. This was in contrast to the VP-B mice which did not express detectable transgene VP RNA in their ovaries. These data suggest that an ovary-specific enhancer element may lie in the regions that flank the 1.25 kb BVP proximal promoter and the coding region. Thus, whilst selective repression may play a dominant role in achieving neuron-specific expression of the BVP gene, an attenuation mechanism involving cell-specific enhancer elements is apparently superimposed over lineage-specific repression in the ovary. Morley and Ivell (1987) have reported that the VP gene is expressed in the bovine ovary, although only aberrant, intron-containing VP cDNA clones could be isolated from a library made using mRNA from the bovine corpus luteum of an early non-pregnant cycle.

Expression of BVP transgenes in the murine hypothalamus

The distribution of hypothalamic vasopressinergic neurons in rat, mouse and cattle is species dependent. In the rat and in cattle, VP secretory cells in the PVN and the SON have a preferential topographical localization which distinguishes them from those neurons expressing the oxytocin (OT) neuropeptide (Swanson and Sawchenko, 1983; Dierickx, 1980). In cattle, the VP and OT neurosecretory cell types also show distinct morphological differences. However, vasopressinergic and oxytocinergic cells in the mouse PVN and SON intermingle (Castel and Morris, 1988). Considering the SCN, the dorsomedial region is the principal location of vasopressinergic neurons in the rat; in the mouse, VP-neurophysin immunoreactive cells are found throughout the SCN but are concentrated in clusters of cells in the dorsomedial, ventrolateral and ventromedial regions (Castel and Morris, 1988). To our knowledge, no studies investigating the distribution of vasopressinergic neurons in the SCN of cattle have been reported.

Serial sections of VP-B2 and VP-C4 brains showed BVP transcript expression patterns in the SON, including its retrochiasmatic division, and in the PVN, which parallel that of the endogenous murine VP gene (Figures 3B and C and 4). In the bovine SON, there is a distinct rostro-caudal compartmentalization of OT- and VP-secretory cells (Dierickx, 1980), whilst our ISHH data showed that BVP-expressing cells were distributed throughout the transgenic mouse SON, mimicking the murine VP expression pattern (Castel and Morris, 1988). The BVP expression pattern in the VP-B2 SCN also resembled that of murine VP. It would seem, therefore, that expression of BVP is 'mouse-like', reflecting the activation of BVP expression by transactivating factors shared with the murine VP transcription machinery.

The expression of the VP-C transgene, which shows the most restricted activity pattern, is limited to a subset of murine vasopressinergic neurons. These cells display a heterogeneity in their level of transgene expression both before and after salt-loading. Such heterogeneity in VP gene expression has also been observed in the vasopressinergic cells of the rat hypothalamus (Meeker *et al.*, 1991). We were not able to detect signals specific for the transgene in the SCN of mice carrying the VP-C transgene. Whilst this may reflect the species differences alluded to above, it may also be a consequence of the transgene lacking further important enhancer element(s). In this context it is important to consider the work of Young *et al.* (1990) who have described appropriate expression of the rat OT gene in transgenic mice. The OT gene is closely linked to the VP gene in all mammalian species examined. In the rat the two genes are only 8 kb apart and are transcribed towards each other from opposite strands of the DNA duplex (Mohr *et al.*, 1988a).

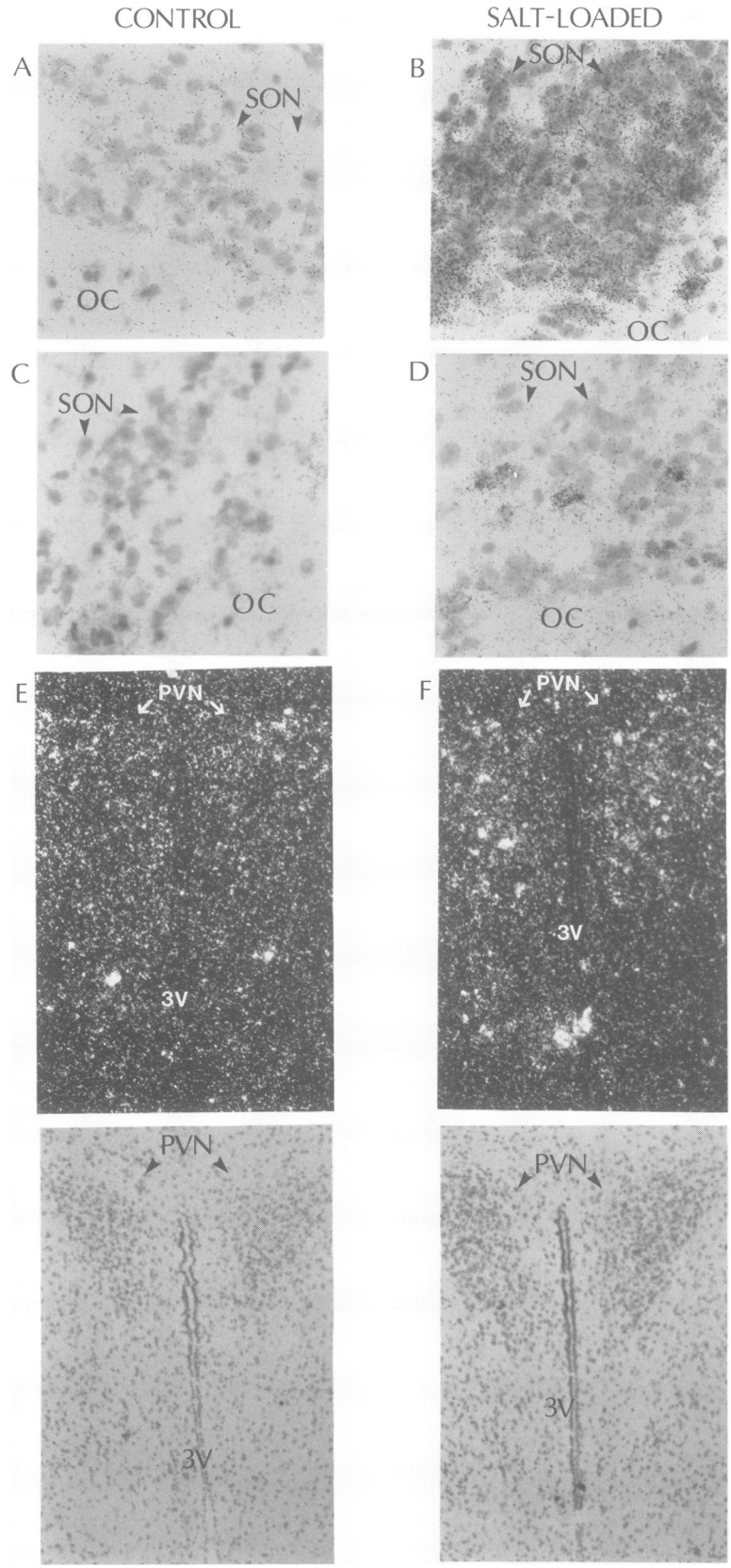


Fig. 6. ISHH of hypothalamic sections from control and salt-loaded VP-B1 and VP-B2 mice. Bright-field microscopy of the SON (~400× magnification) from control (**A**) and salt-loaded (**B**) VP-B2 transgenic brains and from control (**C**) and salt-loaded (**D**) VP-B1 brains. Dark-field (upper panel) and bright-field (lower panel) microscopy of the PVN (~100× magnification) of control (**E**) and salt-loaded (**F**) VP-B1 brains. Similar results (not shown) were obtained with transgene expression in PVN of control and salt-loaded VP-B2 brains. As a consequence of the osmotic stimulus of chronic 2% sodium chloride intake, the transgene signal in the SON and the PVN increases. The sections were counter-stained with 1.2% neutral red. Abbreviations: third ventricle (3V) and optic chiasm (OC).

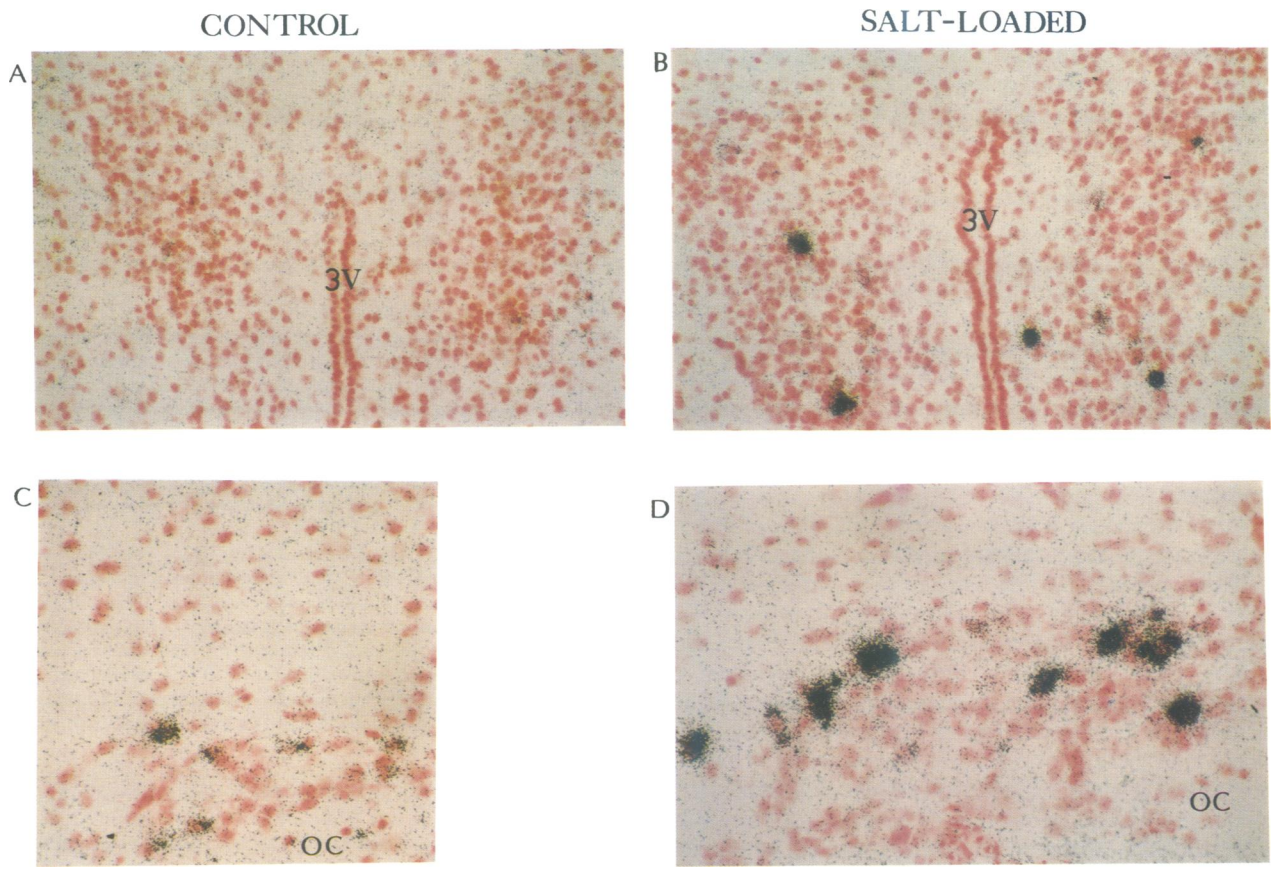


Fig. 7. ISHH of hypothalamic PVN and SON sections from control and salt-loaded VP-C4 animals. Bright-field microscopy of the PVN (~200× magnification) from control (**A**) and salt-loaded (**B**) transgenic brains and of the SON (~300× magnification) from similar control (**C**) and salt-loaded (**D**) animals. As a consequence of the osmotic stimulus of chronic 2% sodium chloride intake, more cells of the SON and the PVN express the transgene and the signal per cell increases. The sections were counter-stained with 1.2% neutral red. Abbreviations: third ventricle (3V) and optic chiasm (OC).

However, VP and OT are generally expressed in mutually exclusive sets of neurons in the SON and PVN (Mohr *et al.*, 1988b), except in circumstances such as chronic dehydration when a small subpopulation (1–2%) of magnocellular neurons expresses both VP and OT (Kiyama and Emson, 1990). Like Ang *et al.* (1991), who described the expression of a bovine OT transgene in mice, Young *et al.* (1990) failed to detect hypothalamic expression of a rat OT transgene. However, the OT component of a 'mini-locus' consisting of the rat OT gene linked to the rat VP gene was appropriately expressed in murine magnocellular neurons, although the VP segment was silent. These data are strongly suggestive of a regulatory interaction between different elements located in the two transcription units of the VP-OT locus.

Physiological regulation of bovine vasopressin transgenes in the mouse hypothalamus

Although the VP-B transgene does not confer an appropriately restricted pattern of expression of BVP in the brain, transgene expression in the PVN and the SON is upregulated by osmotic challenge. The VP-C transgene shows both restricted and appropriate expression in subsets of neurons within the mouse hypothalamus and also contains sufficient regulatory elements to mediate a physiological change in its expression pattern. The osmotic challenge of salt-loading resulted in a significant increase in BVP RNA in the hypothalami of two VP-C lines, an upregulation which paralleled that of the endogenous gene. Further, ISHH of hypothalami from the VP-C4 line showed that, after salt-loading, more cells in the SON, PVN and accessory magnocellular neurons contained detectable BVP RNA and that the signal per cell was stronger. These data suggest that the regulation of the BVP gene in response to osmotic challenge involves elements common to both VP-B and VP-C transgenes. We have previously demonstrated that levels of cAMP increase in the rat SON as a consequence of a hyperosmolar stimulus (Carter and Murphy, 1989c). Other studies have also implicated cAMP as a mediator of the osmotic regulation of the VP gene and, by transfection into a heterologous cell line, we have described a cAMP response element (CRE) of the CREB/AP-1/ATF family located in the proximal BVP promoter (Pardy *et al.*, 1992). Nuclear proteins from rat SON have been shown to bind to the region of the BVP promoter containing the CRE (Pardy *et al.*, 1992). As the CRE element is present in the VP-B and VP-C transgenes, and they are both responsive to a hyperosmolar stimulus, it follows that the CRE may be important for osmotic regulation. VP-C derivatives lacking the BVP CRE are currently being introduced into mice to directly test the importance of this element in the physiological regulation of the BVP gene.

In the rat SON, the increase in VP mRNA level consequent to an osmotic stimulus is accompanied by a dramatic increase in poly(A) tail length (Carrazana *et al.*, 1988; Zingg *et al.*, 1988; Carter and Murphy, 1989a). However, in the VP-C

mice, the increase in BVP RNA level upon salt-loading was not accompanied by an increase in message size (Figure 5). Similarly, the endogenous murine VP RNA fails to increase in size upon osmotic challenge (Figure 5; Murphy *et al.*, 1989; Murphy and Carter, 1990), indicating that, at least in this species, poly(A) tail length modulation plays no role in the adaptive response of the animal to dehydration. It is unclear whether the lack of poly(A) tail length shifts is a consequence of an absence of poly(A) tail length regulatory factors in the mouse hypothalamus, or is a function of the *cis*-acting sequences contained (or not) within both the murine and the bovine transcripts. It is also not known whether an osmotic stimulus regulates the poly(A) tail length of the BVP RNA when expressed in its homologous species environment.

Summary

The following model of BVP gene expression is consistent with our results. The proximal promoter contains little or no tissue-specific information and is influenced by neighbouring elements. The activity of this promoter is selectively repressed in non-neuronal peripheral tissues and neuronal extrahypothalamic brain regions by factors interacting with at least two silencer elements located in the structural gene and flanking regions. Enhancer elements also reside within these flanking regions; at least one is ovary-specific. The activity of other enhancers is mediated by physiological stimuli. For example, elements shared by VP-B and VP-C mediate the osmotic upregulation of the BVP gene. Transgenic analysis of the BVP gene thus reveals a hierarchy of elements, not only repressor and enhancer elements which mediate cell-specific expression, but also elements that mediate responses to physiological cues in a cell-specific manner. Such complexity may engender a plasticity consistent with the varied, cell-specific roles of VP in the maintenance of homeostasis.

Materials and methods

Constructs for microinjection

VP-A (Figure 1A). This transgene was derived from plasmid pbAVPCAT1.25 (Murphy *et al.*, 1987). The plasmid was digested to completion with *Bam*HI and partially with *Sal*I. The 2.85 kb insert excised contained 1.25 kb of the VP proximal promoter region fused to the CAT reporter gene, the SV40 small t-antigen intron and the SV40 early region polyadenylation and transcription termination sequences.

VP-B (Figure 1B). This transgene contained 3.45 kb of BVP 5′ and coding sequences. It was derived from two plasmids: pUCVP2A (Murphy *et al.*, 1987), which contains the 1.25 kb VP proximal promoter, and pbAVP5.5E (Ruppert *et al.*, 1984), a BVP genomic sub-clone containing the entire BVP coding sequences and 2.5 kb of sequences upstream of the start of transcription. pbAVP5.5E was digested with *Xba*I, giving two fragments. The 2.4 kb fragment, which contained the coding region of the VP gene, was ligated to *Xba*I-linearized pUCVP2A. The transgene contained the promoter sequences linked to the coding region in the native orientation. However, because of the enzyme sites chosen for the subcloning, the recombinant plasmid contained a direct repeat of promoter sequences, defined by *Xba*I and *Sal*I sites, located downstream of the BVP coding region. For microinjection, the recombinant plasmid was digested completely with *Sal*I and then partially digested with *Xba*I. The 3.45 kb fragment thus obtained excluded the repeated fragment.

VP-C (Figure 1C). The 13.4 kb genomic fragment of the VP gene was cloned into the EMBL 3 replacement vector at the *Sal*I sites flanking the stuffer fragment and designated λVpNpII and was kindly given by Dr Siegfried Ruppert (Ruppert *et al.*, 1984). It contained 9 kb of 5′ sequences, 2.2 kb of the coding region and 1.5 kb of 3′ sequences.

Production of transgenic mice

Transgenic mice were generated by microinjecting fertilized one-cell mouse oocytes obtained from F1 (CBA/J×C57BL/10) mice with the constructs described above. DNA fragments were introduced into the male pronuclei and viable eggs were then transferred into the oviducts of pseudopregnant surrogates according to procedures described by Murphy and Hanson (1987). Transgenic pups were identified by Southern analysis of restriction-digested genomic DNA isolated from tail biopsies. Founders were outbred with wild type F1 mates to establish independent lines with single transgene DNA integration events. Only Southern blot positive heterozygotes were used for expression analysis and in the physiological experiments.

Northern analysis of tissue RNA

Northern analysis was used to describe the tissue distribution of transgene expression. Eight males and four females from each transgenic line were sacrificed for the dissection of tissues. Extraction of total cellular RNA from mouse tissues and analysis of RNA on Northern blots were performed according to the methods described by Murphy *et al.* (1989).

The following oligonucleotide probes were employed. All were synthesized in the laboratory of Dr Ben Li (National University of Singapore) and end-labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs).

The BVP probe. This was a 35mer antisense synthetic oligonucleotide complementary to nucleotides 2132−2166 (sequences in exon III encoding the C-terminus of neurophysin and the N-terminus of the glycopeptide) of the published genomic sequence (Ruppert *et al.*, 1984; Genbank accession no. X00503). This probe was specific for the BVP transcript and did not cross-hybridize with the RVP message. Hybridization was carried out overnight at 55°C and washing at room temperature for 5 min and then at 65°C for 10 min.

The RVP probe. This was an antisense 48mer synthetic oligonucleotide and corresponded to nucleotides 2254−2301 of the RVP genomic sequences, which are from exon III and encode the last 16 amino acids of the VP precursor (Schmale *et al.*, 1983; Genbank accession no. X01637). Hybridization and washing were performed at 65°C.

The α-tubulin probe. This 20mer oligonucleotide was designed by NEN−Dupont (Boston, MA). Hybridization and washing were performed at 65°C.

One double stranded DNA probe was used. The 1.6 kb CAT probe was derived from pbAVPCAT1.25 (Murphy *et al.*, 1987) by digesting with the enzymes *Hin*dIII and *Bam*HI. The fragment was labelled by the random primer method using [α-^{32}P]dCTP (Feinberg and Vogelstein, 1983). Hybridization and washing were performed at 65°C.

The levels of transgene and endogenous VP RNA were determined by densitometric scanning of autoradiograms using the Visage 110 (Bioimage) with the Sunview program and corrected against the level of α-tubulin RNA. Note that short exposure, linear range autoradiograms were used for densitometric scanning. The significance of changes in RNA levels was determined by applying the Mann−Whitney U-test.

In situ hybridization

The protocols used have been described (Funkhouser, 1992; adapted from Young and Zoeller, 1987). 10 μm serial sections of brains from F1 (CBA/J×C57BL/10), VP-B1, VP-B2, VP-C3 and VP-C4 mice and 10 μm sections of adrenal gland of F1, VP-B1 and VP-B2 were used. Oligonucleotide probes used in these experiments were the same as those used in the Northern analysis. Probes were 3′ end-labelled using [^{35}S]dATP and terminal deoxynucleotidyl transferase. Hybridization of the slides was carried out at 43°C for BVP and RVP probes. Washing was performed at 60°C for 4 h in 0.1×SCC. In every experiment, tissue sections of F1 (CBA/J×C57BL/10) mice were used as controls.

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References

Ang,H.-L., Ungefroren,H., de Bree,F., Foo,N.-C., Carter,D., Burbach,J.P., Ivell,R. and Murphy,D. (1991) *Endocrinology*, **128**, 2110–2117.

Brownstein,M., Russel,J. and Gainer,H. (1980) *Science*, **207**, 373–378.

Buijs,R.M., Kalsbeek,A., van der Woude,T.P. and van Heerikhuize,J.J. (1990) *Soc. Neurosci. Abstr.*, **16**, 292.15.

Burbach,J.P.H., de Hoop,M.J., Schmale,H., Richter,D., de Kloet,E.R., Ten Haaf,J.A. and de Wied,D. (1984) *Neuroendocrinology*, **39**, 582–584.

Carrazana,E.J., Pasieka,K.B. and Majzoub,J.A. (1988) *Mol. Cell. Biol.*, **8**, 2267–2274.

Carter,D.A. and Murphy,D. (1989a) *J. Biol. Chem.*, **264**, 6601–6603.

Carter,D.A. and Murphy,D. (1989b) *Mol. Brain Res.*, **6**, 233–239.

Carter,D.A. and Murphy,D. (1989c) *Brain Res.*, **487**, 350–356.

Carter,D.A. and Murphy,D. (1990) *Neurosci. Lett.*, **109**, 180–185.

Carter,D.A. and Murphy,D. (1991a) *Neuroendocrinology*, **53**, 1–6.

Carter,D.A. and Murphy,D. (1991b) *Mol. Brain Res.*, **12**, 315–321.

Castel,M. and Morris,J.F. (1988) *Neuroscience*, **24**, 937–966.

Dierickx,K. (1980) *Int. Rev. Cytol.*, **62**, 119–185.

Feinberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6–13.

Funkhouser,J. (1992) In Murphy,D. and Carter,D.A. (eds), *Transgenesis Techniques: Principals and Protocols*. Humana Press, Clifton, NJ, in press.

Gillies,G.E., Linton,E.A. and Lowry,P.J. (1982) *Nature*, **299**, 355–357.

Goldfien,A. (1986) In Greenspan,F.S. and Forsham,P.H. (eds), *Basic and Clinical Endocrinology*. Lange Medical Publications, Los Altos, CA, pp. 326–344.

Hara,Y., Battey,J. and Gainer,H. (1991) *Mol. Brain Res.*, **8**, 319–324.

Herman,J.P., Schafer,M.K.-H., Watson,S. and Sherman,T.G. (1991) *Mol. Endocrinol.*, **5**, 1447–1456.

Horn,A.M, Robinson,I.C.A.F. and Fink,G. (1985) *J. Endocrinol.*, **104**, 211–224.

Ivell,R. (1987) In Turner,A.J. (ed)., *Neuropeptides and Their Peptidases*. Ellis Horwood Ltd, Chichester, UK, pp. 31–60.

Ivell,R. and Burbach,J.P.H. (1991) *J. Neuroendocrinol.*, **3**, 583–585.

Ivell,R. and Richter,D. (1984) *EMBO J.*, **3**, 2351–2354.

Kiyama,H. and Emson,P.C. (1990) *J. Neuroendocrinol.*, **2**, 257–260.

Leeson,T.S., Leeson,C.R. and Paparo,A.A. (1988) In Wonsiewicz,M. (ed.), *Atlas of Histology*. International edition. W.B.Saunders, Philadelphia, PA, pp. 586–593.

Levy,A., Lightman,S.L., Carter,D.A. and Murphy,D. (1990) *J. Neuroendocrinol.*, **2**, 329–334.

Lightman,S.L. and Young,W.S.,III (1987) *J. Physiol.*, **394**, 23–39.

McKnight,R.A., Shamay,A., Sankaran,L., Wall,R.J. and Hennighausen,L. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6943–6947.

Meeker,R.B., Greenwood,R.S. and Haywood,J.N. (1991) *Neuroendocrinology*, **54**, 236–247.

Mercer,E.H., Hoyle,G.W., Kapur,R.P., Brinster,R.L. and Palmiter,R.D. (1991) *Neuron*, **7**, 703–716.

Mohr,E., Schmitz,E. and Richter,D. (1988a) *Biochimie*, **70**, 649–654.

Mohr ,E., Bahnsen,U., Kiessling,C. and Richter,D. (1988b) *FEBS Lett.*, **242**, 144–148.

Mohr,E., Fehr,S. and Richter,D. (1991) *EMBO J.*, **10**, 2419–2424.

Moore,R.Y. (1983) *Fed. Proc.*, **42**, 2783–2789.

Morley,S.D. and Ivell,R. (1987) *Mol. Cell. Endocrinol.*, **53**, 255–258.

Murphy,D. and Carter,D. (1990) *Mol. Endocrinol.*, **4**, 1051–1059.

Murphy,D. and Hanson,J. (1987) In Glover,D.M. (ed.), *DNA Cloning: A Practical Approach*. IRL Press, Oxford, Vol. III, pp. 213–248.

Murphy,D., Bishop,A., Rindi,G., Murphy,M.N., Stamp,G.W.H., Hanson,J., Polak,J.M. and Hogan,B.L.M. (1987) *Am. J. Pathol.*, **129**, 552–566.

Murphy,D., Levy,A., Lightman,S. and Carter,D. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 9002–9005.

Murphy,D., Ang,H.-L., Zeng,Q., Ho,M.Y., Funkhouser,J. and Carter,D. (1992a) *Prog. Brain Res.*, **92**, 77–96.

Murphy,D., Funkhouser,J., Ang,H.-L., Foo,N.-C. and Carter,D.A. (1993) *Ann. N.Y. Acad. Sci.*, in press.

Pardy,K., Adan,R.A.H., Carter,D.A., Seah,V., Burbach,J.P.H. and Murphy,D. (1992) *J. Biol. Chem.*, **267**, 21746–21752.

Rehbein,M., Hillers,M., Mohr,E., Ivell,R., Morley,S. Schmale,H. and Richter,D. (1986) *Biol. Chem. Hoppe-Seyler*, **367**, 695–704.

Richter,D. (1988) *Am. J. Physiol.*, **255**, F207–F219.

Rivier,C. and Vale,W. (1982) *Endocrinology*, **113**, 937–942.

Robinson,B.G., Frim,D.M. Schwartz,W.J. and Majzoub,J.A. (1988) *Science*, **241**,342- 344.

Ruppert,S., Scherer,G. and Schutz,G. (1984) *Nature*, **308**, 1–3.

Russo,A.F., Crenshaw,E.B., Lira,S.A., Simmons,D.M., Swanson,L.W. and Rosenfeld,M.G. (1988) *Neuron*, **1**, 311–320.

Schmale,H., Heinsohn,S. and Richter,D. (1983) *EMBO J.*, **2**, 763–767.

Sherman,T.G., Day,R., Civelli,O., Douglass,J., Herbert,E., Akil,H. and Watson,S.J. (1988) *J. Neurosci.*, **8**, 3785–3796.

Sodersten,P., de Vries,G.J., Buijs,R.M. and Melin,P. (1985) *Neurosci. Lett.*, **58**, 37–41.

Stefaneanu,L., Rindi,G., Horvath,E., Murphy,D., Polak,J.M. and Kovacs,K. (1992) *Endocrinology*, **130**, 1789–1795.

Swanson,L.W. and Sawchenko,P.E. (1983) *Annu. Rev. Neurosci.*, **6**, 269–324.

Uhl,G.R. and Reppert,S.M. (1986) *Science*, **232**, 390–393.

van den Pol,A.N. (1980) *J. Comp. Neurol.*, **191**, 661–702.

Watts,A.G.and Swanson,L.W. (1987) *J. Comp. Neurol.*, **258**, 230- 252.

Wolfson,B., Manning,R.W., Davis,L.G., Arentzen,R. and Baldino,F. (1985) *Nature*, **315**, 59–61.

Wuenschell,C.W., Mori,N. and Anderson,D.J. (1990) *Neuron*, **4**, 595–602.

Young,W.S. and Zoeller,R.T. (1987) *Cell. Mol. Neurobiol.*, **7**, 353–366.

Young,W.S., Mezey,E. and Siegel,R.E. (1986) *Mol. Brain Res.*, **1**, 231–241.

Young,W.S., Reynolds,K., Shepard,E.A., Gainer,H. and Castel,M. (1990) *J. Neuroendocrinol.*, **2**, 1–9.

Zimmerman,E.A. and Silverman,A.-J. (1983) *Prog. Brain Res.*, **60**, 493–504.

Zingg,H.H., Lefebvre,D. and Almazan,G. (1986) *J. Biol. Chem.*, **261**, 12956–12959.

Zingg,H.H., Lefebvre,D. and Almazan,G. (1988) *J. Biol. Chem.*, **263**, 11041–11043.

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