Species- and tissue-specific physiological regulation of vasopressin mRNA poly(A) tail length

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Si-Hoe, San-Ling, David Carter, and David Murphy. Species- and tissue-specific physiological regulation of vasopressin mRNA poly(A) tail length. Physiol Genomics 5: 1–9, 2001.—Transgenic experiments can be used to test the extent to which genes from different species can be swapped around, but still retain function, and be appropriately regulated. A vector has been developed that directs the expression of foreign genes to specific groups of vasopressin (VP) hypothalamic neurons in transgenic rats. Using this vector, we have expressed the bovine VP (bVP) RNA in the rat brain. In contrast to the situation in a mouse host, but like its endogenous rat counterpart, the mRNA encoded by the bVP transgene is subject to posttranscriptional physiological regulation in the hypothalamus; its poly(A) tail dramatically lengthens as a consequence of 3 days of dehydration. Transgene expression is also seen in the adrenal cortex, but here, despite a marked increase in transgene RNA levels with dehydration, there is no change in poly(A) tail length. These data suggest that the mouse hypothalamus and the rat adrenal gland do not have the transcript recognition or enzymatic machinery required for the physiologically responsive poly(A) tail length modulation seen in the rat brain.

Mammals respond to changes in osmolarity of their extracellular fluid by altering their behavior and physiology. The behavioral response entails regulation of the salt and water intake through changes in sodium appetite and thirst. The physiological response involves the modulation of the renal excretion of water, which is achieved through changes in the plasma concentrations of the 9-amino acid neuropeptide vasopressin (VP), the antidiuretic hormone (36, 40). This, in turn, is achieved through regulation of VP secretion from the posterior pituitary (5). Renal V2 receptors mediate VP-induced tubular reabsorption of water via induction of intracellular cAMP production in collecting duct cells (34), thereby conserving stores of bodily fluid during times of restricted intake.

VP is synthesized in, and secreted from, a specialized part of the brain called the hypothalamoneurohypophysial system (HNS), which consists of specific neuronal cell bodies in the hypothalamus and their axonal projections, which terminate in the posterior pituitary gland. VP is synthesized in the cell bodies of 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. VP neurons are found in the magnocellular supraoptic nucleus (SON) and in the paraventricular nucleus (PVN). The closely related neuropeptide oxytocin (OT) is also expressed in magnocellular neurons of the SON and PVN, but VP and OT are rarely found in the same cell (24, 27). It is the magnocellular neurons that are involved in osmoregulation. Within these neurons, VP gene expression is upregulated to compensate for the depletion of posterior pituitary stores that accompanies a physiological stimulus (7, 32). Osmotic stimuli such as dehydration (fluid deprivation) or salt loading (the normal tap water diet is replaced with a solution of 2% wt/vol NaCl) result in an increase in VP gene transcription (29) and a concomitant increase in VP mRNA abundance (3, 6, 20, 25, 29, 39, 54). The OT gene is similarly regulated by osmotic stimuli (10, 11, 25, 42), despite being expressed in distinct magnocellular cells.

In the rat, VP and OT gene expression is also modified at the posttranscriptional level by an osmotic stimulus; the poly(A) tail length of the rat VP and OT RNAs increases in length from around 200 to 400 residues (9, 11, 29). This process is specific to the VP and OT RNAs and does not affect coexpressed messenger RNAs (11). However, in the mouse, equivalent osmotic stimuli do not alter the poly(A) tail lengths of either the VP or OT RNAs (29).

Transgenic experiments can be used to test the extent to which genes from different organisms can be swapped around but still retain function (26). Thus, using transgenic mice harboring bovine VP (bVP) genes, Ang et al. (2) saw no increase in the poly(A) tail length of the transgene RNAs in the host hypothalamus following an osmotic stimulus, but it was unclear whether this was due to the lack of such a system in cattle, or the presence of the system in cattle, but the...
lack thereof in mice. The fate of the endogenous bVP mRNA poly(A) tail within the physiologically stimulated bovine hypothalamus is not, and probably will never, be known. While being of great agricultural interest, cattle represent a somewhat problematic experimental model. We therefore sought a more tractable experimental model organism and have used a novel transgenic expression vector system to direct the transcription of bVP RNA in rat VP neurons. We show that the bovine transgene encoded VP RNA undergoes tissue-specific poly(A) tail lengthening in the rat host.

EXPERIMENTAL PROCEDURES

Availability. All original materials described in this study are available to the research community, subject to the conditions of appropriate Materials Transfer Agreements.

Transgene construction. We have previously described our success in generating transgenic rats bearing a VP transgene that shows an appropriate cell-specific and physiological expression (47, 51). This transgene construct (5-VCAT-3), consists of the rat VP structural gene containing a chloramphenicol acetyl transferase (CAT) reporter sequence in exon III, flanked by 5 kbp of upstream and 3 kbp of downstream VP gene sequence (Fig. 1B). 5-VCAT-3 is expressed in VP, but not OT, magnocellular neurons, within which it is subject to dramatic upregulation by osmotic stimuli (47, 51). Site-directed mutagenesis was used to derive 5-VCAT-3-Sal (Fig. 1, C and D). Using a modification of the method of Deng and Nickoloff (14), we replaced the three translation initiation codons of the VP prepropeptide with a unique SalI restriction endonuclease recognition site (46; Fig. 1D). We have previously described the expression in mice of the bovine vasopressin transgene VP-B, which was derived from genomic sequences (37). VP-B is a 3.45-kbp VP transgene fragment consisting of the structural gene flanked by ~1.25 kbp of upstream and 0.2 kbp of downstream sequences (2). In mice, this transgene was expressed throughout the central nervous system, including the hypothalamus (2). Within the SON and PVN, VP-B RNA was upregulated by osmotic stimuli that increase the level of the endogenous VP RNA (2). VP-B was cut with XbaI to liberate a DNA fragment consisting of the bVP structural gene flanked by ~0.2 kbp of upstream and 0.2 kbp of downstream sequences. This 2.4-kbp fragment was introduced, via XhoI linkers, into the SalI site of 5-VCAT-3-Sal to give 5-VCAT-3-bVP (Fig. 1E). The transgene fragment, consisting of the bVP gene within the 5-VCAT-3 background, was excised from vector with NotI and microinjected into fertilized one-cell rat eggs. Of the four independently derived transgenic founders generated using the 5-VCAT-3-bVP construct, one rat failed to transmit the transgene, leaving three lines, which were designated 5-VCAT-3-bVP1–3. Line 5-VCAT-3-bVP3 was lost upon transfer from Singapore to the United Kingdom.

Animals. Animals were cared for in accord with National Institutes of Health guidelines and UK Home Office regulations. Transgenic rats were generated by microinjecting fertilized one-cell eggs obtained from Sprague-Dawley donors. All procedures have been described (31, 44, 45). Dehydration of rats involved total fluid deprivation for up to 72 h. Glucocorticoids were depleted with 11β-hydroxylase inhibitor metyrapone. Rats were treated with either metyrapone (Sigma, 100 µg/kg sc) or vehicle [dimethyl sulfoxide (DMSO)] following the protocol described by Burke et al. (8). An initial injection of metyrapone was administered at 1800 on day 1, followed by a second injection at 0900 on day 2, after which rats were killed 3 h postinjection. In the combined metyrapone-dehydration treatment, the first metyrapone injection was given on the first day of dehydration.

Expression analysis. Only Southern blot-positive obligate heterozygous male transgenic animals were used for expression analysis. Total cell RNA was extracted from tissue samples using Trizol (Life Technologies, Gaithersburg, MD) and was analyzed by Northern blotting (1, 16, 28). In some
experiments, poly(A) tails were removed from RNAs by hybridization to oligo dT (Pharmacia, Uppsala, Sweden), followed by RNase H digestion (9). Northern blots were controlled for equal loading and even transfer in two ways. First, following transfer, the ethidium bromide-stained filter was viewed and photographed under ultraviolet light. Second, filters were routinely reprobed with control oligonucleotides corresponding to the mRNAs encoding either glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or α-tubulin. The abundance of these RNAs does not change in the hypothalamus following physiological stimulation and can be thus used as quantitation standards. Note that α-tubulin and GAPDH RNAs are expressed at different levels in different tissues and that the relative pattern of expression observed is consistent between animals. The oligonucleotide probes specific for the rodent VP (rVP) and bVP RNAs have been described (1, 16, 28). In some experiments, sections were simultaneously hybridized with radiolabeled and digoxigenin-labeled probes to reveal the relative localization of two different RNA species (45). Each experiment was performed at least twice on both the 5-VCAT-3-bVP/1 and 5-VCAT-3-bVP/2 lines, with three groups each time. Similar results were obtained for both lines, although data for line 5-VCAT-3-bVP/1 is mainly shown here.

RT PCR analysis. RT-PCR analysis of VP expression was performed essentially as described previously (8). Total cellular adrenal RNA (2 μg) from wild-type rats was treated with DNase I to eliminate genomic contamination and then reverse transcribed using a SuperScript preamplification system (Life Technologies). cDNAs were amplified on an MJ Research model PTC-100 thermal cycler (Genetic Research Instruments). Amplification reactions contained 2 μl cDNA (of 20 μl total synthesized), 2 μl DMSO, 0.5 μl MgCl2 (25 mM), 2 μl PCR reaction buffer (Promega), 1 μl dNTPs (Pharmacia Biotech, 2 mM), 0.5 μl Taq polymerase (Promega), 2 μl each of forward and reverse primers (50 pmol, Genosys), and sterile water to a final volume of 20 μl. Primers used were: forward, 5'-CCGCCGCCGGCATCTGCTGCAACGAT-3', and reverse, 5'-GGGGAGGCGTGGGGGGCGATG-3', which were predicted to amplify a 223-bp fragment from rat cDNA. This amplified fragment size is specific for cDNA as the primer pair spans an intron in the genomic sequence. The PCR amplification reaction of 92°C for 30 s, 60°C for 30 s, and 75°C for 90 s was repeated for 35 cycles. Taq polymerase was added to the amplification mix during an initial denaturation step (94°C, 3 min) to reduce nonspecific amplification, and a final, prolonged extension step (75°C, 5 min) was also included. Aliquots of the amplification reactions were electrophoresed on 1% (wt/vol) agarose gels containing ethidium bromide, and size estimates were made with reference to a 1-kbp DNA marker (Life Technologies). Resolved DNA bands were transferred to a nylon membrane (Hybond-N, Amer sham) and processed for Southern analysis as described (8) using a 32P-labeled oligonucleotide specific for a rodent AVP sequence (1, 8, 16, 28).

RESULTS

Transgene expression. Two independently derived lines (5-VCAT-3-bVP/1 and 5-VCAT-3-bVP/2) were analyzed in this study, thus eliminating the possibility that a transgenic phenotype was due to the integration of the transgene into a particular chromosomal site. Following the identification of transgenic founders, lines were generated through crosses with wild-type Sprague-Dawley mates. At each generation, individual obligate heterozygous rats were identified by Southern blot hybridization analysis of tail genomic DNA, using a bVP-specific probe. Line 5-VCAT-3-bVP/1 contained 30 copies of the transgene per haploid genome, whereas line 5-VCAT-3-bVP/2 contained 4 copies. Using Northern blotting, we observed a high level of transgene expression in the adrenal gland of both lines (Fig. 2). In situ hybridization analysis showed specific expression of the bVP transgene in the rat adrenal cortex, most prominently within the concentric zone corresponding to the glucocorticoid producing zona fasciculata (Fig. 3). Neither Northern blotting nor in situ hybridization could detect endogenous wild-type rat VP mRNA in the adrenal gland (data not shown); only the sensitivity of RT-PCR could reveal expression (Fig. 4). Lower levels of transgene expression were also seen in lung and heart of line 5-VCAT-3-bVP/2 and in spleen, heart, thymus, kidney, testis, and liver of line 5-VCAT-3-bVP/1. In control (euthydrated) animals, very low expression of transgene RNA was observed in a dissection of the brain that excluded the hypothalamus (Fig. 2). Northern blotting (Fig. 5) revealed expression in the hypothalamus of line 5-VCAT-3-bVP/1, and in situ hybridization analysis of coronal brain sections (Fig. 6) showed this to be confined to the SON and PVN. Although Northern blotting was not sufficiently sensitive to detect expression in the hypothalamus of line 5-VCAT-3-bVP/2, in situ hybridization analysis of coronal brain sections (Fig. 6) revealed

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specific expression in the SON and PVN. As in the 5-VCAT-3 lines (51), no expression was seen in the suprachiasmatic nucleus of either lines (not shown).

**Physiological regulation of transgene expression.** Dehydration for 3 days markedly and specifically increased the abundance of transgene transcripts in the hypothalamus of line 5-VCAT-3-bVP/1, as revealed by Northern blotting (Fig. 5) and in situ hybridization (Fig. 6). Upregulation of transgene expression was also seen in line 5-VCAT-3-bVP/2, particularly in the PVN (Fig. 6).

Double in situ hybridization analysis revealed that, within the SON and PVN of dehydrated rats, expression of the bovine VP transgene was confined to cells expressing the endogenous rat VP gene (Fig. 7). The abundance of transgene RNA in the brain dissection that excluded the hypothalamus was not altered by dehydration (Fig. 2). Unexpectedly, the same paradigm also resulted in an increase in transgene expression in the adrenal gland, as revealed by Northern blotting (Figs. 5 and 8) and in situ hybridization (Fig. 3).

To test the hypothesis that glucocorticoids may be responsible for the osmotic effect on adrenal transgene expression, glucocorticoid synthesis in the adrenal cortex was blocked by treatment with metyrapone (an 11β-hydroxylase inhibitor). Metyrapone treatment in a normally hydrated rat resulted in a marked upregulation of the level of adrenal transgene mRNA compared with vehicle-treated controls (Fig. 8). Vehicle treatment did not affect transgene mRNA levels compared with untreated control rats (data not shown). The role of glucocorticoids on the upregulation of expression with dehydration was then examined. Metyrapone administration over the course of a 3 days of dehydration did not abolish, but rather exaggerated, the osmotic response of adrenal transgene transcripts (Fig. 8).

**Tissue-specific elongation of the poly(A) tail on dehydration.** In the hypothalamus, the size of the transgene RNA increased following 3 days of dehydration (Fig. 5), in parallel with the endogenous rat VP message (Fig. 5). This increase was a consequence of a lengthening of the poly(A) tail; when the poly(A) tract was removed, the transgene core RNAs, like the endogenous VP...
mRNAs, from control and dehydrated rats comigrated (Fig. 5). No such increase in transcript size was detected with the adrenal transgene transcripts, despite their increase in abundance with dehydration (Figs. 5 and 8).

**DISCUSSION**

We have described a rat VP expression vector that is able to direct the cell-specific and physiological expression of foreign genes to specific neurons in the rat hypothalamus. Using this vector, we have directed the expression of the bVP RNA to rat VP magnocellular neurons and to the cells of the adrenal cortex. Hypothalamic mRNA encoded by the bVP transgene is subject to posttranscriptional physiological regulation in the rat host; its poly(A) tail increases as a consequence of 3 days of dehydration, a contrast to the situation in both the transgenic mouse hypothalamus and the transgenic rat adrenal. Differential polyadenylation is thus species and tissue specific.

The 5-VCAT-3-Sal expression system that we have developed is able to direct the expression of certain types of foreign gene to the VP magnocellular neurons and to the cells of the adrenal cortex. Hypothalamic mRNA encoded by the bVP transgene is subject to posttranscriptional physiological regulation in the rat host; its poly(A) tail increases as a consequence of 3 days of dehydration, a contrast to the situation in both the transgenic mouse hypothalamus and the transgenic rat adrenal. Differential polyadenylation is thus species and tissue specific.

The 5-VCAT-3-Sal vector (46) failed to elicit any expression. In contrast, we now show that an intact structural gene, which includes its own intron and transcription termination sequences, is expressed. We suggest that the lack of expression of cDNAs is due to the recognition, by the host, of a premature termination codon introduced into exon I and the subsequent degradation of the message by nonsense-mediated decay (NMD; 19). Mammalian NMD discriminates between premature translation stop codons and normal stop codons by assessing their position relative to a point of reference defined by the final exon/intron junction. Premature termination codons located 50 bases 5' of the final splice junction promote NMD, whereas mRNAs with a premature termination codon 3' to this boundary are stable (41, 52, 53). Thus NMD renders unstable any coding sequence inserted into exon I of the VP gene, but not exon III. Indeed, the 5-VCAT-3 transgene was efficiently expressed in transgenic rats despite the insertion of bacterial chloramphenicol acetyl transferase sequences, including a new translational termination codon, into exon I of the VP gene, but not exon III. Indeed, the 5-VCAT-3 transgene was efficiently expressed in transgenic rats despite the insertion of bacterial chloramphenicol acetyl transferase sequences, including a new translational termination codon, into exon III (52), a position downstream of the final exon/intron junction that would not elicit NMD. Similar results have been obtained in transgenic mice bearing mouse OT gene-derived expression vectors (50). Transgenes with insertions in exon I were not expressed efficiently, but transgenes with insertions in exon III were robustly expressed in the PVN and SON. These results demonstrate that, like the VP gene, the OT gene is inefficiently expressed if exon I is disrupted by sequences without introns. However, like the VP gene, disruption of OT exon III is tolerated.

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Following the onset of an osmotic stimulus, the poly(A) tail length of the rat VP mRNA increases in length from around 200 to 400 residues (9). The osmotic effect on the VP mRNA poly(A) tail length is species specific, having been observed in the rat (9) and chicken (12) but not in the mouse (29). Two hypotheses can explain these species differences: either the differences reside in the gene sequences that mediate the recognition of these mRNAs by the enzymatic machinery mediating poly(A) tail shifts, or the differences reside in the machinery itself. Transgenesis experiments, involving the transfer of genes between species, have allowed these hypotheses to be directly tested. A rat VP transgene mRNA increases in abun-
The VP mRNA poly(A) tail, but had no effect on VP kinase C (PKC) pathway also increased the length of poly(A) tail length (15). Stimulation of the protein mRNA abundance and an increase in the VP mRNA resulted in an increase in the abundance of the VP thalamic cultures with stimulators of the PKA pathway. This is unlike poly(A) tail whose position of a putative precursor molecule is also shown.

The VP mRNA poly(A) tail length modulation. Evidence from Xenopus suggests that the length of the poly(A) tail is governed by cis-acting elements within the nuclear pre-mRNA that presumably interact with factors that control the polyadenylation machinery (13, 35). There is also little information regarding the signaling pathways that mediate the poly(A) tail effect in rats. Treatment of fetal hypothalamic cultures with stimulators of the PKA pathway resulted in an increase in the abundance of the VP mRNA and an increase in the VP mRNA poly(A) tail length (15). Stimulation of the protein kinase C (PKC) pathway also increased the length of the VP mRNA poly(A) tail, but had no effect on VP mRNA abundance (15). It is noteworthy that a specific isoform of PKC, PKC-ζ, is predominantly expressed in the SON, and its mRNA is upregulated by osmotic stimuli (49).

In both of the 5-VCAT-3-bVP lines studied, transgene expression in the adrenal gland was much greater than that observed in the hypothalamus. Because the adrenal expression of the 5-VCAT-3 bVP transgene was so marked, it became an obvious system with which to compare the studies in the hypothalamus and merits further comment. Whereas VP-B expression in the mouse is restricted to the adrenal medulla, which is derived from the primitive cells of the neural crest (2), 5-VCAT-3-bVP expression is restricted to the adrenal cortex, which arises from the fetal mesoderm. Both the rat (18) and bovine (18, 33) adrenal cortex have been reported to contain immunoreactive VP, and we show here that VP transcripts can be detected in the rat adrenal gland by RT-PCR. Thus 5-VCAT-3-bVP adrenal expression may thus represent an appropriate overexpression. Unexpectedly, 72 h of dehydration resulted in a dramatic upregulation in adrenal transgene mRNA expression, but unlike the hypothalamus, no increase in the adrenal transcript size was detected. Differential polyadenylation in response to an osmotic stimulus is thus tissue specific. We note that a hyperosmotic stimuli failed to increase thymic expression of both transgene and endogenous VP (22). This indicates that the adrenal cortex cells, like hypothalamic VP neurons, are under regulatory control by osmotic stimuli that do not affect the thymic VP-expressing cells.

Dehydration for 60 h results in an increase in plasma corticosterone (23). We therefore asked if glucocorticoids are mediating the osmotic response of the transgene in the adrenal glands of 5-VCAT-3 bVP rats. Transgenic rats were treated with the 11β-hydroxylase inhibitor, metyrapone. Northern analysis revealed that inhibition of glucocorticoid synthesis enhanced basal transgene expression and exaggerated the upregulation of the transgene on dehydration. These results are consistent with those of Burke et al. (8), who showed that depletion of glucocorticoids resulted in a dexamethasone-reversed increase in adrenal VP-B transgene mRNA levels. We have also tested the possibility that adrenal cortex cells may be intrinsically osmosensitive. Despite the extremely high levels of expression observed in vivo, explant adrenal cultures demonstrated no transgene expression under either control conditions or when exposed to varying osmolalities (data not shown). It is thus possible that basal expression of the transgene is thus dependent upon systemic inputs, and it was therefore not possible to address the question of intrinsic osmosensitivity. The systemic inputs responsible for maintaining the expression and stability of the transcripts may include sympathetic inputs, acetylcholine, or neurotrophic factors. Interestingly, adrenal sympathetic nerve activity increases following injection of hypertonic saline into the third ventricle (4).

To conclude, we have provided evidence that the sequences within the 5-VCAT-3-bVP transgene are
able to mediate multiple modes of physiological regulation in vivo) 1) an appropriate osmotic response in magnocellular neurons, 2) a tissue- and stimulus-specific increase in poly(A) tail length, and 3) a negative regulation by glucocorticoids in the adrenal cortex. Transgenic experiments reveal that the mouse probably lacks the transcript recognition required for physiologically responsive poly(A) tail length modulation. Furthermore, this differential polyadenylation is tissue specific probably as a consequence of the different physiological pathways via which hormonal transcription is activated. There is no information about the physiological role of the VP mRNA poly(A) tail length shift in the rat, although, in accord with well-established notions regarding the function of the poly(A) tail of eukaryotic mRNAs, it has been suggested that translational efficiency and/or message stability may be affected (9–11). The poly(A) tail of the growth hormone mRNA increases in length in hypothyroid rats, and evidence has been presented that supports a role in increasing transcript stability (30, 43). Whatever the function, if any, in the rat, it is clear that the mouse does not need to lengthen the poly(A) tail of the VP mRNA to adapt to the challenge of an osmotic stimulus.

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