The Identification of a cis-Acting Element Involved in Cyclic 3',5'-Adenosine Monophosphate Regulation of Bovine Vasopressin Gene Expression*

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Cyclic adenosine 3',5'-monophosphate (cAMP) has been implicated as an intracellular messenger mediating osmotic regulation of expression of the gene encoding the neuropeptide vasopressin (VP) in the hypothalamus. We have used a heterologous transient transfection system to demonstrate that cAMP regulates the bovine VP gene promoter following transfection into CV1 cells. Mutational analysis identified a bovine VP cAMP-responsive element (BVP-CRE) 120–112 base-pairs upstream of the start of transcription. DNase I footprint analysis using nuclear protein extract from CV1 cells showed protection at the site of the BVP-CRE. Protection of the BVP-CRE was also observed using purified AP1 protein, while there was a weak interaction with the BVP-CRE using purified rat CREB protein. Nuclear proteins purified from the rat supraoptic nucleus bind to the BVP-CRE. As transgenic mouse studies have shown that the bovine VP gene is subject to appropriate physiological regulation in the mouse hypothalamus (Ang, H. L., Funkhouser, J., Carter, D. A., Ho, M. Y., and Murphy, D. (1991) Soc. Neurosci. Abstr. 513, 12), these data indicate a role for the BVP-CRE element in regulating VP gene expression in vivo. These data demonstrate that cAMP regulates bovine VP gene expression in vitro via a cis-acting element within the VP promoter, and this activation may be mediated by members of the AP1/ATF/CREB family of transcription factors.

The gene encoding the neuropeptide vasopressin (VP)1 is expressed within discrete neuronal cell groups of the hypothalamus and is subject to differential regulation by a variety of physiological stimuli. VP produced within the magnocellular neurons of the supraoptic nucleus (SON) and paraventricular nucleus is transported to the neural lobe of the pituitary, from where it is released into the circulation. A rise in plasma osmotic pressure stimulates release of VP, which promotes water retention in the renal collecting ducts. Levels of VP mRNA in the SON and paraventricular nucleus are increased following an osmotic stimulus produced either by salt loading (1–7) or dehydration (8). This effect was recently shown to be a consequence of an increase in the level of transcription of the VP gene (9, 10).

Little is known about the intracellular factors and cis-acting elements which mediate the physiological regulation of VP gene expression, the study of which has been hindered by the lack of appropriate VP-producing cell lines in which to perform gene transfer studies. However, a number of indirect studies have implicated cyclic 3',5'-adenosine monophosphate (cAMP) as a potential second messenger mediating activation of VP gene expression. A role for cAMP in mediating osmotic induced changes in VP gene expression has been suggested, as osmotic stimulation causes an increase in cAMP levels within the SON and paraventricular nucleus in vivo (11, 12). Using primary cultures of hypothalamic neurons from fetal rats, Oeding et al. (13) have demonstrated an increase in the number of VP mRNA containing cells following treatment with cAMP activators. Enhancement of VP mRNA levels by cAMP have also been observed in the human VP-expressing small cell lung carcinoma cell line, GLC-8, and an increase in promoter activity of the human VP gene by cAMP has been reported using a heterologous transfection system (14).

We have previously shown that the bovine VP gene is expressed in a tissue-specific manner in the hypothalamus of transgenic mice and that the level of transgene expression is appropriately regulated by osmotic stimulation (15). In this study we have investigated directly whether the bovine VP promoter is cAMP-responsive and mapped the sequences of the cis-acting elements responsible. We have also characterized possible DNA-protein interactions which may be involved in mediating the cAMP response in vitro and further, have shown that proteins extracted from the rat SON interact with the same region of the bovine VP promoter.

MATERIALS AND METHODS

Cell Culture and DNA-mediated Transfection—CV1 cells (ATCC) were maintained in growth medium consisting of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were incubated at 37 °C in a humidified atmosphere of 95% air, 5% CO2 (v/v).

Plasmid DNA used in transfection experiments was prepared by alkaline lysis followed purification using a single CsCl gradient (16). CV1 cells were transfected using the lipfection method (17). Cells were grown to 90% confluence in 35-mm tissue culture dishes and then washed with phosphate-buffered saline. OptiMEM I RSM
(GIBCO) (2 ml) was added to each dish, and transfection was performed by adding 100 μl of a mixture containing 12 μg of BPV CAT DNA, 1 μg of RSVLUC as an internal control for transfection efficiency and 30 μg of lipofectin (Bethesda Research Laboratories) in 100 μl of H2O. Cells were incubated in the transfection medium for approximately 24 h, after which the transfection medium was withdrawn and replaced with 2 ml of growth medium. The cells were returned to the incubator for 20 h, at which time experimental drugs were added to the medium. The cells were treated with either 20 μM forskolin, 0.5 μM 3-isobutyl-1-methyl-xanthine (IBMX) or 20 μM forskolin + 0.5 μM IBMX. Control plates of cells were incubated in the presence of the vehicle alone. After a further 28 h incubation at 37 °C, the cells were washed and harvested. The cell lysate was assayed for chloramphenicol acetyltransferase (CAT) activity using the method of Sleigh (18) and luciferase activity using the method of de Wet et al. (15).

A time course of the effect of forskolin (20 μM) + IBMX (0.5 μM) on pBVPCAT1250 activity was performed using CV1 cells. The level of CAT activity was increased after 16 h of incubation in the presence of cAMP activators and was maximal after a 28 h of incubation. A 28-h incubation in the presence of cAMP activators was therefore chosen for all subsequent experiments investigating cAMP regulation of the bovine VP promoter following cotransfection into CV1 cells. The sequence of the primers is shown in Table I. All constructs were prepared as follows. Male Sprague-Dawley rats (250-300 g) were housed in a controlled environment with food and water available ad libitum. The SON were dissected from the brains of either 100 control rats or 100 rats which had been salt-loaded by replacing drinking water with 2% NaCl (w/v) for 5 days. The SON from each group of 100 animals were pooled and placed in 5 ml of buffer A (10 mM HEPES, 10 mM KCl, 0.2% Triton X100, 0.2 mM EDTA, 0.2 mM EGTA, 1 μg/ml penicillin A, 10 μg/ml leupeptin, 0.1 mM pepsin A, 10 μg/ml aprotinin) containing 25% glycerol (v/v). The tissue was homogenized on ice with 10 strokes using a Dounce A homogenizer and layered onto a 5-mL cushion composed of 50% glycerol (v/v) in buffer A. The addition of 20% sucrose was spun in a 5000 × g for 10 min at 4 °C and the pellet was resuspended in 500 μl of buffer A, with NaCl added to a final concentration of 0.4 M NaCl. The suspension was vortexed, incubated on ice for 10 min, and then spun in an Eppendorf centrifuge at 14,000 rpm for 10 min at 4 °C. The supernatant was dialyzed against 2 x 1 liter of 10 mM HEPES, 200 mM KCl, 3 mM MgCl2, 1 mM dithiothreitol, 0.2 mM EDTA, 0.1 mM EGTA, 25 μg/ml glycerol (v/v) at 4 °C for 3 h. The nuclear extracts were spun at 14,000 rpm in an Eppendorf centrifuge at 4 °C for 2 min. The supernatant was divided into 50-μl aliquots and stored at −80 °C.

The HindIII-PstI fragment (position −30 to −200 of the bovine VP promoter) was cloned into HindIII-PstI-digested pUC18, labeled bovine VP 5′-flanking region was digested with EcoRI and filling in the sites with Klenow enzyme in the presence of [α-32P]dATP and unlabeled dNTPs. The labeled products were then digested with HindIII, and the VP 5′-flanking regions purified from a 1% agarose gel using a Qiaex gel purification kit (Qiagen). Nuclear protein extract from CV1 cells (10–20 μg) or 5 μg recombinant CREB protein was incubated in 100 μl of buffer containing 3 mM spermin, 4 mM MgCl2, 80 mM KC1, and 200 ng of poly(dI-dC) for 15 min on ice. In experiments using purified AP1 and AP2 proteins (Promega), 1 μg (footprint unit, as defined by the manufacturer) was incubated as above in 10 μl of buffer without the addition of poly(dI-dC). Approximately 5 μg of labeled VP 5′-flanking region was used and incubated for 20 min on ice. After this 20-min incubation period, 2 μl of DNase I (Worthington) was added and the incubation continued for a further 1.5 min at 25 °C. The concentration of DNase I used was determined empirically for each probe and protein extract. The reaction was terminated by the addition of 0.5 μl of 0.5 M EDTA (w/v) and the 5′-end fragments of DNA were separated on 1% agarose gel, 20 μg sodium acetate, 50 mM EDTA, 270 ng/μl (w/v) RNA. The mixture was then extracted with phenol, precipitated with ethanol, and the resulting pellet resuspended in 4 μl of loading buffer (90% formamide, 1 mM EDTA, 0.05% (w/v) bromophenol blue, 0.5% (w/v) xylene cyanol). The labeled products (approximately 4000 cpm/lane) were then run on a 7% sequencing gel (19:1; 2% agarose gel, 100 mM TBE, Tris-base, EDTA buffer). DNA sequencing gels were exposed to x-ray film, and autoradiographs analyzed by radiography. The A+G ‘tanes were generated by Maxam and Gilbert sequencing using cleavage at purine residues (23).

**Table 1**

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<tr>
<th>Oligonucleotide primers used in the PCR amplification of specific regions of the bovine VP promoter</th>
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<td>Bovine VP constructs</td>
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<td>5′ Primers</td>
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**RESULTS**

We have investigated the effect of cAMP on VP gene expression in vitro using a heterologous transfection assay system. The monkey kidney CV1 cell line was chosen to perform these experiments as this cell line has been used previously in the study of the regulation of other neuropeptide genes (29). Significant stimulation of the activity of a construct consisting of a 1.25 kilobase of the bovine VP promoter linked to CAT was observed using 20 μM forskolin (247 ± 18.8%, p < 0.05) and a combination of forskolin (20 μM) +
IBMX (0.5 μM) (249.5 ± 28.5%, p < 0.05), while there was no significant effect of IBMX alone (109.7 ± 1.29%). A series of 5' deletion mutants were constructed in order to identify regions which mediate the cAMP response of the VP gene. The constructs containing 1.25 kilobase pair, 300 bp, 155 bp, and 131 bp of 5'-flanking sequence all showed a significant increase in the level of CAT activity after incubation in the presence of forskolin (Fig. 1). A minimal promoter construct containing 36 bp of VP 5'-flanking sequence, which includes the TATA box, did not show a significant response to forskolin, indicating that the BVP-CRE lies between -131 and -36 (Fig. 1).

We then investigated whether interactions occur between CV1 nuclear proteins and the CRE-containing region of the bovine VP promoter. DNase I footprint analysis of a bovine VP promoter fragment consisting of nucleotides -300 to -36 using CV1 nuclear protein extract (Fig. 2) revealed the presence of a weak DNA-protein interaction in the region of -113 to -122, with some further protection upstream to -129, and a strong interaction in the region of -65 to -76.

In order to assess the role of these nuclear protein-binding sites in cAMP regulation of the BVP gene, deletion constructs were analyzed in which putative transcription factor binding sites revealed by the DNase I footprint analysis were excised from the pBVPCAT155 and pBVPCAT131 constructs, thus generating the plasmids pBVPCAT155A142-63 and pBVPCAT131A112-63. The pBVPCAT155A142-63 construct showed no significant response to forskolin (Fig. 3). However, deletion of nucleotides -112 to -63 from the pBVPCAT131 construct to form pBVPCAT131A112-63 did not abolish the response to cAMP activators (Fig. 3). Thus, nucleotides -131 to -112 harbor a putative CRE. Mutations within this putative CRE containing region were then analyzed in order to assess the contribution of this region to the BVP cAMP enhancer. A putative CRE located between nucleotides -120 and -112 was deleted in the construct pBVPCAT131Δ120-112. This construct showed no significant response to forskolin (Fig. 3). A point mutation within the CRE at position -116 (pBVPCAT131CM1) resulted in only a slight reduction in the response of the VP promoter to cAMP and a significant response to forskolin was retained (p < 0.05) (Fig. 3). However, a second mutation of the CRE sequence at positions -115 and -114 (pBVPCAT131CM2) resulted in no significant response to forskolin (Fig. 3). The BVP-CRE was thus shown to be located between nucleotides -112 and -120.

We then asked about the nature of the protein interacting with the BVP-CRE by performing DNase I footprint experiments using purified recombinant AP1 (c-jun), AP2, and rat CREB proteins. AP1 bound to the region between -113 and -139, which overlaps the BVP-CRE (Fig. 4A) and corresponds to the location of a weak footprint seen using CV1 nuclear extract. AP2 bound to a site proximal to the BVP-CRE (-96 to -119) which contains an element resembling...
FIG. 3. Analysis of mutations of putative transcription factor binding sites within the bovine VP promoter. Deletion of the nucleotides −142 to −63 from pBVPCAT155 and deletion of nucleotides −112 to −63 from pBVPCAT131 was performed, thus removing putative AP2-binding sites from the bovine VP promoter. Deletion of nucleotides −120 to −112 from pBVPCAT131 was performed, thus removing a putative CRE from the bovine VP promoter. Point mutations were introduced into the putative −120 to −112 CRE as follows: a C to G exchange at position −116 produced the construct pBVPCAT131CM1, while mutation of a G to an A residue and a C to a G residue at positions −115 and −114 produced the construct pBVPCAT131CM2. The effect of treatment with 20 pM forskolin on expression of these mutant constructs in CV1 cells is shown. Relative CAT activity was calculated as for Fig. 1, and each point represents the mean ± S.E. of at least five separate experiments.

We then investigated whether nuclear proteins from the rat SON bound to the BVP-CRE region of the bovine VP promoter. DNase I footprint analysis of the BVP promoter using nuclear protein extracts prepared from the SON of control animals revealed the presence of protected regions between nucleotides −65 to −75 (Fig. 5A). This region, which is not involved in mediating the response to cAMP, is also protected with extracts from salt-loaded rats and from CV1 cells (Fig. 5B). Protection of a distal region between −113 and −145 (Fig. 5A). The pattern of protection in this region was compared using extracts prepared from control and salt-loaded SON and CV1 cells and by varying the amount of DNase I in the cleavage reactions (Fig. 5B). Although the footprints differ, the enhancements engendered at −134 and −145 by the SON extracts are not apparent with the CV1 extracts; the region of the BVP-CRE is protected by all three extracts. Protection at −122 is seen with the CV1 extracts and the control SON extracts irrespective of DNase I concentration and with salt-loaded SON extracts at the higher DNase I concentration.

In a separate series of experiments, the basal expression of constructs used in this study were compared. The relative basal expression of the 5′ deletion mutants of the bovine VP promoter was expressed as a percentage of the activity of pBVPCAT1250 and is shown in Fig. 6. A significant reduction in basal expression was observed in the minimal promoter construct pBVPCAT36 (16.0 ± 5.1%, p < 0.05). Furthermore, a similar reduction in basal activity was observed in the constructs pBVPCAT155Δ142−63 (26.0 ± 8.7%, p < 0.05) and pBVPCAT131Δ112−63 (14.0 ± 5.6%, p < 0.05), indicating the presence of a basal enhancer within the region −112 to −63. The proximal −65 to −76 element which is protected in DNase I footprint experiments using both CV1 and SON nuclear extracts (Figs. 2 and 5) may act as a basal enhancer of BVP gene expression.

DISCUSSION
In this study, we have demonstrated cAMP activation of the bovine VP gene promoter in a heterologous transfection system.
assay system, suggesting a direct role for cAMP in the regulation of the VP gene transcription.

Cyclic AMP is a well described regulator of gene expression (26, 27), including the transcriptional regulation of several neuropeptide genes (25, 28). The first cAMP-responsive element (CRE) to be described in eukaryotes was that of the somatostatin gene which binds to the transcription factor CREB (29, 30). The ATF family of transcription factors are structurally related to CREB and are also regulated by cAMP (31). Furthermore, proteins of the AP1, ATF, and CREB have been shown to form heterodimers which may be important in mediating the differential effects of cAMP on gene expression (32–34). Another nuclear protein which has also been implicated in mediating the effects of cAMP is the transcription factor AP2 and two putative CRE sites (Fig. 7), each of which are potential candidates as mediators of the cAMP response. In this study, analysis of 5' promoter deletion mutants revealed that a BVP-CRE is located between positions -112 and -120. This element, although clearly necessary for the transcriptional response to cAMP in vitro, may represent only one component of a larger, more complex cAMP response unit.

The BVP-CRE contains the core sequence TGACG, which represents the 5-bp core sequence of the palindromic CREB-binding site first identified in the somatostatin gene (29, 30). Further comparison of the BVP-CRE with that of other neuropeptide gene promoters reveals several similarities (Fig.

FIG. 5. DNase I footprint analysis of the bovine VP promoter using SON nuclear protein extracts (A) and comparison with CVI extracts (B). DNase I footprint analysis of the bovine VP promoter was performed using nuclear protein extract (approximately 10–15 μg) from the SON of control (CSON), salt-loaded (or dehydrated, DSON) rats and from CVI cells (CVI). A+G lanes were generated by Maxam and Gilbert sequencing using cleavage at purine residues and (−) lanes generated by DNase I treatment in the absence of nuclear protein extract. In B, the arrows at the top of the figure indicate increasing DNase I in the cleavage reactions: CSON, 2, 4, and 8 μg; DSON, 4, 6, and 8 μg; CVI, 1.5, 2 μg. Vertical parallel lines indicate the limits of protection. Prominent enhancements at −134 and −145 are indicated by arrows to the left of the figures.

FIG. 6. Comparison of the relative basal expression of 5' deletion mutants of the bovine VP promoter. The levels of basal CAT activity were measured for each bovine VP CAT construct, normalized with respect to RSVLUC activity, and expressed as a percentage of the normalized expression of the pBVP CAT1250 construct. Each value represents the mean ± S.E. of four experiments and an asterisk shows a statistically significant difference from the basal expression of the native pBVP CAT300 construct.

FIG. 7. Putative transcription factor binding sites within the bovine VP promoter. Putative transcription factor binding sites within 500 bp of bovine VP 5'-flanking sequence are indicated, with their position aligned relative to the start of transcription of the bovine VP gene (+1). The TATA box is shown by the sequence TAAATA, the sequence of putative cAMP-responsive elements is underlined and putative AP2 sites (comprising of the sequence CTGGGGA) are shown by blocks with arrows indicating the orientation of the sequence within the VP promoter.
The proenkephalin promoter contains two CRE sites termed ENKCRE-1 and ENKCRE-2, both of which are essential for cAMP activation of the proenkephalin gene (25). The BVP-CRE (nucleotides -121 to -114) is identical in sequence to the ENKCRE-2 (nucleotides -91 to -84), which is in the opposite orientation relative to the start of transcription (Fig. 8) and has been shown to bind to the transcription factor AP1 (37). The VIP gene promoter contains a 25-bp cAMP regulatory region, which, like the BVP-CRE, includes the 5-bp CGTCA core element and also another inverted copy of this motif (38).

We have used DNase I footprint analysis to identify the nature of the DNA-protein interactions which occur at the BVP-CRE. We have demonstrated that a minimum of two factors present in CV1 nuclear protein extracts bind to the bovine VP promoter: one in the region of the BVP-CRE and the other at a putative basal enhancer element (Fig. 2). The use of purified transcription factor proteins in DNase I footprint experiments showed that AP1 (c-jun homodimer) binds to the region corresponding to the BVP-CRE (Fig. 4A) and may reflect the similarity of this element to ENKCRE-2, which also binds to AP1 (37). Purified recombinant CREB also shows a weak protection in the region of the BVP-CRE (Fig. 4C). A strong interaction of recombinant CREB protein with the bovine VP promoter was observed at a second proximal site (nucleotides -64 to -48), which we have shown by deletion analysis not to be involved in mediating the BVP response to cAMP (Fig. 4C). This region contains an element which resembles the CREB consensus binding site (CCACGTCA), but no protection of this element was seen using CV1 nuclear extracts, indicating that in this context this region is not important in the cAMP regulation of BVP gene expression. Purified AP2 also bound to the BVP promoter at a site proximal to the BVP-CRE which resembles the consensus sequence of the AP2-binding site (Fig. 4B). However, we have shown by mutational analysis that this region is not involved in mediating the BVP response to cAMP. Furthermore, no corresponding footprint was observed using CV1 nuclear extracts (Fig. 2). Sequence analysis of the VP promoter has previously revealed two further downstream potential AP2-binding sites which are present in both the bovine (Fig. 7) and rat VP promoters (39). However, we did not observe specific binding of AP2 protein to these putative elements, indicating that they do not function as AP2-binding sites within the VP promoter. We suggest that transcription factor(s) related to the AP1/APF/CREB family are responsible for mediating cAMP activation of the BVP gene via the -120 to -120 promoter region, which is part of a cis-acting element conferring cAMP responsiveness. Members of the AP1 family of transcription factors have previously been implicated in the physiological regulation of VP gene expression. The levels of transcription of the c-fos and c-jun genes are elevated in the rat SON following an osmotic stimulus, which is a potent stimulus of VP gene expression in this area of the hypothalamus (40–42).

In order to further investigate whether the BVP-CRE is relevant to the regulation of VP gene expression in vivo, we performed DNase I footprint experiments on the BVP promoter using nuclear extracts prepared from the rat SON. The SON is a major expression site of VP in the hypothalamus and chronic osmotic stimulation results in an elevation of both VP mRNA (1–7, 43) and cAMP levels (11, 12) in the SON. We note that the extent of the transcriptional increase in VP gene expression in the hypothalamus is very similar to the degree of induction of BVP promoter usage by cAMP in transected CV1 cells (2–3-fold, 9). We have previously shown that the BVP gene is expressed in the rodent SON using the transgenic mouse model and that the expression of this transgene is also appropriately regulated by an osmotic stimulus (15). DNase I footprint analysis using rat SON nuclear protein extract revealed the presence of a DNA-protein interaction at the BVP-CRE (Fig. 5A), which may indicate that this element is involved in regulating BVP gene expression in vivo. Protections and enhancements in the region of the BVP-CRE were seen using nuclear protein extracts from control and osmotically stimulated animals (Fig. 5). However, differences in the pattern of protection between the two extracts (Fig. 5B) have to be interpreted with caution given that the methodology employed is not quantitative. Activation of pre-existing transcription factors (for example, by phosphorylation) following an osmotic stimulus, or quantitative differences in the level of transcription factors within control and salt-loaded SON would not be detected using DNase I footprint analysis. We are currently constructing transgenes incorporating the deletion of the BVP-CRE described in this study, in order to assess the role of this element in the osmotic stimulation of the bovine VP transgene expression in mice.

The data we present here demonstrate that cAMP activates VP gene expression in vitro and that this effect is mediated at least in part via a cis-acting element located between nucleotides -120 and -112 of the bovine VP promoter. Transcription factors of the AP1/APF/CREB family appear to be mediators of this response. The binding of a rat SON nuclear factor to the same BVP-CRE region suggests that regulation of the VP gene by cAMP may be of physiological importance in mediating osmotically induced changes in VP gene expression.

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cAMP Regulation of Vasopressin Gene Expression