Vasopressin gene expression is stimulated by cyclic AMP in homologous and heterologous expression systems

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The possible role of cyclic AMP (cAMP) in the regulation of the vasopressin (VP) gene was tested in two cellular expression systems: one cell line with endogenous VP expression and the other which was transiently with a VP promoter-luciferase fusion gene. 8,Bromo-cAMP stimulated the VP mRNA content about 4-fold in the human VP-expressing small cell lung carcinoma cell line GLC-8. The luciferase activity in P19 embryonal carcinoma cells which were transiently transfected with −174 to +44 of the 5'-flanking region of the human VP gene linked to the firefly luciferase gene, was stimulated about 2-fold by the cAMP analogue. The results indicate that cAMP plays a role in the upregulation of the VP gene and hence point to several putative nucleotide motives in the promoter functionally conferring this response.

Vasopressin gene expression; Gene regulation; cyclic AMP; Small cell lung carcinoma cell line; AP-2

1. INTRODUCTION

The hormonal control of fluid homeostasis involves vasopressin (VP) which originates from magnocellular neurons in the hypothalamus. These neurons are clustered in the supraoptic (SON) and paraventricular (PVN) nuclei and express the VP gene [1,2]. VP gene products are transported to the neural lobe of the pituitary gland, where they are secreted into the circulation [3].

In the SON and the PVN, VP gene expression is critically altered by changes in osmotic balance as shown by the increase in VP mRNA content in salt loaded or dehydrated rats [4-6]. Furthermore, changes in VP gene expression have been found during development, gestation and lactation [7-9]. Electrophysiological and morpholocial studies have indicated that the activity of VP neurons is controlled by neuronal inputs [10,11], thus pointing to an involvement of second messengers in mediating effects of transmitters on gene expression. Three lines of experiments suggest that cAMP may be one of the involved second messengers. Firstly, hyperosmolality caused an increase in the levels of cAMP [12] and of the G protein α-subunit mRNA [13]. Secondly, cAMP increased the number of VP mRNA containing neurons in a primary culture [14], and thirdly, the 5' flanking regions of human and rat genes contain nucleotide sequences which resemble AP-2 binding elements [15-17]. To directly investigate the influence of cAMP on VP mRNA synthesis, we here use the human small cell lung carcinoma (SCLC) cell line GLC-8 with endogenous expression of the VP gene (Verbeeck et al., unpublished data). Here we report the effect of cAMP on VP mRNA levels in this cell line and on VP promoter activity with a promoter-luciferase fusion gene in a heterologous expression system.

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Abbreviations: cAMP, cyclic AMP; VP, vasopressin; SON, supraoptic; PVN, paraventricular; Ec, embryonal carcinoma; SCLC, small cell lung carcinoma; IBMX, isobutyl-methylxanthine; 8,Br-cAMP, 8,Bromo-cAMP

2. MATERIALS AND METHODS

2.1. *Genomic analysis*

GLC-8 cells (5×10^7) were washed in phosphate-buffered saline (pH 7.4) and DNA was prepared from isolated nuclei [18]. 15 μg was digested with restriction enzymes *Eco*RI, *Bam*HI or *Hin*dIII, loaded on 0.7% agarose gel and run at 1 V/cm. The gel was then blotted on a nylon filter (Zeta probe, Bio-Rad, Richman, CA) in 2 M sodium hydroxide for 5 h. The Southern blot was prehybridized at 70°C in 1.5 × SSPE (0.18 M sodium chloride, 10 mM sodium phosphate (pH 7.0) and 1 mM EDTA), 2% SDS, 1% defatted milk powder (Refit, Campina, Eindhoven, The Netherlands) and 1 mg/ml denatured herring sperm DNA for 5 h. Hybridization was performed simultaneously with two ^{32}P-labelled genomic probes (3×10^6 cpm/ml) in 1.5 × SSPE, 1% SDS, 0.5% milk powder, 10% dextran sulphate and 0.5 mg/ml denatured herring sperm DNA at 70°C for 16 h. One probe was the *Bam*HI-*Pst*I fragment of the human VP gene containing 169 bp of the first exon, and the other was the *Hgi*AI-*Bam*HI genomic fragment containing 255 bp of the last exon of the VP gene [19]. Blots were washed successively in 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1% SDS at room temperature for 20 min, in 0.5 × SSC at 50°C for 30 min and in 0.1 × SSC at 50°C for 10 min. Blots were exposed to X-ray film (Kodak, X-OMAT) with an intensifying screen at −80°C for 20 h.

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2.2. *RNA isolation and analysis*

Total cellular RNA of $(2.5\text{–}5) \times 10^7$ cells was prepared as described by Wilkinson [20]. Cells ($(2.5\text{–}5) \times 10^7$) were resuspended in 400 μl ice-cold Tris-saline (25 mM Tris (pH 7.4), 130 mM NaCl, 5 mM KCl). 100 μl ice-cold NND buffer (1% Nonidet P-40, 0.5% sodium deoxycholate and 0.01% dextran sulphate made up in Tris-saline) was gently mixed. The homogenate was spun in an Eppendorf centrifuge at 4°C for 30 s. The pellet was stored and used later for the determination of the DNA content [21]. The supernatant was extracted twice with an equal volume of phenol/chloroform/isoamylalchol (25:24:1). RNA was precipitated overnight at −20°C after supplementing 1 ml 100% ethanol. After precipitation the pellets were dissolved in 20 mM Tris (pH 7.4), 1 mM EDTA and the amount of RNA was determined spectrophotometrically by UV absorption at 260 nm.

For Northern blotting total RNA (20 μg) was denatured with glyoxal and dimethylsulphoxide in 10 mM phosphate buffer (pH 6.5) at 55°C for 60 min, cooled on ice, loaded on a 1.4% agarose-10 mM phosphate gel and run at 10 V/cm for 3 h. Transfer of RNA from the gel to a nylon membrane (Hybond-N, Amersham, UK) was carried out overnight in 25 mM phosphate buffer (pH 6.5). The blot was briefly washed in 2×SSC and baked at 80°C for 2 h. Filters were prehybridized in 50% (v/v) formamide, 6×SSC, 8% dextran sulphate, 0.5 mg/ml denatured herring sperm DNA and 0.5% (w/v) defatted milk powder at 50°C for at least 6 h. Denatured ^{32}P-labelled DNA probes were added to the prehybridization mix to a final concentration of at least 2×10^6 cpm/ml at 50°C and incubated overnight. The VP-specific probe was the *Hgi*AI-*Bam*HI genomic fragment containing 225 bp of the last exon of VP [19]. The blots were also separately hybridized with the rat β-actin specific probe, which was the *Pst*I fragment (about 1500 bp) of the rat cDNA clone [22]. The filters were washed twice in 2×SSC, 0.1% SDS at room temperature for 5 min, followed by one wash at 50°C for 30 min, one wash at 60°C for 20 min and a final wash in 0.1×SSC, 0.1% SDS and 1 mM EDTA at 65°C for 20 min. The filters were then exposed to X-ray film (Kodak X-OMAT) with an intensifying screen at −80°C for various lengths of time. The optical density of the lanes on the films was determined with an one-dimensional densitometric scanner (Zeiss KM3, Oberkochen, FRG).

2.3. *Treatment and transfection of cell lines*

SCLC GLC-8 cells [23] were cultured in RPMI medium (Gibco

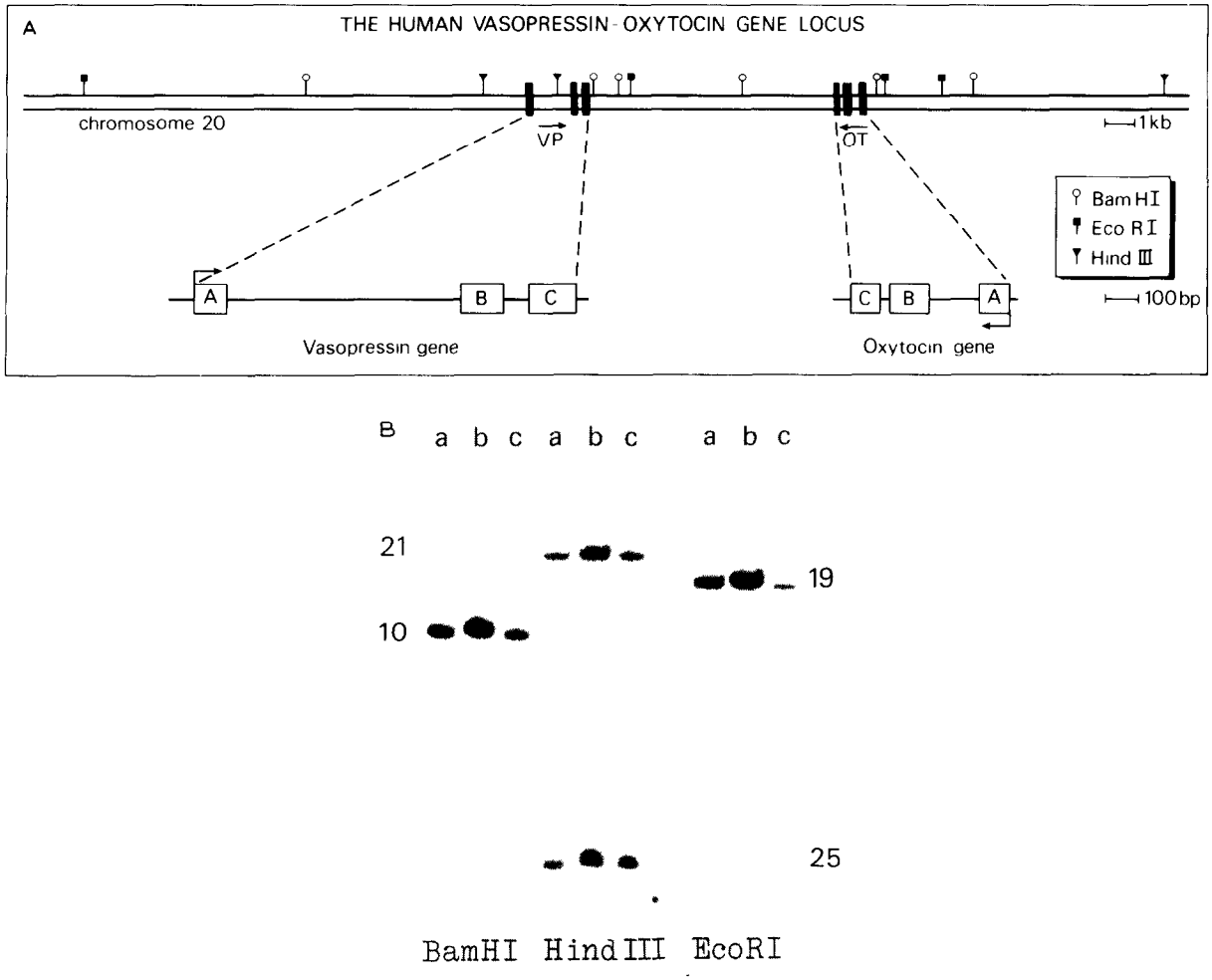

Fig. 1. Analysis of the VP locus in the cell line SCLC GLC-8. (A) Genomic map of the VP gene locus. In the upper part of the figure exons A, B and C are represented by ■. The position of the oxytocin gene and several restriction sites have been taken from Sausville et al. [19]. Other sites were determined by Southern analysis (see panel B). (B) Genomic fragments containing VP sequences of human placenta and SCLC cells. Samples of genomic DNA (15 μg) of GLC-8 passage 27 (a), GLC-8 passage 91 (b) and human placenta (c) were digested with *Bam*HI, *Hin*dIII or *Eco*RI, electrophoresed and transferred to a nylon membrane. The Southern blot was hybridized with two probes simultaneously: one probe of 345 bp containing a part of exon A of VP, the other of 525 bp containing a part of exon C of VP. The exposure time was 24 h. For details see Materials and Methods.

Europe, Breda, The Netherlands), supplemented with 10% calf serum (High Clone, Greiner Laboratories, Life Technologies Inc., Grand Island, New York, USA), 2 mM glutamine (Gibco), 100 units/ml penicillin-streptomycin (Gibco), 0.5 mM β-mercaptoethanol (Merck, Darmstadt, FRG), 1 mM sodiumpyruvate (Sigma Chemical Co., St. Louis, USA), 2.5 μg/ml fungizon (Gibco) and 60 μg/ml gentamycin (Gibco). Before incubation with 0.5 mM, 8,Bromo-cAMP (Boehringer-Mannheim, FRG) and 0.5 mM isobutyl-methylxanthine (IMBX) (Boehringer-Mannheim), the cells were kept on stripped, steroid-free serum for 24 h. The vehicle for 8,Br-cAMP and IBMX was ethanol. After 8,Br-cAMP and IBMX treatment for 50 h samples of about 5×10^7 cells were harvested.

P19 embryonal carcinoma (EC) cells [24] were cultured in DMEM (Gibco) supplemented with 7.5% fetal calf serum. The 5'-flanking region of the human VP gene, from position -174 to $+44$ [19], was fused to the luciferase gene in p19LUC [25] to create pHVPLUC. 10 μg of pHVPLUC was transfected with the calcium-phosphate precipitation method as described [26]. One day after transfection the medium was replaced and the cells received fresh medium containing 0.5 mM 8,Br-cAMP and/or 0.5 mM IBMX. After 24 h the cells were harvested in 350 μl 100 mM potassium phosphate (pH 7.8) with 1 mM dithiothreitol (Boehringer-Mannheim) and luciferase was measured in 60 μl of extract according to the protocol of de Wet [27], using a Lumac/3m biocounter M2010A luminometer.

3. RESULTS AND DISCUSSION

The SCLC cell line GLC-8 has been identified before among three VP-expressing SCLC cell lines as the one with the highest levels of VP mRNA (Verbeeck et al., unpublished data). Before use in studies on the regulation of the VP gene, the VP locus in this cell line was analyzed on genomic blots. The three restriction enzymes used produced the same restriction fragments of GLC-8 DNA from two passages as normal placenta DNA (Fig. 1A). The intensity of the signals was approximately the same. This result indicates that there are no serious rearrangements or amplifications of the VP gene locus over about 15 kb 5' upstream to 20 kb downstream of the VP gene in this cell line (Fig. 1B). Thus, SCLC GLC-8 was then used to study the role of cAMP in the regulation of endogenous VP mRNA levels.

Treatment of SCLC GLC-8 cells with 0.5 mM of the cAMP analogue 8,Br-cAMP in the presence of 0.5 mM IBMX and ethanol as vehicles caused a marked increase in the VP mRNA level as compared to cells which were cultured under identical conditions without 8,Br-cAMP (Fig. 2A). IBMX or ethanol alone had no significant effect on the VP mRNA level as compared to untreated cells, but the compounds together caused a reduction of the VP mRNA content. On the basis of the β-actin mRNA content, 8,Br-cAMP stimulated the VP mRNA content approximately 4-fold (average of three experiments; Fig. 2B). There was no significant effect of 8,Br-cAMP on the β-action mRNA and total RNA contents per cell, nor an effect on the total cell number. The results thus show that the steady-state level of VP mRNA is increased by the cAMP analogue.

To investigate whether transcriptional regulation was involved in the increase in VP mRNA level, the effect of the cAMP analogue on the activity of the VP promoter

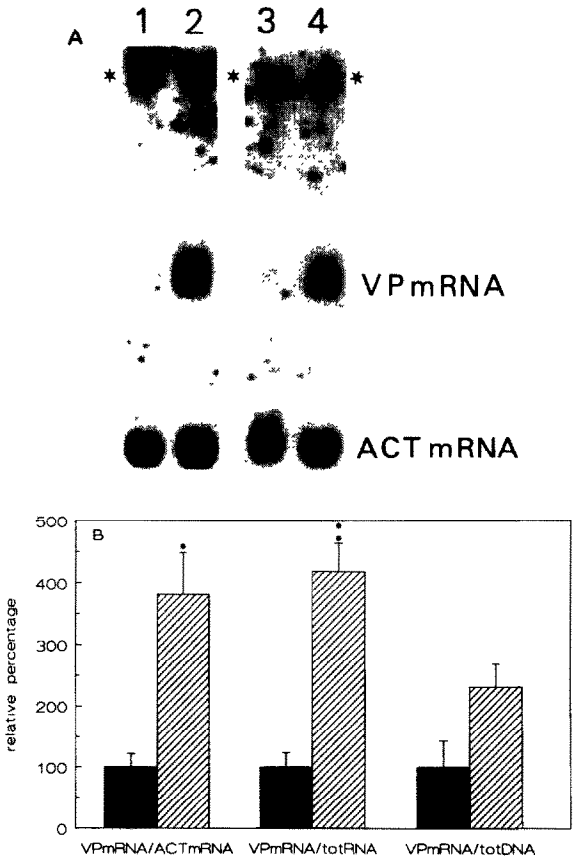

Fig. 2. Effect of 8,Br-cAMP treatment on VP mRNA levels in SCLC GLC-8 cells. (A) Northern blot with 20 μg total RNA of SCLC GLC-8. Lanes 1 and 3 represent two GLC-8 cultures separately treated with 0.5 mM IBMX alone, lanes 2 and 4 represent two GLC-8 cell cultures separately treated with 0.5 mM IBMX and 0.5 mM 8,Br-cAMP. Both treatments lasted for 50 h. The filter was hybridized with random primer labelled VP-specific probe containing VP exon C [19] and later with a β-actin-specific probe [22]. The exposure time was 24 h. For details see Materials and Methods. (*marks the 28 S ribosomal RNA; ACT, actin.) (B) Relative levels of VP mRNA in SCLC GLC-8 cells. The striped bars represent the relative levels of VP mRNA after treatment with 0.5 mM 8,Br-cAMP and 0.5 mM IBMX which are expressed as percentage of the levels measured after treatment with 0.5 mM IBMX as vehicle alone, represented by the black bars. The values are the average (± SEM) of six determinations done in three separate experiments. Statistics were performed with the Student's t-test ($P<0.001$). ACT, actin; tRNA, total RNA; tDNA, total DNA.

was tested in a heterologous expression system. The 5'-flanking region from nucleotides -174 to $+44$ of the human VP gene [19] was cloned in front of the firefly luciferase gene in plasmid p19LUC and transfected into mouse P19 EC cells. This fusion gene had a consistent basal expression which was 2–3-fold higher than the promoterless plasmid p19LUC. Treatment of transfected cells with 0.5 mM 8,Br-cAMP increased luciferase activity about 2-fold (Fig. 3). There was no effect of 8,Br-cAMP on luciferase activity when the promoterless vector P19 was used (data not shown). IBMX alone had no effect on luciferase activity (Fig. 3). This result shows that the 5'-flanking region

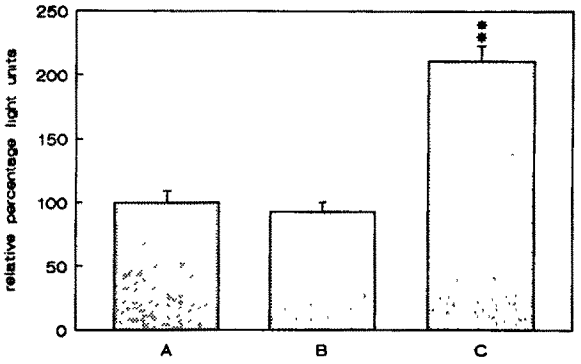

Fig. 3. Effect of 8,Br-cAMP on luciferase activity in P19 EC cells after transfection of a VP gene luciferase construct. P19 EC cells were transiently transfected with the 5'-flanking region (−174 to +44) to the human VP gene fused to the luciferase gene in plsmid p19LUC. Transfected cells, not treated (A) and transfected cells treated with 0.5 mM IBMX (B) or 0.5 mM IBMX and 0.5 mM 8,Br-cAMP (C). The values are the average (± SEM) of three separate determinations. Statistics were performed with the Student's *t*-test ($P<0.001$).

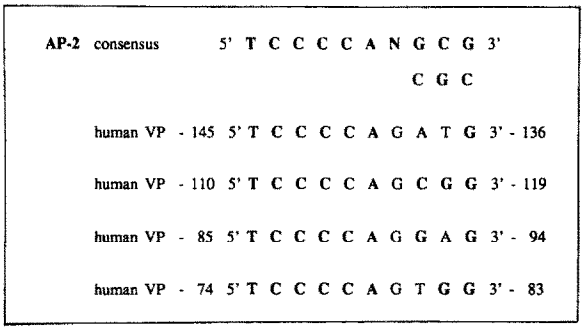

Fig. 4. Putative AP-2 binding elements in the 5'-flanking region of the human VP gene. AP-2, activator protein 2. The numbering indicates the position of the elements upstream of the transcriptional start site. The consensus sequences of AP-2 binding sites [17] and the homologous sequences in the human VP gene [19] are shown in bold.

of the VP gene can confer responsiveness to cAMP and correlates with the increase in VP mRNA in the SCLC GLC-8 cell line after supplementation of the cAMP analogue.

In these experiments we have directly investigated the putative involvement of cAMP in mediating effects on VP gene expression. Previously, the increase in cAMP levels and the G protein α-subunit mRNA in SON and PVN after osmotic stimulation [12,13] as well as the effect of cAMP on the number of VP mRNA-containing neurons in primary hypothalamic cultures [14] have been taken as indications of an involvement of cAMP in VP gene regulation. This hypothesis is supported by the presence of putative AP2 binding elements in the 5'-flanking regions of mammalian VP genes [15–17]. From the present results which were obtained in two different, independent expression systems, it is concluded that the human VP gene is responsive to cAMP. Putative AP-2 binding elements are located in the VP promoter construct used in these experiments at positions −145 to −136, −110 to −119, −86 to −95 and −75 to −84 (Fig. 4). Promoter deletion studies and direct tests of protein-DNA interactions can now be performed to identify the *cis* and *trans*-acting elements involved in the response to cAMP. Preliminary experiments indicate that the rat gene is similarly responsive to cAMP (Adan et al., unpublished results), which is not surprising in view of the high degree of homology between the 5'-flanking regions of the human and rat VP genes. The cAMP responsiveness of the VP gene may function in situations of hyperosmolality to increase VP mRNA levels and VP biosynthesis. In vivo it has been observed that the increase in VP mRNA is accompanied by an increase in poly A tail size [28,29]. In our in vitro experiments with SCLC GLC-8 a change of poly A tail size in the VP transcript was not observed after 8,Br-cAMP treatment. This suggests that cAMP does not mediate the effect on mRNA size. Therefore, still other signal transduction pathways may be involved in the full control of VP gene expression during osmotic stress. The SCLC GLC-8 cell line may also provide a useful model system to define these factors.

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