Transcriptional Regulation of the Pituitary Vasopressin V1b Receptor Involves a GAGA-binding Protein*

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The role of CT repeats (inverted GAGA box) in the rat vasopressin V1b receptor (V1bR) promoter in the transcriptional regulation of this gene was studied in H32 hypothalamic cells, which express endogenous V1bR. Transfection of a 2.5-kb V1bR fragment (2161 bp upstream and 377 bp downstream of the proximal transcriptional start point) into a luciferase vector (V1bRp2.5-Luc) results in promoter activity in these cells. The 670-bp proximal promoter fragment containing the GAGA box showed maximal promoter activity, whereas deletion of the GAGA box abolished transcription. Drosophila GAGA-binding protein increased V1bR promoter activity by 11-fold when cotransfected with V1bRp2.5-Luc and increased endogenous V1bR expression. Electrophoretic mobility shift assay showed specific binding of putative nuclear extracts to radio-labeled GAGA oligonucleotides, which increased following restraint stress in rats, a condition associated with V1bR up-regulation. DNA-binding activity involved a protein complex because it was abolished by deoxycholate. Size-exclusion column chromatography showed a complex of 127 kDa, which dissociated into ~70-kDa components after deoxycholate/Nonidet P-40 treatment. This study demonstrates that interactions of GAGA-binding proteins with the GAGA box of the V1bR promoter activate V1bR gene expression and provides a potential mechanism for physiological regulation of V1bR transcription.

The hypothalamic nonapeptide arginine vasopressin is an important regulator of pituitary ACTH secretion (1, 2). The pituitary actions of vasopressin are mediated by vasopressin receptors of the V1b subtype (V1bR), located in the plasma membrane of the pituitary corticotroph (3). The vasopressin V1bR is a G protein-coupled receptor associated with phospholipase C/calcium/phospholipid-dependent signaling mechanisms (4). This receptor is structurally different from the V1a receptor, which mediates vasoconstrictor and metabolic actions of vasopressin, and the V2 receptor responsible for water conservation in the kidney, which is coupled to adenylate cyclase (5).

Previous studies have shown that a good correlation exists between vasopressin binding and pituitary responsiveness, suggesting that the number of vasopressin V1b receptors in the corticotroph plays an important role in determining the sensitivity of the ACTH responses during stress (6–8). To study the regulation of the V1bR, we (10) and others (9) have recently isolated and characterized the 5’-flanking region of the rat V1bR. Computer analysis of the putative promoter region (bp 2161 to +377, corresponding to 2.1 kb of the putative promoter, exon 1, intron 1, and 164 bp of exon 2) showed no TATA box, but several potential sites for glucocorticoid, AP1, and cAMP regulation. In addition, the promoter region of the rat V1bR includes structural features not common in G protein-coupled receptors, such as CA repeats (CACA box) and an unusually large stretch of 134 CT repeats (inverted GAGA box).

GAGA repeats were first described in Drosophila heat shock protein and histone gene promoters, where they were found to bind a protein called the GAGA factor, encoded by the essential Trithorax-like (Trl) gene (11–14). The GAGA factor has been implicated in chromatin structure and remodeling and normal expression of several developmental genes in Drosophila (11, 15). Involvement of GAGA-binding protein in transcription has also been reported for vertebrate genes such as the serine protease inhibitor-2.1 gene, the type 1 angiotensin II receptor gene, and the Xenopus stromelysin-3 gene (16–18). The consensus GAGA binding sequence consists of ~3.5 GA repeats, but longer repeats have been described in some promoters, including hsp26, hsp70, his3, and his4 (13, 19).

Because the V1bR promoter contains a prominent GAGA box near the transcriptional start point (10), in this study, we address the question whether a GAGA-binding protein plays a role in V1bR transcription. Using V1bR promoter-driven luciferase constructs and a Drosophila GAGA expression vector in rat H32 hypothalamic cells, which express endogenous V1b receptors, we provide evidence that a GAGA-binding protein complex is involved in the transcriptional regulation of the V1bR.

MATERIALS AND METHODS

V1bR Promoter Constructs—A construct containing a 2.5-kb NcoI fragment of the V1bR gene from bp −2161 to +377 (+1 was assigned to the proximal transcriptional start point; corresponding to 2.1 kb of the putative promoter, exon 1, intron 1, and 164 bp of exon 2) cloned into the luciferase reporter vector pGL3-basic (Promega) (10) was used to prepare the following restriction fragments of the V1bR promoter: a 830-bp fragment (bp −453 to +377; obtained by SmaI digestion) spanning the SmaI sites in the multiple cloning site of the vector and bp −453 in the V1bR promoter region, a 670-bp fragment (bp −294 to +377; produced by BglII digestion) from the multiple cloning site to the BglII site at bp −294 in the V1bR promoter, and a 470-bp fragment (bp −90 to +377; obtained by digestion with MluI) from the multiple cloning site to BseHI at bp −90 in the V1bR promoter region. The restriction fragments were separated on a 1% agarose gel; the bands

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¶ The abbreviations used are: ACTH, adrenocorticotropic hormone; V1bR, V1b receptor; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; FPLC, fast performance liquid chromatography.
were excised; and the DNA was eluted using a QIAquick gel extraction kit (QIAGEN Inc., Valencia, CA) and ligated using T4 DNA ligase (Promega) into pGL3-basic to obtain the constructs pGL3-V1bRp830, pGL3-V1bRp670, and pGL3-V1bRp470, respectively. An additional construct, pGL3-V1bRp830/H11004 GAGA (bp/H11002 453 to bp/H11002 303 and bp/H11002 90 to bp/H11001 377), was created by BglII/BssHII digestion of pGL3-V1bRp830 and ligation after generation of blunt ends using Pfu Turbo® (Stratagene).

The Drosophila GAGA factor cDNA was obtained by PCR from a clone in the pET-14b vector (kindly provided by Dr. Jordi Bernues, Departamento de Biologia Molecular Celular, Instituto de Biologia Molecular, Barcelona, Spain). The cDNA fragment (pCR3.1-GAGA construct) was generated using primers 5′/H11032-ggcagccatatgtcgctgccaa-3′ (forward) and 5′-cgggctttgttagcagccggat-3′ (reverse), spanning 13 bp of the pET-14b vector and 9 bp of the 3′-untranslated region of Drosophila GAGA, and subcloned into the mammalian expression vector pCR3.1 (Invitrogen). PCR was performed using 100 ng of plasmid template DNA and 2.5 units of Platinum® Pfx DNA polymerase (Invitrogen). Amplification was performed using an initial cycle at 94 ºC for 2 min, followed by 30 PCR cycles at 94 ºC for 30 s, 65 ºC for 1 min, and 72 ºC for 1 min and a 10-min final extension at 72 ºC.

The ligation reaction was used to transform TOP 10F® competent cells (Invitrogen) following the manufacturer’s protocol. All constructs were purified using Wizard Plus® Miniprep (Promega) or Maxiprep (QIAGEN) procedures. The accuracy of the PCR product was verified by sequencing (Veritas, Rockville, MD).

**Animal Procedures**—Male Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighing 250–300 g were housed three per cage in a controlled environment with free access to food and water. After at least a 5-day stabilization period, rats were killed by decapitation, and tissues were removed and rapidly frozen on dry ice for preparation of nuclear extracts. To determine whether the up-regulation of the V1bR observed after stress (6) is associated with changes in...
GABA-binding activity, groups of rats were subjected to restraint stress for 15–90 min using 2.5 × 6-inch plastic restrainers (20). Rats were killed by decapitation 15, 30, and 90 min after initiation of the stress. Pituitaries were rapidly removed, dissected from the neurointermediate lobes, and frozen in dry ice for nuclear protein extraction. Fragments of liver from control and stressed rats were collected as a control.

Cell Culture, Transfections, and Luciferase Assay—The hypothalamic cell line H32 (produced by Dr. Joachim Spiess, Max Planck Institute for Experimental Medicine, Gottingen, Germany) (21) was cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 10% horse serum, and 1% penicillin/streptomycin (Invitrogen). Preliminary experiments in our laboratory using RT-PCR showed that this hypothalamic cell line expresses the Drosophila GABA factor (pcR3.1-GAGA) or the empty expression vector pcR3.1. Promoter activity is expressed as the percentage of pGL3-basic luciferase activity, and data were analyzed by analysis of variance and Fisher’s Protected Least Significant Difference test. * p < 0.05 versus V1bR830; #, p < 0.01 versus respective control. Bars represent the means ± S.E. of results from three different experiments.

Preparation of mRNA and Quantitative RT-PCR—For quantitative RT-PCR of V1b mRNA, cells were plated in 10-cm Petri dishes, transfected with 4 μg of pcR3.1-GAGA expression vector or the empty vector pcR3.1, and cultured for 24 h before mRNA isolation using RNAzolTM B (Tel-test, Inc.) following the manufacturer’s protocol. Poly(A)+ RNA was isolated from the total RNA using oligo(dT) Poly-ATtract mRNA isolation systems (Promega). RNA was subjected to digestion with 1 unit of DNase I, Amplification Grade (Invitrogen) to eliminate contaminant endogenous DNA and quantified by UV spectrometry.

Five aliquots of each poly(A)+ preparation (50–100 ng) were used for synthesis of double-stranded DNA and PCR amplification using the SUPERSCRIPT™ One-Step RT-PCR Platinum™ Taq system (Invitrogen) and rat V1b primers 5‘-ggagagagaggggccactacta-3′ (forward) and 5‘-ggagagagaggggccactacta-3′ (reverse). Rat cyclophilin primers used as internal controls were as follows: 5‘-ggagagagaggggccactacta-3′ (forward) and 5‘-ggagagagaggggccactacta-3′ (reverse). Single-stranded cDNA was synthesized at 55 °C for 30 min, followed by 3 min at 94 °C. The five aliquots of each sample were subjected to a different number of PCR cycles (20, 25, 30, 35, and 40) to determine the linear range for quantification of the endogenous rat V1b after transfection with Drosophila GABA protein. Each cycle consisted of 40 s at 94 °C, 40 s at 55 °C, and 40 s at 72 °C, followed by a 10-min extension at 72 °C. The PCR products were separated and visualized on 2% Tris acetate/EDTA agarose gel containing ethidium bromide and sized using PCR markers (Promega). The image was captured electronically, and the bands were quantified using Kodak 1D image analysis software.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from different rat tissues were prepared using NE-PER™ nuclear and cytoplasmic extraction reagent (Pierce) according to the manufacturer’s instructions.

FIG. 2. Effect of Drosophila GAGA-binding protein on V1bR promoter activity. H32 cells were cotransfected with luciferase reporter plasmid V1bRp2.5, V1bRp830, or V1bRp3802GAGA and the expression vector containing the Drosophila GABA factor (pcR3.1-GAGA) or the empty expression vector pcR3.1. Promoter activity is expressed as the percentage of pGL3-basic luciferase activity, and data were analyzed by analysis of variance and Fisher’s Protected Least Significant Difference test. * p < 0.05 versus V1bR830; #, p < 0.01 versus respective control. Bars represent the means ± S.E. of results from three different experiments.

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Evaluation of Transfection Efficiency by Southern Blotting—To determine whether differences in transfection efficiency could account for the increases in promoter activity of the V1bR promoter-luciferase deletion constructs, the amount of luciferase DNA was quantitated by Southern blot analysis after transfection of pGL3-V1bRp830, or pGL3-V1bRp830GAGA and 25–50 ng of pcR3.1-GAGA or the empty expression vector. After transfection, H32 cells were maintained in Dulbecco’s modified Eagle’s medium with 2.5% fetal bovine serum/horse serum for 24 h and then lysed using 1× reporter lysis buffer (Promega) and processed for luciferase assay (Promega).

Preparation of mRNA and Quantitative RT-PCR—For quantitative RT-PCR of V1b mRNA, cells were plated in 10-mm Petri dishes, transfected with 4 μg of pcR3.1-GAGA expression vector or the empty vector pcR3.1, and cultured for 24 h before mRNA isolation using RNAzol™ B (Tel-test, Inc.) following the manufacturer’s protocol. Poly(A)+ RNA was isolated from the total RNA using oligo(dT) Poly-ATtract mRNA isolation systems (Promega). RNA was subjected to digestion with 1 unit of DNase I, Amplification Grade (Invitrogen) to eliminate contaminant endogenous DNA and quantified by UV spectrometry.

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Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts from different rat tissues were prepared using NE-PER™ nuclear and cytoplasmic extraction reagent (Pierce) according to the manufacturer’s instructions.

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nition, unlabeled oligonucleotides (5-, 10-, 30-, and 50-fold) were added to the reaction and incubated for 20 min at room temperature. For competition experiments (nonlabelled competitors) for 45 min at 37 °C.

Bars represent the means ± S.E. of values from three experiments. *p < 0.05 versus cells transfected with the empty vector.

Fig. 3. Effect of GAGA-binding protein on endogenous V1bR mRNA expression in H32 cells. A, cells were transfected with the empty expression vector pCR3.1 or pCR3.1-GAGA. V1bR and cyclolin (Cyph) mRNAs were measured by quantitative RT-PCR. Samples without the reverse transcription step (−RT) were used as controls to exclude genomic contamination. B, quantitative analysis of the bands was performed using the Kodak 1D image analysis system. Bars represent the means ± S.E. of values from three experiments. *p < 0.05 versus cells transfected with the empty vector.

Nonidet P-40 (Fluka). Comparison of GAGA-binding activity between control and stressed rats was performed using 1 μg of nuclear extract prepared from pools of two anterior pituitaries. In these experiments, protein concentrations were determined using the sensitive BCA protein assay (Pierce) before and after adjusting the protein concentration to 0.3 μg/μl. Reaction products were electrophoresed on a 5% polyacrylamide gel (29:1 acrylamide:bisacrylamide) in 0.5 × Tris borate/EDTA at room temperature for 90 min at 150 V. Gels were vacuum heat-dried, and bands were visualized and quantified using the Molecular Dynamics PhosphorImager after 2 h of exposure at room temperature.

Post-Performace Liquid Chromatography (FPLC) Fractionation of Nuclear Extracts—To determine the apparent molecular mass of the GAGA-binding protein complex, ~500 μg of rat pituitary nuclear extract in 250 μl were subjected to size-exclusion chromatography on a Sephadex G-75 column using an FPLC system (Amersham Biosciences) at 4 °C. The column was equilibrated with buffer containing 20 mM HEPES (pH 7.9), 20% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, and 0.5 mM DTT at a flow rate of 0.5 ml/min and then clarified by multiple runs with protein standards (ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), and albumin (67 kDa); Amersham Biosciences). A standard curve was obtained from a plot of the distribution coefficient Kav versus log(molecular mass). The nuclear extract, diluted in the equilibration buffer, was then run on the column, and fractions of 400 μl were collected for analysis of GAGA-binding activity by EMSA. Prior to each run, freshly prepared 4-(2-aminoethyl) benzenesulfonfluoride (0.5 mM; ICN, Costa Mesa, CA) was added to the equilibration buffer to prevent low level proteolysis of the nuclear proteins during the runs. To test whether the apparent mass of the binding activity could be shifted in the presence of 0.8% deoxycholate (a mild ionic detergent known to disrupt protein-protein interaction), the fractionation was repeated in the presence of this detergent, and the fractions were analyzed for GAGA-binding activity after reconstitution of the proteins with Nonidet P-40. The apparent molecular masses of the peaks of GAGA-binding activity were calculated using the standard curve.

RESULTS

Transcriptional Activity of the Rat V1bR Promoter Deletion Constructs—A 2.5-kb fragment of the 5′-flanking region of the rat V1bR containing 2.1 kb upstream of the transcriptional start point, exon 1, intron 1, and 164 bp of exon 2 was previously shown to have promoter activity in several heterologous cell lines (10). To determine the minimal promoter sequence required for transcriptional activation, we examined the activity of the full-length 2.5-kb fragment and several deletion fragments linked to a luciferase reporter gene after transfection into the hypothalamic cell line H32 (21), which expresses V1bR mRNA. As shown in Fig. 1A, the 2.5-kb construct (V1bRp2.5-Luc) showed 5-fold higher luciferase activity than the pGL3-basic empty vector. In addition, as previously described in other cell lines (10), a V1bR promoter construct lacking the intron had no promoter activity in H32 cells (data not shown).

The 830- and 670-bp proximal fragments (V1bRp830-Luc and V1bRp670-Luc, respectively) showed significantly higher luciferase activity than the full-length 2.5-kb fragment, with 11- and 12.5-fold increases, respectively. To determine whether the higher luciferase activity was due to higher promoter activity or to an increase in transfection efficiency, we used Southern blot analysis to evaluate the amount of plasmid DNA after transfection of H32 cells with the V1bRp2.5-Luc, V1bRp830-Luc, and V1bRp670-Luc constructs. The transfection efficiency of V1bRp830-Luc and V1bRp670-Luc was similar to that of the 2.5-kb construct, indicating that V1bRp830-Luc and V1bRp670-Luc have higher promoter activity (Fig. 1, B–D). The 470-bp proximal fragment lacking the GAGA box had no promoter activity. This suggests the presence of repressor elements upstream of bp −294 and that the region between bp −294 and +377 (containing an AP2 site, the GAGA and CACA boxes, and intron 1) is responsible for minimal promoter activity. Deletion of the GAGA box (bp −303 to −90; V1bRp830ΔGAGA) from the 830-bp construct abolished pro...
moter activity, suggesting that the GAGA box is important for V1bR gene transcription.

To determine whether a recognized GAGA-binding protein can affect V1bR promoter activity, we cotransfected the 2.5-kb V1bR promoter-luciferase construct (V1bRp2.5-Luc) with an expression vector containing the Drosophila GAGA factor (pCR3.1-GAGA) into H32 cells. As shown in Fig. 2, cotransfection of V1bRp2.5-Luc and pCR3.1-GAGA into H32 cells caused a 11-fold increase in luciferase activity compared with cotransfection with the empty expression vector. Cotransfection of pCR3.1-GAGA had no effect on luciferase activity of the pGL3-basic empty reporter vector. Deletion of the GAGA repeats in the V1bR promoter significantly decreased basal V1bR promoter activity and abolished the stimulatory effect of the Drosophila GAGA factor.

Effect of the Drosophila GAGA Factor on Endogenous Expression of the V1bR—Because H32 cells express endogenous V1bR, we investigated the effect of Drosophila GAGA-binding protein on the expression of the endogenous V1bR gene. To address this issue, we transfected H32 cells with the pCR3.1 empty vector or the construct containing the GAGA protein (pCR3.1-GAGA) and measured V1bR mRNA by quantitative RT-PCR using cyclophilin as a control and under conditions yielding PCR products for both genes in the linear range. As shown in Fig. 3 (A and B), transfection of Drosophila GAGA had no effect on the 250-bp cyclophilin band, but it increased the 509-bp band corresponding to the V1bR mRNA by 6-fold. Similar increases in endogenous V1bR mRNA were found after transfection of MCF-7 cells with the Drosophila GAGA factor (data not shown).

V1bR GAGA Repeats Bind Nuclear Proteins—To determine whether the V1bR GAGA box interacts with pituitary nuclear proteins, we performed EMSA after incubation of pituitary nuclear extracts with (GAGA)$_3$ or (GAGA)$_5$ repeat oligonucleotides flanked by the sequences found in the V1bR promoter. A shifted band of increasing intensity was observed with increasing amounts of nuclear extract from 0.5 to 3 μg; binding maintained a plateau up to 10 μg of protein. A similar binding pattern was observed using either the (GAGA)$_3$ or (GAGA)$_5$ repeat probe, but the amount of probe shifted was higher.
for the (GAGA)$_5$ oligonucleotide than for the (GAGA)$_3$ repeats (Fig. 4).

Binding of pituitary nuclear extracts to radiolabeled (GAGA)$_3$ and (GAGA)$_5$ repeats was effectively competed by the homologous unlabeled oligonucleotide (Fig. 5A). For both probes, addition of five times the molar concentration of unlabeled oligonucleotide caused $\sim 80\%$ inhibition of the shifted bands. A progressive but slower binding inhibition was observed with higher concentrations of unlabeled oligonucleotide, with $\sim 5\%$ of the binding remaining in the presence of 50 times the molar concentration of competitors (Fig. 5B). In contrast, no inhibition of the binding of the (GAGA)$_3$ probe to nuclear proteins was observed after addition of a mutant oligonucleotide (Fig. 5C). Furthermore, the radiolabeled mutant oligonucleotide did not bind nuclear proteins (Fig. 5C).

The time course of the binding of GAGA repeat oligonucleotides to pituitary nuclear proteins is shown in Fig. 6A. Binding was already marked after 3 min, reached equilibrium by 18 min, and maintained a plateau for up to 1 h. Consistent with the data in Fig. 4, using equimolar concentrations of probe, the percentage of radiolabeled probe shifted was much higher for the (GAGA)$_5$ repeat oligonucleotide. Dissociation of DNA-protein complexes by addition of the competitor at the time when binding of radiolabeled (GAGA)$_3$ oligonucleotides to pituitary nuclear extracts had reached equilibrium was very rapid. One min after addition of unlabeled (GAGA)$_3$ and (GAGA)$_5$ repeats, 55 and 63% binding inhibition was already observed, respectively (Fig. 6B). A slow further decline in binding was observed from 1 to 16 min after addition of the competitor. GAGA-binding activity was not confined to pituitary nuclear proteins, but was present in nuclear extracts from a number of rat tissues (Fig. 7). The highest binding was observed in the brain cortex, hypothalamus, and kidney, followed by moderate binding in the pituitary, liver, thymus, and ovary and very low binding in the spleen.

Effect of Acute Stress on GAGA-binding Activity—To determine the possible involvement of GAGA-binding proteins in V1hR transcription in vivo, we used EMSA to compare the GAGA-binding activity of pituitary nuclear proteins from controls and rats subjected to restraint stress, a condition associated with up-regulation of V1hR expression. PhosphorImager

![Fig. 5. Specificity of the binding of pituitary nuclear extracts to (GAGA)$_3$ and (GAGA)$_5$ oligonucleotides. A, inhibition of binding by addition of increasing amounts of unlabeled homologous oligonucleotide; B, quantification of the intensity of the bands in the gel shown in A; C, EMSA following incubation of pituitary nuclear extracts with (GAGA)$_3$ (3x-GAGA) or a (GAGA)$_3$ mutant oligonucleotide (3x-GAGAmut) used as a probe or competitor. 5x-GAGA, (GAGA)$_5$; AU, arbitrary units.](image)

![Fig. 6. A, time course of the binding of radiolabeled (GAGA)$_3$ or (GAGA)$_5$ repeats to pituitary nuclear extract. Quantification of the shifted bands was performed using the PhosphorImager. B, binding dissociation curve in the presence of unlabeled (GAGA)$_3$ (3x-GAGA) or (GAGA)$_5$ (5x-GAGA) repeats added at binding equilibrium. The intensity of shifted bands was quantified, and the results are expressed as the percentage of total binding in the absence of competitor. B/Tx100, % bound/total.](image)
analysis of the shifted bands revealed an increase in binding of pituitary nuclear proteins from stressed rats to radiolabeled GAGA oligonucleotides compared with nuclear extracts from non-stressed control rats. The increases in binding were minor by 15 min, reached a significant 40% by 30 min, and declined again by 90 min (Fig. 8). In contrast, no increases in intensity of the shifted bands were observed in EMSA using liver nuclear extracts (data not shown).

GAGA-binding Activity of Pituitary Nuclear Extracts Corresponds to Protein Complexes—To determine whether the GAGA-binding activity observed in pituitary nuclear extracts involves a single protein or a protein complex, binding of the nuclear extracts to the radiolabeled GAGA oligonucleotide was performed in the presence of increasing concentrations of sodium deoxycholate. It has been shown that this treatment dissociates protein-protein complexes without affecting DNA-protein interaction, an effect that is reversible by addition of the nonionic detergent Nonidet P-40 (22, 23). As shown in Fig. 9A, disruption of protein-protein interaction by deoxycholate inhibited the binding of nuclear proteins to DNA; shifted bands were markedly decreased in the presence of 0.8% deoxycholate and were abolished with 1.2%. Addition of 1% Nonidet P-40 on its own to the binding reaction had no effect on the shifted bands (Fig. 9A, last lane), but considerably reduced the inhibitory effect of deoxycholate (Fig. 9B). To determine whether dissociation of protein-protein complexes by deoxycholate affected the absolute binding activity or the affinity of protein monomers for DNA, we incubated increasing amounts of pituitary nuclear extract with (GAGA)3 repeats in the presence or absence of 0.8% deoxycholate. As shown in Fig. 10 (A and B), the inhibitory effect of deoxycholate was not overcome by increasing protein concentrations, suggesting that protein complex formation is essential for DNA binding.

The apparent molecular mass of the protein complex was examined by size-exclusion chromatography of pituitary nuclear extracts in the presence or absence of sodium deoxycholate. In the absence of deoxycholate, the binding activity was observed in the included volume with a peak in fraction 21 (Fig. 11A and B). The apparent molecular mass of this binding activity was calculated to be $\pm 127 \times 12$ kDa. There was no binding detected in the fractions from the run performed in the presence of 0.8% deoxycholate (data not shown). However, when Nonidet P-40 was added to reconstitute protein complexes, the binding activity was then observed in fractions 21–25, with a peak in fraction 23 corresponding to a calculated apparent molecular mass of $\pm 70$ kDa (Fig. 11C). Collectively, these data suggest that a protein complex of $\pm 127 \times 12$ kDa is responsible for DNA binding to GAGA repeats and that this protein complex may include two subunits, each with an apparent molecular mass of $\pm 70$ kDa.

**DISCUSSION**

The recent characterization of the 5′-flanking region of the V1bR has made it possible to study the transcriptional regulation of the receptor. Previous studies in this laboratory have shown that a 2.5-kb V1bR gene fragment (bp -2161 to +377 from the proximal transcriptional start point, corresponding to 2.1 kb of the putative promoter, exon 1, intron 1, and 164 bp of exon 2) has promoter activity in cell lines not expressing the V1bR (10). We now show that this same DNA fragment is capable of driving reporter gene expression in the hypothalamic cell line H32 (21), which expresses the V1bR. The increase in luciferase activity after deletion of sequences upstream of bp -294 suggests that the shorter fragments have higher promoter activity. The demonstration by Southern blotting of similar transfection efficiency for all constructs strongly suggests that the region between bp -294 and -2161 of the V1bR promoter contains repressor elements. Previous studies in AtT-20 cells have suggested the presence of repressors in the V1bR promoter (10). It is also clear from the data that the GAGA box is essential for full promoter activity.

The proximity of an inverted GAGA box to the transcriptional start point of the V1bR gene, in conjunction with the dramatic decrease in basal promoter activity following deletion of the GAGA box, suggested to us that this sequence is important for the transcriptional regulation of the receptor (10). The present study provides evidence supporting this hypothesis. First, a recognized GAGA-binding protein, the *Drosophila* GAGA factor, stimulates V1bR promoter activity. This increase in V1bR promoter activity by the *Drosophila* GAGA factor is probably mediated by the GAGA box in the promoter because the effect was abolished by deletion of bp -305 to -90 (V1bRp830GAGA), which include the main stretch of GAGA repeats, preserving the upstream regulatory elements. In addition, the fact that transfection of the GAGA factor increased the expression of endogenous V1bR provides strong evidence that a GAGA-binding protein is involved in the regulation of the V1bR.

It has been reported that a GAGA-binding protein is involved in the transcriptional regulation of other vertebrate genes,
including the human type 1 angiotensin II receptor gene, the rat serine protease inhibitor-1 gene, and the *Xenopus* stromelysin-3 gene (16–18). In the case of serine protease inhibitor-1 and the type 1 angiotensin II receptor, GAGA-binding activity is stimulated by growth factors, and the GAGA box appears to mediate growth factor-mediated gene transcription (16, 17). GAGA box-mediated transcriptional activation of the V1bR promoter could also involve growth factors because recent experiments in our laboratory have shown increases in GAGA-binding activity after incubation of H32 cells with the epidermal growth factor. The marked reduction in basal promoter activity following deletion of the main GAGA box from the V1bR minimal promoter suggests that the GAGA repeats are required for initiation of transcription, as has been postulated for type 1 angiotensin II receptor and serine protease inhibitor genes (17, 24).

The shift of radiolabeled GAGA repeat oligonucleotide

![Graph](image-url)
probes by pituitary nuclear proteins indicates that endogenous nuclear proteins have the ability to bind to the GAGA box of the V1bR. The markedly higher binding activity of the (GAGA)$_5$ repeat compared with the (GAGA)$_3$ repeat could be due to higher affinity of the larger repeats for the binding proteins. However, this possibility is not supported by the kinetics of interaction between the two probes and nuclear extracts, with similar association and dissociation patterns. The GAGA box of the V1bR, comprising 134 bp, is unusually large. Although the consensus GAGA binding sequence consists of ~3.5 GA repeats, atypical sequences frequently found in other promoters have also been shown to bind GAGA-binding proteins (16–18).

**Fig. 10.** Increasing protein concentration does not restore binding of nuclear extracts to GAGA oligonucleotides in the presence of deoxycholate. A, EMSA following binding of (GAGA)$_3$ oligonucleotides to 1–10 µg of nuclear extract with and without 0.8% deoxycholate (DOC); B, quantitative analysis of the shifted bands. AU, arbitrary units.

**Fig. 11.** A, gel shift analysis of FPLC fractions from pituitary nuclear extract after incubation with (GAGA)$_3$ repeats. Control, pituitary nuclear extract not subjected to fractionation. B, gel shift analysis of pituitary nuclear extract pretreated with 0.8% sodium deoxycholate before FPLC fractionation. After fractionation, protein complexes were reconstituted by addition of 1% Nonidet P-40 before binding to the (GAGA)$_3$ repeat probe. C, quantification of the intensity of the shifted bands for the native protein (---) and for the sodium deoxycholate (DOC)/Nonidet P-40-treated extracts (⋯⋯). The apparent molecular mass of the bound protein was calculated using the formula $y = 1.163 - 0.225x$ ($r^2 = 0.99$, where $x = \log($molecular mass$)$ and $y = K_{av}$), obtained after calibration of the Sephadex G-75 column with different standards. AU, arbitrary units.
The V1bR promoter contains several short sequences for GAGA upstream of the major GAGA box, which may explain a minor stimulatory effect of the cotransfected GAGA factor on promoter activity of the construct lacking the GAGA box.

This study clearly shows that pituitary nuclear proteins can interact with GAGA repeats. The finding of GAGA-binding activity in a number of tissues in the rat is consistent with the view that GAGA repeats may be involved in the transcriptional regulation of a variety of mammalian genes (25). The rat GAGA-binding protein is structurally different from the Drosophila GAGA factor because an antibody recognizing the latter (24, 26) failed to supershift the band in EMSA (data not shown). Although the exact identity of the GAGA-binding protein interacting with the V1bR promoter remains to be elucidated, it is clear from the data that DNA-binding activity requires the formation of a protein complex, which can be dissociated by deoxycholate. Although the native nuclear protein complex showed an apparent molecular mass of \( \approx 127 \pm 12 \) kDa after size-exclusion chromatography, Nonidet P-40 reconstitution of the complex after fractionation of the deoxycholate-dissociated extract showed that the binding activity was recovered in the \( \approx 70-70 \) kDa range. This indicates that all components of the complex were eluted in the same fractions and suggests that the active complex may correspond to a homo- or heterodimer of proteins of \( \approx 70 \) kDa. The present findings are consistent with characteristics described for Drosophila GAGA-binding proteins (25). A number of cDNA species have been described for Drosophila GAGA factor, encoding isoforms of 70–75 kDa (GAGA-519) and 80–90 kDa (GAGA-581). The two isoforms have been shown to form both homo- and heteromeric complexes (25).

An important question is whether GAGA-binding activity in nuclear extracts in the pituitary corticotropin is regulated during physiological conditions leading to changes in V1bR expression (6). Because we have previously shown that restraint stress causes rapid increases in pituitary V1bR mRNA, it was important to determine whether this stress paradigm could increase GAGA-binding activity in the pituitary. The present demonstration that pituitary extracts from stressed rats indeed display higher GAGA-binding activity supports a role for the GAGA box in the physiological regulation of V1bR transcription. Moreover, this effect appears to be specific for the pituitary because no differences between control and stressed rats were found in EMSA analysis of liver nuclear proteins. The effect of stress on GAGA-binding activity of pituitary extracts was relatively small. Because corticotrophs represent \(<10\%\) of the pituitary cell population (27), GAGA-binding proteins present in other pituitary cell types would mask changes in GAGA-binding activity in the corticotroph. It is noteworthy that the increases in GAGA-binding activity during stress were early (30 min) and transient. This is consistent with the possibility of activation of GAGA-binding proteins by phosphorylation, as has been reported for growth factor-mediated regulation of the type 1 angiotensin II receptor promoter (17).

In conclusion, these studies provide evidence that GAGA-binding proteins stimulate transcription of the V1bR by interaction with GAGA repeats present in the promoter of the V1bR. This is supported by the marked increase in V1bR promoter-driven luciferase after cotransfection with the GAGA factor, the ability of transfected GAGA protein to activate endogenous V1bR expression, and the ability of endogenous pituitary nuclear proteins to bind GAGA repeat oligonucleotides. The binding protein corresponds to a protein complex with an apparent molecular mass of \( \approx 127 \) kDa, which may comprise two 70-kDa subunits.

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