Transcriptional Activation of the Rat Vesicular Monoamine Transporter 2 Promoter in Gastric Epithelial Cells

REGULATION BY GASTRIN*

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Vesicular monoamine transporter 2 is important for the accumulation of monoamine neurotransmitters into synaptic vesicles and histamine transport into secretory vesicles of the enterochromaffin-like cell of the gastric corpus. In this study we have investigated the mechanisms regulating the transcriptional activation of the rat vesicular monoamine transporter 2 (VMAT2) promoter in gastric epithelial cells. Maintenance of basal levels of transcription was dependent on the presence of SP1, cAMP-response element (CRE), and overlapping AP2/SP1 consensus sequences within the region of promoter from −86 to +1 base pairs (bp). Gastrin stimulation increased transcriptional activity, and responsiveness was shown to be dependent on the CRE (−33 to −26 bp) and AP2/SP1 (−61 to −48 bp) consensus sites but independent of the SP1 site at −86 to −81 bp. Gastrin-induced transcription was dependent on the cooperative interaction of an uncharacterized nuclear factor of −23.3 kDa that bound to the putative AP2/SP1 site, CRE-binding protein (CREB), and CREB-binding protein/p300. Gastrin stimulation resulted in the increased binding of phosphorylated CREB to the promoter, but it did not result in the increased binding of the AP2/SP1-binding protein. The gastrin responsiveness of the promoter was shown to be dependent on both the protein kinase C and mitogen-activated protein kinase/extracellular signal-regulated kinase-signaling pathways, which may converge on the AP2/SP1-binding protein.

The transport protein vesicular monoamine transporter 2 (VMAT2) plays a central role in cellular physiology. It is responsible for the accumulation of monoamine neurotransmitters into synaptic vesicles and has been implicated in the synthesis and storage of histamine in the interleukin-3-dependent cell line Ea3.123 (1–5). VMAT2 may play a significant role in locomotor stimulation and/or the behavioral reward produced by amphetamines, and malfunctions of monoamine transport have been implicated in psychiatric disease (6, 7).

Studies on VMAT2 knockout mice support the idea that vesicular monoamine transport is important in maintaining neuronal function with heterozygous VMAT2+/− mice having dysfunctional monoamine storage and release parameters (6, 8). VMAT2 is also likely to mediate histamine transport into the secretory vesicles of the enterochromaffin-like cell (ECL) of the gastric corpus (2, 4, 9–11). In the rat stomach the ECL cell is tightly regulated by the peptide hormone gastrin, with gastrin stimulation leading to the release of histamine and the resultant production of gastric acid from the parietal cell (12–14). Histamine is synthesized within the cytosol of the ECL cell from L-histidine by the action of the enzyme L-histidine decarboxylase (HDC) (15) and is then sequestered by VMAT2 into secretory vesicles that are stabilized by chromogranin A (CgA), a multi-functional acidic protein expressed both in neuroendocrine cells and in ECL cells (16–21). Evidence suggests that VMAT2 may be up-regulated to accommodate the increased histamine biosynthesis and secretion that accompanies ECL stimulation. Hypergastrinaemia-induced degranulation of the ECL cell is accompanied by a parallel secretion of histamine and CgA followed by enhanced production of both molecules, and in the pre-B cell line Ea3.123, the mRNA abundance for both VMAT2 and HDC is increased in a parallel fashion after mobilization of their intracellular calcium levels or by activation of protein kinase C (4, 5, 20–23). In addition, increases in the abundance of mRNA for HDC, VMAT2, and CgA were observed in animal models of hypergastrinaemia, suggesting that the transcriptional activity of these genes could be regulated by gastrin in vivo (24–26).

Indeed, gastrin has been shown to transcriptionally activate both the HDC and CgA promoters (27–29). Regulation of the human HDC promoter by gastrin involves a protein kinase C-dependent, MAP kinase/ERK-dependent, and AP1-dependent pathway and involves the binding of distinct nuclear factors to two cis-acting overlapping binding sites (GAS-RE1, +1 to +19; and GAS-RE2 +11 to +27) (30–32). Gastrin transactivation of the mouse CgA promoter was found to be dependent on the binding of Sp1 to an Sp1/Egr motif located at −88 to −77 bp and a CRE-like element at −71 to −64 bp of the mCgA promoter (29). Gastrin stimulation resulted in an increased binding of both Sp1 and CREB to their consensus sequences within the mCgA promoter, and overexpression of either Sp1 or phosphorylated CREB transactivated the promoter. Coexpression of both transcription factors resulted in an additive mCgA promoter response, suggesting that the effect of gastrin was brought about by their cooperative action.

In the present study we have investigated the transcriptional regulation of VMAT2 in the gastric epithelial cell line AGS-Gr. This cell line has been permanently transfected with the CCKRβ-gastrin receptor (33). It has been shown to express a functional CCKβ receptor and utilizes signaling pathways that...
are common to the ECL cell (33, 13). This has allowed us to examine the controls of both basal and gastrin-stimulated VMAT2 transcriptional activity in these gastric epithelial cells. Maintenance of the basal level of transcription was dependent on the presence of SP1, CRE, and overlapping AP2/SP1 consensus sequences within the region of promoter from −86 to +1 bp, whereas full gastrin responsiveness of the promoter depended on the presence of the intact CRE and AP2/SP1 consensus sequences. Regulation of the rat VMAT2 promoter was found to involve the protein kinase C-dependent and MAP kinase/ERK-dependent pathway, binding of a distinct nuclear factor of estimated molecular mass 23.3 kDa to the putative AP2/SP1 consensus site and the binding and phosphorylation of CREB to the CRE site within the promoter. Thus, our study demonstrates that the rat VMAT2 promoter can, like the HDC and mCgA promoters, be transcriptionally regulated by gastrin, but the mechanism of this regulation is distinct from that observed for these other gastrin-sensitive promoters.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ham’s F-12, fetal bovine serum, and penicillin/streptomycin were from Life Technologies, Inc. Gastrin (10 nM from Peninsula Laboratories, Inc.) was obtained from Biotalia, and BiotAQ DNA polymerase was obtained from Boehra, London, UK. A gel shift assay system containing HeLa and AP2 nuclear extracts and consensus oligos for AP1, OCT1, CREB, NF-Kβ, and TFIID was obtained from Promega. Additional oligonucleotides were synthesized by Sigma-Genosys, and all antibodies were purchased from Santa Cruz Biotechnology, Inc. apart from the phospho-CREB antibody, which was from Upstate Biotechnology and peroxidase-conjugated anti-goat IgG from Sigma. Complete, mini-protease inhibitor mixture table diagrams were from Roche Molecular Biochemicals. The inhibitors GF109303X and PD98059 were obtained from Calbiochem. All other reagents were obtained from Sigma.

**DNA Constructs and Plasmids**—The promoter region of the rat VMAT2 gene had been previously cloned, and a subclone pV2404-8 was generated in the pGEM-TEasy vector (Promega, Southampton, UK) as described previously (5). The subclone pV2404-8 was sequenced in both directions by an automated dyeodeoxy method and used to make a series of VMAT2 promoter 5′-deletional constructs in the pGL3- Basic vector (Promega) that contained 1632, 1289, 943, 609, 223, 86, and 36 nucleotides upstream of the start site together with 55 bp of exon 1. Polymerase chain reaction products from pV2404-8 were directionally cloned between the SacI and XhoI sites of pGL3-Basic, and the resultant constructs were sequenced in both directions to confirm their integrity. In addition, polymerase chain reaction products from pV2404-8 that contained 86 and 36 nucleotides upstream of the transcriptional start site together with 55 bp of exon 1 were directionally cloned between the BamHI and XhoI sites of the reporter luciferase-reporter vector FXP2. Again, the integrity of these constructs and the sequences of mutated constructs generated by polymerase chain reaction were confirmed before their use in experiments. The mutations generated in these constructs are indicated in the appropriate figures and their legends.

**Cell Culture and Transfection Studies**—AGS-Gc cells, which were permanently transfected with the human CCK2Gastrin receptor (33) driven by the EF1-α promoter under pyruvycin selection, were grown in Ham’s F-12 medium supplemented with 10% (fetal bovine serum) and penicillin/streptomycin (100 IU/ml) at 37 °C in 5% CO2. Transient transfections were carried out using TransFastTM transfection reagent from Promega. Cells were plated out at a density of 5 × 104 cells/65-mm well 24 h before transfection. During transfection, cells were incubated for 1.5 h in 2 ml of transfection mix in serum-free media. The transfection mix consisted of 3.4 μl of TransFastTM, 1.5 μg of luciferase reporter construct, and 0.1 μg of Renilla luciferase (pRL-TK) control vector (Promega) per well. The quantity of firefly luciferase reporter was reduced to 1 μg/ml when additional expression constructs were added. Transfected cells were then incubated for 0.5 μg/ml. Cells were stimulated 24 h post-transfection as described in the figure legends and harvested at appropriate time points after stimulation. Luciferase activity was determined using the dual luciferase reporter system (Promega), and each individual transfection was assayed in duplicate.

**Electrophoretic Mobility Shift Assays**—Nuclear extracts from AGS-Gc cells and rat gastric corpus epithelium were prepared using the methodology described in Schreiber et al. (34). Double-stranded oligonucleotides were radiolabeled with [α-32P]dATP, and EMSAs were performed with 10 μg of nuclear extracts in a final volume of 20 μl of binding buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 μg of poly(dA-dT), 10% glycerol, and 10 μM Zn2+. Mixtures were incubated with 10 fmol of double-stranded oligonucleotides for 20 min on ice. After addition of the supplier shift assays, nuclear extracts were incubated with 1 μl of anti-phospho-CREB, anti-ATF, and anti-AP2 antibodies but 2 μl of anti-Sp family antibodies. In addition, the Sp supershifts required the addition of 4 μg of bovine serum albumin to the binding reactions. DNA-protein complexes were electrophoresed on a 6% nondenaturing polyacrylamide gel. The dried gel was exposed to a phosphor storage screen, and the image was analyzed with a PhosphorImager (Molecular Dynamics, Sevenoaks, Kent, UK).

**UV Cross-linking**—20-μg aliquots of AGS-Gc nuclear extract were incubated with 10 fmol of Klenow [α-32P]dATP end-labeled probes (A, B, and E, as described in the legend to Fig. 6) in EMSA binding buffer for 30 min on ice. The samples were exposed to UV light for 60 min before addition of an equal volume of 2× SDS sample buffer and heating to 99 °C for 5 min. 10 μg of each sample and prestained molecular weight markers were electrophoresed on a 12% SDS-polyacrylamide gel in Tris/glycine electrophoresis buffer. The gels were dried, and radioactivity associated with the proteins was visualized using a PhosphorImager.

**Western Blot Analysis**—5 × 105 AGS-Gc cells were plated in 65-mm culture dishes containing 5 ml of complete medium. 18 h later, the medium was removed and replaced with 5 ml of serum-free medium, and the cells were incubated in the absence or presence of gastrin (5 × 10-8 M) for 3, 6, 12, and 24 h before preparation of a total cell lysate. To prepare the cellular lysate AGS-Gc cells were washed twice with ice-cold phosphate-buffered saline and resuspended in 250 μl of lysis buffer containing 20 mM Tris (pH 7.8), 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 10 mM NaF, 15 mM benzamide, 8.5 mM sodium orthovanadate, 0.5 mM dithiothreitol, 10 mM/ml phenylmethylsulfonyl fluoride, and one protease inhibitor mixture tablet/10 ml of lysis buffer. 40 μg of the lysates were electrophoresed on a 12% SDS-polyacrylamide gel. After electrophoresis, the proteins were blotted on nitrocellulose membranes, and immunodetection of the proteins was performed. The primary antibody used was obtained from Santa Cruz Biotechnology and is specific for mouse, rat, and human VMAT2 but is non-cross-reactive with VMAT1. The secondary antibody was a peroxidase-conjugated anti-goat IgG. Enhanced chemiluminescence (SuperSignal West Pico chemiluminescent substrate; Pierce) was used to identify the VMAT2 protein.

**Statistical Analysis**—Results were analyzed for statistical significance using the Student’s t test for independent samples.

**RESULTS**

**Basal Transcriptional Activity of the Rat VMAT2 Promoter in the AGS-Gc Cell Line.**—The basal transcriptional activity of the rat VMAT2 promoter in the gastric environment was characterized by ligating polymerase chain reaction-generated segments of the 5′-flanking region of the VMAT2 gene upstream of the gene for firefly luciferase in the reporter vector pGL3-Basic and transfection of the resultant constructs together with a Renilla luciferase vector into AGS-Gc cells. The AGS-Gc cell line has been permanently transfected with the CCK2-Gastrin receptor and has been demonstrated to show specific activation by gastrin (33). As shown in Fig. 1, transfection of all the constructs resulted in significant luciferase activity with the largest construct v1632pg (encompassing nucleotides −1632 to −55 bp) showing an approximate 10.3-fold increase in luciferase activity over that seen in the promoterless vector. Activity was maximal in the construct v223pg (−223 to −55 bp), which showed an average 44.7-fold increase in luciferase activity over promoterless vector. This level of expression was maintained in the construct v86pg (−86 to −55 bp). The increase in luciferase activity observed in the v223pg construct as compared with v609pg (−609 to −55 bp) suggests the presence of at least one negative regulatory element in the region from −609 to −223 bp. The schematic illustrated in Fig. 2a shows the sequence of the 5′-flanking region of the rat VMAT2 gene from −86 to −55 bp, with bases numbered relative to the transcriptional start.
Increased Transcriptional Activation of VMAT2 by Gastrin

Basal Transcriptional Activity of rat VMAT2 Promoter

FIG. 1. Basal transcriptional activity of the rat VMAT2 promoter in AGS-Gi2 cells. AGS-Gi2 cells were transfected with 5′-deletional VMAT2 promoter/luciferase constructs containing from 1632 down to 36 bp of promoter together with 55 bp of exon 1 in the promoterless vector pGL3b (constructs v1632pg, v1289pg, v943pg, v609pg, v223pg, v86pg, and v36pg are shown schematically on the left). Transcriptional activity is expressed as luciferase activity, and the results are representative of nine independent experiments assayed in duplicate.

FIG. 2. a, sequence of the 5′-flanking region of the rat VMAT2 gene. Bases are numbered relative to the transcriptional start site (+1), which was determined previously by primer extension analysis (5). Putative cis-regulatory elements (SP1, AP2/SP1, and CRE) are underlined; the 55 bp of exon 1 included in the VMAT2 promoter/luciferase constructs are double-underlined. The schematic in b illustrates the deletional and mutated VMAT2 promoter/luciferase constructs used in this study. v, VMAT2; 86, 48, or 36 is the number of bases of promoter in the construct; px, PXP2; pg, pGL3b. The sites mutated are indicated both in the construct name and in their associated line diagrams and are as follows: v86sp1pg, mutation of authentic SP1 site GGCCCGG to GAGCTC; v86ap2px, mutation of AP2/SP1 composite site CCCCTCCGCCC to AACGTCAGAAC; v86crepx, mutation of CRE site CTATTCGAGCTCAGGACGGCGGACTGAGGTCAGAAGGGGCTGAGG; v36mpx/pg, mutation of CRE site TGACGGCTGAGGTCAGAAGGGGCTGAGG.

Gastrin Responsiveness of the Rat VMAT2 Promoter in the AGS-Gi2 Cell Line—AGS-Gi2 cells were transiently transfected with the VMAT2-promoter luciferase constructs before stimulation with gastrin. Constructs that contained from 1632 to 86 bp of VMAT2 promoter in the vector pGL3b all showed significant increases in luciferase activity after stimulation with gastrin (Fig. 4a) compared with their basal levels of activity. The luciferase activity of the largest construct, v1632pg, was increased 8.2 ± 0.9-fold (mean ± S.E., n = 9) over basal activity after stimulation with gastrin (5 × 10−8 M) for a 24-h period. Truncation of the promoter region of this construct down to 86 bp did not lead to significant reductions in response. Gastrin stimulation led to a 7.1 ± 1.5-fold (mean ± S.E., n = 9) increase in the luciferase activity of the construct v609pg, which was not significantly different from that of construct v86pg, which was on average increased 5.2 ± 0.4-fold (mean ± S.E., n = 9). Stimulation of the v86px construct with 5 × 10−8 M gastrin increased the transcriptional activity of the promoter within 4 h of stimulation and was maximal at 24 h post-stimulation. Maximal responses were seen with 5 × 10−8 M gastrin, but significant stimulation was seen with 5 × 10−9 M gastrin (data not shown). Gastrin responsiveness was inhibited by the gastrin antagonist L740093, and Gly-extended gastrin was unable to increase the transcription of the v86px construct (data not shown). Western blot analysis demonstrated VMAT2 protein in the AGS-Gi2 cell line, but its abundance was not significantly elevated after gastrin stimulation of the cells (data not shown).
The luciferase activity of the promoterless pGL3b-empty vector was modestly increased after stimulation with $5 \times 10^{-8}$ M gastrin (2.67 ± 0.6-fold over basal levels, mean ± S.E., n = 9; Fig. 4a). Although this increase was not comparable with those observed with constructs containing the rat VMAT2 promoter segments, new constructs were made in the PXP2 luciferase vector to confirm and extend the data on gastrin responsiveness of the promoter. Stimulation with gastrin did not increase the intrinsic luciferase activity of the promoterless PXP2 vector (Fig. 4b). However the construct v86px showed a 4.44 ± 0.8 (mean ± S.E., n = 11) fold increase in luciferase activity over basal levels after gastrin stimulation (Fig. 4b), confirming our findings originally made using the pGL3b vector that the region of rat VMAT2 promoter from −86 to +55 bp was capable both of sustaining basal transcription and of increasing its transcriptional activity in response to gastrin. The gastrin responsiveness of constructs v48px (48 to +55 bp) and v36px was significantly reduced when compared with that of v86px.
The gastrin-induced luciferase activity of the mutated constructs was expressed as a percentage of that observed for the wild type (100%). Results are the mean ± S.E. and are representative of a minimum of six separate experiments. The n value for each construct pair is given in the text. AGS-GR cells were transiently transfected with the mutated VMAT2 promoter/luciferase constructs v86ap2/sp1px, v86ap2px, v86sp1/2px, and vcrepx or the parental construct v86px before gastrin stimulation, as described under “Experimental Procedures.” Luciferase activity is expressed as a fold increase relative to unstimulated controls (=1.0), and results are representative of a minimum of six independent experiments assayed in duplicate. n values are included in the text.

**Conclusion**

The gastrin responsiveness of the rat VMAT2 promoter was confirmed through its mutation from within the construct v86px (Fig. 5b). Gastrin increased the luciferase activity of the resultant vcrepx construct by only 1.3 ± 0.2-fold (mean ± S.E., n = 14) over basal levels, which was significantly less than the responsiveness of the parental v86px construct (4.4 ± 0.8-fold over basal levels; mean ± S.E., n = 11). However, the gastrin-stimulated luciferase activity of the construct v86ap2sp1px, in which the complete AP2/SP1 site was mutated, also showed a significantly reduced gastrin responsiveness (2.26 ± 0.44-fold over basal levels; mean ± S.E., n = 6) as compared with v86px, indicating that this site also might regulate the gastrin response (Fig. 5b). When only the AP2 part of the putative AP2/SP1 site was mutated in the construct v86ap2px, the gastrin response was not significantly different from that of the v86px construct. In contrast, when the SP1 part of the AP2/SP1 site was mutated in the construct v86sp1/2px, the gastrin responsiveness was significantly reduced compared with that of v86px (2.23 ± 0.25-fold increase over basal; mean ± S.E., n = 12). Thus, both the CRE and part of the composite AP2/SP1 site may be important in achieving full transcriptional activation after gastrin stimulation.

**Binding of Nuclear Proteins from the AGS-Gr Cell Line and Rat Gastric Corpus to the AP2/SP1 Consensus Sequence—EMSA analysis showed that a nuclear protein from the AGS-Gr cell line could bind to the putative AP2/SP1 Consensus Sequence (Fig. 6c).** Binding of this protein could be inhibited by inclusion in the binding reaction of a 100-fold excess of unlabeled wild type probe. However a 100-fold excess of competitor in which the AP2/SP1 site is completely mutated
isolated from rat gastric corpus also specifically bound to the putative AP2/SP1 site, as shown in Fig. 6c. The similar mobilities of the protein/AP2/SP1 site complexes that are observed for both the AGS-GR cell line and for the rat corpus suggest that the proteins are probably the same. A cellular extract enriched for AP2-binding protein was used to demonstrate the ability of AP2 protein to bind specifically to a canonical AP2 binding site in our assay system (Fig. 6b). A supershift of the bands binding to the AP2 oligonucleotide was observed in the presence of AP2o antibody but not by AP2β or -γ antibodies (Fig. 6c). None of these antibodies could supershift the band produced by nuclear extracts from the AGS-GR cells binding to the AP2/SP1 site (Fig. 6d). In addition, we were unable to detect a specific AP2 protein in the AGS-GR nuclear extracts that recognized our AP2 probe (data not shown), suggesting that this protein may not be abundant in this cell line. Specific binding was observed of AGS-GR nuclear proteins to a radiolabeled authentic SP1 probe (Fig. 7). In two cases this binding was shown to be specific. One of the proteins was supershifted by a SP1 antibody, whereas the binding of another protein was inhibited in the presence of a SP3 antibody. The AGS-GR protein, which bound to the AP2/SP1 site, was, however, not supershifted by any of the SP family antibodies (Fig. 7). In addition the mobility of the AP2/SP1-binding protein as observed in the gel-shift assay suggests that the protein is smaller than SP family proteins.

**Binding of Nuclear Proteins from the AGS-GR Cell Line to Regulatory Elements Present in the HDC and mCgA Promoters**—Fig. 8 shows the pattern of protein/DNA binding that is obtained using EMSA when nuclear extracts from AGS-GR cells are incubated with radiolabeled probes corresponding to the AP2/SP1 consensus sequence found in the rat VMAT2 promoter, an AP2/SP1 consensus site, which is found in the mCgA promoter (−90 to −77 bp) and where it has been reported that SP1 protein binds to regulate transcription (29), and the GAS-RE (+1 to +27 bp) of the HDC promoter, which has been shown to regulate gastrin responsiveness through the binding of two as yet uncharacterized nuclear proteins (32). Binding of proteins to the (−90 to −77 bp) fragment of the mCgA promoter (lane 3) was inhibited by a 100-fold excess of unlabeled wild type probe (lane 4) but not by a 100-fold excess of the AP2/SP1 sequence (lane 5). In addition we used EMSA to investigate the binding of AGS-GR nuclear extracts to the −100 bp to −43 bp region of the mCgA promoter and found no evidence for binding of the uncharacterized AP2/SP1 protein to this region (data not shown). The pattern of AGS-GR protein/DNA binding observed with the GAS-RE (+1 to +27 bp, lane 6) was also inhibited with a 100-fold excess of its unlabeled wild type probe but not by a 100-fold excess of the AP2/SP1 probe. These results indicate that the uncharacterized AP2/SP1-binding protein, which is important in the gastrin responsiveness of the VMAT2 promoter, is unlikely to be involved in regulation of the mCgA and human HDC promoters.

**Estimation of the Molecular Weight of AP2/SP1-Binding Protein from AGS-GR Cells**—To investigate the size of the AGS-GR nuclear protein that bound to the putative AP2/SP1 consensus sequence, UV cross-linking studies were performed as described under “Experimental Procedures.” The molecular weight of the oligonucleotide probes was subtracted from the molecular weight of the protein. As shown in Fig. 9 the wild-type probe (A), which contained the AP2/SP1 consensus sequence, bound to a protein of 23.3 kDa that did not bind to the mutant probe (B) or the SP1 probe (E). In addition, two proteins with molecular weights compatible with them being members of the SP family bound to the SP1 probe (E).
The protein that recognizes the AP2/SP1 putative site is not SP1. See Fig. 6 for sequences of double-stranded oligonucleotides used. Lane 1, binding of AGS-GR nuclear extracts to radiolabeled SP1 probe (E). Lane 2, binding of AGS-GR nuclear extracts to radiolabeled probe (E) in the presence of a 100-fold excess of unlabeled wild type competitor (E). Lanes 3–6, binding of AGS-GR nuclear extracts to radiolabeled probe (E) in the presence of anti-SP family antibodies as indicated. Lane 7, binding of AGS-GR nuclear extracts to radiolabeled wild type AP2/SP1 probe (A). Lane 8, binding of AGS-GR nuclear extracts to radiolabeled AP2/SP1 probe (A) in the presence of a 100-fold excess of unlabeled wild type competitor A. Lanes 9–12, binding of AGS-GR nuclear extracts to radiolabeled AP2/SP1 probe (A) in the presence of anti-SP family antibodies as indicated.

Binding of Nuclear Proteins from the AGS-GR Cell Line to the CRE Consensus Sequence in the Rat VMAT2 Promoter—We investigated the binding of nuclear proteins from the AGS-GR cells to a radiolabeled probe that contained from −36 to +16 bp of rat VMAT2 promoter. The radiolabeled probe was bound by a complex of proteins as shown in Fig. 10 (lane 1), and binding was inhibited in the presence of a 100-fold excess of unlabeled probe (Fig. 10, lane 2). Binding specificity was shown by the failure of a 100-fold excess of unlabeled competitor in which the CRE was mutated to inhibit binding (Fig. 10, lane 3). This was confirmed by use of an ATF family antibody to supershift the complex (Fig. 10, lane 4). Stimulation of the AGS-GR cells with gastrin (5 × 10⁻⁸ m) for 15 min before the extraction of nuclear proteins led to an increase in the binding of phosphorylated CREB to the promoter as determined using a phospho-CREB antibody (Fig. 10, lanes 5 and 7). However, gastrin stimulation did not lead to a detectable increase in the quantity of CREB bound to the promoter and neither did it increase the binding of the AP2/SP1 protein to its consensus sequence (data not shown).

Effect of Protein Kinase C Inhibitor GF109303X and MAPK Inhibitor PD98059 on the Gastrin Responsiveness of the Rat VMAT2 Promoter—The increase in transcriptional activity of the construct v86px, which was observed after gastrin stimulation (4.2 ± 0.39-fold over basal; mean ± S.E., n = 15), was reduced (2.61 ± 0.5-fold over basal; mean ± S.E., n = 11) to a level comparable with that observed for the construct v36px in the presence of the protein kinase C inhibitor GF109303X (Fig. 11a). However, the response of the v36px construct was not significantly affected by the presence of this inhibitor and nei-
The gastrin responsiveness of the v86px construct (66% ± 3% of original response; mean ± S.E., n = 6; Fig. 12b). These data thus in part support the concept of MAPK-dependent signaling through the AP2/SP1-binding protein. The overexpression of acidic CREB was used to confirm CREB transcriptional activity of the VMAT2 promoter (Fig. 12c). An acidic extension of A-CREB interacts with the basic region of CREB, forming a coiled-coil extension of the leucine zipper and, thus, preventing the basic region of wild type CREB from binding to DNA (36). Overexpression of A-CREB led to a 56 ± 2% (mean ± S.E., n = 6) reduction in the gastrin responsiveness of the construct v86px (Fig. 12c).

**The Adenoviral Oncoprotein E1A Inhibits the Gastrin Responsiveness of the Rat VMAT2 Promoter**—E1A is an adenoviral oncoprotein that binds to and inactivates p300/CBP (35). The gastrin-induced transcription of the construct v86px was inhibited by coexpression of wild-type E1A (44.5% ± 5.6% reduction of stimulated value; mean ± S.E., n = 6) but not by a mutant of E1A (ΔΔ2-36E1A) that is unable to bind CBP (Fig. 13). Thus, the effect of gastrin on transcription requires the involvement of p300/CBP.
stimulation with 5 dominant-negative (dn) ERK1, or dominant-negative (dn) ERK2 before stimulation with $5 \times 10^{-8}$ M gastrin. Panel c, AGS-Gc cells were transfected with v86px together with either vehicle control or acidic CREB before gastrin stimulation. Luciferase responses to gastrin are expressed as a percentage of the unstimulated activity (=100%) of each construct. Results are representative of at least six independent experiments assayed in duplicate. Individual $n$ values are given in the text.

The key physiological regulator of gastric acid secretion is histamine, whose synthesis, storage, and secretion from the ECL cell is regulated by the antral hormone gastrin (14). In the gastric mucosa of humans and rodents, the ECL cell is the major source of the histamine that is generated in the cytosol by HDC and sequestered into the ECL cell secretory vesicles by VMAT2 (2–4, 9–13). The abundance of mRNAs encoding VMAT2, HDC, and CgA are increased to accommodate the increased histamine biosynthesis and secretion that accompanies ECL-cell stimulation by gastrin (4, 5, 20–26). Elegant studies have clearly demonstrated that gastrin can regulate the human and rat HDC promoters together with the mouse CgA promoter (27–29), and in this study, we have investigated the possibility that gastrin can also regulate transcription of VMAT2. We had previously cloned and characterized the 5'-flanking region of the rat VMAT2 gene (5) and found it to contain no TATA or TATA-like sequence upstream of the transcriptional start site, but we identified multiple potential cis-regulatory elements. Of particular interest is the region of promoter from −86 to +1 bp, which contains SP1, CRE, and putative overlapping AP2/SP1 consensus sequences.

To investigate both basal and gastrin-stimulated transcriptional regulation of the VMAT2 promoter, we generated a series of 5'-flanking deletional constructs of the VMAT2 gene in the luciferase reporter vectors pGL3b and PXP2. All constructs generated were capable of driving the transcription of the VMAT2 gene in the AGS-Gc cell line, and responses were maintained in the construct v86pg, which contained the SP1, AP2/SP1, and CRE regulatory elements. Mutagenesis of either the SP1, AP2/SP1, or CRE consensus sequence significantly reduced basal activity of the parental construct, suggesting that basal transcription is maintained through interaction of a combination of transcription factors with these cis-regulatory elements. Gastrin stimulation resulted in the transactivation of all rat VMAT2-promoter luciferase constructs with significant responsiveness (−4–5-fold over basal) again being maintained in constructs that contained from −86 to +55 bp of the gene. Mutagenesis of the intact SP1 site at position −86 to −81 bp from within construct v86px did not affect the gastrin responsiveness of the promoter; however, mutation of the CRE from within the construct v36px did significantly reduce the gastrin response. The importance of the CRE in gastrin-stimulated transactivation of the VMAT2 promoter was confirmed by the significant reduction in responsiveness of the v86crepx construct when the CRE site was also mutated and the inhibition of the gastrin responsiveness of the v86px construct in the presence of acidic CREB. Moreover, using EMSA supershift analysis it was demonstrated that CREB bound to the CRE consensus sequence and that it was phosphorylated after gastrin stimulation. Because stimulation of CREB-dependent transcriptional activity is generally achieved by phosphorylation of the transcription factor, this was an important observation (37). We did not detect, however, any increase in the amount of CREB binding to the CRE site. Although our data indicate that CREB is necessary for gastrin responsiveness of the VMAT2 promoter, it also suggests that an additional factor that might act cooperatively with CREB is necessary for full gastrin responsiveness. Total mutagenesis of the putative AP2/SP1 site significantly reduced the gastrin responsiveness of the construct v86px as did partial disruption of this site through mutation of the SP1 section of this AP2/SP1 sequence. Mutation of the AP2 portion of the AP2/SP1 site did not inhibit the response of the v86px construct. We showed using EMSA supershift analysis that the protein that bound specifically to this site was unlikely to be a member of either the AP2 or SP family. Additionally the finding that the binding of the protein was not totally dependent on the presence of zinc and that the mobility of the protein-DNA complex was much greater than the SP family-DNA complexes suggests that it is smaller than an SP protein. This was confirmed by UV-cross-linking experiments, which estimated the molecular mass of the protein to be 23.3 kDa. A protein from rat gastric corpus nuclear extracts also
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bound to the AP2/SP1 consensus sequence, and the mobility of the corresponding protein-DNA complex as observed in EMSA suggested that the protein found in the corpus is the same as that found in the AGS-Ga cells. The AGS-Ga cells utilize similar signaling pathways to the ECL cell (13, 30, 33), and the finding that they contain transcription factors common to cells in the corpus make them an appropriate cell type to use in this study. The overexpression of the adrenocortical oncogene E1A inhibited the gastrin responsiveness of the v86px construct, whereas overexpression of a mutant form that is unable to bind p300/CBP failed to do so. These data imply that gastrin might transcriptionally activate the rat VMAT2 promoter through a mechanism involving the cooperative interaction of CREB, the AP2/SP1-binding protein, and p300/CBP.

A growing number of studies demonstrate a role for p300/CBP in connecting CREB to different transcription factors and the MAP kinase/ERK intracellular signaling pathways (41–44). The use of inhibitors and dominant-negative ERKs demonstrated that the gastrin response of the rat VMAT2 promoter was potentially regulated by both the protein kinase C and MAPK signaling pathways, and the putative target of these signaling systems is the protein that binds to the AP2/SP1 site. Activation of our constructs in which only the CRE consensus sequence remained intact (truncated and mutated constructs v36px and v86sp1px) was less dependent on these signaling pathways. Our previous studies showed that mobilization of intracellular calcium can increase the transcriptional activity of the VMAT2 promoter in a manner dependent on the CRE consensus sequence, and since activation of the CCK_{0}-gastrin receptor is known to lead to an increase in free cytosolic calcium, it is possible that in the absence of the AP2/SP1 consensus site, activation of the rat VMAT2 promoter may be largely through this pathway. The finding that the gastrin response of the construct v36px was slightly inhibited by the overexpression of the dominant-negative ERK-2 is not inconsistent with the finding that it is largely the activity of the AP2/SP1 protein, which is regulated by the protein kinase C/MAPK pathways, as it is possible that there is some interaction of the AP2/SP1-binding protein and p300/CBP in the absence of DNA binding. Indeed, c-Jun can stimulate CBP-mediated transcription in a manner that is independent of its ability to bind DNA (35). However, it is recognized that CREB phosphorylation through activation of Ca_{2+}+calmodulin or MAP kinase-dependent pathways can occur (38–40). Gastrin-dependent regulation of the mCgA promoter has been shown to involve the cooperative interaction of both SP1 and CREB transcription factors (29), but the signal transduction pathways regulating these proteins in this system remain to be elucidated.

The gastrin-stimulated transcription of the rat VMAT2 promoter is similar to that of the mCgA promoter in that it involves the phosphorylation of CREB, but unlike the mCgA promoter, the gastrin responsiveness of the VMAT2 promoter appears to be independent of the SP1 transcription factor. The −100 to −43-bp region of the mCgA promoter is transcriptionally responsive to gastrin, and within this region, the SP1 protein binds to a consensus sequence contained within a larger AP2 consensus site (Fig. 8). This sequence is different from the AP2/SP1 consensus sequence, which we have described for the rat VMAT2 promoter, and the AP2/SP1-binding protein, which is described in this study did not bind to the CgA consensus sequence or to any part of the −100 to −43-bp region. The gastrin-dependent regulation of the HDC promoter is dependent on the protein kinase C/MAPK pathways (28, 30, 31) and is mediated by two distinct nuclear factors of 52- and 35-kDa, which remain to be characterized (32). The gastrin responsiveness of the rat VMAT2 promoter shares with the HDC promoter its dependence on the protein kinase C/MAPK-signaling pathways, but we have shown that the AP2/SP1 binding protein does not recognize the consensus sequence through which gastrin responsiveness of the HDC promoter is mediated (GAS-RE; +1 to +27 bp). The present study demonstrates that the rat VMAT2 promoter can, like the HDC and mCgA promoters, be transcriptionally regulated by gastrin, but although the mechanism of this gastrin regulation shares some similarities with that observed for each of these promoters, there are important differences. Thus, gastrin-dependent regulation of the rat VMAT2 promoter involves the cooperative interaction of the CREB transcription factor not with the SP1 transcription factor, as seen for the mCgA promoter, but with an unidentified factor of 23.3 kDa, which binds to a putative AP2/SP1 site and possibly interacts with CREB utilizing p300/CBP. The amount of protein binding to the AP2/SP1 site was not increased after gastrin stimulation, but the observation that gastrin responsiveness of the rat VMAT2 promoter is regulated by the protein kinase C/MAPK pathways suggests that the activity of the protein may be regulated by phosphorylation. Further work will therefore be necessary both to identify the AP2/SP1-binding protein and to test this hypothesis. The cluster of cis-regulatory elements and transactivating factors identified here to be important in gastrin-stimulated VMAT2 gene transcription is likely to have significant roles in the regulation of VMAT2 gene expression in a number of systems. The widespread importance of VMAT2 means that it is pertinent to investigate the controls of VMAT2 transcriptional regulation in diverse physiological environments and to determine whether the uncharacterized AP2/SP1-binding protein is of universal importance.

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REFERENCES

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Transcriptional Activation of the Rat Vesicular Monoamine Transporter 2 Promoter in Gastric Epithelial Cells: REGULATION BY GASTRIN

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