Five Discrete Cis-active Domains Direct Cell Type-specific Transcription of the Vasoactive Intestinal Peptide (VIP) Gene*  

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Vasoactive intestinal peptide (VIP) is a neuromodulator expressed with great anatomical specificity throughout the nervous system. Cell-specific expression of the VIP gene is mediated by a tissue specifier element (TSE) located within a 2.7-kilobase (kb) region between −5.2 and −2.5 kb upstream from the transcription start site, and requires an intact promoter proximal VIP-CRE (cyclic AMP-responsive element) (Hahm, S. H., and Eiden, L. E. (1997) J. Neurochem. 67, 1872–1881). We now report that the TSE comprises a 425-base pair domain located between −4.7 and −4.2 kb containing two AT-rich octamer-like sequences. The 425-base pair TSE is sufficient to provide full cell-specific regulation of the VIP gene, when fused to the 5′ proximal 1.55 kb of the VIP gene. Mutational analysis and gel shift assays of these octamer-like sequences indicate that the binding of proteins related to the ubiquitously expressed POU-homeodomain proteins Oct-1 and/or Oct-2 to these octamer-like sequences plays a central role for the function of the TSE. The TSE interacts with three additional discrete domains besides the cAMP response element, which are located within the proximal 1.55 kb of the VIP gene, to provide cell-specific expression. An upstream domain from −1.55 to −1.37 kb contains E-boxes and MEF2-like motifs, and deletion of this domain results in complete abrogation of cell-specific transcriptional activity. The region from −1.37 to −1.28 kb contains a STAT motif, and further removal of this domain allows the upstream TSE to act as an enhancer in both SH-EP and HeLa cells. The sequence from −1.28 to −0.9 kb containing a noncanonical AP-1 binding sequence (Symes, A., Gearan, T., Eby, J., and Fink, J. S. (1997) J. Biol. Chem. 272, 9648–9654), is absolutely required for TSE-dependent cell-specific expression of the VIP gene. Thus, five discrete domains of the VIP gene provide a combination of enhancer and repressor activities, each completely contingent on VIP gene context, that together result in cell-specific transcription of the VIP gene.

VIP† is distributed throughout the central and peripheral nervous systems and functions as a neuromodulator, growth regulator, and neuroendocrine releasing factor (1, 2). It is a 28-amino acid peptide that is processed from a larger precursor peptide that also encodes a second VIP-like peptide called PHI in rodents and PHM in humans (3). VIP is found in high concentrations in specific regions of the brain including cerebral cortex, hypothalamus, and hippocampus (4), and in the peripheral nervous system in cholinergic and sensory nerves (5). VIP is a co-transmitter mediating vasodilatation in the brain, gut, and other organs. VIP is also expressed in the anterior pituitary and functions in the regulation of endocrine homeostasis (2, 6). An important role for VIP in development is suggested by striking growth regulatory effects of exogenously administered VIP during mouse embryogenesis (7). The anatomical and temporal specificity of VIP expression throughout the neuroendocrine axis is therefore critical to neuronal function and endocrine homeostasis. Understanding how this specificity is obtained, at the level of the cis-acting sequences of the VIP gene itself, should provide experimental access to the trans-acting factors that interact with these sequences, and ultimately to the signal transduction pathways that developmentally and physiologically link VIP gene expression within VIPergic cells with the extracellular environment.

We have relied on neuroblastoma cell lines, subclones of which recapitulate various aspects of VIP gene regulation in vivo (8–10), to study the mechanism of cell-specific regulation of the VIP gene underlying its anatomically precise expression in vivo. Subclones from the SK-N-SH neuroblastoma cell line have been characterized with respect to cell type-specific modes of basal and inducible regulation of the VIP gene. In particular, the SH-EP subline constitutively expresses high levels of VIP mRNA (10, 11). Using VIP-luciferase reporter constructs in transient expression assays, we have previously shown that cell type-specific expression of the VIP gene in SH-EP cells requires at least two different cis-acting sequences within the VIP 5′-flanking region. An upstream tissue-specifier element (TSE) located between −4.6 and −4.0 kb from the transcription start site was absolutely required for a cell type-specific expression of the VIP gene. A deletion in this region abolished high level expression of the reporter gene in SH-EP cells (11). Inactivation of a 17-bp promoter proximal element (the VIP-CRE), originally defined by its ability to mediate cAMP-dependent induction of the VIP gene (12), caused an approximately 60% decrease in the level of expression of the reporter gene in SH-EP cells (11), indicating that the upstream TSE cannot fully direct cell-specific expression of the VIP gene without the participation of the promoter-proximal CRE.

We hypothesized that additional elements in the VIP gene might be required for full tissue-specific gene expression directed by the TSE. Stepwise deletion of domains of the VIP gene between the TSE and the promoter proximal CRE, and assay of cell-specific transcription in SH-EP versus HeLa cells, now show this hypothesis to be correct. Cell type-specific ex-
pressure of the VIP gene requires combinatorial effects from multiple cis-acting sequences that both repress transcriptional activity of the TSE in HeLa cells, and enhance its activity in SH-EP cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture reagents, LipofectAMINE, and synthetic oligonucleotides were obtained from Life Technologies, Inc. (Gaithersburg, MD), fetal bovine serum from BioWhittaker (Walkersville, MD), and culture plates from Costar Corp. (Cambridge, MA). pG3 vectors, pGEM vectors, luciferase assay reagents, reporter lysis buffers, and core footprinting primer DNA were purchased from Promega Corp. (Madison, WI). DNA ligation was kit was from Boehringer Mannheim Corp. (Indianapolis, IN). Sequencing reagents were purchased from U. S. Biochemical Corp. and Sequagel-6 ready to use 6% sequencing gel solutions were from National Diagnostics (Atlanta, GA). Oct-1 and Oct-2 supershift antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

**Cell Culture**—SH-EP neuroblastoma cells and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium with 4.5 g of glucose per liter, containing 10% fetal bovine serum (heat-inactivated) supplemented with glutamine (0.03%), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were maintained in a humidified 95% air, 5% carbon dioxide atmosphere. Culture medium was changed every 3–4 days to a fresh medium and SH-EP cells were subcultured before the cells reached approximately 70% confluency.

**Construction of VIP-Luciferase Reporter Plasmids**—The methods used to construct VIP-luciferase reporter plasmids were essentially as described previously (11). All VIP-luciferase reporter constructs contained the first exon, the first intron, and the second exon extending to 5′ flank of the VIP gene. The promoter proximal 251 nucleotides upstream of the VIP transcription initiation site, which includes the minimal VIP promoter and the VIP-CRE, demonstrated previously to be required for full cell-specific as well as inducible expression in SH-EP and SK-N-SH cells is designated domain e in Fig. 1. To this minimal VIP gene was fused various segments b–d of the VIP gene lying between the TSE (designated a in Fig. 1) and the proximal promoter e. These constructs were assayed for transcriptional activity as described below, both with and without the TSE fused to the 5′-end of the recombinant transcriptional unit. The minimal TSE (−4,656 to −4,231 bp) was defined by both 5′ and 3′ progressive deletions of the −5.2 to −2.5 HindIII fragment previously shown to be required for cell-specific expression of VIP (10) fused to −1.88 to −0.984 minimal promoter-enhancer and transfected into SH-EP cells.

**Transient Expression Assays**—SH-EP and HeLa cells were transfected as described previously (11) with some modifications. Cells were seeded in Costar 12-well tissue culture plates at densities of 8 × 10^4 and 1 × 10^5 cells/well for SH-EP and HeLa cells, respectively, in 1.0 ml of culture medium. Cells were allowed to grow to approximately 60–70% confluence and were transfected with 0.5 μg of LipofectAMINE polycationic liposome reagent/well for 5 h in 1 ml of serum-free medium. After transfection, medium was removed and replaced with complete medium. Cells were incubated for an additional 36–40 h before they were harvested in 200 μl of the reporter lysis buffer. For the luciferase assay, 20 μl of cell lysate was mixed with 100 μl of the luciferase substrate and light units were counted for 20 s using a luminometer (Lumat LB9501, Berthold).

**In Vitro Footprinting Analysis**—SH-EP and HeLa cell nuclear extracts were prepared according to Ausubel et al. (13). Fragments DNA used in the footprinting analysis represent different parts of the 425-bp TSE sequence covering each of the two 9-bp AT-rich sequences of the pG3-basic vector, in addition to various regions of 5′ flank of the VIP gene. The promoter proximal 251 nucleotides upstream of the VIP transcription initiation site, which includes the minimal VIP promoter and the VIP-CRE, demonstrated previously to be required for full cell-specific as well as inducible expression in SH-EP and SK-N-SH cells is designated domain e in Fig. 1. To this minimal VIP gene was fused various segments b–d of the VIP gene lying between the TSE (designated a in Fig. 1) and the proximal promoter e. These constructs were assayed for transcriptional activity as described below, both with and without the TSE fused to the 5′-end of the recombinant transcriptional unit. The minimal TSE (−4,656 to −4,231 bp) was defined by both 5′ and 3′ progressive deletions of the −5.2 to −2.5 HindIII fragment previously shown to be required for cell-specific expression of VIP (10) fused to −1.88 to −0.984 minimal promoter-enhancer and transfected into SH-EP cells.

**Electrophoretic Mobility Shift Assay**—SH-EP and HeLa cell nuclear extracts were prepared according to Schreiber et al. (16) with minor modifications. Cells were grown in T150 flasks until they reached approximately 65% confluency. Cells grown in two T150 flasks were collected and resuspended in 1.2 ml of cold buffer A (10 mM HEpes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 2 μg/ml pepstatin). Cells were allowed to swell on ice for 25 min and 75 μl of 10% Nonidet P-40 was added. Cell homogenate was spun in a Microfuge 1530 at 15,000 × g for 30 s and the nuclear pellet was resuspended in 150 μl of ice-cold buffer C (20 mM HEpes, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 2 μg/ml pepstatin). Nuclear proteins were extracted on ice by vigorously shaking on a rocker for 30 min. The extract was centrifuged at 16,000 × g for 5 min at 4 °C, and the supernatant, which contains the nuclear proteins, was transferred to a fresh tube. Protein concentrations were determined by Bio-Rad protein assay, according to the manufacturer’s protocol, and nuclear extracts were frozen by submerging in liquid nitrogen and stored at −70 °C in small aliquots. Synthetic oligonucleotides were annealed and labeled using [γ-32P]ATP by T4 polynucleotide kinase. Approximately 100,000 cpm of probe (−1.0 ng) was mixed with 10 μg of SH-EP or HeLa nuclear extract in a total volume of 10 μl containing 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 50 μg/ml poly(dI-dC). Samples were incubated at room temperature for 20 min and loaded onto a 3.75% polyacrylamide gel in 0.5 × TBE (22 mM Tris-HCl, pH 8.3, 22 mM boric acid, 0.6 mM EDTA). Gels were pre-electrophoresed at 100 V for 1–2 h and electrophoresed at 300 V for approximately 1.5 h at 7 °C. Gels were dried and autoradiographed. For supershift assays, 2 μl of TransCruz Oct-1 or Oct-2 antibody was added to the binding reaction. Oligonucleotides used for the VIP gene upstream and downstream AT-rich sequences were: CATGTTTTCGTACGTGCA and CTGGATTGTTT- CATTAATTG (and their 3′ complements), respectively. SP1 consensus oligonucleotide used as a nonspecific competitor was ATTCGAGGCGGGCAGAGGAGC and its 3′ 5′ complement.
**RESULTS**

We have previously shown from a deletional analysis that sequences within the region −5.2 to −2.5 kb upstream from the transcription start site of the human VIP gene are absolutely required for cell-specific expression of a VIP-luciferase reporter gene in SH-EP neuroblastoma cells (11). A 425-bp sequence (referred to here as the VIP tissue-specifier element, or TSE), spanning two PsII sites located at −4,656 bp and −4,231 bp from the start of transcription (Fig. 1), completely recapitulated cell-specific transcription imparted by 2.5 kb upstream from the transcription start site of the VIP2.5 constructs. SH-EP cells were transfected for 5 h using LipofectAMINE and harvested 40 h after transfection for lysis and harvest. The results are expressed as mean ± S.E.M. of light units per extract aliquot (see "Experimental Procedures") from triplicate wells. Experiments were repeated twice with similar results.

**Fig. 2. Effect of the 425-bp TSE of the VIP gene on reporter gene expression.** Transient expression assays were done in SH-EP human neuroblastoma cells using VIP-luciferase reporter constructs in which 5.2 and 2.5 kb of the VIP 5′ gene flank (VIP5.2 and VIP2.5, respectively (11)) are fused to the luciferase expression vector pGL3-basic. The VIP2.5-TSE construct contains a 425-bp TSE fragment subcloned in front of the VIP2.5 constructs. SH-EP cells were transfected for 5 h using LipofectAMINE and harvested 40 h after transfection for the luciferase assay. The results are expressed as mean ± S.E.M. of light units per extract aliquot (see "Experimental Procedures") from triplicate wells. Experiments were repeated twice with similar results.

**VIP octamer-like sequences:**
- Upstream sequence: ccatgaATTTTCAATgttca
- Downstream sequence: tctggaATTTTCAATaatgt

**GnRH Oct-1 binding site:**
- ctgagaATTTTCAATaggcc

**Octamer consensus sequences:**
- H2B (histone gene): atttATTGATCAaag
- Ad2 ori (adenovirus origin of replication): atttATTATCATatt

**Pit-1 binding sites:**
- rGH1 (rat growth hormone gene): atttATTATTTGATggc
- rGH2 (rat growth hormone gene): atttATTATTTGATaag
- p3D (prolactin gene): catTTATTATTTGATcat

**Overall consensus:**
(A/T)4TNCAT

**Fig. 3. Comparison of the VIP gene AT-rich 9-bp repeats with other POU homeodomain protein binding sequences.** The 9-bp VIP AT-rich sequences and the GnRH Oct-1-binding site (17) are underlined. Consensus octamer sequences found in histone H2B gene, adenovirus origin of replication, and the growth hormone and prolactin gene Pit-1 binding sequences are underlined.

GnRH gene, the consensus octamer sequence, and Pit-1 binding sites found in some other genes. The 9-bp AT-rich sequence of the human VIP gene differs from the Oct-1 binding sequence of the GnRH gene by only 1 nucleotide (ATTTTCCAT versus ATTTTACAT), and matches the overall consensus of Pit-1, (A/T)4TNCAT (18).

Mutations in either of the AT-rich sequences of the VIP gene significantly decreased reporter gene expression, suggesting that both of these AT-rich sequences are required for cell-specific expression of the VIP gene. The results in Fig. 4 show that while the TSE can fully recapitulate cell-specific expression when fused 5′ to 1.55 kb of the VIP gene 5′ flank (VIP1.55-TSE, mutating four nucleotides within the upstream AT-rich sequence of the TSE (VIP1.55-Attt1.m4 construct) caused an approximately 50% decrease in the effect of the TSE. Similar 4-base mutations in the downstream AT-rich sequence (VIP1.55-Attt2.m4 construct) decreased the level of reporter gene expression driven by the TSE by approximately 80% in SH-EP cells. Also, a deletion of 93 bp from the 3′ region of the TSE that removes the downstream AT-rich sequence (VIP1.55-TSE.A2 construct) caused a more than 80% decrease in transcription. These results indicate that both of the AT-rich sequences play an important role for the function of the TSE.

Electrophoresis mobility shift assays were performed using synthetic oligonucleotides spanning each of the AT-rich repeats of the VIP gene, to investigate nuclear proteins that interact with these sequences. The Attt-2 probe, spanning the downstream 9-base AT-rich sequence of the VIP gene, produced multiple complexes with both SH-EP and HeLa nuclear proteins (Fig. 5). Formation of these complexes was specific as a 100-fold molar excess of an unrelated SP-1 consensus oligonucleotide failed to compete with the wild type Attt-2 probe. Addition of an Oct-1 or an Oct-2 antibody in the binding reaction caused a supershift of the bands marked as Oct-1 and Oct-2, respectively. Unlabeled oligonucleotide spanning the upstream AT-rich repeat (Attt-1) competed with labeled Attt-2 for binding to those complexes supershifted with an Oct-1 antibody. Attt-1 did not compete with labeled Attt-2 binding in those complexes recognized by an Oct-2 antibody. Likewise, an Oct-1 consensus oligonucleotide specifically competed for the formation of the complex with Attt-2 that is supershifted with Oct-1 antibody, and not the complex recognized by the Oct-2 antibody (Fig. 5). These Oct-1 and Oct-2 complexes were observed in both SH-EP and HeLa nuclear preparations. The Attt-2/m4 oligonucleotide (containing 4 single-base mutations...
within the AT-rich sequence) failed to compete for the formation of these complexes, indicating that Oct-1 and Oct-2 cannot bind to this mutated AT-rich sequence. In the experiment shown in Fig. 4, the same mutations within the downstream AT-rich sequence caused an 80% decrease in cell-specific VIP gene transcription mediated by the TSE, indicating that binding of Oct-1 and Oct-2 to the AT-rich sequence is critical for the function of the TSE. When an oligonucleotide spanning the upstream AT-rich sequence was used as a probe (Att1-1 probe) for the gel shift assay, three major complexes were observed in both SH-EP (Fig. 6) and HeLa (data not shown) nuclear extracts. All were specific based on competition with unlabeled Att1-1. Unrelated oligonucleotide (SP-1 consensus) and the Att1-1 oligonucleotide containing 4-base mutations (Att1-1/m4) failed to compete for formation of these complexes. Addition of Oct-1 specific antibody caused a supershift of the band marked as Oct-1 in Fig. 6. This complex, but not the other two complexes, was competed by an addition of excess unlabeled Oct-1 consensus oligonucleotide (Fig. 6). No Oct-2/Att1-1 specific complex was detectable in this assay. Thus, unlike the downstream AT-rich sequence (Att1-2) which recognizes both Oct-1 and Oct-2 proteins, Att1-1 oligonucleotide spanning the upstream AT-rich sequence binds only Oct-1. Since the core 9-base sequences of Att1-1 and Att1-2 are identical, sequences flanking the 9-bp AT-rich repeats may be critical for determining the differential specificity of these two elements.

In vitro footprinting analysis was performed to better define protein-binding regions within the TSE. The result in Fig. 7 show that the downstream AT-rich sequence was protected by either SH-EP or HeLa nuclear proteins. In addition, an approximately 30-bp region spanning the immediate 5′ flank of the downstream AT-rich element was protected specifically by SH-EP nuclear proteins. This suggests that a multiprotein complex may be formed at this region of DNA to support cell-specific enhancer function of the TSE, including POU-homeodomain proteins Oct-1 and Oct-2 and other proteins, some of which are expressed specifically in VIP-expressing SH-EP cells.

In addition to potentially complex protein interactions within the TSE itself, the dependence of TSE function on intact cAMP response element-binding protein-binding sequences (VIP-CRE) within the proximal promoter of the VIP gene (11) suggested that other regions of the VIP gene between the TSE and VIP core promoter could be involved in mediating TSE function as well. This became evident as a minimal VIP-luciferase construct containing the TSE fused directly to the VIP 0.94 construct (contains 94 bp of the VIP gene 5′-flanking sequence including the VIP-CRE) failed to fully recapitulate cell-specific expression of the reporter gene in SH-EP cells (Fig. 8). The VIP 0.94-TSE construct was expressed at a significantly lower level than the full-length VIP 5.2 construct in SH-EP cells. In five separate experiments, the VIP 0.94-TSE was expressed on average at approximately 37% of the level of the full-length VIP 5.2 construct, indicating that both the TSE and VIP-CRE are necessary but not sufficient for a maximum cell-specific expression of the VIP gene.

To define additional cis-acting sequences required for full cell-specific expression of the VIP gene, transient transfection/ expression assays were performed using reporter constructs in which the TSE was fused to VIP gene 5′-flanking sequences with progressive deletions from −2.5 kb to −94 bp (Fig. 8). Full cell-specific expression of the reporter gene was achieved in SH-EP cells only when the TSE was fused upstream of at least 1.55 kb of promoter proximal VIP gene 5′ flank. These constructs had appropriately low transcriptional activity in non-VIP-expressing HeLa cells. Removal of 270 bp from −1.55 to −1.28 kb from the VIP gene 5′ flank caused a decrease in reporter gene transcription more than 50% in SH-EP cells. More significantly, deletion of the VIP gene 5′ flank below −1.55 kb caused a large increase in expression of the reporter gene in HeLa cells, such that these constructs no longer exhibited cell-specific transcriptional activity.

To better define the role of the 270-bp domain from −1.55 to −1.28 kb, a construct was made by removing the upstream 180-bp portion of the 270-bp domain from the VIP 1.55-TSE construct. The resulting VIP 1.37-TSE construct showed a complete loss of cell-specific expression in SH-EP cells (Fig. 9), indicating that sequences located between −1.55 and −1.37 kb enhance the effect of TSE in SH-EP cells but not in HeLa cells. Further deletion of the 5′ flank down to −1.28 kb (VIP 1.28-TSE construct) caused an increase in the level of reporter gene expression in both SH-EP and HeLa cells, indicating that the region between −1.37 and −1.28 kb contributes repressor activity in non-VIP-expressing cells.

In addition, when the effect of the 180-bp domain (domain b) between −1.55 and −1.37 kb was examined in the absence of the downstream 90-bp domain (domain c) between −1.37 and −1.28 kb (by deleting domain c from the VIP 1.55-TSE construct), the resulting VIP abde construct was expressed at a
sequences of the VIP TSE. \textsuperscript{32}P-Labeled oligonucleotides spanning the downstream AT-rich sequence of the VIP gene was used as a probe (Att-2). The Att-2/m4 oligonucleotide contains 4-bp substitutions within the AT-rich sequences (from ATTTTCCAT to AgcTTgCAg). Binding reactions were carried out using either (A) SH-EP or (B) HeLa nuclear extract. For competition assays, 100-fold molar excess of unlabeled oligonucleotides were added in the reactions. SP-1 consensus sequence was used as a non-specific competitor. For supershift assays, either an Oct-1 or an Oct-2 antibody was added in the binding reactions.

Fig. 5. Electrophoretic mobility shift analysis of the proteins binding to the downstream AT-rich sequences of the VIP TSE. \textsuperscript{32}P-Labeled oligonucleotides spanning the downstream AT-rich sequence of the VIP gene was used as a probe (Att-2). The Att-2/m4 oligonucleotide contains 4-bp substitutions within the AT-rich sequences (from ATTTTCCAT to AgcTTgCAg). Binding reactions were carried out using either (A) SH-EP or (B) HeLa nuclear extract. For competition assays, 100-fold molar excess of unlabeled oligonucleotides were added in the reactions. SP-1 consensus sequence was used as a non-specific competitor. For supershift assays, either an Oct-1 or an Oct-2 antibody was added in the binding reactions.

level less than 50% of the VIP1.55-TSE construct (or VIP-abcde) in SH-EP cells (Fig. 11A). This indicates that sequences between −1.37 and −1.28 kb (c) are also required for the function of the TSE (a).

The region spanning −1.55 to −1.28 kb (b) of the human VIP gene is highly homologous to the corresponding region of the mouse VIP gene (19), and contains two E-boxes, two MEF2 (myocyte enhancer factor-2)-like sequences, a STAT site, and a pair of 8-bp dyad symmetry element (Fig. 10). The STAT site has previously been identified as a critical determinant in VIP gene up-regulation after exposure of NBFL neuroblastoma cells to ciliary neurotrophic factor (20). The 270-bp domain between −1.55 and −1.28 kb (bc), however, when fused downstream of the TSE (a) but directly upstream of 251 bp (PacI site) of the VIP gene promoter proximal sequence (e) failed to recapitulate a full cell-specific expression of the reporter gene (VIP-abcde construct, Fig. 11B). Instead, the VIP-abcde construct was expressed at an intermediate level both in SH-EP and HeLa cells, like the VIP251-TSE construct (VIPae) lacking the 270-bp (bc) domain. This indicates that additional sequences, located within domain d (between the 270-bp domain and the promoter proximal VIP-CRE), are required for full cell-specific expression.

Finally, to better analyze the d domain, an additional construct was made by deleting approximately 650 bp (between −904 and −251 bp) of d from the VIP-abcde construct, leaving an approximate 370-bp upstream region of the d domain containing the non-canonical AP-1-binding site (21). The deletion of 650 bp from the d domain did not significantly affect cell-specific expression of the reporter gene (VIP-abcde' construct, Fig. 11A). Therefore, a full cell-specific expression of the VIP gene requires the upstream TSE (a), promoter proximal VIP-CRE (e), and the entire region between −1.55 kb and −904 bp of approximately 640 bp (bcde').

DISCUSSION

We have shown here that at least five distinct regions of the human VIP gene 5' flank work in concert to mediate maximal cell-specific expression of the reporter gene in SH-EP neuroblastoma cells. This analysis is summarized schematically in Fig. 12. A 425-bp TSE (or domain a) located at approximately −4.3 kb can fully recapitulate cell-specific transcription when fused to at least 1.55 kb of the VIP gene promoter proximal sequence, in VIP-expressing cells (Fig. 12). The VIP TSE contains two 9-bp AT-rich sequences, designated here as Attt-1 and Attt-2. Binding of proteins of the POU-homeodomain family (Oct-1 for Attt-1 and Oct-1 and Oct-2 for Attt-2) or closely related proteins to the TSE is likely to be required for transcriptional activation of the VIP gene. This is based on the observation that Oct-1-immunoreactive protein is present in nuclear extracts of SH-EP cells, and that mutations in the TSE that abrogate Oct-1 binding also abolish the cell-specific enhancer activity of this element. Whyte et al. (1995) have reported that block replacement mutations in a similar Oct-1 binding motif in the GnRH gene decreased reporter gene expression by 95% in the GnRH-secreting hypothalamic neuronal cell line GT1. Therefore, binding of Oct-1 or a closely related POU homeodomain protein to the AT-rich sequences plays a major role in cell-specific transcription of at least two neuropeptide-encoding genes, those for VIP and GnRH.

While Oct-1 is generally believed to be ubiquitously ex-
pressed in vivo, it has been shown to interact with other factors that are expressed in a lineage-restricted manner, to mediate cell-specific transcriptional regulation. For example, B-cell-specific transcription of immunoglobulin genes requires binding of the ubiquitous Oct-1 (or Oct-2), but also requires a B-cell-specific co-activator OCA-B, also called Bob-1 or OBF-1 (18, 22–24). Oct-1 may interact with similar factors expressed selectively in neural crest-derived cells to mediate a constitutive expression of the VIP gene in this neuronal cell lineage. Indeed, footprinting of the VIP TSE suggests that protein factor(s) specifically expressed in SH-EP but not in HeLa cells may participate in a protein complex responsible for the specificity of VIP gene expression in neuroblastoma cells.

Region b (between −1.55 and −1.37 kb) plays a critical role in conferring cell-specific enhancer activity to the TSE. This domain contains an E-box and two MEF2-like sequences. Available evidence suggests that interactions between MEF2 proteins and cell type-specific basic helix loop helix proteins are important for tissue-specific transcription in both muscle and neuronal cell lineages (25–29). The mammalian achaete-scute homologue 1 (MASH1) is a neurogenic basic helix loop helix protein that is expressed in subsets of cells in the central and peripheral nervous systems during mouse embryogenesis, and plays an important role in the early development of the nervous system (30, 31). It has been shown that MASH1 forms a heterodimer with ubiquitously expressed basic helix loop helix transcription factors known as E proteins (25, 32). MEF2 interacts with MASH1/E-protein heterodimers to synergistically activate transcription. The E-box and MEF2-like sequences in this domain of the VIP gene may mediate a similar type of transcriptional regulation by members of the basic helix loop helix protein family and MEF2 or related proteins.
The downstream domains from \(-1.37\) to \(-1.28\) kb (region c) and \(-1.28\) to \(-2.251\) kb (region d) contain repressor elements, as progressive removal of these regions increases TSE-mediated expression of VIP gene constructs in neuroblastoma cells. However, this increased transcriptional activation is no longer cell-type specific. These data may explain previous results in which tissue specific activity was attributed to upstream sequences including the TSE, fused directly to the VIP core promoter, based on assessment of transcriptional activity in neuroblastoma cells without concomitant assessment in a VIP non-expressing cell line (33). Elements between \(-1.37\) and \(-2.51\) of the VIP gene may function mainly to silence transcription of the VIP gene in VIP non-expressing cells. Contribution from the \(-1.28\) to \(-2.51\)-kb domain (region d) is required for both TSE-mediated maximum expression in SH-EP cells and full repression of TSE-containing reporter constructs in HeLa cells. The region from \(-1.37\) to \(-1.28\) kb overlaps with the 5'-end of the 180-bp cytokine responsive element which mediates induction of the VIP gene by ciliary neurotrophic factor (20, 34). This region contains a binding site for STAT1 and STAT3 proteins, which play important roles in cytokine-mediated up-regulation of transcription in diverse systems (20, 35). The role of this STAT site and other regions of the cytokine responsive element in cell-specific or second messenger-inducible expression of the VIP gene is currently under investigation.

Overall, these experiments demonstrate that the participa-
tion of five separate cis-active domains within the VIP promoter/enhancer are required for full cell-specific expression of the VIP gene in neuroblastoma cells. Each domain is likely to bind multiple components, including ubiquitously expressed proteins such as Oct-1, Oct-2, and cAMP response element-binding protein and as yet unidentified cell-specific transcription factors that mediate both transcriptional activation in VIP-expressing cells and transcriptional repression in VIP non-expressing cell types. The apparent complexity of cell-specific regulation of VIP transcription at the level of the gene itself may reflect the requirement for integration of multiple intra- and extracellular inputs to the appropriate developmental and physiological expression of the VIP gene in vivo.

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REFERENCES
1. Brenneman, D. E., Nicol, T., Warren, D., and Bowers, L. M. (1990) J. Neurosci. Res. 25, 386–394
2. Gozes, I., and Brenneman, D. E. (1989) Mol. Neurobiol. 3, 1–36
3. Mutt, V. (1988) in Vasoactive Intestinal Polypeptide and Related Peptides (Said, S. I., and Mutt, V., eds) pp. 1–19, The New York Academy of Sciences, New York
4. Emson, P. C., Gilbert, R. F. T., Loren, I., Fahrenkrug, J., Sundler, F., and Muckadell, O. B. S. D. (1979) Brain Res. 177, 437–444
5. Fahrenkrug, J. (1979) Digestion 19, 149–169
6. Rostene, W. H. (1984) Prog. Neurobiol. 22, 103–129
7. Gressens, P., Hill, J. M., Gozes, I., Fridkin, M., and Brenneman, D. E. (1993) Nature 362, 155–158
8. Cooper, M. J., Hutchins, G. M., Cohen, P. S., Helman, L. J., Mennie, R. J., and Israel, M. A. (1990) Cell Growth Differ. 1, 149–159
9. Waschek, J. A., Pruss, R. M., Siegel, R. E., Eiden, L. E., Bader, M.-F., and Aunis, D. (1987) Ann. N. Y. Acad. Sci. 493, 308–323
10. Waschek, J. A., Hou, C.-M., and Eiden, L. E. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2547–2551
11. Hahn, S. H., and Eiden, L. E. (1996) J. Neurochem. 67, 1872–1881
12. Fink, J. S., Verhaue, M., Kasper, S., Tsukada, T., Mandel, G., and Goodman, R. H. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6662–6666
13. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994) Current Protocols in Molecular Biology, Vol. 2, Unit 12.1, pp. 1–8, John Wiley & Sons, New York
14. Ohlsson, H., and Edlund, T. (1986) Cell 45, 35–44
15. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419
17. Clark, M. E., and Mellon, P. L. (1995) Mol. Cell. Biol. 15, 6169–6177
18. Herr, W., Sturm, B. A., Clerc, R. G., Corcoran, L. M., Baltimore, D., Sharp, P. A., Ingraham, H. A., Rosenfeld, M. G., Finney, M., Ruvkun, G., and Horvitz, H. R. (1988) Genes Dev. 2, 1513–1516
19. Sena, M., Bravo, D. T., Agoston, D. V., and Waschek, J. A. (1994) DNA Seq. 5, 25–29
20. Symes, A., Lewis, S., Corpus, L., Rajan, P., Hyman, S. E., and Fink, J. S. (1994) Mol. Endocrinol. 8, 1750–1763

Fig. 12. Schematic representation of the combinatorial and individual activities of five discrete domains of the VIP gene 5′ flank contributing to cell-specific expression. Five discrete domains of the VIP gene 5′ flank (a, b, c, d, and e) that are involved in cell-specific expression are shown in the upper panel. Relative importance of each of the domains is summarized in the lower panel that shows the level of reporter gene expression from reporter constructs containing various combinations of the VIP gene domains in both SH-EP and HeLa cells, expressed as % of the level achieved by the VIP1.55-TSE construct in SH-EP cells. Data summarize the results depicted in Figs. 8, 9, and 11.
21. Symes, A., Gearan, T., Eby, J., and Fink, J. S. (1997) *J. Biol. Chem.* **272**, 9648–9654
22. Cepek, K. L., Chasman, D. I., and Sharp, P. A. (1996) *Genes Dev.* **10**, 2079–2088
23. Gstaiger, M., Knoepfel, L., Georgiev, O., Shaffner, W., and Hovens, C. M. (1995) *Nature* **373**, 360–362
24. Strubin, M., Newell, J. W., and Matthias, P. (1995) *Cell* **80**, 497–506
25. Mao, Z., and Nadal-Ginard, B. (1996) *J. Biol. Chem.* **271**, 14371–14375
26. Black, B. L., Ligon, K. L., Zhang, Y., and Olson, E. N. (1996) *J. Biol. Chem.* **271**, 26659–26663
27. Molkentin, J. D., and Olson, E. N. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 9366–9373
28. Molkentin, J. D., Black, B. L., Martin, J. F., and Olson, E. N. (1995) *Cell* **83**, 1125–1136
29. Jones, E. G. (1986) *J. Neurosurg.* **65**, 135–153
30. Kageyama, R., Sasai, Y., Akazawa, C., Ishibashi, M., Takebayashi, K., Shimizu, C., Tomita, K., and Nakanishi, S. (1985) *Clin. Rev. Neurobiol.* **9**, 177–188
31. Guillemot, F., Lo, L. C., Johnson, J. E., Auerbach, A., Anderson, D. J., and Joyner, A. L. (1993) *Cell* **75**, 463–476
32. Johnson, J. E., Biren, S. J., Saito, T., and Anderson, D. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3596–3600
33. Agoston, D. V., Bravo, D. T., and Waschek, J. A. (1990) *J. Neurosci. Res.* **27**, 463–476
34. Symes, A. J., Rao, M. S., Lewis, S. E., Landis, S. C., Hyman, S. E., and Fink, J. S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 572–576
35. Darnell, J. E. (1997) *Science* **277**, 1630–1635
36. Symes, A., Corpus, L., and Fink, J. S. (1995) *J. Neurochem.* **65**, 1926–1933
37. Whyte, D. B., Lawson, M. A., Belsham, D. D., Eraly, S. A., Bond, C. T., Adelman, J. P., and Mellen, P. L. (1995) *Mol. Endocrinol.* **9**, 467–477
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