Biochemical and Biophysical Research Communications **284,** 211–218 (2001)
doi:10.1006/bbrc.2001.4948, available online at http://www.idealibrary.com on IDEAL®

VIP Gene Transcription Is Regulated by Far Upstream Enhancer and Repressor Elements

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Received April 19, 2001

SK-N-SH human neuroblastoma subclones differ widely in basal and second messenger induction of the gene encoding the neuropeptide vasoactive intestinal peptide (VIP). These differences were recapitulated by a chimeric gene which consisted of 5.2 kb of the human VIP gene 5′ flanking sequence fused to a reporter. Subsequent gene deletion experiments revealed several regulatory regions on the gene, including a 645-bp sequence located approximately 4.0 upstream from the transcription start site. Here we examined this upstream region in detail. Inhibitory sequences were found to be present on each end of the 645-bp fragment. When removed, basal transcription increased more than 50-fold. Subsequent deletion/mutation analysis showed that the 213-bp fragment contained at least two enhancer elements. One of these was localized to an AT-rich 42-bp sequence shown by others to bind Oct proteins in neuroblastoma cells, while the other corresponded to a composite AP-1/*ets* element. In addition to these enhancers, a 28-bp sequence on the 213-bp fragment with no apparent homology to known silencers inhibited transcription. The studies provide molecular details of a complex regulatory region on the VIP gene that is likely to be used to finely tune the level of gene transcription *in vivo*. © 2001 Academic Press

Neuropeptide genes are expressed in distinct subpopulations of cells in the central and peripheral nervous system. After expression is activated at appropriate developmental stages, the level of gene activity must be actively regulated in a cell-specific way in response to hormones, injury, and other environmental stimuli. A high degree of phenotypic diversity and plasticity in gene expression is accomplished by combinations of transcription factors which act in specific ways on subsets of genes, either through transcriptional enhancement or repression.

In earlier studies, we analyzed by transfection the expression of a chimeric gene consisting of 5.2 kb of the human VIP gene promoter fused to the reporter gene chloramphenicol acetyltransferase (1). The activity of this fusion gene paralleled the levels of endogenous VIP gene expression in individual subclones of the human neuroblastoma cell line SK-N-SH, and recapitulated the subclone-specific responses of the gene to phorbol ester and cAMP. When the sequences were progressively deleted from the 5′ distal flanking region, basal reporter activity was selectively reduced in SH-IN cells, the SK-N-SH subclone which expressed the highest level of endogenous VIP. Thus, upstream sequences appeared to contain elements which selectively conferred a high level of cell-type specific expression in SK-N-SH-IN cells. Subsequent deletion studies (2, 3) indicated that significant enhancer activity resided somewhere on a 645 bp fragment located approximately 4.0 to 4.6 kb upstream from the VIP transcription start site. In the current studies, we analyzed this upstream region in detail in SH-IN cells and report that it contains multiple positive- and negative-acting transcriptional control domains, some of which correspond to consensus sequences that typically bind members of known transcription factor families. The studies set the stage for the identification of the specific transcription factors that interact with these sequences, and the determination of how they interact in a cell specific way to regulate VIP gene transcription.

MATERIALS AND METHODS

VIP/luciferase plasmid constructions. In previous studies, we created a plasmid called p5TCVL, which consisted of an upstream 645-bp human VIP gene *Xba*I/*Taq*I fragment inserted into a parent vector called pCVL (2). pCVL contained a short human VIP gene segment consisting of promoter/enhancer and 5′ untranslated se-

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0006-291X/01 $35.00

quence (nucleotides −500 to +142) fused to the gene encoding firefly luciferase. In the current studies, we created a new parent vector which contained a smaller VIP promoter/enhancer sequence fused to luciferase in a vector background that allowed high and consistent plasmid yields to be obtained. This was accomplished by digesting p5TCVL with *Nco*I and *Sal*I, which removes nucleotides −500 to −97. The digestion products were polished with DNA polymerase I, separated by gel electrophoresis, and recircularized by ligation. The resulting plasmid, called p5THC1VL, contained the 645 bp upstream VIP gene fragment and a truncated VIP promoter/enhancer separated by a polylinker. To transfer the minimal promoter/enhancer to a new vector background, p5THC1VL was digested with *Hin*dIII, which cuts just upstream from the minimal VIP promoter/enhancer and with *Cla*I, which cuts within the luciferase gene. This fragment containing the minimal VIP promoter fused to part of the luciferase gene was isolated, then ligated with *Hin*dIII/*Cla*I fragment of pGL2-B (Promega). The pGL2-B fragment contained the distal part of the luciferase gene, the SV40 virus polyadenylation signal, and remaining vector sequences. The resulting plasmid (pHC2VL) contained the polylinker of pGL2-B situated upstream from the minimal VIP core promoter/enhancer (−96 to +142), followed by the fully-reconstituted luciferase gene and SV40 polyadenylation signal.

The 645-bp upstream VIP gene fragment was then subcloned into pHC2VL. To do this, the 645-bp *Xba*I/*Taq* fragment was excised from pVIPCAT5.2 (1) and ligated into the *Xba*I/*Acc*I site of pUC18. The resulting plasmid was digested with *Hin*dIII and *Sma*I, and treated with Klenow to fill the *Hin*dIII 5′ overhang. The products were separated on an agarose gel, and the 645-bp fragment ligated in both orientations into the *Sma*I site of pHC2VL. Deletions from the 5′ and 3′ end of the 645 fragment in were made by *Exo*III digestion followed by religation using the Nested-Deletion kit (Pharmacia, 27-1691-01). *Exo*III treatment was preceded by digesting with *Hin*dIII, filling in with α-phosphorothiolates to create an *Exo*III-resistant end, then digestion with *Mlu*I to create an *Exo*III-compliant end. Appropriately sized recombinants were identified by restriction digestion, then sequenced to identify the deletion endpoint.

In other experiments, selected portions of the 645-bp fragment were subcloned into pHC2VL. p213 was created by digesting deletion mutant 5A (Fig. 1) with Pst I, and isolating the 213-bp fragment. This contained sequences −4438 to −4226 of the VIP gene, and was inserted into the *Pst*I site of pHC2VL. Short sequences containing potential regulatory elements were made by obtaining synthetic oligonucleotides (Gibco/BRL) containing *Mlu*I and *Pst*I restriction sites at the 5′ and 3′ ends, respectively. These were annealed, and ligated in a + orientation into the respective sites of pHC2VL. All plasmids examined were sequenced to confirm identity.

Site-directed mutagenesis. The "QuickChange Site-Directed Mutagenesis Kit" (Catalog 200518, Stratagene) was used to change specific nucleotides in p213. Mutagenesis primers (purified by PAGE) were obtained from Operon (Alameda, CA). In general, the mutagenesis kit instructions were followed. Plasmids obtained were sequenced to confirm that the desired mutations had been accomplished.

Transfection and expression assays. SK-N-SH-IN cells were obtained from Ross and Spengler (4), and were cultured as described (1). Cells were plated at 50–75,000 cells/35 mm^2 well on the day before transfection. In initial studies, VIP/luciferase plasmids were transfected into cells by the method of Chen and Okayama (5), scaled down to 35 mm^2 wells and 2 ml of medium. The total amount of DNA per well was 4 μg, which included 100 ng RSVcat (6), which was used to control for transfection efficiency. On the morning following transfection, cells were rinsed with medium, then replaced with fresh medium. Three to six hours later, medium was replaced again. On the following day, cells were harvested by rinsing with PBS, then addition directly to the plate 350 μl of buffer containing 25 mM glycylglycine, 10 mM $MgSO_4$, and 1 mM DTT. Cells were subjected to a total of three 5 min −70/37°C freeze/thaw cycles. The lysate was transferred from the plate to a microfuge tube, and centrifuged at 14,000*g* for 1 min. Ten to fifty microliters of sample was assayed for luciferase activity using the Promega Luciferase Assay kit (Cat. E1500). Twenty microliters of the same lysate was also analyzed for CAT activity in 125 μl buffer containing ^{14}C chloramphenicol (ICN), 0.25 M Tris–HCl (pH 8.0) and *n*-butyryl coenzyme A (0.2 mg/ml) for 2 to 24 h, and then extracted with xylene as described in Promega technical bulletin 084. The activities of the different plasmids, corrected for transfection efficiency, was calculated as luciferase activity/CAT activity.

In some transfection experiments cells were transfected using Lipofectamine2000 (Gibco/BRL) rather than the calcium phosphate method. In these cases, a dual luciferase reporter system was used. VIP/luciferase plasmids were transfected in these experiments along with pRL-SV40 (Promega), which contains the Renilla luciferase gene under the control of the SV40 viral promoter. Firefly and Renilla luciferase were measured using the manufacturer's kit (Dual-Luciferase Reporter Assay System, Promega).

Gel shift assays. Nuclear proteins were extracted by the method of Digham *et al.* (7). Complimentary oligonucleotides were synthesized by BRL/Gibco, purified on a 10% polyacrylamide gel, annealed, radiolabeled using [γ-^{32}P]ATP and T4 kinase (VSE-1) or [α-^{32}P]dCTP and Klenow (AP-1/*ets*), then purified again by either gel electrophoresis or on G50 Sephadex columns. Labeled oligonucleotides (20,000 to 50,000 cpm) were incubated for 10–15 min at room temperature in 20 μl reaction buffer (Hepes (pH 7.9) 20 mM, KCl 50 mM, $MgCl_2$ 1 mM, EDTA 0.1 mM, DTT 1 mM, glycerol 10%) containing 0–4.5 μg nuclear protein and 6 μg poly(dldC), then run on a 6% polyacrylamide nondenaturing gel. Gels were dried and exposed for autoradiography.

RESULTS

Our previous studies indicated that sequences between 2.5 and 5.2 kb upstream from the VIP gene transcription start site conferred a high level of transcriptional activity in SK-N-SH-IN cells (1), and that enhancer-like activity was present on a 645-bp *Xba*I/*Taq*I fragment located approximately 4.0 to 4.6 kb upstream from the transcription start site (2). The exact position of this fragment on the VIP gene corresponds to −4662 to −4018 upstream from the designated transcription start site (8). In the current studies, we inserted the 645-bp fragment into a new parent vector, called pHC2VL, which consisted of a "minimal" human VIP core promoter/enhancer and 5′ untranslated sequences (−96 to +142) fused to the gene encoding firefly luciferase. The 645-bp fragment was inserted in both orientations and subjected to a limited *Exo*III deletion analysis to determine the approximate location of enhancer sequences. Progressive deletion from the 5′ end revealed the presence of inhibitory sequences at the 5′ end, and enhancer-like sequences towards the center of the fragment. Deletion of the 224 upstream-most nucleotides (from −4662 to −4438) resulted in luciferase activity that was more than 8-fold higher than that of the intact fragment (Fig. 1, p645 vs deletion 5A). Further deletion from −4438 to −4361, and from −4361 to −4299 (5B and 5C, respectively) resulted in a step-wise loss in luciferase activity, sug-

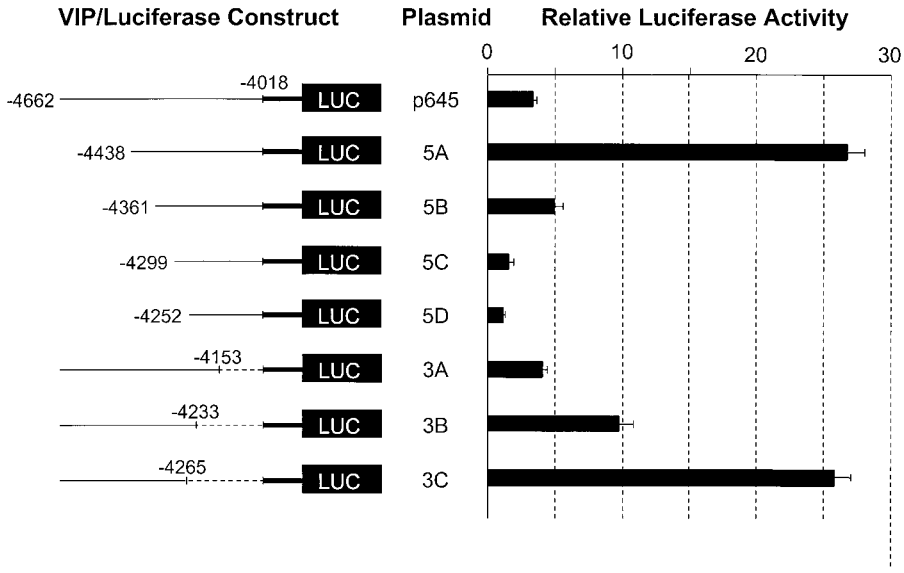

FIG. 1. Luciferase activity in SH-IN human neuroblastoma cells transfected with 5′ and 3′ deletions of the upstream 645-bp human VIP gene enhancer region and an internal 213-bp fragment. Values are expressed relative to that of the parent plasmid (pHC2VL), which consisted of a core human VIP promoter/enhancer region (−94 to +142, represented by the thickened line) fused to the firefly luciferase gene (LUC). Deletion endpoints are indicates. Luciferase activity is corrected for transfection efficiency as described in the methods section. Constructs were tested in triplicate wells, in three to eight separate transfection experiments. Values (±SEM) represent the mean of all experiments.

gesting the presence of enhancer-like elements in this region. Deletion of 5′ sequences beyond this did not reveal the presence of additional regulatory elements, although 5′ deletion analysis beyond −4228 was not performed. In contrast, deletion of sequences from the 3′ end indicated that inhibitory sequences were also present on the 3′ part of the 645-bp fragment. Significant increases in expression were observed when sequences were removed between −4153 and −4233 (3A vs 3B), and between −4233 and −4265 (3B vs 3C). Overall, this initial deletion analysis indicated that enhancer sequences resided in the middle part of the fragment, and that these were flanked by inhibitory sequences.

The above analysis indicated that significant enhancer activity was present downstream of −4438 (the deletion endpoint in construct 5A (Fig. 1)). An internal 213-bp fragment (−4438 to −4226) was then inserted into the parent vector pHC2VL. The 213-bp fragment contained the putative enhancer, but not the inhibitory sequences at the 5′ and 3′ end of the original 645-bp fragment. The activity of this plasmid (p213) was found to be 55-fold higher than the parent vector pHC2VL (Fig. 2), indicating that p213 contained strong enhancer-like sequences which are modulated by inhibitory sequences at each end. Moreover, the enhancer activity of 213-bp fragment was much higher in SH-IN cells than in SH-SY-5Y cells, a subclone derived from the same cell line that expresses much lower levels of VIP (1). Thus the large differences in endogenous VIP gene expression in SH-IN cells and SH-SY-5Y cells may thus be explained in part by specific enhancer sequences on p213.

Subsequent analyses focused on the identification of specific regulatory elements on the 213-bp fragment. A comparison of the sequence of this fragment with consensus sequences of known regulatory elements revealed that certain portions of this fragment have high homology to *cis*-acting elements on other genes (Fig. 3). These include a composite atypical AP-1/*ets* element, and an AT-rich region containing a sequence (ATTTTCCAT) previously shown to bind Oct-1 and Oct-2 homeobox proteins in SH-EP neuroblastoma cells (14). The AP-1 sequence (TGATTCA) is identical to a sequence on the tyrosine hydroxylase (TOH) gene (9), and is atypical in the sense that it differs from the classical AP-1 site (10) by one nucleotide. The adjacent *ets* sequence conforms to a consensus sequence that typically binds to members of the large family of *ets* transcription factors (11). A symmetrical dyad overlaps this composite element. Composite AP-1/*ets* elements have been noted on a number of genes, and cooperativity between AP-1 and *ets* sites with respect to transcriptional activity has been demonstrated (11–13).

A comparison of the transfection data in Fig. 1 with the location of consensus sequences and deletion endpoints (Fig. 3) indicated that the AP-1/*ets* and Oct sequences might function as enhancers in SH-IN cells. Because the Oct consensus-containing sequence had already been shown to function as an enhancer in neuroblastoma cells, we focused most of our attention on the composite AP-1/

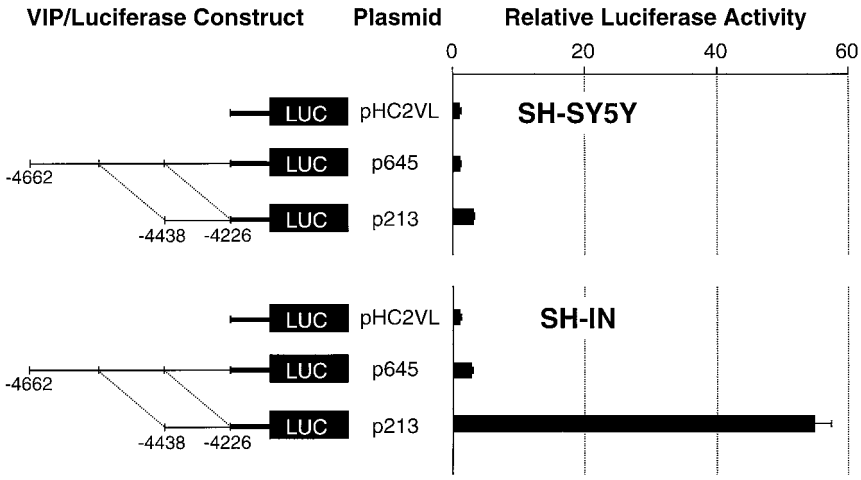

FIG. 2. Cell-specific enhancer activity of plasmids containing the full 645-bp upstream VIP gene sequence (p645) or the 213 bp subfragment (p213) in SH-SY-5Y (top) vs SH-IN (bottom) human neuroblastoma cells. The parent plasmid (pHC2VL) and other details are described in the legend to Fig. 1.

ets element. The activity of this element was tested by site-directed mutagenesis of p213. Mutation of five nucleotides of the AP-1/*ets* element in p213 (**TGA**TTCA-**CA**TCCC to **GTG**TTCA**AC**TCC) (p213m) resulted in a more than 95% loss of luciferase activity in SH-IN cells (Fig. 4). In contrast, the same mutation resulted in only a 26% decrease in expression in SH-SY-5Y cells.

We then tested the possibility that the AP-1/*ets* and Oct-1/2 elements could function as enhancers independent of surrounding sequences on p213. To do this, 25 bp complimentary oligodeoxynucleotides containing these elements were synthesized (Table 1) and inserted into the parent vector pHC2VL. Insertion of the AP-1/*ets* sequences in pHC2VL resulted in activity that was about 8-fold higher than the parent vector (Fig. 5). Mutations of five central nucleotides on the AP-1/*ets* element (mAP-1/*ets*) completely abolished its activity. In contrast, the plasmid containing the Oct sequences (Oct-1/2) showed no activity above the parent vector. However, when 31 nucleotides 5′ to the Oct-1/2 site were included (Oct-Ex), a 3-fold increase in reporter activity was observed. Thus the Oct-1/2 element requires a significant number of 5′ sequenced to function independently as an enhancer. The data are consistent with that of Hahm and Eiden (14), who showed by footprint analyses that these additional sequences are bound by nuclear proteins in neuroblastoma cells.

The above analyses show that two distinct enhancers are present on the 213-bp VIP gene fragment. However, the initial deletion analysis indicated that the

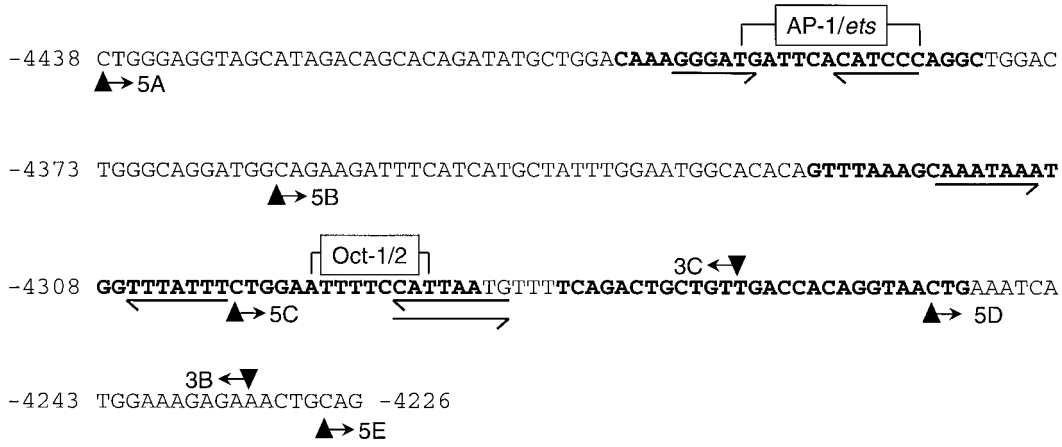

FIG. 3. Nucleotide sequence of the 213-bp human VIP gene fragment showing AP-1/*ets* consensus and Oct-1/2 binding sequences. Also shown are palindromes and dyads (indicated by arrows). Sequences in bold correspond to oligonucleotides that exhibited enhancer or silencer activity when inserted into a parent vector (Fig. 5 and text). Numbers on the left indicate the nucleotide position relative to the VIP gene transcription start site.

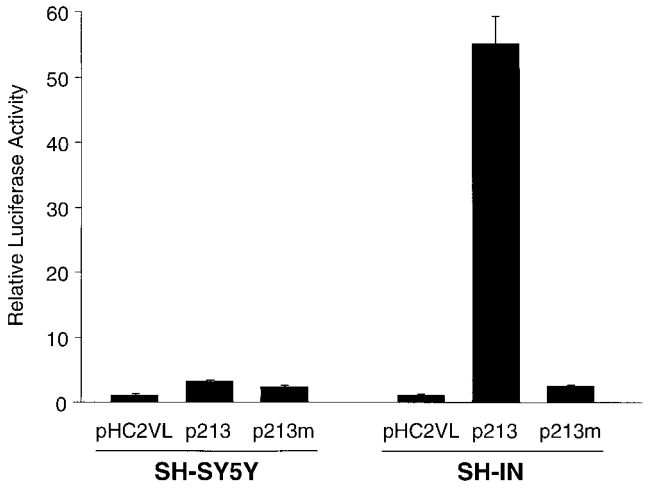

FIG. 4. Loss of cell-specific enhancer activity by mutation of the AP-1/*ets* sequence on the upstream 213-bp VIP gene fragment. p213 contains the wild type gene fragment in the parent vector pHC2VL. p213m contains five nucleotides of the AP-1/*ets* sequence changed by site-directed mutagenesis (**TGA**TTCA**CA**TCCC to **GTG**TTCA**AC**TCC, see Fig. 3). Plasmids were transfected into SH-SY-5Y (left) and SH-IN (right) human neuroblastoma cells. Other details are described in the legend to Fig. 1.

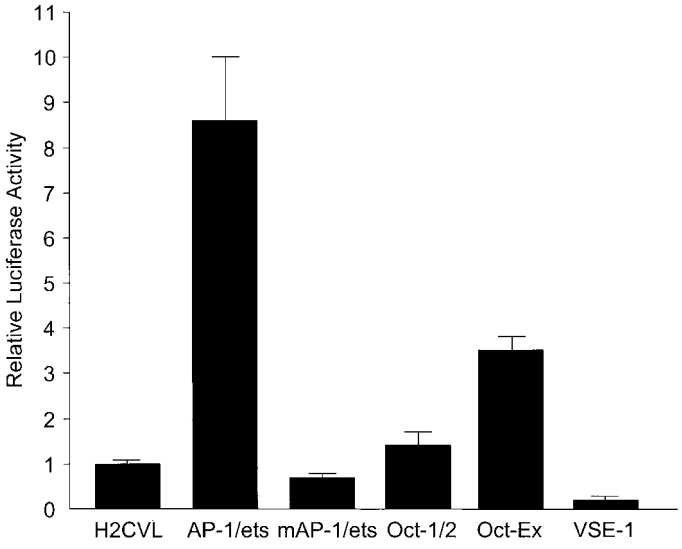

FIG. 5. Luciferase activity in SH-IN cells transfected with the parent plasmid pHC2VL, and the parent plasmid containing oligonucleotides corresponding to potential regulatory sequences on the VIP gene (Table 1).

213-bp fragment might also contain a negative regulatory element located at its 3′ end, just upstream of −423 (compare the activity of 3B vs 3C in Fig. 1). A 28-bp oligonucleotide containing sequences in the deleted region, designated VSE-1 (Table 1) was synthesized and inserted into the parent vector pHC2VL. Insertion of VSE-1 reduced reporter activity by 85% compared to that of the parent vector (Fig. 5), indicating that it can act as a silencer element independent of surrounding sequences on p213. Moreover, when seven nucleotides on VSE-1 were mutated on p213 by site-directed mutagenesis (CCACAGG to TACGTTA), reporter gene expression was increased by 50% compared to p213 (data not shown). A similar increase in expression due to mutation of VSE-1 was observed when the plasmids were transfected into SH-SY-5Y cells and human embryonic kidney 293 cells (data not shown). Together the data indicate that VSE-1 functions to inhibit gene expression in neuroblastoma cells, but that the silencer activity of VSE-1 is not accentuated in cells that express little or no VIP.

TABLE 1

Sequences Tested for Enhancer or Silencer Activity[a]

Name	Sequence
AP-1/*ets*	CAAAGGGATGATTCACATCCCAGGC
mAP-1/*ets*	CAAAGGGA**GTG**TTCA**AC**TCCCAGGC[b]
Oct-1/2	TCTGGAATTTTCCATTAATGTTTTC
Oct-Ex	GTTTAAAGCAAATAAATGGTTTATTTCTGGAATTTTCCATTAA
VSE-1	TCAGACTGCTGTTGACCACAGGTAACTG

[a] See Fig. 3 for location of sequences.
[b] Mutated nucleotides in mAP-1/*ets* are indicated in bold.

The above transfection analyses confirmed that the Oct-1/2 sequence on the VIP gene acts as an enhancer in neuroblastoma cells, but identified two new elements, an AP-1/*ets* sequence, and VSE-1, that regulate gene expression in neuroblastoma cells. We therefore tested the ability of the latter two sequences to bind nuclear proteins from SH-IN cells. The AP-1/*ets* sequence specifically bound nuclear proteins in a gel-shift assay (Fig. 6A), indicating that it interacts with nuclear proteins in these cells, most likely to one or more proteins in the large families of AP-1 and *ets* transcription factors. VSE-1 also specifically bound nuclear proteins from SK-N-SH-IN cells (Fig. 6B). A search of the TFSITES.DAT transcription factor binding site data base (15) indicated that VSE-1 contained no sequences with significant homology to consensus sequences known to bind transcriptional repressors. Thus, VSE-1 might interact with a novel family of transcriptional repressors.

DISCUSSION

The studies reported here identify three distinct far upstream regulatory elements that control the level of basal VIP gene transcription in SH-IN neuroblastoma cells. In previous studies, we identified a 645-bp enhancer region located approximately 4.0 to 4.6 kb upstream from the VIP gene transcription start site (1, 2). Here we examined this upstream region in detail and

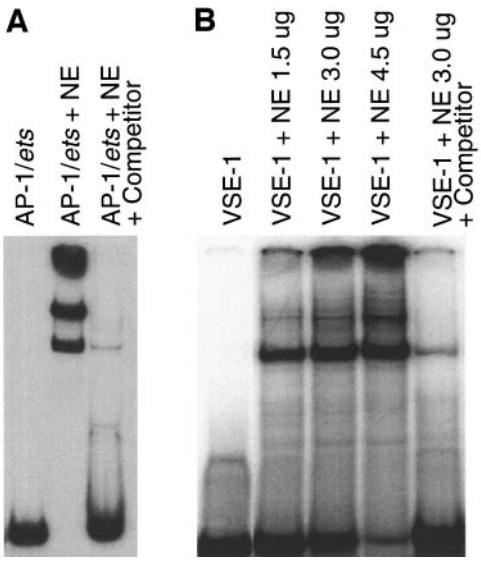

FIG. 6. Gel shift assay showing specific binding of nuclear proteins to the AP-1/*ets* (A) and VSE-1 (B) sequences. (A) Lanes 1 and 2 show the migration of the AP-1/*ets* sequence in the absence and presence of nuclear proteins (2 μg), respectively. Lane 3 is the same as reaction as in lane 2, except that a 100-fold excess of unlabeled AP-1/*ets* sequences was included as a competitor. (B) Lane 1 shows the migration of VSE-1 in the absence of nuclear proteins. Lanes 2–4 show VSE-1 incubated with increasing amounts of nuclear extracts (NE, in μg protein). Lane contains radiolabeled VSE-1 with plus 3 μg SK-N-SH-IN nuclear proteins plus a 100-fold excess of unlabeled VSE-1.

report its involvement in determining constitutive VIP gene transcription. Deletion of sequences from either the 5′ or 3′ end of this 645-bp fragment resulted in an increase in reporter activity, suggesting that silencer elements are present on each end of the fragment. The 213-bp fragment showed much stronger enhancer activity in SH-IN cells, which exhibit a high degree of basal VIP gene transcription, than in the sister subclone SH-SY5Y, which expresses very low amounts of VIP. Enhancer elements on this 213-bp fragment may thus explain in part the large difference in VIP gene transcription in these subclones.

Subsequent mutation analysis of the 213-bp fragment showed that it contains at least two distinct enhancers and, surprisingly, one silencer. One of the enhancers corresponded to an AP-1/*ets* consensus sequence. Remarkably, mutation of this sequence by site-directed mutagenesis resulted in a 95% decrease reporter activity in SH-IN cells (Fig. 4). The same mutation had only a small effect in SH-SY5Y cells, indicating that this element plays an important role in regulating the different levels of VIP gene transcription in these different subclones. The other enhancer identified in these studies was a 43-bp AT-rich sequence which contained an element previously shown by Hahm and Eiden (14) to bind Oct-1 and Oct-2 transcription factors in SH-EP cells. Like SH-IN, the SH-EP subclone expresses high amounts of VIP. Mutation of this Oct-1/2 consensus site resulted in decreased reporter expression in SH-EP cells, indicating the Oct-1/2 site was necessary for full VIP gene transcriptional activity. In our studies, we found that the Oct-1/2 element (and 6–9 flanking nucleotides) was insufficient on its own to act as an enhancer in SH-IN cells (Fig. 5). However, when 31 nucleotides 5′ to the Oct-1/2 consensus sequence were included along with the Oct-1/2 element, enhancer activity was clearly demonstrated. That these 31 nucleotides may be required for enhancer activity seems in line with the data of Hahm and Eiden (14), who showed by footprint analyses that these additional sequences are bound by nuclear proteins in SH-EP cells.

In addition to the enhancer elements, we localized one inhibitory sequence on the VIP gene, designated VSE-1, which specifically bound nuclear proteins from SK-N-SH-IN cells. Silencer elements in some cases are used to restrict gene expression within a particular lineage (16–18). It may be relevant that tyrosine hydroxylase (TOH) and VIP are both expressed initially in catecholaminergic precursor cells. However, as development proceeds, VIP expression is extinguished in the great majority of these catecholaminergic precursors (19). VSE-1 or other silencer elements on the VIP gene might be used to extinguish or restrict VIP expression in certain neural crest lineages. The protein that interacts with VSE-1 remains to be determined. VSE-1 does not contain consensus sequences that bind to known transcriptional repressors. In addition, it had no significant homologies to silencers thought to be relevant to gene expression in neuroblasts, for example, the two E-box elements in the choline acetyltransferase (ChAT) which restrict ChAT expression in adrenergic, but not cholinergic neuroblastoma cell lines (20). There is also no homology to either the E-box/dyad in the TOH gene, or the upstream regulator element of N-CAM (21), both of which bind the homeodomain protein CDP2/cux (in the cut family of relievable transcription repressors). Finally, there is no significant homology to the Pax motif which has been shown to repress the Ng-CAM gene in neuroblastoma and other cell lines (22), or to the consensus sequence that binds REST/NRSF (23). The lack of homology of sequences on VSE-1 to known consensus sequences involved in transcriptional silencing suggest that VSE-1 might interact with a novel family of transcription repressors.

These and other studies that suggest multiple mechanisms are used to establish the temporal and spatial pattern of VIP gene expression during development, and to actively regulate expression of the gene in response to hormones, injury, and other external stimuli. The proximal promoter has been analyzed in greatest detail, and appears to be involved in setting basal cell-specific expression as well as response to cAMP/protein kinase A

and protein kinase C (1, 8, 24–26). Another region located approximately 1.1 to 1.3 kb upstream from the transcription start site is highly conserved between the human and mouse genes (27) and has been found to mediate induction by several cytokines (28, 29), and to be required for full cell type-specific expression (14, 30). The studies reported here, as well as those of Hahm and Eiden (14), are beginning to reveal the molecular details of a regulatory region on VIP gene located more than 4 kb upstream from the transcription start site that is likely to be used to finely tune the level of transcription in different neuronal lineages *in vivo*.

ACKNOWLEDGMENTS

The work was supported by National Institutes of Health HD06576, HD04612, HD07032 (K.K.), and the American Paralysis Association.

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