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# Lineage-specific regulation of the vasoactive intestinal peptide gene in neuroblastoma cells is conferred by 5.2 kilobases of 5'-flanking sequence

(SK-N-SH cell line/neuropeptide/transacting factor/transcription regulatory element/enhancer–promoter)

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**ABSTRACT The expression of a transfected plasmid containing 5.2 kilobases (kb) of 5' regulatory DNA sequence of the human vasoactive intestinal peptide (VIP) gene attached to coding sequences of the reporter gene chloramphenicol acetyltransferase (CAT) was compared with endogenous VIP expression in subclones of the human neuroblastoma cell line SK-N-SH. These subclones vary widely in basal and inducible quantities of VIP and its precursor mRNA and can be interconverted under specified culture conditions. Endogenous VIP immunoreactivity, detectable in all subclones, was lowest in the neuronal subclone SH-SY-5Y, whereas 15- to 25-fold higher levels were observed in the epithelial-appearing SH-EP and intermediate SH-IN subclones. Treatment with 10 nM phorbol 12-myristate 13-acetate (PMA) stimulated VIP peptide levels ≈5-fold in SH-SY-5Y cells but did not increase appreciably VIP levels in the other subclones. Treatment with 2.5 μM forskolin resulted in <50% stimulation of VIP expression in all subclones. Levels of mRNA encoding the VIP precursor generally paralleled these differences in VIP immunoreactivity. In cells transfected with the VIP/CAT fusion gene, CAT activity reflected closely these differences in basal VIP expression and the changes in response to PMA and forskolin. Deletion of 2.7 kb of the most upstream sequences resulted in an 80–90% reduction in basal CAT activity in SH-IN, but not SH-SY-5Y cells, and resulted in an 80% reduction in PMA stimulation in SH-SY-5Y cells. Deletion to within 74 nucleotides of the transcription start site resulted in CAT expression in SH-IN cells that was only 3% of that seen with the full 5.2-kb flanking sequences and further diminished the remaining PMA responsiveness in SH-SY-5Y cells. The data indicate that important cell-type-specific transcription regulatory sequences reside >2.5 kb upstream from the VIP transcription start site.**

Several important eukaryotic DNA regulatory sequences have been identified in recent years as well as some of the proteins that act on them (1–7). DNA sequences responsible for the constitutive and inducible expression of a number of genes have been identified by deletion and/or mutation analysis of transfected DNA (7–10). Analysis of tissue or cell-type-specific expression usually involves comparison of transfected gene activity in a cell line that endogenously expresses the gene of interest with that in an unrelated control cell line that does not express the gene (11–17). The appropriateness of such a comparison relies on the assumption that differences in endogenous transcription rates are controlled actively by regulatory factors present in one or both cell lines.

A number of neuroblastoma cell lines display distinct cell subtypes that mimic properties of various neural crest derivatives (18, 19). We show here that SK-N-SH human neuroblastoma subtypes, which have been stably subcloned and characterized (18), display large differences in basal and phorbol ester-stimulated levels of vasoactive intestinal peptide (VIP). Because these subclones can interconvert under specified culture conditions with respect to morphology, VIP content, and other biochemical markers (ref. 18 and unpublished observations), it is apparent that their phenotypic specialization does not arise from irreversible structural alterations in their genome. Thus, basal and inducible expression of a transfected VIP gene could be investigated in cells in which the endogenous VIP gene is regulated actively and in which differences in endogenous peptide and mRNA could be quantified. We report here that DNA sequences >2.5 kilobases (kb) upstream from the VIP transcription start site account for most of the observed differences in basal and inducible VIP expression in the subclones.

## MATERIALS AND METHODS

**Cell Culture.** The primary subclones of SK-N-SH (SH-SY-5Y, SH-IN, and SH-EP) were originally isolated and described by Ross *et al.* (18). Experiments were carried out on these cells between the 40th and 50th passage after cloning for the SH-SY-5Y cells and between the 16th and 22nd passage for the SH-IN and SH-EP subclones. Cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g of glucose per liter (Whittaker M.A. Bioproducts, Walkersville, MD) and 10% heat-inactivated fetal calf serum (also Whittaker), supplemented with glutamine (0.03%), penicillin (100 units/ml), and streptomycin (100 μg/ml), and were maintained in a humidified 95% air/5% carbon dioxide atmosphere. For VIP peptide and mRNA measurement and for transfection experiments, cells of each subclone were plated in 35-mm wells at densities resulting in approximately equivalent total protein content per well: SH-SY-5Y at 2.0–5.0 × $10^5$ cells per well and SH-IN and SH-EP at 0.6–1.0 × $10^5$ cells per well. In all experiments, drugs were added 3 days after plating of cells.

**Isolation of the Human VIP-Encoding Gene.** A Charon 4A human library (20) was screened using a full-length human VIP cDNA probe (21). A phage containing a >14-kb insert was isolated and found to contain six complete exons and >6 kb of 5' flanking sequences of the VIP-encoding gene with a restriction-digest pattern and sequence data in agreement with that reported by Tsukada *et al.* (22).

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Abbreviations: VIP, vasoactive intestinal peptide; CAT, chloramphenicol acetyltransferase; PMA, phorbol 12-myristate 13-acetate; CREB, cAMP response element-binding protein.

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**Transfection Method and Assay of Promoter Activity.** Plasmids were transfected 2 days after plating the cells. Approximately 5 $\mu$g of total plasmid DNA was transfected by the method of Chen and Okayama (23), scaled down for addition of DNA to 2 ml of medium. Sixteen hours after DNA addition, cells were rinsed, and fresh medium was added. After 8–10 hr, drugs were added, and in another 20 hr, cells were harvested with trypsin/EDTA. Cells were then pelleted, washed, and resuspended in the assay buffer (100 mM potassium phosphate, pH 7.8/1 mM dithiothreitol). Lysates were analyzed for chloramphenicol acetyltransferase (CAT) activity as described (24). The lysate volume analyzed was adjusted to keep conversion below 25% of the added [$^{14}$C]chloramphenicol but at least 5 times background values.

## RESULTS

Large differences in basal and stimulated VIP expression were seen among SK-N-SH subclones (Fig. 1). The neuronal-appearing SH-SY-5Y cells contained 1.6 ± 0.3 ng of VIP per mg of protein, whereas SH-IN and SH-EP cells contained 15- to 25-fold higher basal levels of this peptide. Because VIP expression in bovine chromaffin cells and other neuroblastoma cell lines can be induced by PMA and by agents that mimic or increase cAMP (27, 28), we investigated this stimulation in SK-N-SH subclones. PMA (10 nM) induced VIP expression >5-fold in SH-SY-5Y cells and caused little or no stimulation of VIP levels in the other subclones. Forskolin (2.5 $\mu$M) increased VIP peptide levels <50% in each subclone, an effect which in each case was more apparent with PMA. Similar differences in basal and inducible VIP expression were found at the level of mRNA encoding the VIP precursor (Fig. 2). VIP mRNA in SH-SY-5Y cells was virtually undetectable but was easily detectable in SH-IN and SH-EP cells. Treatment with PMA resulted in a large increase in VIP mRNA in SH-SY-5Y cells, no effect in SH-IN cells, and a small increase in SH-EP cells. Forskolin modestly stimulated VIP mRNA in each subclone.

Transfection of the fusion gene VIPCAT5.2 (Fig. 3) resulted in CAT expression that paralleled the behavior of the endogenous VIP gene with respect to both basal expression in subclones and inducibility by forskolin and PMA (Fig. 4). Basal CAT activity was lowest in SH-SY-5Y cells (0.034 ± 0.3 CAT units/mg of protein, not visible as a bar above the abscissa on Fig. 4). Basal CAT levels in SH-IN and SH-EP

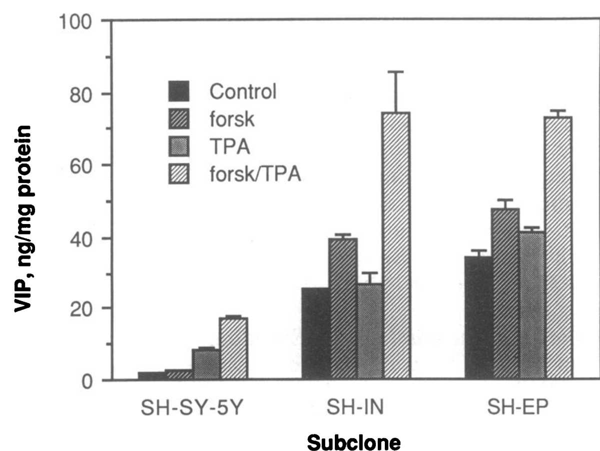


FIG. 1. Levels of VIP immunoreactivity in subclones of SK-N-SH after 20-hr treatment with either 2.5 $\mu$M forskolin (forsk), 10 nM PMA (TPA), or the combination of the two drugs (forsk/TPA). Values (cell extracts plus medium) are mean of three wells ± SEM. Cells were rinsed with phosphate-buffered saline, harvested in 0.1 M HCl, and measured by RIA (25), with N10 VIP antiserum (26). Total protein was measured using the Bio-Rad protein standard and bovine serum albumin.

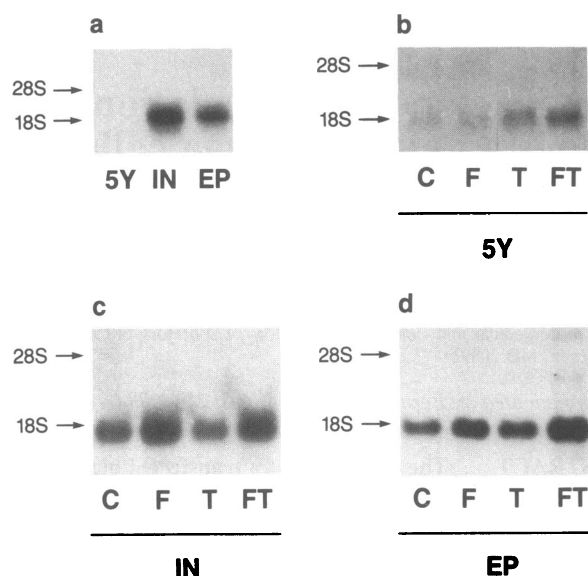


FIG. 2. Autoradiographs of Northern blots showing basal and stimulated levels of mRNA encoding the VIP precursor in subclones of the human neuroblastoma SK-N-SH. Within each subset *a–d*, equivalent amounts of RNA were applied. (*a*) Basal VIP mRNA in SH-SY-5Y (5Y), SH-IN (IN), and SH-EP (EP). (*b–d*) VIP mRNA in SH-SY-5Y, SH-IN, and SH-EP cells, respectively, after 14-hr drug treatment. Lanes: C, untreated; F, 2.5 $\mu$M forskolin; T, 10 nM PMA; and FT, combination of these drugs. In *b–d*, cells were plated as described and RNA was extracted by proteinase K digestion (29). In *a*, RNA from flasks of cells at near confluence was extracted by the guanidinium thiocyanate method (30). RNA was run on agarose gels, and the gels were photographed under UV transillumination. RNA was then transferred to GeneScreen and baked at 80°C for 90 min. Blots were prehybridized and hybridized at 45°C with a 450-base-pair (bp) *Ban* I/*Eco*RI human VIP cDNA fragment (31) $^{32}$P-labeled by nick-translation, and washed at 55°C in the buffers described (29).

subclones were ≈500-fold higher than in SH-SY-5Y cells. Forskolin produced 2- to 3-fold increases in CAT activity in all subclones. CAT activity in response to PMA increased 80-fold in SH-SY-5Y cells, decreased 65% in SH-IN cells, and increased ≈2-fold in the SH-EP cells. Synergy between forskolin and PMA was seen in SH-SY-5Y and SH-EP subclones, whereas the apparently counteracting individual effects of these drugs in SH-IN cells resulted in no net effect on CAT expression driven by the VIPCAT fusion gene. The data obtained with VIPCAT5.2 in SK-N-SH subclones are in close agreement with what was found with the endogenous gene with one exception: A significant decrease in CAT expression driven by VIPCAT5.2 was seen after PMA treatment, a potential negative effect on transcription that was not apparent by measurement of VIP peptide or its mRNA.

Analyses of a limited series of deletion mutations made on the basis of convenient restriction sites allowed mapping of the general location of DNA sequences responsible for cell type-specific basal and drug-stimulated expression in SK-N-SH subclones. At least three short DNA sequences located <100 nucleotides upstream from the VIP transcription start site (Fig. 3*b*) can be considered potentially important regulatory elements on the VIP gene because they have homology to cAMP- or PMA-responsive consensus sequences mapped on several genes. Specifically, the sequences correspond to binding sites for three recently purified DNA-binding proteins: (*i*) AP-1, which in some tissues can be activated by PMA (3, 34), (*ii*) AP-2, which in some tissues can be stimulated by PMA or cAMP (4), and (*iii*) cAMP response element-binding protein (CREB), which is activated by cAMP (5). The sequence on the VIP gene that could interact with this latter protein is responsive to cAMP in rat pheochromocytoma (PC12) cells (10).

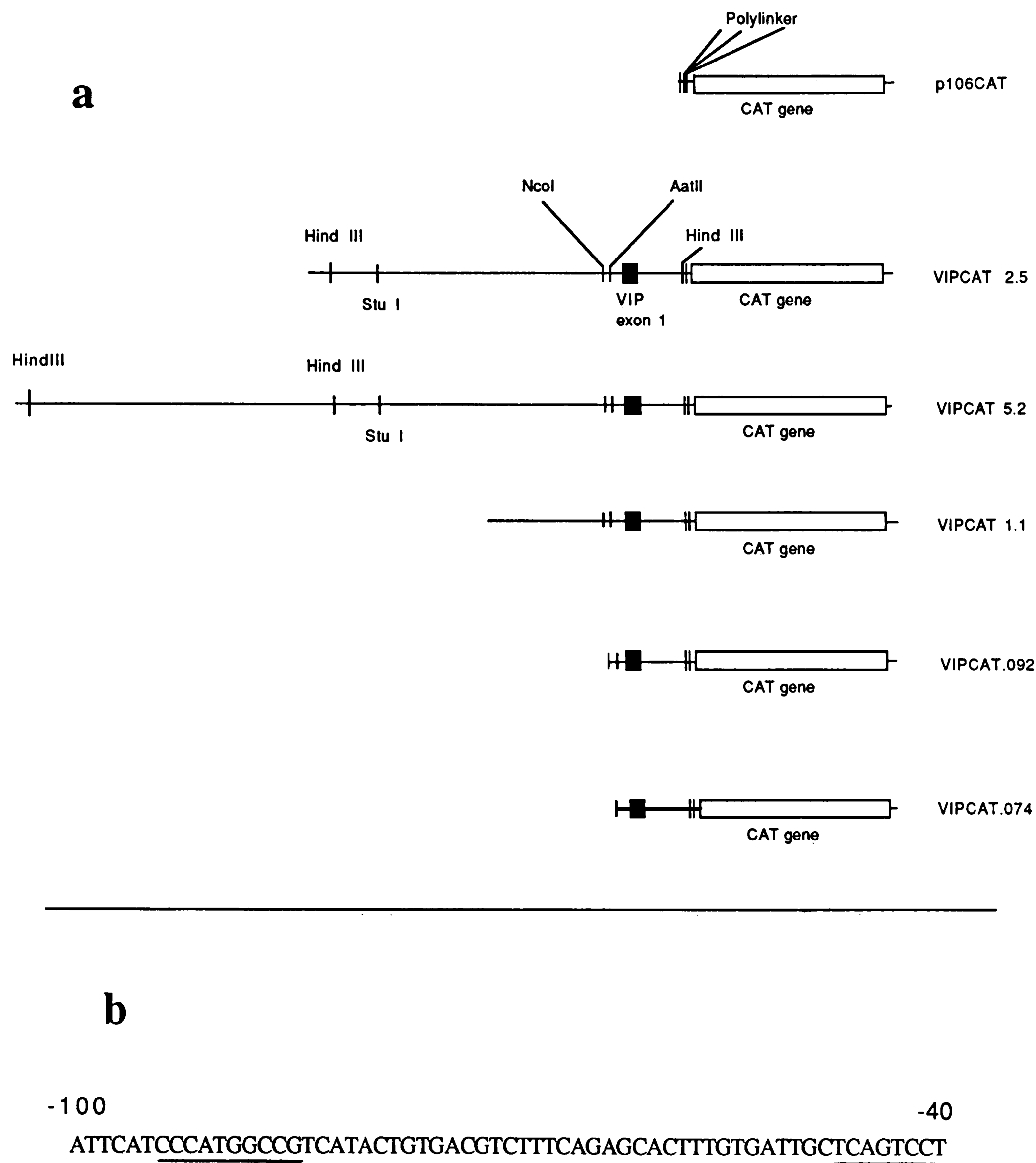


FIG. 3. (*a*) Relevant portions of plasmids used in transfection studies. The plasmid p106CAT is the promoterless parent vector that contains the coding sequences of *Escherichia coli* CAT downstream from a polylinker (32). In VIPCAT2.5, a 3.2-kb *Hin*dIII fragment of the VIP gene containing 2.5 kb of VIP 5′-flanking sequences, the first exon (denoted by black box), and ≈600 bp of the first intron was inserted into the polylinker. To minimize chances of aberrant transcription or translation due to the presence of a 5′, but not a 3′, RNA splice sequence in the fusion gene, complementary synthetic oligomers corresponding to the DNA sequences at the junction of the first intron and second exon of the VIP gene (22) were inserted into the *Sal* I/*Sma* I site in the polylinker just downstream from the inserted *Hin*dIII fragment. VIPCAT5.2 was prepared by partial digestion of VIPCAT2.5 with *Hin*dIII and insertion of the next upstream VIP *Hin*dIII fragment. Proper orientation of the correct 2.7-kb *Hin*dIII fragment was ensured by reconstitution of the same *Eco*RI digestion and hybridization pattern seen with the phage DNA isolate and the same *Eco*RI digestion pattern reported by Linder *et al.* (33) in this region of the gene. VIPCAT1.1 was prepared by digesting VIPCAT2.5 at a unique *Stu* I site, followed by BAL-31 digestion and self-ligation. VIPCAT.092 was prepared by ligation of two fragments made from VIPCAT2.5. The CAT-encoding portion was obtained by complete digestion with *Hin*dIII, filling in with Klenow fragment, digestion with *Sal* I, and isolation of the fragment containing the CAT-encoding and synthetic splice sequences. The VIP gene-containing portion was made by digestion of VIPCAT2.5 with *Nco* I, filling in the ends with Klenow fragment, digestion with *Sal* I, and isolation of the fragment containing the VIP gene sequences. VIPCAT.074 was prepared by double partial digestion of VIPCAT2.5 with *Hin*dIII and *Aat* II, filling in the ends with Klenow fragment, and isolation of the proper fragment followed by self-ligation. (*b*) Published sequence of nucleotides on the VIP gene located 40–100 bases upstream from the transcriptional start site on the VIP gene (22). Underlined portions are similar to consensus sequences purported to bind transcription regulatory factors. From left to right, the sequences correspond to binding sites for AP-2 (ref. 4, sequences here in the inverted form), CREB (5), and AP-1 (see TRE M65/66/TK CAT in ref. 34).

VIPCAT5.2, VIPCAT2.5, and VIPCAT1.1 preserve all of the above mentioned potential regulatory elements. VIPCAT.092 preserves the potential AP-1 and CREB-binding sequences but contains a single base change in the potential AP-2 site (the recombinant procedure replaced the CC at positions −94 and −93 on the VIP gene with a CT). In VIPCAT.074, both AP-2 and cAMP-responsive consensus sequences are deleted, but the potential AP-1 binding consensus sequence is preserved. The above described VIPCAT plasmids were transfected into SH-IN and SH-SY-5Y cells and analyzed for basal CAT expression.

In SH-IN cells, deletion of the farthest upstream 2.7 kb of VIP 5′-flanking sequences resulted in an 80–90% reduction in basal CAT expression (Fig. 5). Subsequent deletions caused smaller but reproducible changes in CAT activity. Deletion to within 92 bp of the transcriptional start site (VIPCAT.074)

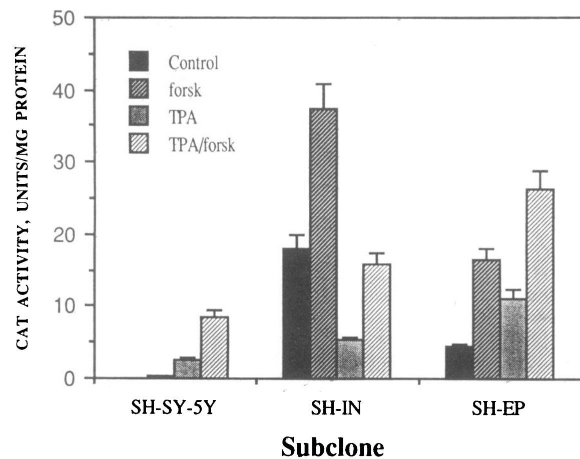


FIG. 4. Stimulation of CAT activity in individual subclones of SK-N-SH transfected with a plasmid containing 5.2-kb VIP 5′-flanking sequences fused to CAT coding sequences (VIPCAT5.2, Fig. 3). Transfected cells were stimulated for 20 hr with either 2.5 $\mu$M forskolin (forsk), PMA (TPA), or a combination of the two drugs (TPA/forsk). Values are mean of three wells ± SEM and are representative of several experiments.

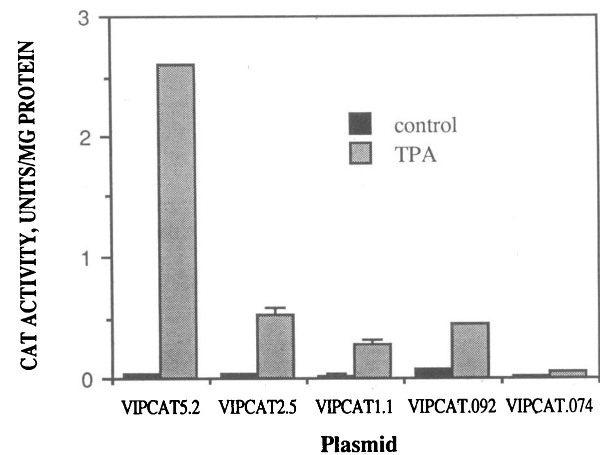


FIG. 6. Stimulation of CAT activity in SH-SY-5Y cells transfected with various lengths of VIP 5′-flanking sequences fused to CAT encoding sequences (Fig. 3) by treatment with 10 nM PMA (TPA) for 20 hr. Values (mean of three wells ± SEM) were corrected for transfection efficiency as in Fig. 5.

caused a further reduction to 3% of the transcriptional activity of VIPCAT5.2. In contrast, little change in basal CAT activity was seen after deletion of these sequences in SH-SY-5Y cells. Thus, the differences in basal expression between SH-IN and SH-SY-5Y cells appears to be primarily due to action of a positive regulatory factor(s) in SH-IN cells acting on a DNA sequence(s) located 2.5–5.2 kb upstream from the transcriptional start site.

Sequences conferring PMA stimulation of VIP expression in SH-SY-5Y cells were also investigated by deletion analysis. Deletion of the upstream 2.7 kb resulted in an 80% reduction in the ability of PMA to stimulate CAT expression. Further deletions produced a small stepwise reduction in ability of PMA to stimulate CAT activity (Fig. 6). When a segment located between −74 and −92 nucleotides relative to the transcription start site was deleted, PMA stimulation of CAT activity was reduced from 6-fold to 2.5-fold.

## DISCUSSION

The most significant finding in this report is that up to 5.2 kb of 5′-flanking sequences on the VIP gene was necessary and

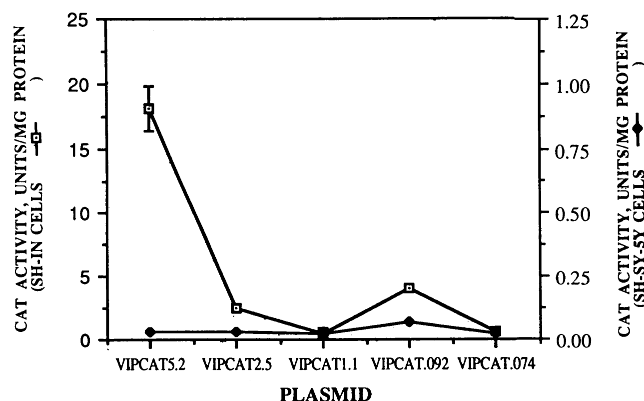


FIG. 5. Basal levels of CAT activity (mean of three wells ± SEM) in SH-IN (▫) and SH-SY-5Y cells (♦) transfected with different lengths of VIP 5′-flanking sequences fused to CAT encoding sequences (Fig. 3). Cells were cotransfected with a plasmid (RSVL) containing the Rous sarcoma virus promoter fused to coding sequences of firefly luciferase (35). Within each subclone, values between different plasmids were corrected for transfection efficiency by comparing luciferase activity in cell extracts (35).

sufficient to recapitulate the transcriptional behavior of the endogenous VIP gene in neuroblastoma subclones that differed widely in their basal and inducible expression of VIP. Because these subclones can reversibly interconvert with respect to morphology and VIP content, differences in VIP expression would appear to be actively controlled by factors acting on regulatory sequences common to both endogenous gene and transfected fusion gene.

SH-IN and SH-EP subclones were found to express endogenous VIP in about 15- to 25-fold greater abundance than in SH-SY-5Y cells. Both phorbol ester and forskolin increased VIP peptide and VIP mRNA levels in SH-SY-5Y and EP cells. Forskolin, but not PMA, stimulated VIP and VIP mRNA expression in SH-IN cells. Thus, the three cell lines display significantly different modes of expression and inducibility of the VIP gene. The behavior of the transfected plasmid containing 5.2 kb of 5′ flanking sequence of the VIP gene fused to the CAT reporter gene sequence was very similar to that of the endogenous gene. It was therefore possible to begin to investigate DNA sequences responsible for the differences in basal and PMA-inducible expression of VIP in the subclones. Deletion of the farthest upstream 2.7 kb of DNA from this plasmid caused an 80–90% fall in basal expression in SH-IN, but not in SH-SY-5Y cells, and a >80% reduction in PMA-inducible expression in SH-SY-5Y cells. Thus, elements between 5.2 and 2.5 kb upstream from the start of transcription of the VIP gene may function as cell-type-specific regulatory element(s) to positively regulate the level of endogenous VIP expression in SH-IN cells and to mediate PMA induction of VIP in SH-SY-5Y cells. Further deletion resulted in smaller changes in both basal transcription in SH-IN and SH-SY-5Y cells and PMA-inducibility in SH-SY-5Y cells. Deletion beyond the region containing sequences homologous to putative cAMP- and PMA-inducible AP-2 and cAMP-inducible CREB binding sites (Fig. 3*b*, refs. 4 and 5) resulted in a reduction in basal CAT activity in both SH-IN and SH-SY-5Y cells and thus does not appear to be of major importance in determining the difference in constitutive VIP expression in the two cell types. Removal of this sequence did cause a reduction of PMA induction of CAT activity in SH-SY-5Y cells. Deletion of further downstream sequences could identify other regulatory elements, for example, the potential AP-1 consensus sequence (Fig. 3*b*).

These data extend those of Tsukada *et al.* (10), who found that sequences between −86 and −70 nucleotides relative to the VIP transcription start site confer responsiveness of a

transfected gene to forskolin in rat pheochromocytoma (PC12) cells. The present report addresses additional questions of tissue specificity and PMA responsiveness, and these experiments were done in cell lines in which VIP peptide and VIP precursor mRNA levels were measured. Deletional analysis of the forskolin response in SH-SY-5Y cells (data not shown) was in general agreement with that of Tsukada *et al.* (10), but the responses of both endogenous VIP and transfected gene in SK-N-SH subclones to forskolin were not large (2- to 3-fold).

CAT expression from VIPCAT5.2 in SH-SY-5Y cells was 2–5 times higher than in HeLa cells (data not shown), in which VIP expression was undetectable (<0.1 ng/mg of protein). Although the higher level of CAT activity in SH-SY-5Y cells, which contain 1–2 ng of VIP per mg of protein, could be interpreted as evidence that expression from VIPCAT5.2 is cell-specific, deletion of all but 74 nucleotides of the 5′-flanking sequence caused only small changes in basal CAT activity in SH-SY-5Y cells. The same set of plasmids transfected into HeLa cells produced a pattern of basal CAT expression similar to that in SH-SY-5Y cells (data not shown). It is possible that sequences on the VIP gene not analyzed here could account for the differences in basal expression in SH-SY-5Y versus HeLa cells. Alternatively, a major differentiation process leading to general activation or repression of transcription from the VIP gene locus could be an irreversible regulatory event, with various cell-specific factors acting on DNA sequences such as those localized here, to regulate transcription more precisely.

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