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A restrictive element 1 (RE-1) in the VIP gene modulates transcription in neuronal and non-neuronal cells in collaboration with an upstream tissue specifier element

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Abstract

The vasoactive intestinal peptide (VIP) gene has been studied extensively as a prototype neuronal gene containing multiple *cis*-active elements that confer responsiveness to cell lineage, neurotrophic, and activity-dependent intrinsic and extrinsic cues. However, reporter genes containing the presumptive complete regulatory region 5′ to the start of transcription do not confer tissue-specific gene expression *in vivo*. We therefore sought *cis*-regulatory elements downstream of the transcriptional start that might confer additional tissue-specific and tissue-restrictive properties to the VIP transcriptional unit. We report here a repressor element, similar to the canonical restrictive element-1 (RE-1), located within the first non-coding exon of the human VIP gene. The ability of this element to regulate VIP reporter gene expression in neuroblastoma and fibroblastic cells was examined. Endogenous VIP expression is high in SH-EP neuroblastoma cells, low but inducible in SH-SY5Y cells, and absent in HeLa cells. Endogenous RE-1 silencer factor (REST) expression was highest in SH-EP and HeLa cells, and significantly lower in SH-SY5Y cells. Transient transfection of a VIP reporter gene containing a mutated RE-1 sequence revealed an RE-1-dependent regulation of VIP gene expression in all three cell types, with regulation greatest in cells (SH-EP, HeLa) with highest levels of REST expression. Serial truncation of the VIP reporter gene further revealed a specific interaction between the RE-1 and a tissue-specifier element located 5 kb upstream in the VIP gene. Thus, REST can regulate VIP gene expression in both neuroblastic and non-neuronal cells, but requires coupling to the upstream tissue specifier element.

Keywords: gene expression, neuroblastoma, repression, signal transduction.

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Vasoactive intestinal peptide (VIP) is a neuropeptide uniquely distributed within the brain and peripheral nervous system with pleiotropic functions in the immune, endocrine and nervous systems. VIP plays an important role in neurodevelopment with mitogenic and trophic effects on embryonic neurons in the CNS and PNS (Brenneman and Eiden 1986; Gressens *et al*. 1993). It is transiently expressed at high levels in rat stellate ganglion *in utero*, possibly regulating neuroblast and glial cell proliferation and survival (Tyrrell and Landis 1994; Waschek 1995). Control and regulation of the VIP gene is likely, therefore, to have far reaching effects on the organism. Although multiple regulatory regions have been mapped on the VIP gene, complete recapitulation of VIP gene expression has not yet been achieved using VIP reporter genes in transgenic animal models, suggesting the presence of other as yet unidentified regulatory regions (Agoston *et al*. 1990; Tsuruda *et al*. 1996; Waschek *et al*. 1999).

The human VIP gene sequence was searched using the web-based transcription factor analysis software package Transfac (Wingender *et al*. 2000), and a restrictive element-1

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Abbreviations used: CRE, cyclic AMP responsive element; CREB, cyclic AMP responsive element binding protein; DBD, DNA binding domain; DBH, dopamine beta-hydroxylase; cDNA, complementary DNA; EMSA, electrophoretic mobility shift assay; β-Gal, beta galactosidase; GAPDH, glyceraldehyde phosphate dehydrogenase; PACAP, pituitary adenylate cyclase activating polypeptide; PHM, peptide histidine methionine; Q-RT-PCR, quantitative RT-PCR; RE-1, repressor element-1; REST, restrictive element-1 silencer factor; RRL, rabbit reticulocyte lysate; RT-PCR, reverse transcription-polymerase chain reaction; TRE, phorbol 12-myristate 13-acetate-responsive element; TSE, tissue specifier element; VIP, vasoactive intestinal polypeptide.

(RE-1)-like site was identified within the first exon. The RE-1 is a *cis*-active repressor element found in many genes (Schoenherr *et al*. 1996) both neuronal and non-neuronal. Present data suggest that restrictive element-1 silencer factor (REST) binds the RE-1 and inhibits expression of neuronal genes in non-neuronal cell types (Chong *et al*. 1995; Mieda *et al*. 1997). During embryonic development, REST is down-regulated in maturing neuroblasts (Nishimura *et al*. 1996) and neurons (Palm *et al*. 1998), although low levels can still be found within the adult brain (Palm *et al*. 1998) and neuronal cell lines (Chong *et al*. 1995; Lonnerberg *et al*. 1996). Several truncated variants due to alternative splicing have been identified, two of which are strictly neuronal. REST4 and REST-N62 are the dominant neuronal variants found in the rat and human, respectively (Palm *et al*. 1998, 1999). Although originally described as having repressor activities (Palm *et al*. 1998), REST4 has more recently been described as having anti-repressor activity on the cholinergic gene locus (Shimojo *et al*. 1999) and may act by inhibiting REST repression. Extensive analysis of REST4 has revealed that it is capable of binding to the RE-1, albeit weakly (Lee *et al*. 2000), and confirmed the formation of hetero-oligomers with REST which inhibit REST binding to the RE-1 (Tabuchi *et al*. 2002). A recent publication has found yet another unique truncated form of REST in small cell lung cancer cells that have high levels of vasopressin (Coulson *et al*. 2000).

REST effects on gene expression appear to be promoter position dependent, as proximal location to the TATA box has been reported to change REST function to an enhancer mode (Bessis *et al*. 1997). Tissue specificity also plays a role, since in transgenic mice containing the L1 gene regulatory sequence minus the RE-1 there was enhanced gene expression in the brain and non-neuronal tissues compared with transgenic mice containing the complete L1 regulatory sequence. Unexpected decreases in some specific brain regions, including the hippocampus and cerebellum, were also found (Kallunki *et al*. 1998). It is currently unknown if the RE-1-like sequence within the VIP non-coding region regulates VIP expression.

A set of experiments was designed to test whether the RE-1-like sequence could regulate VIP gene expression in either non-neuronal or neuroblastoma cell lines. VIP reporter gene constructs, including 5.2 kb of upstream regulatory sequence plus the first exon, first intron and first 13 bp of the second exon, were generated with and without either a six or 12 bp mutation of the putative RE-1. Reporter constructs were transiently transfected into neuroblastoma cell lines, derivatives of the neural crest that display distinct cell karyotypes with either neuron-like or epithelial/glial-like morphology (Biedler *et al*. 1978; Ross *et al*. 1983). Mutation of the RE-1 led to increased VIP reporter gene activity in both fibroblastic and neuroblastic cell types. Gene expression paralleling that of the endogenous VIP gene, however, required the combined action of the RE-1 with an OCT-1-containing upstream element previously characterized as conferring tissue-specific VIP gene expression via an enhancer-like activity in neuronal cells.

Materials and methods

Neuroblastoma cell culture, plasmid preparation and transient transfections

SH-EP, SH-SY5Y, SK-N-SH and HeLa cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g/L glucose containing 10% fetal bovine serum (heat inactivated) supplemented with glutamine (0.03%), penicillin (100 units/mL) and streptomycin (100 μg/mL). SH-SY5Y cells were plated in 12-well dishes at 4×10^5 cells/well, SK-N-SH at 2.5×10^5 cells/well, SH-EP and HeLa at 1×10^5 cells/well, in 1 mL medium and allowed to grow to 70% confluence (approximately 48 h). Cells were transfected with 0.5 μg VIP reporter, 0.1 μg pSV-β-galactosidase (β-Gal) control vector (Promega, Madison, WI, USA) and 3 μL lipofectamine/well (Invitrogen, Carlsbad, CA, USA) in media free of all serum and antibiotics for 5 h, followed by a change to complete medium. After 36–40 h, cells were rinsed once in 1 × phosphate-buffered saline (PBS), harvested by addition of 200 μL lysis buffer (Promega) and stored at −70°C. Co-transfections with the VIP reporter gene and REST dominant negative expression plasmid (provided by Gail Mandel) were performed in the same manner with 0.25 μg each of reporter and dominant negative DNA. Luciferase activity was determined using 20 μL cell lysate with 50 μL luciferase substrate (Promega), counted for 10 s in a Berthold Lumat 9501 luminometer (Berthold Technologies, Oak Ridge, TN, USA). Reporter gene activity was corrected for β-gal activity as measured by the Promega protocol No. TB097 to normalize for transfection efficiency.

The VIP reporter gene constructs used were as previously described, using 5.2 kb of the VIP upstream promoter plus the first non-coding exon, first intron and 13 bp of the second exon fused to the luciferase gene (Hahm and Eiden 1998a) with or without a 6 or 12 bp mutation of the putative RE-1 (TTCAGCAgaTAttAgAtCTCC, or TTgAcCggacAttcgctCTCC, respectively). The RE-1 was mutated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and verified by sequencing. The REST DNA binding domain (DBD) plasmid was created from the REEX1 plasmid (a gift from Dr Gail Mandel, containing REST cloned into the EcoRI site of pcDNA1) by cutting out the HincII to EcoRI site containing the first seven zinc fingers of the translated REST protein.

Reverse transcription-polymerase chain reaction (RT-PCR)

Cells were grown in six-well plates and harvested for total RNA using the RNAqueous kit (Ambion, Austin, TX, USA) as previously described (Hamelink *et al*. 2002). DNA was removed by treatment with RNase-free DNAse I, and complementary DNA (cDNA) was prepared with the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen). RT-PCR was carried out for determination of REST splice variant expression in all four cell lines using primers that span intron 5 (Table 1). PCR was performed in a 30 μL volume with 2 μg cDNA, 1 × PCR buffer (Invitrogen), 5 mM $MgCl_2$, 0.5 mM dNTP, 0.15 μM primers, with 0.5 μL *Taq* polymerase per

Table 1 Sequence of primers and probes for PCR

	Primers and probes for PCR
hVIP f	5′CCGCCTTAGAAAACAAATGGC3′
hVIP r	5′CTAACTCTTCTGGAAAGTCGGGAG3′
hVIP p	6FAM-ATTCTGAATGGAAAGAGGAGCA GTGAGGGAG-TAMRA
hGAPDH f	5′CACCAACTGCTTAGCACCCC3′
hGAPDH r	5′TCTTCTGGGTGGCAGTGATG3′
hGAPDH p	6FAM-ACAACTTTGGTATCGTGGAAGGACT CATGACC-TAMRA
hREST f	5′TGCACAATACACAACAGTGAGCG-3′
hREST r	5′TTAAGAGGTTTAGGCCCATTGTG3′

reaction. PCR was performed for 5 min at 95°C for polymerase activation, followed by 34 cycles of 30 s at 95°C, 1 min at 65°C and 70 s at 72°C, with a final 10 min extension at 72°C. PCR products were visualized by running on a 2% agarose gel stained with ethidium bromide.

Real time quantitative RT-PCR (Q-RT-PCR) was performed on cDNA obtained by reverse-transcription of 1 μg of total RNA using the Taq-Man 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), and using hVIP and hGAPDH primer sets from Biosource International (Camarillo, CA, USA) (Table 1). Reaction buffer contained 1 × Amplitaq gold PCR buffer (Applied Biosystems), 1% Tween 20 and 0.02% gelatin (Sigma P-9416 and G-1393, St Louis, MO, USA), 0.2 μM 5′FAM-AAAAT-Rox3′ reference dye (Biosource International), 3.5 mM $MgCl_2$, 200 μM dNTPs, 90 nM primers, 100 nM probe and 1.25 units *Taq* gold polymerase per sample (50 μL). VIP quantification was determined from a standard curve generated using dilutions of spectrophotometrically quantified purified VIP amplicon and internally corrected with the GAPDH cDNA signal for variations in amount of input mRNA. Table 1 shows primer and probe sequences.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from cells with the Sigma Nu-CLEAR extraction kit, essentially as described, using a hypotonic lysis buffer and stored at −70°C until use. REST and CREB (cyclic AMP responsive element binding) proteins were *in vitro* translated with the T7 polymerase TNT coupled reticulocyte lysate system (Promega) from REEX1 (provided by Gail Mandel) and rCREB (Anouar *et al*. 1999) expression plasmids, and verified by ^{35}S-labelled methionine incorporation. REST DBD was made by subcloning REEX1 expression plasmid EcoRI to HincII fragment into pcDNA3 vector. Consensus RE-1, VIP-specific RE-1 and VIP RE-1 6 bp mutated oligos were labelled with γ-^{32}P ATP by T4 polynucleotide kinase and purified in STE select-D G-25 columns (Eppendorf-5 Prime, Boulder, CO, USA). Nuclear extracts (2–5 μg protein) or *in vitro* translated proteins (5 μL of the 50 μL translation reaction) were assayed for their binding to DNA oligos by gel mobility shift assay as described (Anouar *et al*. 1999). Nuclear extracts or *in vitro* translated proteins were combined with cold competitors or antibodies for 10 min. Labelled probe (30 000 cpm) was added and reactions incubated for a further 20 min before resolving on a 5% non-denaturing polyacrylamide gel in 0.5 × Tris borate EDTA buffer at 4°C. Rabbit reticulocyte lysate (RRL) with no expression plasmid was included as a control for *in vitro* protein synthesis in place of REST or CREB to identify non-specific binding. Double-stranded oligos containing the VIP RE-1, VIP RE-1/m6, VIP cyclic AMP responsive element (CRE) and the enkephalin (ENK) phorbol 12-myristate 13-acetate-response element (TRE) (see Table 2 for nucleotide sequences) were used as cold competitors in 100-fold molar excess. For supershift, 1 μL of a specific polyclonal REST antibody (provided by Gail Mandel) was incubated with nuclear extracts prior to addition of labeled probe. Gels were dried and apposed to Biomax MR film (Kodak) at −70°C for 12–24 h and film developed in a Kodak (Rochester, NY, USA) X-OMAT 2000 A processor.

Statistics

Transient transfection data presented are means plus or minus SEM for samples run in triplicate. All experiments were repeated at least twice with similar results. All data were analyzed using the superANOVA software package. Data were analyzed by one-way ANOVA with Scheffe's post hoc analysis. Significance was set at $p < 0.05$.

Results

RE-1 found in neuropeptide genes

Regulation of gene transcription is often analyzed by use of reporter constructs composed of the regulatory region of the gene of interest fused to easily monitored reporter genes such as β-Gal, chloramphenicol acetyltransferase or luciferase. When making these constructs there are no defined rules as to how much of the transcribed portion of the gene should be included upstream of the reporter, other than the principle that the reporter should be regulated in parallel to the transcriptional behavior predicted for the endogenous gene. VIP, typical of neuropeptides in general, is synthesized from a gene with multiple exons encoding a precursor peptide. The VIP gene contains seven exons, only one of which encodes VIP. The first exon codes for an untranslated leader sequence, the second for a putative signal peptide, and another for the related peptide histidine methionine (PHM) (Bodner *et al*.

Table 2 Sequence of primers for EMSA oligos

	Primers for EMSA oligos
hVIP-RE-1 f	CCAGGACTTCAGCACCTAAGACAGCTCCA AAACAA
hVIP-RE-1 r	TTGTTTTGGAGCTGTCTTAGGTGCTGAAGTC CTGG
hVIP-RE-1/m6 f	CCAGGACTTCAGCAGATATTAGATCTCC AAAACAA
hVIP-RE-1/m6 r	TTGTTTTGGAGATCTAATATCTGCTGAA GTCCTGG
RE-1 consensus f	CCAGGACTTCAGCACCACGGACAGCGCC AAAACAA
RE-1 consensus r	TTGTTTTGGCGCTGTCCGTGGTGCTGAA GTCCTGG

1985; Linder *et al*. 1987). We decided to examine more closely the entire transcribed portion of the VIP gene for regulatory regions. A putative RE-1 was identified within the first non-coding exon.

Due to the significant sequence identity between VIP and other members of the VIP/secretin family of neuropeptides, we chose to examine other neuropeptides for the occurrence of the RE-1-like sequence. A bioinformatics search of over 30 neuropeptide genes from the human genome revealed an RE-1-like site in several neuropeptide genes, some of which had not previously been identified (Table 3a). About 20 kb of chromosomal gene sequence centered on the coding exons of the neuropeptide of interest was downloaded into Transfac and analyzed for the presence of an RE-1 using 75% core similarity and 85% matrix similarity with the MatInspector tool (Wingender *et al*. 2000), based on a four base essential core (AGCA) defined as critical for function (Schoenherr *et al*. 1996). Of the neuropeptide genes surveyed, 10 were found to contain an RE-1-like sequence (Table 3a), suggesting a common mechanism for gene regulation. RE-1-like sequences were found in many individual members of neuropeptide gene families, but were not found to be ubiquitous in any one family. For example, although VIP and pituitary adenylate cyclase activating polypeptide (PACAP) share significant sequence identity, an RE-1 was found in the VIP gene but not the PACAP gene, nor in any other growth hormone-releasing hormone superfamily member. However, preliminary data on the presence of two RE-1-like sequences in the PACAP gene have recently been reported (Sugahara *et al*. 2003), although both RE-1-like sequences in the human PACAP gene contained 10 mismatches from the Schoenherr 21 bp consensus sequence. The majority of the putative RE-1s found within the neuropeptide genes by Transfac analysis were found within an intron, while three were found in the 3′ downstream region.

Since conservation of sequence across species reflects functional importance, the VIP genes of the mouse, rat (XM_217838 similar to VIP precursor) and cow were compared to that of the human. The mouse and rat VIP genes were also found to contain an RE-1-like sequence (Table 3b) with a high degree of sequence similarity compared to human. The bovine VIP gene sequence was not recognized to contain an RE-1 using Transfac with the criterion specified above due to 6 bp mismatches from the consensus sequence, although only three mismatches were seen with the human VIP RE-1. Both the mouse and human VIP RE-1 sequences were found within the first non-coding exon. Although it is difficult to analyze the location of the

Table 3 (a) Human neuropeptide RE-1 sequences. Human neuropeptide genes were examined for the presence of an RE-1 sequence. Table shows divergence from consensus sequence and location of the RE-1 within the gene. The SST gene was found to contain two RE-1 like sequences. (b) VIP RE-1 sequences across species. The VIP gene from human, mouse, rat, cow and frog were compared for the presence of an RE-1 and sequence divergence

(a)

Consensus	TTC	AGC	ACC	ACG	GAC	AGC	GCC	Location
VIP	TTC	AGC	ACC	taa	GAC	AGC	tCC	Exon 1
PENK	TTC	AGC	ACC	tCG	GAt	AGC	aCt	intron
SST	TTC	AGC	CC	tgG	GtC	AGC	GCt	intron
	gTC	AGC	ACC	AgG	GAt	AGa	cGC	intron
CRH	TTC	AGC	ACC	gCG	GAC	AGC	GCC	intron
TRH	acC	AGC	ACC	tgG	GAC	AGC	GCC	3′
UCN	TTC	AGC	ACC	AtG	GAC	AGC	ctC	5′
HCRT	TTC	AGC	ACC	ttG	GAC	AGC	caC	3′
CART	gTC	AGt	ACC	tgG	GAC	AGC	GCC	intron
AGRP	ccC	AGC	ACC	AaG	GAC	AGa	ata	3′
BNP	aTC	AGC	ACC	A G	GAC	AGC	GgC	5′

(b)

	VIP RE-1 sequences across species						
Human	TTC	AGC	ACC	taa	GAC	AGC	tCC
Mouse	TTC	AGC	ACC	cta	GAC	AGC	tgC
Rat	TTC	AGC	ACC	cCa	GAC	AGC	tCC
Bovine	TTC	AaC	ACC	tga	GAC	AGC	tCt
Xenopus	TTC	AGC	ACC	ACG	GAt	AGC	GCC

VIP, vasoactive intestinal polypeptide; PENK, proenkephalin; SST, somatostatin; CRH, corticotropin releasing hormone; TRH, thyroid releasing hormone; UCN, urocortin; HCRT, orexin; CART, cocaine and amphetamine regulated transcript; AGRP, agouti-related protein; BNP, brain natriuretic peptide.

RE-1 within the rat and cow gene due to a more rudimentary understanding of the genome in these species, VIP sequence alignment of the four organisms shows a similar approximate 75 bp upstream location (in the cDNA) from a potential translation start site. Comparison with the consensus RE-1 (Schoenherr *et al*. 1996) revealed three, four, five and six nucleotide differences for the mouse, human, rat and cow, respectively (Table 3b). Finally, the VIP sequence from the African clawed frog was compared with the human and consensus sequences. The frog sequence was found to contain an RE-1 with only one mismatch from the consensus RE-1 sequence, also located within the transcribed portion of the gene, upstream of a putative translation start site.

Specificity of RE-1 site

EMSA analysis of the VIP RE-1 in comparison with a consensus RE-1 was carried out to determine which proteins bind the putative RE-1. The proteins REST, REST DBD and CREB were synthesized *in vitro* (Fig. 1a) and allowed to interact with the RE-1. A consensus RE-1 oligo bound to the *in vitro* transcribed REST protein (Fig. 1b). Binding was abolished in the presence of a 100-fold molar excess of unlabeled consensus RE-1 oligo, as well as by unlabeled VIP-specific RE-1, but not by excess unlabeled CRE or TRE oligos. In contrast to the clear binding of the VIP RE-1 oligo to the REST and REST DBD proteins, the mutant VIP RE-1 oligo showed significantly less binding to either REST or REST DBD (Fig. 1c). Specificity of the REST binding to the VIP RE-1 was demonstrated by a failure of CREB to bind the RE-1, which demonstrated a non-specific binding pattern similar to the rabbit reticulocyte lysate.

Endogenous VIP and REST quantification

Four cell lines were compared for levels of endogenous VIP and REST mRNA. HeLa, a fibroblast cell type, was compared with SK-N-SH, a neuroblastoma cell line, and two of its subclones SH-EP and SH-SY5Y. The SH-EP line has been characterized as large substrate adherent cells lacking neuritic processes with a general epithelial or glial-like morphology, while SH-SY5Y cells are small cells with a neuroblast-like appearance and small to medium radially-protruding neurites. Karyotype analysis of the SH-EP and SH-SY5Y cells has demonstrated that despite their morphological divergences, these cells come from a common ancestral lineage and can interconvert in a bidirectional manner, albeit at a slow rate (Ross *et al*. 1983). Q-RT-PCR confirmed previously reported high levels of VIP mRNA in SH-EP cells and low levels in SH-SY5Y cells (Waschek *et al*. 1988). The parent cell line, SK-N-SH contained VIP levels intermediate to SH-EP and SH-SY5Y VIP mRNA levels. No VIP mRNA was found in HeLa cells (Fig. 2a). VIP mRNA concentrations in SH-EP cells were 1000-fold greater than those in SH-SY5Y and 50-fold greater than those in SK-N-SH cells.

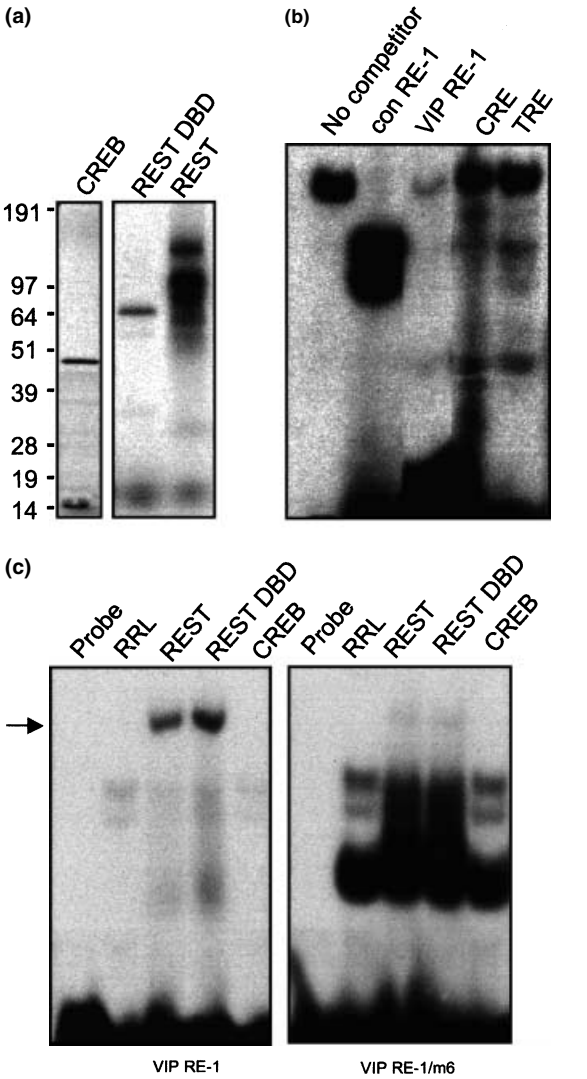

Fig. 1 VIP RE-1 binds REST. (a) *In vitro* translation products for CREB, REST and the DNA binding domain of REST (REST DBD) were synthesized by the T7 polymerase TNT coupled reticulocyte lysis system with ^{35}S-methionine and run on a 10% Tris-Bis gel. (b) Electrophoretic mobility shift assay (EMSA) with γ-^{32}P ATP labeled consensus RE-1 oligo (Table 1) and *in vitro* translation product REST in all lanes. 100-fold molar excess unlabeled competitors in lanes 2–5 with consensus RE-1 oligo, VIP specific RE-1 oligo, CRE oligo and TRE oligo. (c) Left: EMSA using labeled VIP RE-1 oligo and *in vitro* translation products in lanes 2–5: rabbit reticulocyte lysate (RRL), REST, REST DBD and CREB. Right: EMSA with labeled VIP RE-1 oligo with six base pair mutation and *in vitro* translation products in lanes 2–5: rabbit reticulocyte lysate (RRL), REST, REST DBD and CREB. Gels shown are from experiments repeated at least three times with similar results.

These same four cell lines were examined for the presence of REST splice variants (Fig. 2b). SH-EP and HeLa cells were found to contain only the full length variant, while SK-N-SH and SH-SY5Y were found to contain both the full

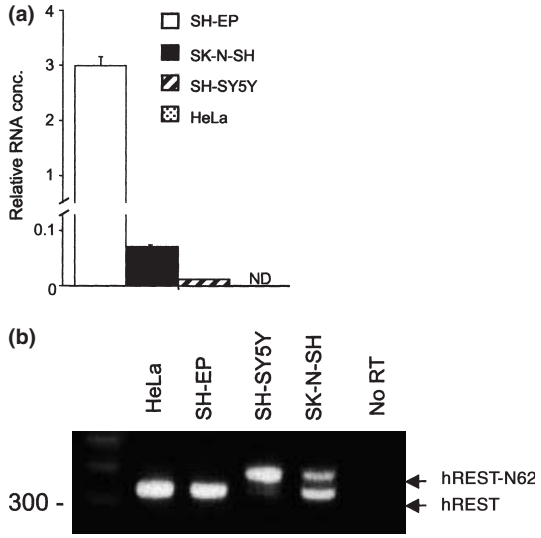

Fig. 2 Endogenous levels of VIP and REST mRNA. (a) Quantitative-RT-PCR for VIP in SH-EP, SK-N-SH, SH-SY5Y and HeLa cells. cDNA prepared from 1 μg total RNA was subjected to real time PCR using primers listed in Table 1. VIP mRNA levels detected were adjusted by GAPDH expression levels to correct for input RNA levels. ND = non-detectable ($n = 3$ independent determinations). (b) RT-PCR for REST in SH-EP, SK-N-SH, SH-SY5Y and HeLa cells using primers that span the fifth intron. Two splice variants detected, the lower band corresponds to the full length REST protein, the upper band to the 62 base pair insert which translates a truncated neuronal protein. No RT = no reverse transcriptase enzyme ($n = 3$ independent determinations).

length and a longer, approximately 60 bp insert, similar to the hREST-N62 neuronal form of REST mRNA described by Palm *et al*. (1999) which codes for a truncated REST protein. SH-SY5Y cells were found to contain predominantly the neuronal-like (N62) variant.

RE-1 mutation leads to increased gene activity

Reporter constructs with or without a 6 bp RE-1 mutation were generated to test the potential effect of this RE-1 sequence on VIP gene expression in SH-EP and HeLa cells. Mutations were targeted towards the invariant nucleotides (Schoenherr *et al*. 1996) (Fig. 3a). Transient transfection of the VIP luciferase reporter genes into these two cell lines revealed significantly greater VIP gene activity in SH-EP cells, demonstrating consistency between transient and endogenous gene activity. Mutation of the RE-1 site in the VIP gene construct, however, led to enhanced VIP gene activity of a similar magnitude in both HeLa and SH-EP cells (Fig. 3b). Co-transfection with a dominant negative REST expression plasmid (CMVp73, kindly provided by Gail Mandel) also increased VIP promoter activity (Fig. 3c).

Gel shift analysis employing nuclear extracts from SH-EP and HeLa cells confirmed that both lines express a REST-like protein which is capable of binding to the VIP gene putative RE-1 element (Fig. 3d). This binding was shown to be

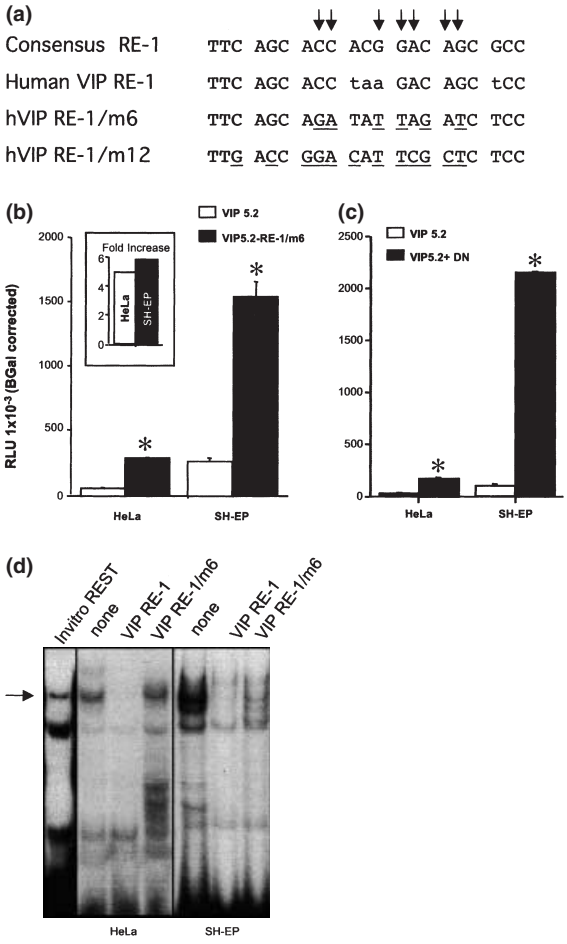

Fig. 3 VIP gene activity increases in HeLa and SH-EP cells upon RE-1 mutation. (a) Comparison of consensus RE-1 sequence (Schoenherr *et al.* 1996) with VIP RE-1 and VIP RE-1 six base pair (hVIP RE-1/m6) and 12 base pair (hVIP RE-1/m12) mutation. Arrows above denote conserved nucleotides for RE-1 sequence in most genes. Lower case letters indicate the four nucleotide differences in the human VIP RE-1 from the consensus sequence, underscored letters show the site-directed base pair mutations. (b) Quantification of VIP reporter gene in HeLa and SH-EP cells. HeLa and SH-EP cells were transfected with the luciferase reporter VIP 5.2 (0.5 μg/well) with or without a six base pair mutation and the co-transfection control pSV-β-galactosidase (β-Gal) plasmids (0.1 μg/well). After 48 h, cells were harvested and analyzed for luciferase and galactosidase activity. Data are presented as luciferase activity normalized to galactosidase activity ($n = 3$ independent determinations). Insert demonstrates fold increases in both cell lines. (c) Reporter gene activity in HeLa and SH-EP cells transfected as above with VIP 5.2 construct and REST dominant negative plasmid (0.25 μg each/well) and 0.1 μg β-Gal. Data are presented as luciferase activity normalized to galactosidase activity ($n = 3$ independent determinations). Asterisk denotes significant increase with six base pair mutation, or dominant negative co-transfection, $p \leq 0.05$ Scheffe's post hoc analysis. (d) EMSA shows HeLa (left) and SH-EP (right) nuclear extracts bind VIP RE-1. Lane 1 contains *in vitro* translated REST protein, arrow denotes REST specific band, lanes 2–4 contain Hela nuclear extract, lanes 5–7 contain SH-EP nuclear extract, all lanes contain labeled VIP RE-1 oligo. Lanes 3 and 6 include VIP RE-1 cold competitor, lanes 4 and 7 include VIP RE-1/m6 cold competitor at 100-fold molar excess.

specific by competition with a 100-fold molar excess of unlabeled VIP RE-1, while the unlabeled VIP RE-1 with the 6 bp mutation competed only weakly. We then tested the ability of REST to inhibit VIP gene expression in the SK-N-SH and SH-SY5Y cells. All cell lines were found to have a REST-like protein which bound to the VIP RE-1 and was supershifted in the presence of a REST antibody (Fig. 4a).

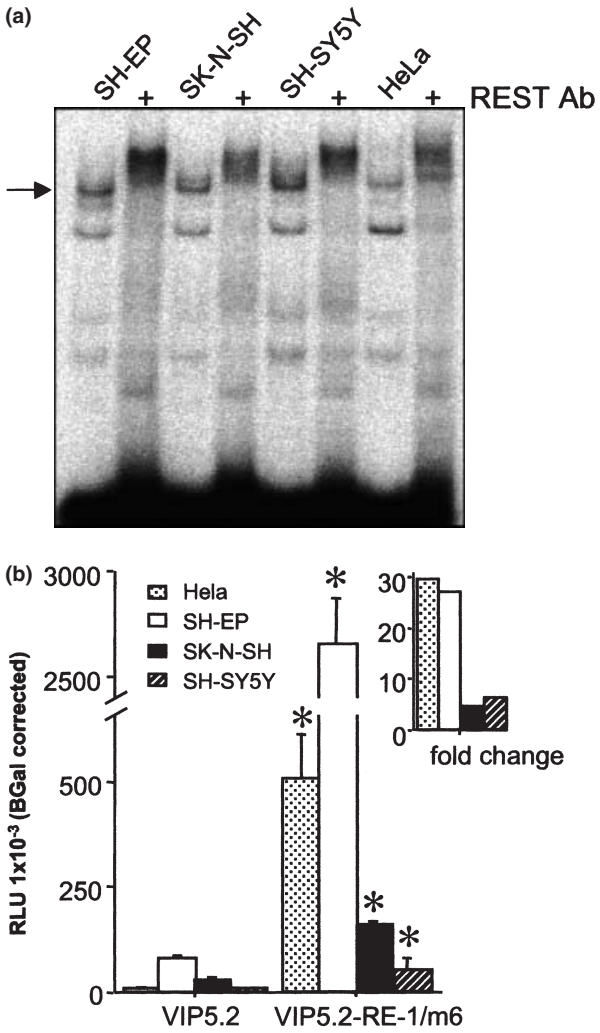

Fig. 4 VIP activity increases with RE-1 mutation. (a) Nuclear extract from SH-EP, SK-N-SH, SH-SY5Y and HeLa cells subjected to EMSA with labeled VIP RE-1 oligo and supershifted by REST antibody (+). (b) Expression of VIP reporter gene in HeLa, SH-EP, SK-N-SH and SH-SY5Y cells. Cells were transiently transfected with a 5.2 kb VIP-luciferase reporter gene (0.5 μg/well) with or without the six base pair RE-1 mutation from Fig. 3a and the co-transfection control pSV-β- galactosidase (β-Gal) plasmid (0.1 μg/well). After 48 h, cells were harvested and analyzed for luciferase and galactosidase activity. Data are presented as luciferase activity normalized to galactosidase activity ($n = 3$ independent determinations), fold increase shown within inset. Asterisk denotes significant difference between VIP 5.2 and VIP 5.2-RE-1/m6 transfection, $p \leq 0.05$ Scheffe's post hoc analysis.

Transient transfections with the VIP 5.2 kb luciferase constructs confirmed the ability of the RE-1 to repress VIP gene expression. Mutation of the RE-1 led to increased gene activity in all cell lines. The greater the amount of full length REST within the cell, the larger the fold change in VIP gene activity following the 6 bp mutation to the RE-1 (Fig. 4b). Mutation of the RE-1 site led to smaller increases in VIP gene activity in neuronal-like cell lines containing less of the full length REST and more of the truncated neuronal splice variant.

RE-1 interacts with other gene elements

To compare further the ability of the RE-1 to influence VIP levels in SH-EP and HeLa cells, serial truncations of the VIP luciferase gene constructs were made with and without the 6 bp RE-1 mutation (Fig. 5). Previous work in these two cell lines demonstrated the absolute necessity for the tissue specifier element (TSE) to impart cell-specific expression of a full length (5.2 kb) VIP reporter gene (Hahm and Eiden 1996, 1998b). Constructs were therefore made with the TSE included. Transient transfection in SH-EP and HeLa cells with the 5.2 kb construct confirmed that the reporter gene paralleled endogenous VIP expression levels in SH-EP (copious) and HeLa (none) cells. The reporter had significant gene activity in SH-EP and minimal activity in HeLa cells. Truncation down to 2.5 kb plus the TSE had no effect on gene activity in either cell line. Further deletions of the VIP promoter led to increased gene activity in the HeLa cell line, culminating in robust VIP gene expression with the minimal promoter (0.5 kb) plus the TSE. The effects of this promoter truncation had opposite effects in the SH-EP cells where the minimal promoter plus the TSE actually had less activity in SH-EP than in HeLa cells (Fig. 5a), similar to results previously reported (Hahm and Eiden 1998a).

A 6 bp mutation within the RE-1 led to significant increases in gene activity with all constructs where the TSE was included, for both cell lines (Fig. 5b). Despite the fact that serial truncation of the promoter region lead to a significant decline in gene activity in the SH-EP cells, mutation of the RE-1 led to significantly increased gene activity for all constructs that included the TSE. The 1.55 kb + TSE construct had even greater promoter activity than the 2.5 kb construct, perhaps suggesting that the region from 2.5 to 1.55 kb contains an additional inhibitory region which, upon removal, allows for expression above that seen in the more complete 2.5 kb + TSE or the 5.2 kb constructs with RE-1 mutation.

Since the 6 bp mutation showed a weak ability to compete with the nuclear extracts for binding to the VIP RE-1, (Fig. 3d) a further six bases were mutated within the VIP RE-1 (Fig. 3a) to create a 12 bp mutation. Transient transfection with a reporter containing the 12 bp mutation revealed a similar increase in reporter activity with the 6 bp mutation (Fig. 6).

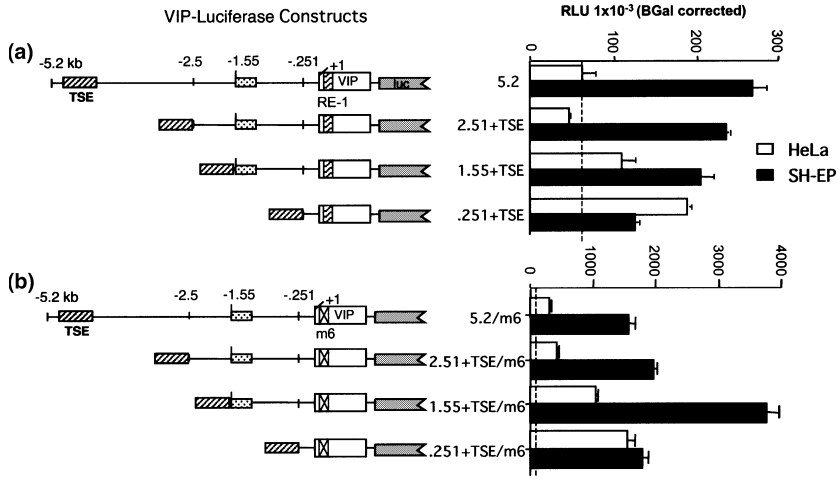

Fig. 5 RE-1 mutation increases reporter activity in all constructs containing the TSE. (a) Quantification of VIP reporter gene activity in HeLa and SH-EP cells. Reporter genes were serially truncated to remove upstream sequence and retain the TSE. Quantification of transient transfections with reporter gene truncations in SH-EP and HeLa cells (0.5 μg/well) and the co-transfection control pSV-β-galactosidase (β-Gal) plasmid (0.1 μg/well). After 48 h, cells were harvested and analyzed for luciferase and galactosidase activity. Data are presented as luciferase activity normalized to galactosidase activity ($n = 3$ independent determinations). (b) Quantification of transient transfections with the same reporter genes containing the RE-1 six base pair mutation from Fig. 3a as per protocol in Fig. 5a ($n = 3$ independent determinations). Dotted line represents the minimum baseline activity seen in HeLa cells with the 5.2 kb construct. Black boxes represent SH-EP cells, white boxes represent HeLa cells.

Finally, we tested the dependence of the RE-1 repressor sequence on the upstream OCT-1 containing TSE. Removal of the TSE from all constructs reduced VIP gene activity significantly in SH-EP cells, with minor differences in HeLa cells as seen previously (Hahm and Eiden 1998b). Mutation of the RE-1 in either the VIP 2.5 kb or VIP 1.55 kb construct lacking the TSE failed to increase reporter gene activity in either cell line (Fig. 7). Further truncation of the VIP gene promoter down to the .25 kb minimal promoter without the TSE decreased gene expression within the SH-EP cells. Mutation of the RE-1 in this construct did, however, lead to a significant increase in reporter activity in SH-EP cells albeit of less magnitude than that seen by mutating the RE-1 in the VIP .25 + TSE construct.

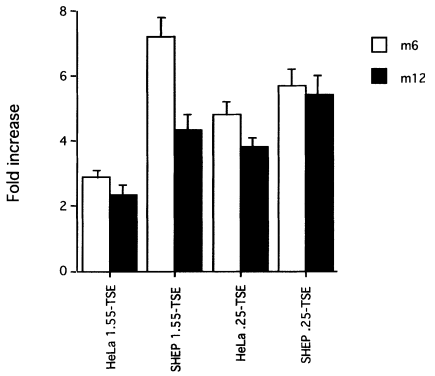

Fig. 6 Twelve base pair RE-1 mutation similarly increases gene activity. Quantification of VIP reporter gene activity in HeLa and SH-EP cells. Cells were transiently transfected with either a 0.25 or 1.55 kb VIP-luciferase reporter gene + TSE construct (0.5 μg/well) with either a six or 12 base pair RE-1 mutation (see Fig. 3a) and the co-transfection control pSV-β-galactosidase (β-Gal) plasmid (0.1 μg/well). After 48 h, cells were harvested and analyzed for luciferase and galactosidase activity. Data are presented as fold increase above 1.55-TSE or 0.25-TSE expression levels ($n = 2$ independent determinations). Black boxes represent the 12 base pair mutation, white boxes represent the six base pair mutation.

Discussion

Here we report the finding of an RE-1 in the VIP gene and several other neuropeptide genes in which the element has not been previously remarked. To test the role of the RE-1 on VIP gene expression, four cells lines were examined: the HeLa cell, a fibroblast cell type, and SK-N-SH and SH-SY5Y, neuroblast-like, and SH-EP, epithelial or glial-like neuroblasts. In the non-neuronal HeLa cell line, endogenous REST levels are high and VIP is not expressed, while mutation of the RE-1 leads to a significant increase in VIP gene activity. Thus, in non-neuronal cells in which VIP mRNA is undetectable, REST seems to contribute partly to this suppression. In the neuroblastoma cell lines, however, REST regulation of VIP is more complex. As these cells have been shown to be capable of interconversion, this is a potentially attractive model for examining the effects of REST in neural crest development (Ross *et al.* 1983). In the SH-EP cell, VIP expression is very high and REST expression is also high, while in the SH-SY5Y cell, VIP

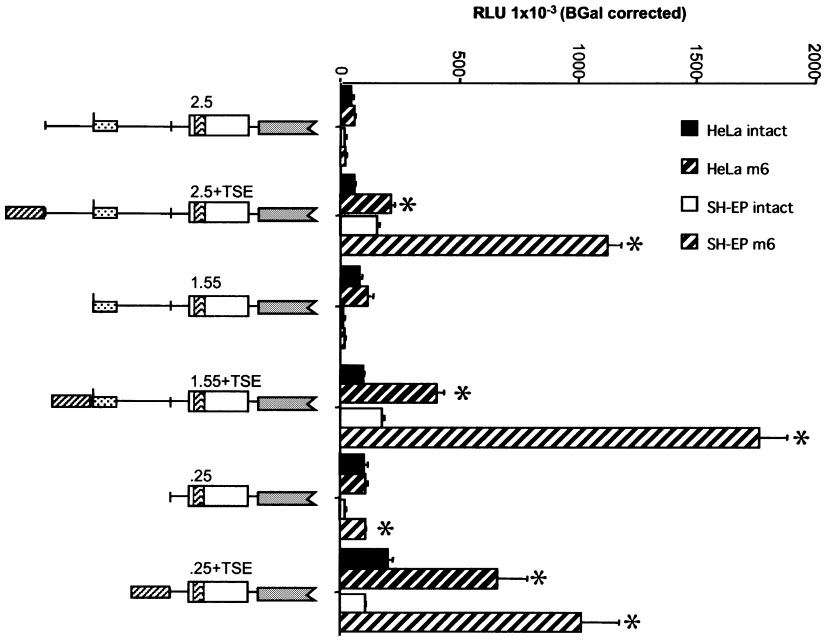

Fig. 7 RE-1 mutation effects require the TSE. Quantification of transient transfections with reporter genes containing the 2.5 kb, 1.55 kb or 0.25 kb VIP promoter with or without the TSE, and with or without the RE-1 six base pair mutation (m6) in SH-EP and HeLa cells. Transfections included the co-transfection control pSV-β-galactosidase (β-Gal) plasmid (0.1 μg/well). After 48 h, cells were harvested and analyzed for luciferase and galactosidase activity. Data are presented as luciferase activity normalized to galactosidase activity ($n = 3$ independent determinations). Asterisk denotes significant difference between the intact and m6 promoter constructs, $p \leq 0.05$ Scheffe's post hoc analysis.

expression is low and REST expression is low. It appears that VIP and REST mRNA levels are dynamic, with an intermediate concentration in the parental cell line SK-N-SH and high levels in the SH-EP neuroblasts with the epithelial-like morphology, and low levels in SH-SY5Y neuroblasts with the neuronal-like morphology. The difference in SH-EP and SH-SY5Y cells are distinguished by differences in full length rest versus N62 rest expression. Decreased rest in SH-SY5Y cells does not lead to an increase in VIP gene expression as previously reported for the cholinergic gene locus (Shimojo *et al*. 1999; Tabuchi *et al*. 2002). Rather, the opposite is true: as the amount of truncated neuronal REST variant increases and the full length variant decreases, the amount of VIP within the cell decreases.

This led us to ask if REST could be acting as an enhancer in these cells. Inhibition of REST action through RE-1 mutation was reported to decrease hippocampal L1 CAM-reporter expression (Kallunki *et al*. 1998). Bessis and colleagues reported that the RE-1 sequence functioned as an enhancer in neuroblastomas when located less than 50 bp upstream of the TATA box or downstream within the 5′ untranslated region (Bessis *et al*. 1997). The possibility of REST functioning as an enhancer for VIP in these cells was tested by site-specific mutation of the RE-1 in reporter gene constructs. VIP reporter constructs containing the RE-1 were compared with a VIP reporter with a 6 bp mutation to the RE-1. Mutation of the RE-1 within the 5.2 kb construct led to an increase in reporter gene activity in all neuroblastoma cell lines tested. In fact, the more REST mRNA a cell contained, the greater the potential for up-regulation by mutation of the RE-1. Similarly, co-transfection of a REST dominant negative expression plasmid with the VIP luciferase reporter increased luciferase activity. Together, these data led us to conclude that REST acts as a *bona fide* repressor of the VIP gene in these neuroblastomas. Over-expression of REST by transfection with the REST expression plasmid REEX1 was unable to reduce endogenous VIP gene expression (data not shown) in rest over-expressing SH-EP cells, probably because REST expression in these cells is already high and the REST-associated effect on attenuaton of VIP transcription is already maximal.

We also asked if the RE-1 is functional in intact neuronal and non-neuronal cells, using EMSA. We have shown that the VIP RE-1 binds to *in vitro* translated REST and REST DBD. Nuclear extracts prepared from all four cells lines bound to the VIP RE-1 oligo, confirming the functionality of the RE-1 as a modulator of VIP expression in these cells.

The neuronal variant of REST, REST4, has been associated with expression of the cholinergic gene locus in PC12 cells whereas the protein kinase A deficient PC12 cell line which has no REST4 has decreased expression of both ChAT and VAChT (Shimojo *et al*. 1999). Although it

is unclear how much full length REST is found in PC12 cells (Andres *et al*. 1999; Ballas *et al*. 2001), REST4 is thought to inhibit REST binding to the VAChT promoter RE-1, thus allowing expression. From this it might be speculated that the SH-SY5Y cells, which contain more of the neuronal splice variant than the full length REST mRNA, would have more VIP mRNA than the SH-EP cells which contain none of the neuronal REST mRNA. This was not found to be true, however. SH-SY5Y cells with the neuron-like morphology and the neuronal splice variant of REST mRNA contain significant DBH and TH activity (Biedler *et al*. 1978), as well as vesicular acetylcholine transporter (VAChT) and choline acetyltransferase (ChAT) mRNA (Hamelink and Eiden unpublished data). We have shown here that these cells have very low levels of VIP mRNA. All of these gene products—DBH, TH, VAChT, ChAT and VIP—are from genes reported to contain an RE-1 regulatory region (Ishiguro *et al*. 1995; Afar *et al*. 1996; Lonnerberg *et al*. 1996; Hahm *et al*. 1997), but expression patterns are very different. Clearly, regulation of neuronal genes is more complicated than inclusion or exclusion of an RE-1 within the gene sequence. Neurons must also have a mechanism for expressing some neuronal genes while repressing others.

Could differential expression of RE-1-containing genes in part be governed by RE-1 interactions with other *cis*-active elements in neuron-specific genes? To test this a series of reporter genes were made with truncations to the VIP promoter. Here we found a striking dependence of the RE-1 repressor activity tied to the upstream OCT-1 containing tissue specifier element. We have previously shown that the TSE is required for the high level of constitutive VIP gene expression in SH-EP cells, hence its designation as the TSE (Hahm and Eiden 1996). Here we show that truncation of the VIP gene promoter down to the minimal .25 kb promoter plus the TSE leads to opposite consequences in SH-EP and HeLa cells. As the VIP gene 5′ sequence is sequentially shortened, gene expression increases in HeLa cells and decreases in SH-EP cells, as seen previously (Hahm and Eiden 1996). Removal of the TSE leads to a complete loss of responsivity to the RE-1 mutation in HeLa cells. Without the TSE, HeLa VIP levels remain at baseline with or without RE-1 mutation, suggesting that the TSE is required for the increases in basal expression that occur with removal of the VIP gene sequence from −2.5kb to –.25kb, as well as the release of repression seen with the RE-1 mutation. Similarly, in the SH-EP cell, effects of mutating the RE-1 depend upon the TSE. Removal of the TSE led to an abrogation of previously seen transcriptional increases upon mutation of the RE-1 in both the 2.5 kb and 1.55 kb constructs. Mutation of the RE-1 led to an increase in reporter activity independently of the TSE only with the VIP .25 construct. It must be pointed out, however, that this construct no longer displays VIP gene activity which parallels the endogenous VIP levels seen in SH-EP and HeLa cells. With this short construct, promoter activity is now greater in the HeLa cell line than in the SH-EP cells. Thus, using reporters which mimic endogenous VIP gene expression, we have shown that the repressive effects of REST upon VIP gene regulation depend upon both the RE-1 sequence and the upstream OCT-1-containing TSE.

In summary, we have identified a new regulatory region within the VIP gene that appears able to repress VIP gene expression in both non-neuronal cells and neuroblastomas. Cells containing the highest level of REST exhibited the largest increases in reporter gene expression upon mutation of the RE-1. Regulation of endogenous VIP expression remains complex, as high levels of REST mRNA and high levels of VIP mRNA were seen in SH-EP cells while low levels of VIP mRNA and full length REST mRNA and a preponderance of the purported anti-repressor neuronal variant (N62) were seen in SH-SY5Y cells. Clarification awaits further study of the combinatorial activation/repression of the VIP gene by the multiple signaling pathways involved in VIP tissue-specific regulation. This may involve the dependence of REST-mediated VIP gene repression upon an upstream OCT-1-containing element previously labeled the TSE.

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