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The CGTCA sequence motif is essential for biological activity of the vasoactive intestinal peptide gene cAMP-regulated enhancer

(neuropeptide/gene regulation/ectopic hormone/trans-acting factor)

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ABSTRACT **cAMP-regulated transcription of the human vasoactive intestinal peptide gene is dependent upon a 17-base-pair DNA element located 70 base pairs upstream from the transcriptional initiation site. This element is similar to sequences in other genes known to be regulated by cAMP and to sequences in several viral enhancers. We have demonstrated that the vasoactive intestinal peptide regulatory element is an enhancer that depends upon the integrity of two CGTCA sequence motifs for biological activity. Mutations in either of the CGTCA motifs diminish the ability of the element to respond to cAMP. Enhancers containing the CGTCA motif from the somatostatin and adenovirus genes compete for binding of nuclear proteins from C6 glioma and PC12 cells to the vasoactive intestinal peptide enhancer, suggesting that CGTCA-containing enhancers interact with similar trans-acting factors.**

DNA control elements that activate transcription in response to intracellular effectors such as cAMP or products of phosphoinositol metabolism have been identified in several cellular and viral genes (1–6). These genetic elements may be particularly important in neuroendocrine cells where the activation of intracellular second messenger pathways may couple secretion and biosynthesis of neuropeptides (7, 8). The genetic element responsible for cAMP-regulated transcription of the human vasoactive intestinal peptide (VIP) gene consists of a 17-base-pair (bp) sequence located between 70 and 86 bp upstream from the transcriptional initiation site (5). This sequence in the VIP gene is similar to the cAMP-responsive elements (CREs) of the rat somatostatin, human proenkephalin, human α-chorionic gonadotrophin, and rat phospho*enol*pyruvate carboxykinase genes (1–5). All of these CREs contain one or multiple copies of the conserved sequence motif CGTCA and several have been shown to have properties of enhancers (2–4). The CGTCA motif is also found in several viral enhancers such as the 18-bp repeat of the cytomegalovirus gene and the E1A-inducible element of the adenovirus E4 promoter (9, 10). The presence of the CGTCA motif in these viral enhancers suggests that similar nuclear factors may activate transcription of both viral and cellular genes. In the VIP CRE, the two CGTCA motifs are present as inverted repeats, a structure characteristic of some other enhancers (11–13). It is not known whether the palindromic structure of the VIP CRE is necessary for biological activity.

In this study, three major issues were addressed. First, we sought to determine whether the VIP CRE could be categorized as an enhancer. Second, we examined the importance of the CGTCA motifs for activity of the DNA element. Finally, we asked whether the VIP element could bind proteins from nuclear extracts and whether DNA sequences from cellular and viral enhancers containing the conserved CGTCA motif could compete for this binding.

PROCEDURES

Cell Culture and DNA-Mediated Gene Transfer. PC12 cells were maintained as described (5). C6 glioma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). For transient expression assays, 2×10^6 PC12 cells were transfected with 40 μg of test plasmid, subcultured into two identical 60-mm dishes (5), treated with 10^{-5} M forskolin (Calbiochem) or 0.1% ethanol, and assayed for chloramphenicol acetyltransferase (CAT) activity after 40 hr (14). Each plasmid was tested in 4–18 separate transfections. To control for variability in transfection efficiency, 40 μg of a Rous sarcoma virus (RSV)-β-galactosidase plasmid (15) was cotransfected with the VIP–CAT plasmids.

To obtain cell lines containing integrated copies of the VIP–CAT fusion genes, 0.5×10^6 C6 glioma cells or 10^6 PC12 cells were transfected with 20 μg of test plasmid and 4 μg of the plasmid pRSVneo (16). Between 50 and 200 G418-resistant clones were pooled for S1 nuclease analysis of the VIP–CAT fusion gene transcripts.

Plasmid Constructions. Oligonucleotides containing the wild-type or mutated CRE were synthesized, annealed, and inserted into a fusion gene containing the RSV promoter linked to the reporter CAT gene (5). Both the wild-type and mutant CREs were synthesized with *Eco*RI and *Sac* I ends and were inserted 22 bp upstream from the RSV "TATA" box. Plasmid VIP31CAT contained the CRE from position −94 and position −64 in its native orientation. Other plasmids containing wild-type and mutated VIP sequences between positions −70 and −86 were designated pVIP17CAT.

To invert the orientation of the VIP regulatory element, an intermediate plasmid was constructed with a 0.4-kilobase (kb) *Sac* I–*Pvu* II fragment from pVIPCAT4, a 1.68-kb *Sac* I–*Acc* I fragment from RSVΔECAT, and a 2.7-kb *Acc* I–*Sma* I fragment from pVIPCAT2 (5). A 1.9-kb *Nar* I–*Nco* I (partial) fragment from this construction was ligated to a 2.5-kb *Nco* I–*Nar* I fragment from another intermediate plasmid, generated by treating pVIPCAT 3 (5) with the enzymes *Pst* I, BAL-31, *Sma* I, and DNA polymerase I (large fragment). The resultant plasmid, pVIP31CAT-R, placed the CRE in reversed orientation with respect to the RSV promoter.

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Abbreviations: VIP, vasoactive intestinal peptide; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase; CRE, cAMP-regulated enhancer; ATF, activating transcription factor.

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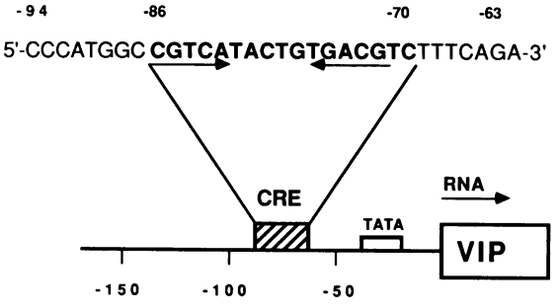

FIG. 1. Location of the VIP CRE. The minimal sequence in the 5′ flanking region of the human VIP gene required for cAMP responsiveness (5) is between positions −70 and −86 (boldface letters). The locations of the 5-bp CGTCA motif and its inverted copy are indicated by the arrows.

To place the CRE on the 3′ side or further on the 5′ side of the RSV promoter, an additional oligonucleotide was synthesized containing VIP sequences from position −94 to position −63, flanked by an *Eco*RI restriction site at the 5′ end and *Bgl* II and *Sac* I restriction sites at the 3′ end. This oligonucleotide was inserted upstream of the RSV promoter to form pVIP32CAT. To place the regulatory element on the 3′ side of the CAT coding sequences (pVIP32CAT-3′), a 4.1-kb *Bgl* II–*Hinc*II fragment from pVIP32CAT was ligated to a 1.7-kb *Bam*HI–*Sac* I fragment from pVIP49CAT (5). To position the regulatory element 300 bp upstream from its native location (pVIP32CAT-300), two 150-bp *Sau*3A fragments from pUC12 were ligated in tandem into the *Bgl* II site of pVIP32CAT.

Repeated copies of the VIP CRE were placed upstream of the RSV promoter by first ligating multiple copies of a synthetic oligonucleotide containing the VIP sequence between positions −70 and −94 into the *Eco*RI site of the plasmid Bluescript (Promega Biotec, Madison, WI). *Bam*HI–*Sma* I fragments from these plasmids were then subcloned into the *Bam*HI–*Sac* I site of pRSVΔECAT (5). Plasmid construction was confirmed by DNA sequencing (17).

Determination of the Transcriptional Initiation Sites Within the Transfected Fusion Genes. The transcriptional start sites for the VIP–CAT fusion genes were determined by S1 nuclease mapping. A 20-bp oligonucleotide complementary to the 5′ end of the CAT mRNA was end-labeled with T4 polynucleotide kinase and [^{32}P]ATP (7000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) and annealed to denatured pVIP25CAT (5). The template was extended with unlabeled deoxynucleoside triphosphates and DNA polymerase I (large fragment) and cleaved with *Pvu* II to generate a 370-nucleotide single-stranded end-labeled probe containing the synthetic VIP oligonucleotide, RSV promoter sequences, and adjacent bacterial sequences. S1 nuclease analysis with this radiolabeled probe (50,000 cpm) and 20 μg of RNA from transfected C6 glioma or PC12 cells was performed as described (18).

DNase I Footprinting Analysis. Nuclear extracts were prepared from C6 glioma and PC12 cells as described by Delegeane *et al.* (19). A plasmid containing VIP 5′ flanking sequences from position –341 to position −30 was labeled with ^{32}P at the *Eco*RI site (position −30) with the large fragment of DNA polymerase I and cleaved with *Hin*dIII (position −341), and the resulting 325-bp probe was purified by agarose gel electrophoresis. DNase I protection assays were performed by using 500 ng of nuclear extract and 10- to 50-fold molar excess of competitor DNA (10). Competition experiments were performed by using unlabeled DNA fragments containing the CREs of the human VIP and rat somatostatin (1) genes or an activating transcription factor (ATF)-binding site of the adenovirus E4 promoter (10). The restriction fragment VIP-409 contained the VIP sequence from position −341 to position −30 as well as 90 bp of the RSV promoter (5). VIP-25 is a 300-bp *Hin*dIII–*Pvu* II restriction fragment prepared from pVIPCAT25 (5) and contains VIP sequences from position −94 to position −70. ATF is a 335-bp *Hin*dIII–*Bst*N1 fragment of pGEM that contained a synthetic oligonucleotide (ACTAAAAAATGACGTAACGGTTAAAGG) corresponding to the ATF-binding site (position −37 to position −63) in the adenovirus E4 promoter (10). The somatostatin competitor was a 311-bp *Sal* I restriction fragment of pSRIF (1) and contained the somatostatin CRE within the sequence from position +61 to position −250.

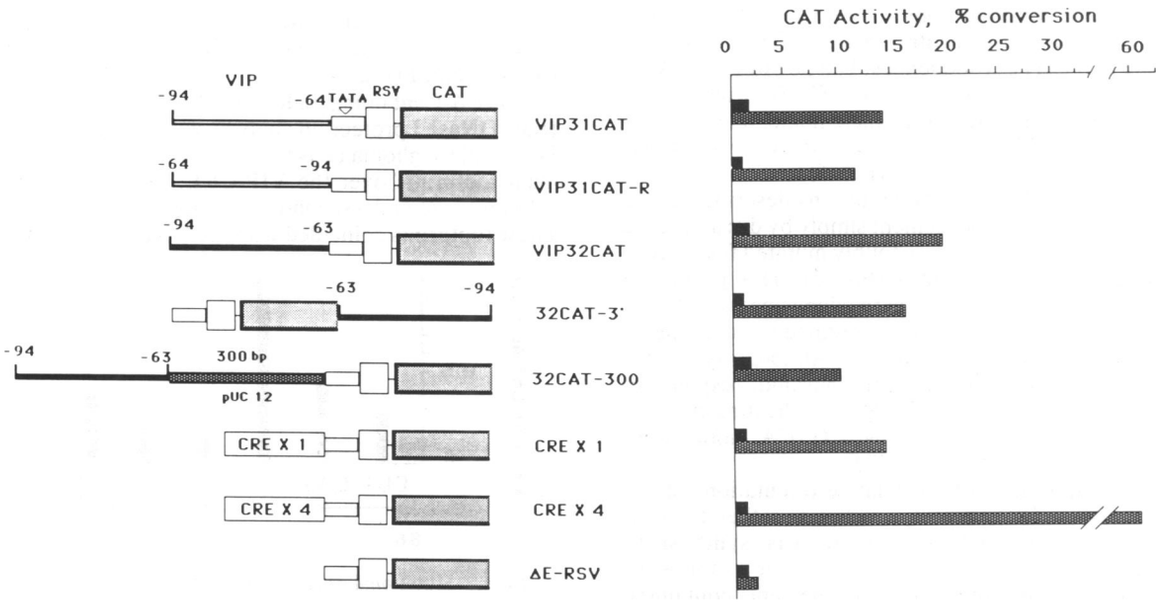

FIG. 2. Enhancer properties of the VIP CRE. (*Left*) Fusion genes containing the VIP CRE are depicted. (*Right*) Corresponding CAT activity after treatment with forskolin (stippled bars) or vehicle (solid bars) is shown. The stimulated CAT activity of VIP31CAT, VIP31CAT-R, VIP32CAT, 32CAT-3′, and CRE×1 did not differ from each other ($P > 0.05$; Mann Whitney U test). CAT activity of 32CAT-300 and CRE×1 were less than VIP32CAT ($U = 1$; $P < 0.05$) and CRE×4 ($U = 0$; $P < 0.05$), respectively (Mann Whitney U test).

RESULTS

The VIP CRE Is an Enhancer. As reported, an element in the 5′ flanking region of the human VIP gene can confer responsiveness to cAMP when placed upstream of a heterologous promoter (ref. 5 and Fig. 1). This sequence also enhances CAT expression in response to forskolin when placed in reversed orientation with respect to the RSV promoter (pVIP31CAT-R; Fig. 2). Similarly, the VIP element confers responsiveness to cAMP when placed either 150 bp (data not shown) or 300 bp upstream from the RSV promoter (pVIP32CAT-300) or on the 3′ side of the CAT gene (pVIP-32CAT-3′). The activity of pVIP32CAT-300 was consistently less than that seen with pVIP32CAT. The position and orientation independence of the VIP CRE indicate that it can be categorized as an enhancer. As observed for many other enhancers, the responsiveness of genes containing multiple copies of the VIP CRE (pCRE×4) was greater than that seen when only a single copy was present (pCRE×1; Fig. 2). Identical results were obtained using either PC12 or C6 glioma cells.

S1 nuclease analysis demonstrated that the VIP–CAT transcripts are initiated correctly at the RSV cap site (Fig. 3). In addition to the correctly initiated transcript, RNA from cells stably transfected with RSV–CAT or pVIPCAT-R protected a larger portion of the radiolabeled S1 probe. The sizes of these larger fragments indicated that there were additional aberrant upstream sites of transcriptional initiation located within the vector sequences in these fusion genes. Although insertion of the CRE in reversed orientation in pVIPCAT-R resulted in a significant level of upstream initiation, only the appropriate transcript was increased by treatment with forskolin (Fig. 3).

Both CGTCA Motifs Are Essential for Activity of the VIP CRE. To test the hypothesis that the CGTCA sequences are essential for biological activity of the VIP CRE, pairs or triplets of nucleotides within the enhancer were altered and the effect on cAMP responsiveness was determined. Mutations either between positions −86 and −81 or between positions −76 and −70 decreased the responsiveness of the enhancer by 55–85% (Fig. 4). The region between positions −86 and −81 corresponded precisely to the upstream CGTCA sequence. The region between positions −76 and −70 included the inverted copy of the CGTCA sequence. Mutations located in the central region of the enhancer, between the two CGTCA sequences, had no effect on cAMP responsiveness. The importance of the CGTCA motifs was underscored by the finding that a single nucleotide substitution at position −72 decreased responsiveness of the pVIP32CAT construction by 80% (Fig. 5).

To test the possibility that the mutations described above disrupted the function of the element simply by disrupting the symmetry of the VIP enhancer, a doubly mutated fusion gene (pVIP17Δ72,86) was synthesized (Fig. 5). This gene, like pVIP32Δ72, contained a guanosine to adenosine substitution at position −72 and, in addition, contained a cytidine to thymidine substitution at position −86. Although symmetry was conserved in the doubly mutated plasmid, responsiveness to forskolin was reduced by 80%, indicating that the specific nucleotide sequences of the CGTCA motifs were important for activity of the VIP CRE.

To test the importance of the relative orientations of the CGTCA motifs on CRE activity, a direct, rather than inverted, repeat of the CGTCA motif was synthesized (pVIP17MD). This mutant was not as effective as the wild-type enhancer but was more active than elements containing only one copy of the downstream motif (pVIP-79--70; Fig. 5).

The center-to-center distance between the two CGTCA motifs is 10 bases, corresponding to one turn of the B form of the DNA helix. Insertion of 5 or 10 bases (corresponding to one-half or one helical turn, respectively) between the motifs (pVIP17+5 and pVIP17+10) resulted in a level of responsiveness intermediate between that of the wild-type CRE and a truncated CRE containing only the downstream CGTCA motif (Fig. 5).

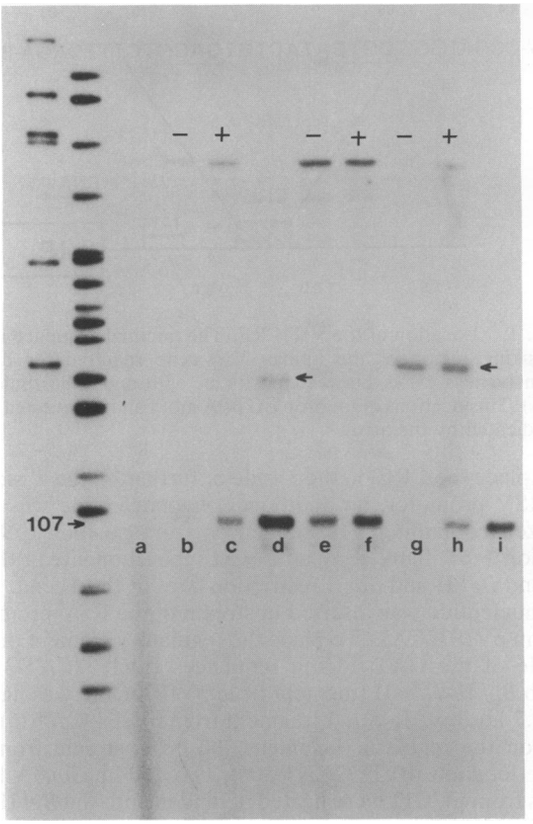

FIG. 3. S1 nuclease analysis of RNA in cells stably transfected with CAT fusion genes. Size markers are in the two lanes to the left. Lanes: a, nontransfected PC12 cell control; b and c, PC12 cells transfected with pVIP31CAT; d and i, C6 glioma cells transfected with plasmid RSV–CAT; e and f, C6 glioma cells transfected with pVIP31CAT; g and h, C6 glioma cells transfected with plasmid VIP31CAT-R. +, Cells treated with forskolin; −, untreated cells. The appropriate 107-nucleotide protected fragment is indicated. Arrows indicate RNA transcribed from cryptic upstream promoters.

CGTCA-Containing Enhancers Bind Related Nuclear Proteins. DNase I protection analysis with nuclear extracts from PC12 and C6 glioma cells (Fig. 6) demonstrated two predominant footprints over the VIP CRE: one between positions −86 and −81 and a second between positions −76 and −63. These footprints coincided with the CGTCA-containing func-

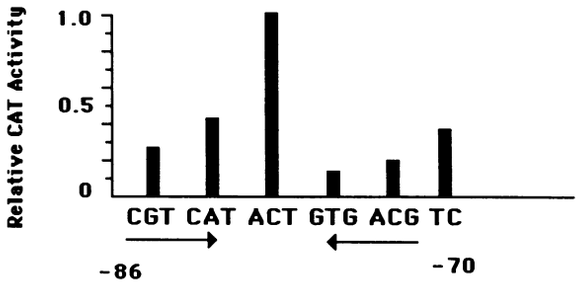

FIG. 4. Mutational analysis of the VIP CRE. The responses of mutated elements to stimulation with forskolin are depicted. Groups of 2- or 3-nucleotide mutations were made in the 17-bp CRE as described in the text. CAT activity is expressed as the relative induction of CAT activity compared to the wild-type CRE (VIP17CAT).

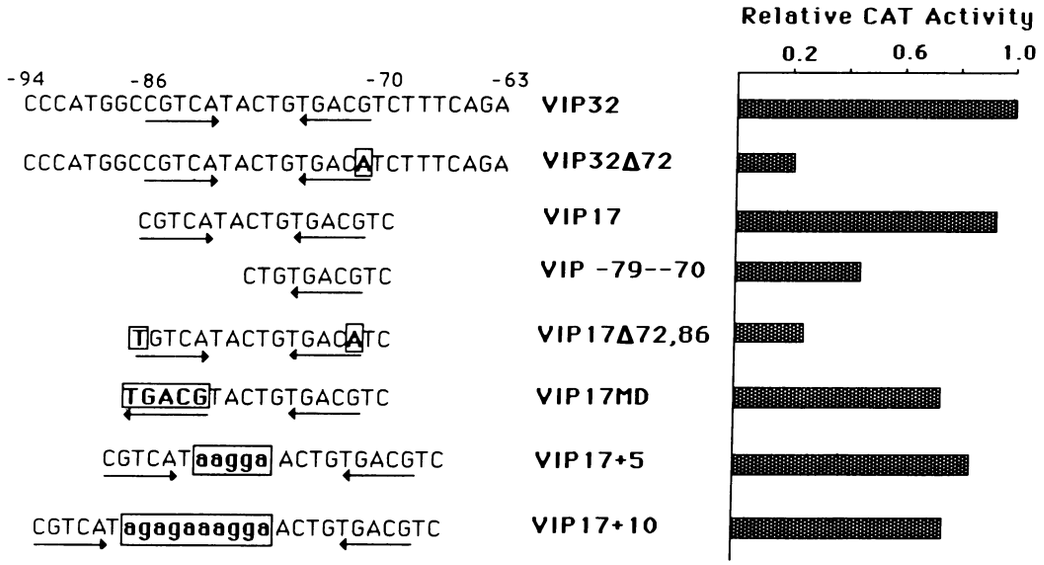

FIG. 5. Structural requirements of the VIP CRE. Mutated nucleotides are indicated by the boxed uppercase letters, and inserted nucleotides are indicated by the boxed lowercase letters. CAT activity is represented as the relative induction of CAT enzyme activity by forskolin compared with VIP32CAT. CAT activity in VIP17MD, VIP17+5, and VIP17+10 is significantly less than VIP17 ($U = 50$, $P < 0.05$; $U = 32$, $P < 0.05$; and $U = 35$, $P < 0.01$, respectively) and greater than VIP-79–70 ($U = 13$, $P < 0.02$; $U = 9$, $P < 0.02$; and $U = 17$, $P < 0.05$, respectively).

tional domains within the CRE. The region of the CRE between the two functional domains was represented by a DNase I hypersensitive site (Fig. 6). DNase I footprinting assays performed in the presence of unlabeled fragments from the VIP, somatostatin, and adenovirus E4 genes indicated that these related enhancers were able to compete with the VIP CRE for binding of nuclear proteins (Fig. 6).

DISCUSSION

The present results demonstrate that the CRE contained within the 5′ flanking region of the human VIP gene can function as an enhancer. The mutational analysis provides direct evidence that the integrity of each of the CGTCA sequences within the VIP CRE is necessary for full activity. Mutations that disrupt either CGTCA motif decrease or abolish activity, indicating that the VIP CRE is composed of two functional domains (Figs. 4 and 5). Mutations between the two motifs do not affect cAMP responsiveness. The downstream functional domain of the VIP CRE contains two additional nucleotides (positions −70 and −71) that are necessary for full activity of the CRE but are not part of the CGTCA motif (Fig. 5). It is possible that these two nucleotides increase cAMP responsiveness by providing a third, incomplete, CGTCA sequence. It has been shown for the vitellogen A2 estrogen responsive element and the simian virus 40 enhancer that imperfect elements, which alone are inactive, cooperate to form functional regulatory elements when duplicated (12, 20). Thus, the incomplete CGTC motif at positions −70 to −73 may be important for the full activity of the VIP CRE when placed in close proximity to the complete CGTCA motifs.

Like the regulatory regions of many other genes, the VIP CRE contains a palindrome. Mutational analysis indicates that the precise nucleotide sequence within the VIP CRE is more important than its palindromic structure for full function of the CRE (Fig. 5). The spatial requirements for cooperativity between the two CGTCA-containing domains were tested by altering the orientation or distance between the two CGTCA motifs. Inverting the orientation of the upstream motif (pVIP17MD) or placing an additional 5 or 10 bases between the functional domains (pVIP17+5, pVIP17+10) resulted in a 11–32% loss of CRE activity, a level which was intermediate between wild-type enhancers and mutant enhancers containing the downstream domain alone. These results indicate that a certain level of cooperativity between the functional domains is preserved despite altering the orientation or distance between the two motifs. Unlike the bacteriophage λ repressor binding site (21), the simian virus 40 early promoter (22), and the *Escherichia coli* Fis binding site (23), placement of the two VIP CRE domains on the same face of the DNA helix does not appear to be essential for cooperativity.

The presence of two copies of the CGTCA motif within the VIP CRE is likely to be important for interaction with nuclear transcription factors. The pattern of the DNase I "footprint" over the CRE correlates with the functional domains determined from mutational analysis: the regions of the CGTCA motifs are protected from DNase digestion but the inactive region between the motifs is not (Fig. 6). The presence of two adjacent functional domains within the VIP CRE also suggests that protein–protein interactions between two CGTCA-binding factors are important for transcriptional regulation. The two domains of the VIP CRE may facilitate interaction between identical regulatory proteins, such as occurs between GCN4 and its recognition sequences in the *HIS3* gene (24) or between various regulatory proteins.

The identity of the protein(s) interacting with the VIP CRE is unknown. One candidate is a 43-kDa CRE binding protein (CREB) isolated from PC12 cells that binds to the CGTCA-containing somatostatin CRE (25). DNA affinity cross-linking studies indicate that a protein of similar size binds to the VIP CRE (K. Walton and R.H.G., unpublished observations). The VIP CRE sequence also resembles the phorbol ester-responsive elements that bind the transcriptional activator protein AP-1 (6). An interaction between AP-1 and the VIP gene might explain the ability of phorbol esters to induce VIP mRNA levels in cultured human neuroblastoma cells (26).

The structural similarity of the VIP CRE with CREs of other cAMP-regulated genes and with the cytomegalovirus and adenovirus enhancers suggests that related nuclear proteins might interact with all of these cis-acting CGTCA-containing DNA transcriptional regulatory elements. The footprinting analyses showing competition of binding to the VIP CRE by DNA fragments containing the somatostatin

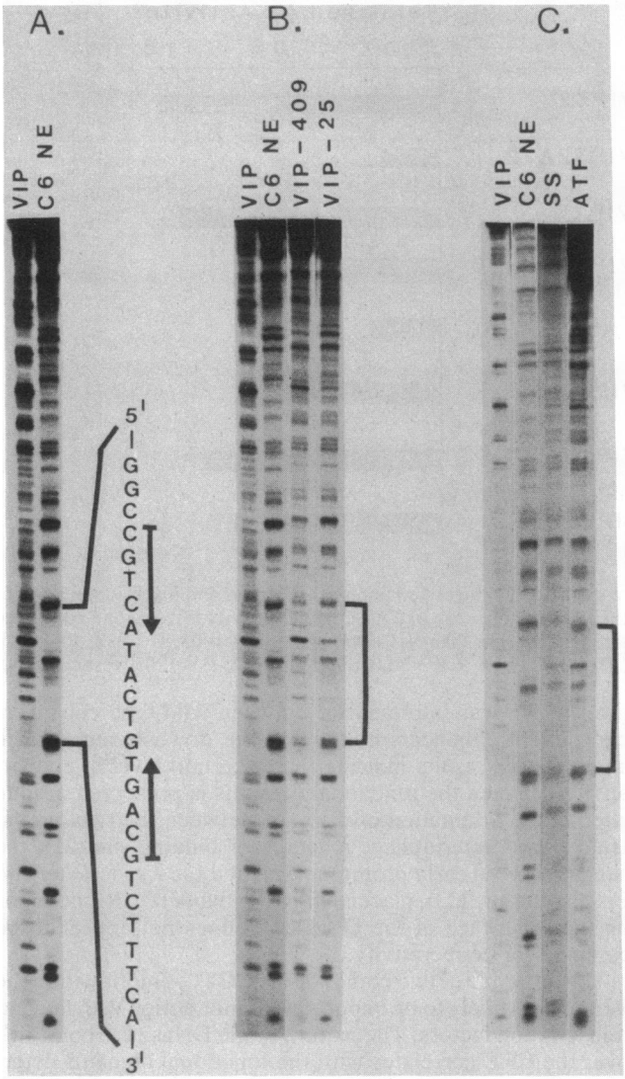

FIG. 6. DNase I footprinting analysis of the enhancer region of the human VIP gene. (*A*) DNase I cleavages of the 325-bp fragment of the VIP 5′ flanking region in the absence (VIP) or presence of 500 ng of C6 glioma nuclear extract (C6NE). (*B* and *C*) Binding of nuclear proteins in the presence of a 10-fold molar excess of the fragment VIP-409 and a 50-fold molar excess of VIP-25, somatostatin (SS), or ATF competitor fragments. Brackets denote boundaries of the CRE.

CRE and an adenovirus E4 enhancer (Fig. 6) are consistant with this hypothesis. Like the proteins that interact with the somatostatin CRE (25) and an adenovirus E4 enhancer (10), the VIP CRE-binding proteins appear to bind to the gene constitutively. Activation of VIP gene transcription by cAMP may depend upon phosphorylation of proteins that interact directly or indirectly with the enhancer. A similar mechanism has been postulated to explain the cAMP-dependent transcriptional activation of the somatostatin CRE by CREB (25). Whether the proteins that bind to the various cellular and viral CGTCA enhancers are identical or related members of a multigene family has yet to be determined.

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