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Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription

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Synthesis of the endogenous opioid precursor, proenkephalin, is regulated by neurotransmitters and membrane depolarization. These events act through second messenger dependent signal transduction pathways via a short inducible DNA enhancer to regulate transcription of the proenkephalin gene. Two DNA elements located within this enhancer are essential for the transcriptional response to cAMP and phorbol ester. Inactivation of either element by mutation or by alteration of their stereospecific alignment eliminates inducible enhancer activity. The promoter distal element, ENKCRE-1, in the absence of a functional adjacent ENKCRE-2 element, has no inherent capacity to activate transcription. However, in the presence of a functional ENKCRE-2 element, this element synergistically augments cAMP and phorbol ester inducible transcription. The promoter proximal element, ENKCRE-2, is essential for both basal and regulated enhancer function. Four different protein factors found in HeLa cell nuclear extracts bind *in vitro* to the enhancer region. ENKTF-1, a novel enhancer binding protein, binds to the DNA region encompassing ENKCRE-1. The transcription factors AP-1 and AP-4 bind to overlapping sites spanning ENKCRE-2, and a fourth transcription factor, AP-2, binds to a site immediately downstream of ENKCRE-2. The binding of ENKTF-1 to mutant ENKCRE-1 sequences *in vitro* correlates with the *in vivo* inducibility of the mutant elements suggesting that ENKTF-1 acts in combination with factors that recognize the ENKCRE-2 domain to regulate cAMP inducible transcription. Together, the two DNA elements, ENKCRE-1 and ENKCRE-2 and the protein factors with which they interact, play a critical role in the transduction and reception of signals transmitted from cell surface receptors to the proenkephalin nuclear transcription complex.

Key words: proenkephalin/cAMP/transcription factor

Introduction

Nerve cells regulate their signalling by controlling both the synthesis and release of their neurotransmitters. The regulation of gene expression by signals received at the synapse (trans-synaptic regulation) is an important mechanism enabling nerve cells to regulate the synthesis of molecules necessary for neurotransmission in response to diverse environmental inputs. Trans-synaptic regulation of transcription may underlie a variety of long-term changes in neuronal plasticity, including changes occurring during learning and memory (Black *et al.*, 1987; Golet *et al.*, 1986; Montarolo *et al.*, 1986. Both the synthesis and release of the opioid neuropeptides, Met- and Leu-enkephalin (Hughes *et al.*, 1976; Comb *et al.*, 1982; Gubler *et al.*, 1982; Noda *et al.*, 1982), are regulated by trans-synaptic events involving the activation of neurotransmitter receptors and second messenger pathways (Eiden *et al.*, 1985; Kanamatsu *et al.*, 1986; Yoshikawa and Sabol, 1986; Young *et al.*, 1986; Comb *et al.*, 1987; Kley *et al.*, 1987). To elucidate the molecular mechanisms mediating trans-synaptic regulation of proenkephalin transcription it is necessary to understand both the pathways transmitting signals from the synapse to the proenkephalin gene, and the factors mediating and regulating transcription in response to these signals.

Signals received at the cell surface are transduced through the plasma membrane by cell surface receptors to G-proteins which regulate the production of second messengers, the gating of ion channels, and eventually the activation of a variety of different protein kinases. Activated protein kinases, in turn, initiate and coordinate complex cellular responses ranging from mitosis to neuromodulation (Nairn *et al.*, 1985; Hunter, 1987). Second messengers such as cAMP and diacylglycerol are known to activate a cAMP dependent protein kinase (protein kinase A) (Nairn *et al.*, 1985) and protein kinase C (Berridge and Irvine, 1984; Nishizuka, 1984) respectively, and the activated kinases are thought to regulate gene transcription by as yet undefined mechanisms.

Transcriptional induction of proenkephalin gene expression by cAMP and phorbol esters has been previously mapped to a short DNA element located in the 5′-flanking sequences of the human gene (Comb *et al.*, 1986). This element confers cAMP and phorbol ester transcriptional responsiveness upon heterologous promoters when placed either upstream or downstream of the RNA start site in an orientation independent fashion, indicating that the element functions as a cAMP and phorbol ester inducible enhancer.

In this report we show that the proenkephalin cAMP and 12-*O*-tetradecanoyl-phorbol-13-ester (TPA) inducible enhancer consists of two functionally distinct elements, ENKCRE-1 and ENKCRE-2, which activate transcription in a synergistic fashion. The ENKCRE-1 element, which itself has little inducing capacity, will greatly augment cAMP and TPA inducible transcription in the presence of the ENKCRE-2 element. The DNA sequence of each element is similar, but not identical, to elements found within other cAMP and phorbol ester inducible genes. At least four distinct proteins bind *in vitro* to the region spanning the enhancer. A novel nuclear factor called ENKTF-1 binds to the ENKCRE-1 element. Two other factors, previously

characterized and purified based upon their affinity for the SV40 enhancer, AP-4 (Mermod *et al.*, 1988) and AP-1 (Angel *et al.*, 1987; Lee *et al.*, 1987), bind to overlapping sites spanning the ENKCRE-2 element. An additional factor, AP-2 (Imagawa *et al.*, 1987; Mitchell *et al.*, 1987), previously shown to mediate both TPA and cAMP transcriptional effects, binds to a site downstream and adjacent to ENKCRE-2. Binding *in vitro* of affinity purified ENKTF-1 to several different single base substitution (SBS) mutants located within ENKCRE-1 correlates with the *in vivo* enhancer activity of each mutant. This suggests that ENKTF-1 mediates inducible enhancer function by binding to the ENKCRE-1 element and synergistically interacting with factors bound at the ENKCRE-2 region. The overlap of AP-1 and AP-4 binding to ENKCRE-2, as well as the lack of precise correlation between the binding of each factor individually to point substitutions within the element and the *in vivo* enhancer activity of each mutant, suggest a complex regulatory scheme at this site which may involve the exchange of different binding factors under different conditions, the binding of several factors in concert, or a role for as yet unidentified factor(s).

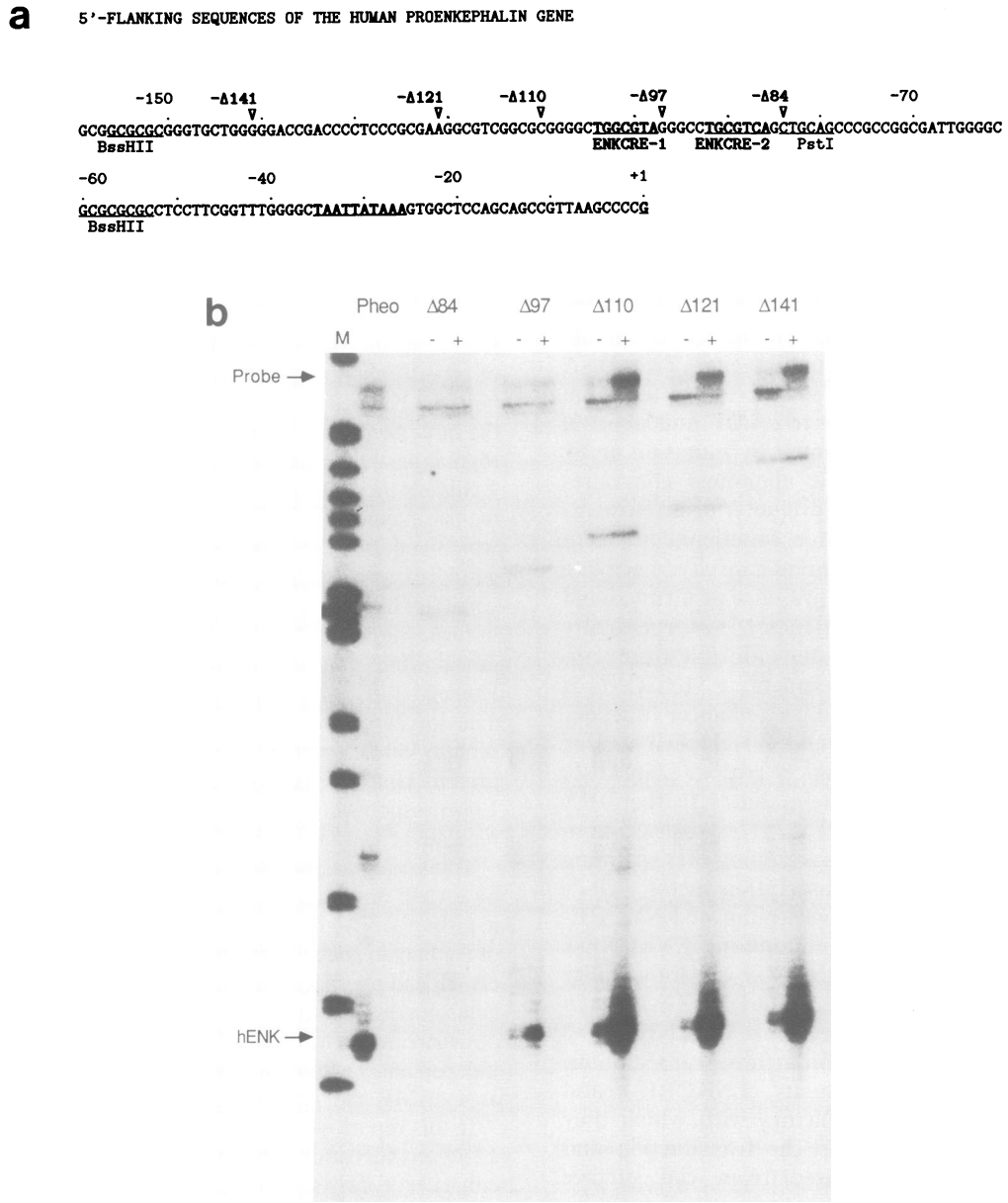

Fig. 1. Deletion analysis of the human proenkephalin promoter/enhancer sequences required for basal and cAMP induced transcription. (**a**) 5′-Flanking sequences of the human proenkephalin gene containing the promoter/enhancer region. Positions of the deletion mutations are indicated by arrows marking the last deleted base, and +1 refers to the start site of transcription initiation. (**b**) Deletion mutants of the human proenkephalin/CAT fusion gene described previously (Comb *et al.*, 1986) whose 5′ end points are indicated in (a) were co-transfected with pRSVNEO (Gorman *et al.*, 1983) into C6-glioma cells, selected with G418, and >1000 independent colonies pooled, treated with CPT-cAMP (200 μM) and IMX (500 μM) for 3 h, RNA isolated, hybridized with a single-stranded DNA probe spanning sequences between −193 and +70, treated with S1 nuclease, and protected DNA analyzed by 7% polyacrylamide−urea gel electrophoresis. Lanes marked M and Pheo refer to mol. wt standards (*Hae*III fragments of pUC18) and 20 μg of human pheochromocytoma RNA. The size of correctly initiated RNA transcripts is indicated with an arrow (70 nt). The remaining lanes contain 40 μg of total RNA isolated from C6-glioma cells transfected with the indicated deletions and treated for 3 h in the presence (+) or absence (−) of CPT-cAMP and IMX. The arrow indicates the size of the probe (263 nt).

Results

Analysis of the human proenkephalin inducible enhancer

Previous deletion and transfer studies have demonstrated that 5′-flanking sequences of the human proenkephalin gene located −193 to −80 bp upstream of the RNA cap site act as a cAMP and phorbol ester inducible enhancer and have mapped the responsive enhancer to a region located between −110 and −72 (Comb *et al.*, 1986). Here we report a detailed analysis of the inducible enhancer region. Analysis of RNA transcripts from C6-glioma cells stably transfected with several different *Bal*31 deletion mutants spanning the enhancer region extending between −141, −121 and −110 produce similar levels of correctly initiated RNA transcripts in the presence or absence of cAMP (Figure 1). Deletions extending to −97 reduce basal transcription several fold and cAMP induction ~10-fold. Deletions extending to −84 totally abolish correctly initiated transcription. This analysis indicates that the 5′end of an element essential for basal transcription lies within the 13 bp region located between nucleotides −97 and −84. Since deletions extending to −110 are fully inducible by cAMP, while deletions extending to −97 exhibit only 2- to 3-fold inductions, it can be concluded that the 13 bp region between −110 and −97 is essential to confer maximal regulation by cAMP on element(s) located downstream of nucleotide −97.

Double-stranded oligonucleotides spanning sequences −110 to −84 were introduced into the *Pst*I site at position −84 of the plasmid pENKAT-Δ84. This plasmid (Figure 1a) contains human proenkephalin sequences to −84 and does not correctly initiate transcription (Figure 1b). As expected, introduction of this 30 base oligonucleotide in either orientation reconstitutes correct basal and regulated transcription (Figure 2b). Insertion, in either orientation, of two copies produces little effect on basal transcription but dramatically increases regulated transcription. In a similar fashion, insertion of 3, 4 and 5 tandem copies of the

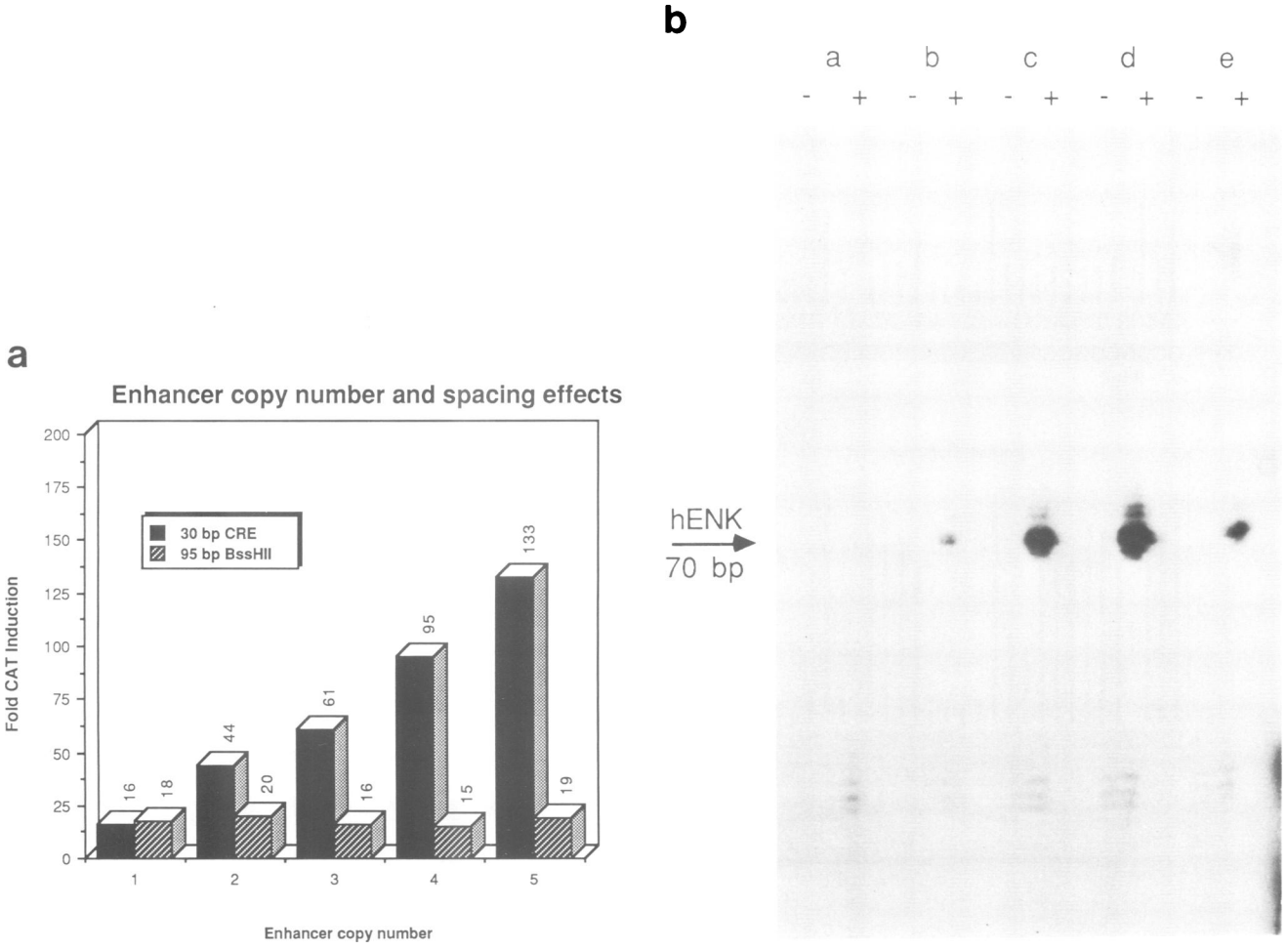

Fig. 2. Effects of enhancer copy number and spacing between tandem repeated enhancer elements. (**a**) The fold CAT induction by cAMP treatment is shown after transfection of CV-1 cells with pENKAT-Δ84 containing the indicated number of enhancer elements: oligonucleotide spanning −85 to −110 (filled bars) or a *Bss*HII restriction fragment, −56 to −155 (stippled bars). In all experiments a plasmid containing the β-galactosidase gene linked to the RSV promoter/enhancer, pRSVBGAL (Edlund *et al.*, 1985) was co-transfected to provide an internal control for differences in transfection efficiency between different precipitates as described previously (Comb *et al.*, 1986). CAT enzyme activities were normalized to β-galactosidase and the fold inductions plotted against the copy number of the introduced enhancer elements, which in each case are inserted in tandem arrays in the + orientation relative to the transcription start site. (**b**) Effects of multiple copies of an enhancer element (−110 to −85) on correctly initiated transcription in the presence and absence of cAMP. C6-glioma cells were co-transfected with pRSVNEO and pENKAT-Δ84 containing the number of inserted enhancer elements indicated below, selected with G418, and RNA isolated from pooled colonies (>1000/transfection) treated in the presence (+) or absence (−) of CPT-cAMP (200 μM) and IMX (500 μM) for 3 h, and analyzed by S1 nuclease protection. Lanes a, b, c and d: 40 μg RNA from cells transfected with pENKAT-Δ84 containing 0, 1, 2 and 3 copies of the 30mer (−110 to −85), all present in the + orientation. Lanes e: 40 μg RNA from cells transfected with pENKAT-12 treated in the presence (+) or absence (−) of CPT-cAMP/IMX, which represents wild-type promoter/enhancer basal and induced expression. Arrow indicates correctly initiated transcription.

oligonucleotide produces an approximately linear increase in cAMP inducible transcription with only small increases in basal transcription (Figure 2a and b). In contrast, insertion of multiple copies of the 97 bp *Bss*HII fragment (−155 to −58), in either orientation, at the *Bss*HII site (−58) results in increased basal transcription (data not shown) but little or no significant increase in cAMP induced transcription (Figure 2a).

SBS analysis defines two elements essential for cAMP inducible enhancer function

A random collection of SBS mutants was generated in order to further define the enhancer. The double-stranded oligonucleotides generated (see Materials and methods) encompass the human proenkephalin cAMP and TPA inducible enhancer region spanning sequences −114 to −84. *Sac*I and *Pst*I compatible termini were added to the 5′ and 3′ ends respectively, to allow directional cloning into the unique *Sac*I/*Pst*I sites of the transcriptionally inactive plasmid pENKAT-Δ84.

Each SBS mutant was co-transfected into CV-1 cells together with the uninducible internal control plasmid, pRSVβGAL (Edlund *et al.*, 1985), for transient analysis of CAT and β-galactosidase expression in the presence or absence of the stable cAMP analogue 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), TPA and the phosphodiesterase inhibitor isobutylmethyxanthine (IMX) (Figure 3a and b). Quantitatively similar results were obtained using CPT-cAMP and IMX, except the overall levels of induction were 1.5- to 2.0-fold lower in the absence of TPA. CAT activities were normalized to the β-galactosidase activities from the same extracts to control for differences in transfection efficiency and the average of three separate experiments is shown in Figure 3. In addition, each SBS mutant was co-transfected into C6-glioma cells together with the plasmid pRSVNEO (Gorman *et al.*, 1983), selected with G418, and >1000 independent G418 resistant colonies were pooled,

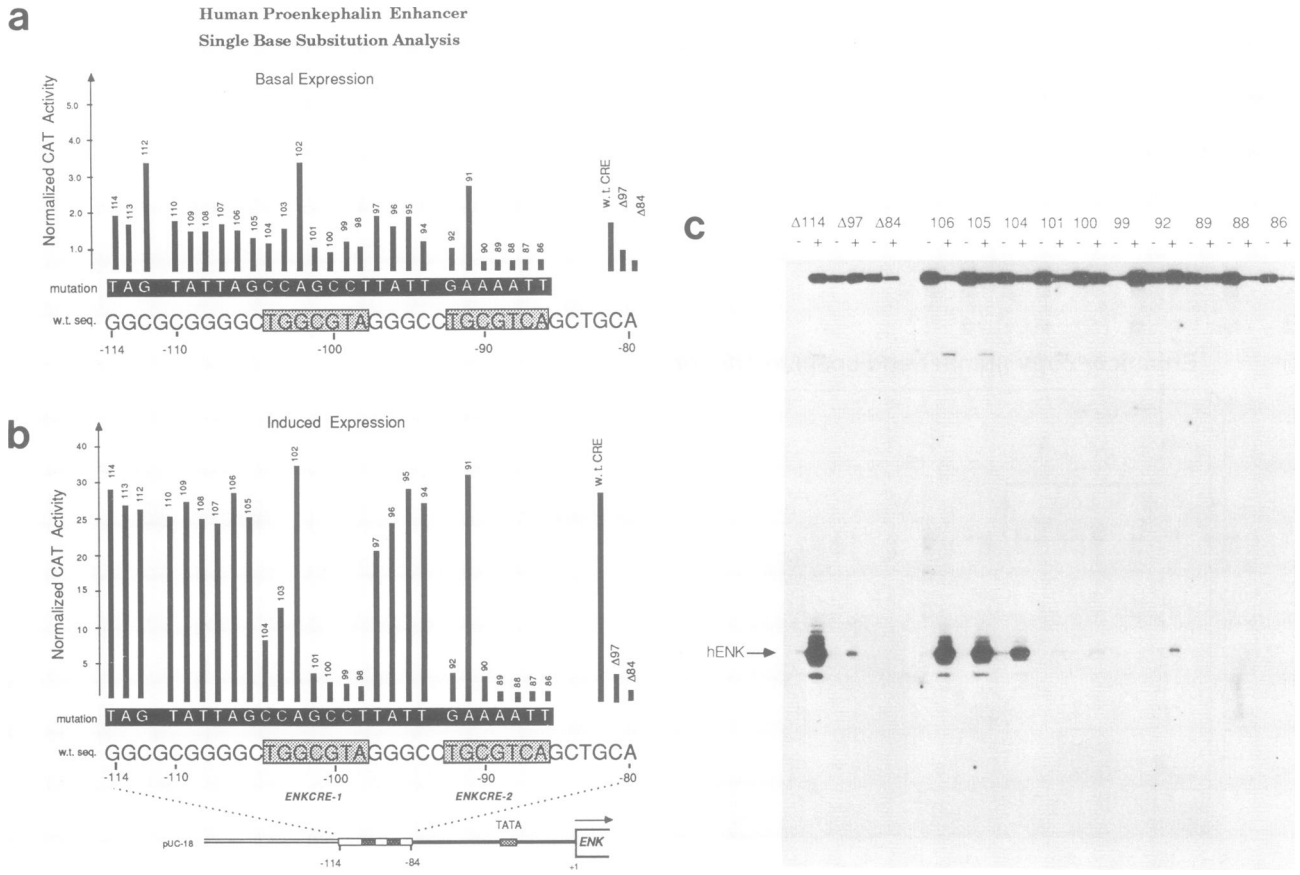

Fig. 3. SBS analysis defines two functionally distinct elements essential for cAMP inducible enhancer function. (**a**) Basal CAT expression of individual SBS mutants following transfection and transient expression in CV-1 cells. The position and base substitution is indicated; black bars indicate arbitrary units of normalized CAT enzyme activity. Basal CAT expression is also shown for the wild-type oligonucleotide construct (w.t.CRE), which is equivalent to a Δ114 deletion, and the Δ97 and Δ84 deletion mutants for comparison. The sequence of the wild-type oligonucleotide inserted into the *Pst*I site at −84 of the plasmid pENKAT-Δ84 is shown and the substituted bases indicated above. CAT activities were normalized to β-galactosidase activities as described in Materials and methods. Indicated values represent the average of three separate experiments. (**b**) Induced levels of CAT enzyme activity following transfection of CV-1 cells with the indicated SBS plasmids and treatment of cells with CPT-cAMP (200 μM), TPA (50 nM) and IM X(500 μM). Regulator treatment was for 6 h starting 18 h after transfection. Note that the same units of normalized CAT activity are used in (a) and (b). Regions sensitive to SBS are boxed and denoted as ENKCRE-1 and ENKCRE-2. Induced CAT activities are also indicated for the Δ114, Δ97 and Δ84 deletions. All CAT activities were normalized to β-galactosidase activities as described in Materials and methods and the indicated values represent the average of three separate experiments. (**c**) S1 analysis of proenkephalin/CAT fusion RNA transcripts from C6-glioma cells stably expressing SBS mutants. Transfection and analysis of RNA transcripts from pools of stably expressing C6-glioma cells was carried out as described in Materials and methods and the legend to Figure 1b. Cells were treated with (+) or without (−) 25 μM forskolin and 500 μM IMX for 2.5 h followed by RNA extraction. The position of correctly initiated RNA transcripts (70 nt) is indicated by the arrow.

and RNA isolated from control and forskolin treated cells. RNA was hybridized with a single-stranded probe from the 5'-flanking region of the human proenkephalin gene and subjected to S1 analysis (Figure 3c) as previously described (Comb *et al.*, 1986). Both transient and stable expression analysis gave similar results (compare Figure 3a and b with c).

Two regions sensitive to SBS mutations are clearly defined. The first region located between nucleotides −104 and −98, called 'Enkephalin cAMP Regulatory Element 1' (ENKCRE-1), spans the sequence TGGCGTA; mutations within this region are characterized by 2- to 15-fold reductions of cAMP and TPA inducible transcription (see Figure 3a−c). SBS in this region reduce both basal and regulated expression. The second SBS sensitive region, 'Enkephalin cAMP Regulatory Element 2' (ENKCRE-2), extends from −92 to −86 and encompasses the related sequence TGCGTCA. As mutations 3' to this site altered the *Pst*I termini and hence inhibited cloning, the 3' end of this element was not defined by this analysis. SBS within ENKCRE-2 result in dramatic reductions in both basal and regulated transcription. For example, substitutions at positions −86, −87, −88 and −89 (see Figure 3a−c) drastically diminish both basal and regulated transcription, little if any correctly initiated transcription is detected on longer exposures of the autoradiogram shown in Figure 3c for the −88 and −86 mutants. We conclude that these two elements act in a synergistic fashion; neither element alone is capable of conferring high levels of inducible expression, but together the two elements strongly enhance cAMP and TPA inducible transcription. The sequence of each element is similar, sharing a common GCGT motif (underlined in the text above), which is separated by 11 bp, one turn of the DNA helix.

SBS at three different positions resulted in increased basal CAT expression with little effect on regulated expression. Substitution of A for G at position −102 and −91 both increase basal transcription 2-fold and results in wild-type regulated transcription. These SBS mutations generate the sequence TACGTCA which is a known binding site for adenovirus transcription factor (ATF) which mediates E1a induction of several adenovirus early genes (Lee,K.A. *et al.*, 1987), and is similar to the binding site of a protein CREB (Montminy and Belezikjian, 1987), which binds the somatostatin cAMP response element. The C to G substitution at position −112 creates an SP1 consensus binding site GGGCGGGG and results in a 2-fold increase in basal transcription (Figure 3a) with little or no effect on regulated transcription (Figure 3b). Substitutions occurring upstream or between these two SBS sensitive domains have little or no effect on transcription.

Effects of SBS mutations on TPA induced transcription

We have previously reported that the transcriptional response of the human proenkephalin gene to TPA in the presence of the phosphodiesterase inhibitor IMX mapped to the same region of DNA responsible for cAMP induction (Comb *et al.*, 1987). The response to TPA alone is small and is greatly potentiated by IMX, and is additive to the effects of cAMP stimulated transcription (S.E.Hyman *et al.*, in preparation and see Discussion). To determine if the effects of cAMP and TPA could be dissociated by point mutations in either of the two elements defined above, the response of enhancers deficient in either ENKCRE-1 or ENKCRE-2 to respond to either cAMP/IMX or TPA/IMX was tested. As shown in Table I SBS mutations in either element produced indistinguishable effects on the transcriptional responses to either cAMP or TPA, indicating that both elements contribute to regulation by either cAMP or TPA.

Effects of altering the spacing between ENKCRE-1 and ENKCRE-2

The spacing between the GCGT motifs of ENKCRE-1 and ENKCRE-2 is 11 bp, one turn of the DNA helix, suggesting that proteins bound at each site would be positioned on the same side of the DNA helix in a favorable position for protein−protein interaction. To test this hypothesis the spacing between elements was altered by integral and half integral helical turns. Insertion of one helical turn between elements (a 10 base sequence containing a *Bgl*II restriction site was inserted between nucleotides −95 and −94) does not significantly alter basal transcription and reduces regulated inductions by forskolin only 2-fold (Figure 4). This level of expression should be compared to the 10-fold reductions in cAMP regulated transcription for the Δ97 deletion mutant where ENKCRE-1 activity has been completely eliminated. Decreasing the size of the inserted DNA from 10 to 6 bases reduces basal and regulated transcription a further 2-fold. Increasing the size of the DNA inserted between ENKCRE-1 and ENKCRE-2 to 14 bases has a very strong inhibitory effect on expression, reducing both basal and regulated expression to levels similar to the Δ97 deletion mutant.

Detection of proteins that bind to the enhancer region

In order to detect protein factors that interact with the elements defined by SBS analysis, we next examined, by DNase I footprinting, the interaction of nuclear proteins isolated from crude human HeLa and rat C6-glioma cell nuclear extracts isolated by the method of Dignam *et al.* (1983). As shown in Figure 5, C6-glioma extracts, at low protein concentrations, protect a 22 bp region spanning nucleotides −110 to −88 from DNase I digestion. The region protected at low protein concentrations is centered around ENKCRE-1 located at −104 to −98. We will refer to the factor(s) protecting sequences between −110 and −88

Table I. Effects of SBS within ENKCRE-1 and ENKCRE-2 on cAMP/IMX and TPA/IMX induced enhancer activity

SBS	CPT-cAMP fold induction	TPA fold induction
Wild-type CRE	10.1	12.7
−101 C to G	3.0	3.9
−100 G to C	2.2	3.1
−89 G to A	1.3	2.2
−86 A to T	1.6	1.8

CV-1 cells transfected with 5 μg of each SBS mutant, 5 μg pRSVβGAL and 10 μg pUC18 were treated in the presence or absence of either 200 μM CPT-cAMP or 50 nM TPA both in the presence of 500 μM IMX for 5 h, and the levels of CAT and β-GAL activity determined as described in Materials and methods. CAT activities were normalized to the relative β-GAL levels and the fold inductions determined.

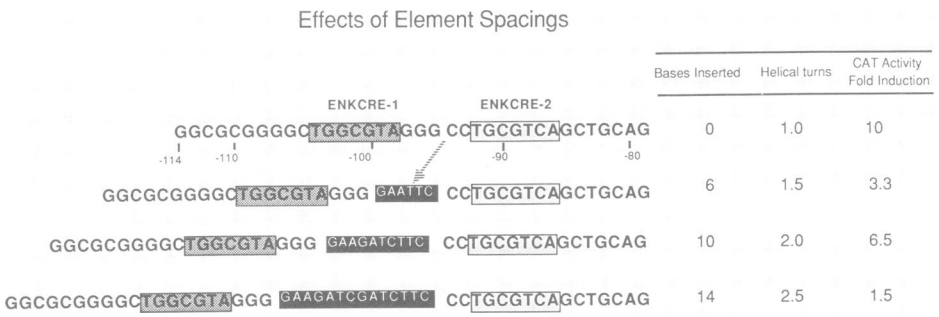

Fig. 4. The effects of changing the spacing between elements on cAMP inducible enhancer function. The spacing between ENKCRE-1 and ENKCRE-2 elements was altered by synthesizing the enhancer region (−114 and −84) with a 10 bp *Bgl*II restriction site inserted between elements at position −94. This oligonucleotide was inserted into the *Pst*I stie of pENKAT-Δ84. Plasmids containing 6 and 14 bp insertions between elements were produced by cleaving the above plasmid with *Bgl*II and treated with either mung bean nuclease to remove the four base overhangs or filled in with the large fragment (Klenow) of DNA polymerase I to generate plasmids containing 6 and 14 bp insertions between elements. The identity of these plasmids was confirmed by DNA sequence analysis. Plasmids were transfected into CV-1 cells, treated with CPT-cAMP (200 μM) and IMX (500 μM) and normalized CAT inductions are indicated.

as ENKTF-1. At higher protein concentrations the region between −87 to −65 is protected. This region, which encompasses ENKCRE-2 (−92 to −86) and sequences downstream, requires 2- to 3-times more C6-glioma protein to give complete protection by DNase I footprinting. Human HeLa cell nuclear extracts give very similar results, protecting sequences between −110 and −65, suggesting that the factors interacting with the enhancer region are not tissue or species specific. Similar patterns of protection are also observed using nuclear extracts prepared from rat PC-12 and mouse AtT-20 cells (data not shown). Because factors interacting with the ENKCRE-2 region appear to be present at higher concentrations in HeLa cells and because we are working with the human gene we have restricted further analysis to HeLa cells.

Footprint competition experiments using synthetic oligonucleotides containing either the ENKCRE-1, or ENKCRE-2 and 3′-flanking sequences as competitors indicated that the DNase I protection observed between −110 and −65 is composed of multiple binding sites for different factors (data not shown). Experiments using crude nuclear extracts to footprint SBS mutants within the ENKCRE-1 element indicated that a factor which binds the wild-type element did not bind to SBS mutants which inactivate inducible enhancer function. However, similar attempts to footprint mutant ENKCRE-2 sites using crude nuclear extracts failed to show altered protection pattern, possibly due to the presence of multiple factors binding this element.

A novel nuclear factor, ENKTF-1, interacts with the ENKCRE-1 element

Proteins interacting with the ENKCRE-1 region were purified by DNA recognition site affinity chromatography, using an oligonucleotide containing the ENKCRE-1 element. Crude HeLa nuclear extract was mixed with competitor DNA and passed 3 times over the column as described by Kadonaga and Tjian (1986). This highly purified preparation (2 footprint units/ng protein, where 1 footprint unit is the amount of protein required to protect 10 fmol of probe from DNase I digestion) was used to footprint the human proenkephalin enhancer region. As shown in Figure 6, this preparation protects a region of the enhancer spanning −110

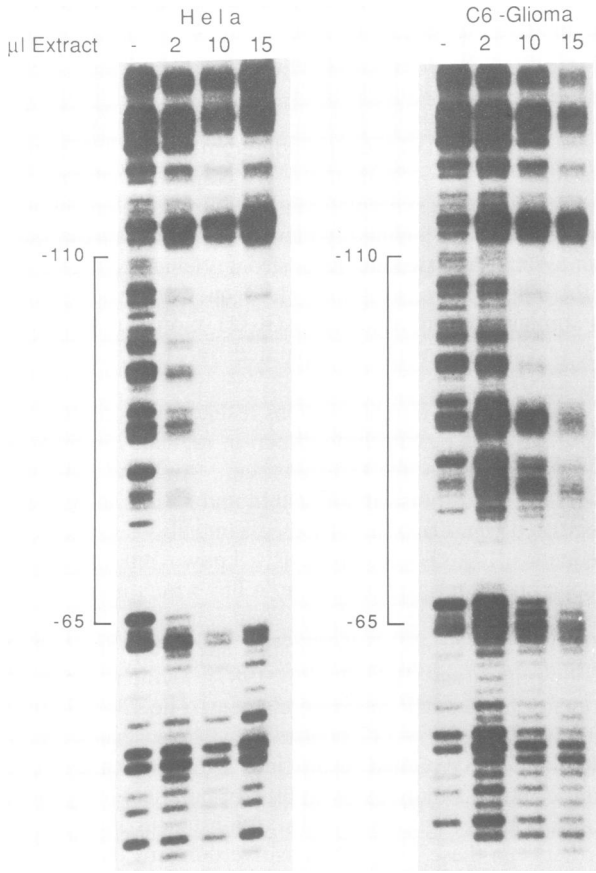

Fig. 5. Binding of nuclear proteins to the human proenkephalin cAMP and phorbol ester inducible enhancer. A DNA fragment containing the human proenkephalin promoter/enhancer region (−193 to +1) was labeled at position +1 and +2 by filling in a *Ava*I restriction site with [^{32}P]dCTP using reverse transcriptase. This generates a probe in which the coding strand is labeled at +1 and +2. Approximately 1 ng of labeled DNA fragment was incubated with the indicated volume (μl of nuclear extract appears above each lane, − refers to the omission of nuclear extract) of crude HeLa (11 mg protein/ml) or C6-glioma (8.9 mg protein/ml) nuclear extracts. DNase I footprinting reactions were carried out as described in Materials and methods, and analyzed on a 7% polyacryamide−urea gel. Protected regions of the DNA (DNase I footprints) are indicated by the lines to the left of the panel.

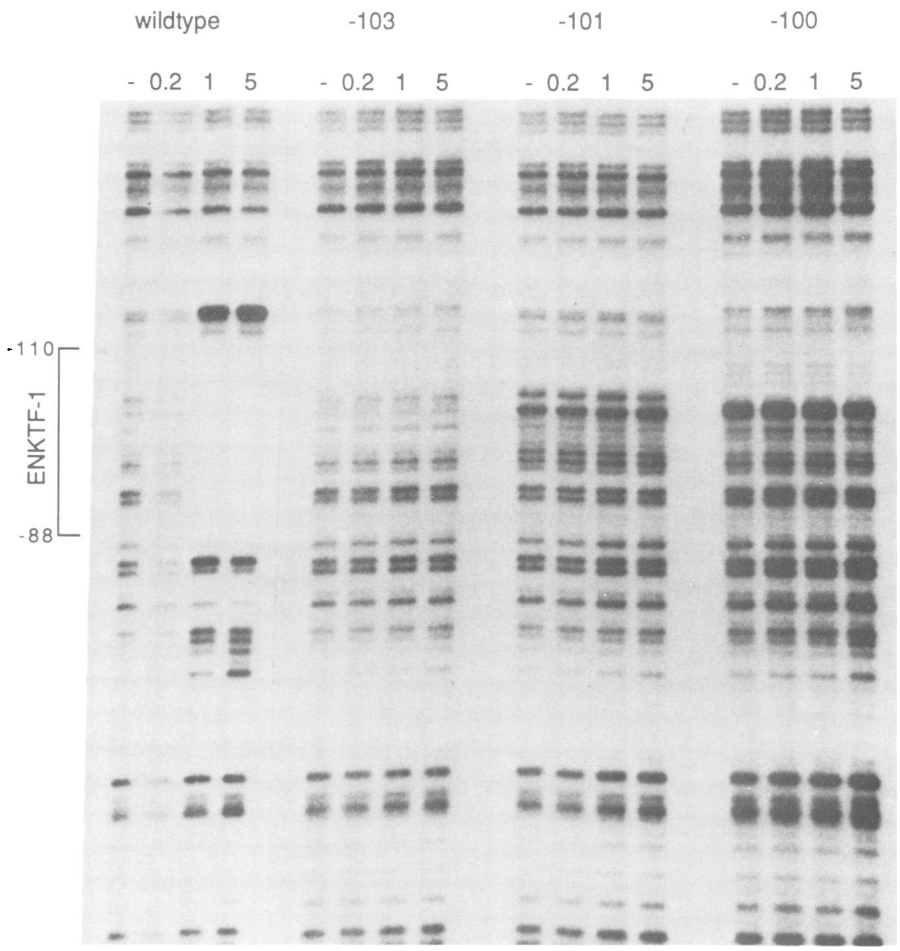

Fig. 6. Binding of affinity purified ENKTF-1 to wild-type and single base substituted human proenkephalin enhancer correlates cAMP inducible enhancer function with ENKTF-1 binding. A DNA fragment containing human proenkephalin sequences between −114 and +1 fused to pUC18 sequences at −114 from the unique *Sac*I site extending upstream to the first *Hae*II site was purified from wild-type and various plasmids containing single base substituted enhancer regions and labeled on the coding strand at positions +1 and +2. The indicated volume (−, 0.2, 1, 5 μl) appears above each lane) of affinity purified ENKTF-1 (two passes over a ENKCRE-1 recognition site affinity column) was incubated with wild-type and the indicated single base substituted probes (see Figure 3b and c for the nucleotide substitutions and *in vivo* activity of these SBS mutant enhancers). The region of the enhancer protected by ENKTF-1 is indicated at the left, spanning sequences from −110 to −88. Affinity purified ENKTF-1 used in these experiments was purified from HeLa cell nuclear extracts as described by Kadonaga and Tjian (1986) using a double-stranded oligonucleotide spanning −110 to −85.

to −88. No other interactions within or surrounding the enhancer region are observed using five times the amount of protein required to give complete protection of the ENKCRE-1 site, suggesting that a single binding activity has been purified.

The *in vivo* enhancer activity of several SBS mutants within the ENKCRE-1 element were next correlated with their ability to bind affinity purified ENKTF-1. As shown in Figure 6, each SBS tested within this region (positions −103, −101 and −100) exhibited little or no protection with ENKTF-1 as determined by DNase I footprinting. These results correlate the binding of ENKTF-1 to the −110 to −88 region of the enhancer with *in vivo* cAMP inducible enhancer function.

Purified transcription factors AP-1 and AP-4 bind to overlapping sites spanning ENKCRE-2

The transcription factors AP-1 and AP-4 bind to sequences in the SV40 and metalothionein II enhancers which are very similar to sequences within and flanking ENKCRE-2 (Angel *et al.*, 1987; Lee *et al.*, 1987a,b; Mermod *et al.*, 1988). Moreover, the transcription factor AP-1 has been shown to bind a short TPA responsive element (TRE) located within the collagenase, SV40 and hMTII genes (Angel *et al.*, 1987; Lee *et al.*, 1987a,b), and the transcription factor AP-4 has been shown to interact synergistically with AP-1 to increase transcription *in vitro* from the SV40 late promoter (Mermod *et al.*, 1988). Therefore, the ability of affinity purified AP-1 and AP-4 to bind the human proenkephalin enhancer was examined *in vitro* by DNase I protection.

Purified AP-1 protects sequences centered directly over ENKCRE-2, extending from −99 to −82, at protein concentrations similar to the levels required to give complete protection of the SV40 high affinity AP-1 binding site (Lee *et al.*, 1987a,b) (Figure 7a). Binding of purified AP-1 to several different SBS mutations spanning ENKCRE-2 was tested. As shown in Figure 7b purified AP-1 does not bind to the −86, −87, −88 or −89 SBS mutations, but binds with wild-type or higher affinity to the −90 mutation, and shows wild-type binding to the −91 and −92 mutations (data not shown). This binding profile agrees with the effects of these mutations on inducible enhancer function

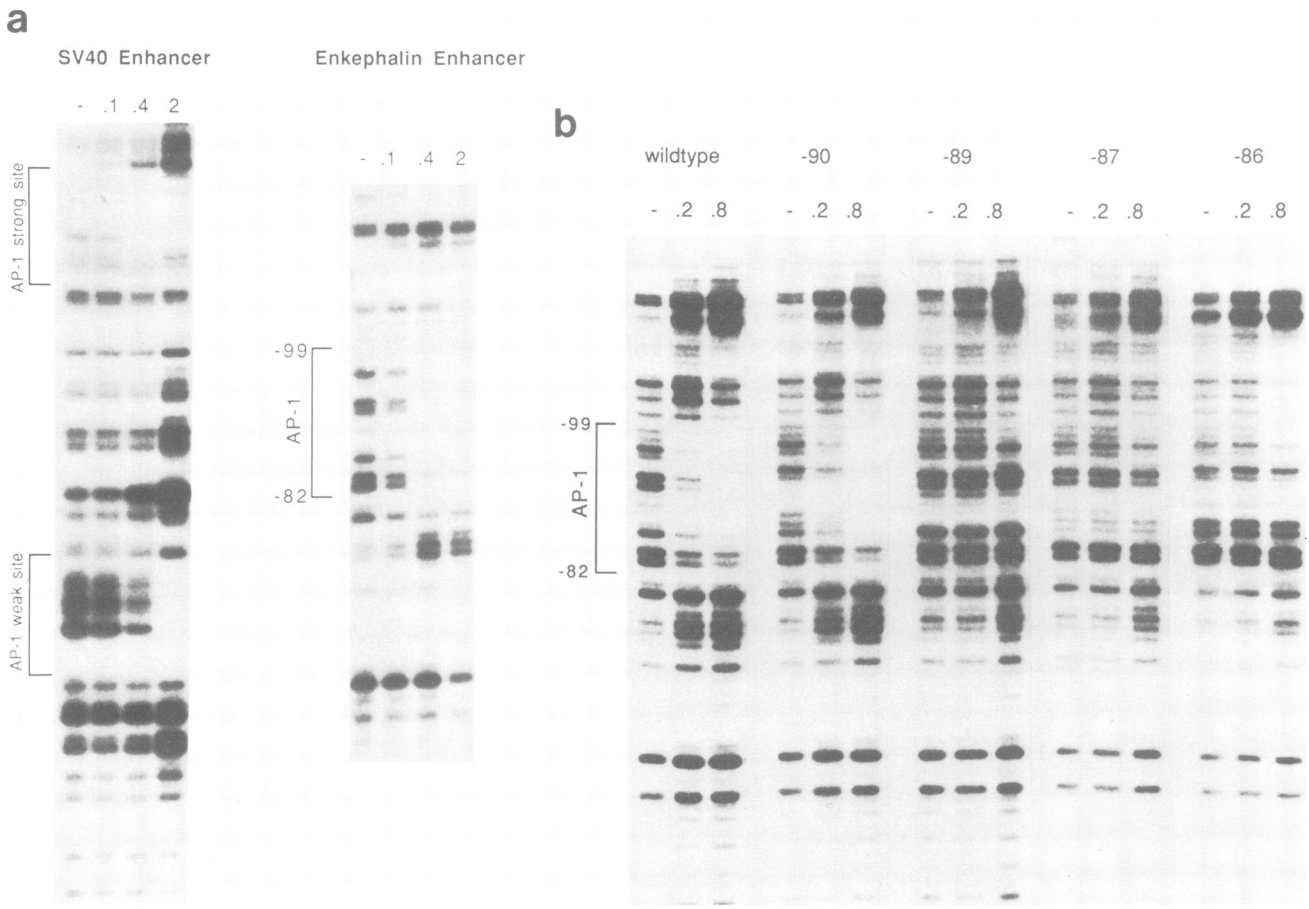

Fig. 7. Binding of affinity purified AP-1 to the SV40 and human proenkephalin enhancers. (**a**) Comparison of DNA binding affinity of purified AP-1 with the human proenkephalin and SV40 enhancers. The indicated volume (μl) of affinity purified AP-1 was incubated with probes spanning the SV40 and proenkephalin enhancers. Binding of AP-1 to the SV40 strong and weak sites (Lee *et al.*, 1987b) and to the proenkephalin enhancer are indicated to the left of the panel. (**b**) Effects of point mutations within the ENKCRE-2 element on protection of the proenkephalin enhancer by affinity purified AP-1. The indicated volumes of affinity purified AP-1 (μl) were incubated with end-labelled DNA probes (labelled on the coding strand at position +1) purified from the indicated ENKCRE-2 SBS mutant. See Figure 3b and c for the *in vivo* effects of each SBS mutation on cAMP inducible enhancer function.

in vivo except for the −90 and −92 mutations. These enhancer mutations both show wild-type or better binding to purified AP-1; however, these mutations *in vivo* strikingly diminish both cAMP and TPA inducible expression (see Figure 3a and b).

AP-4 was purified from HeLa cells by several rounds of affinity chromatography based on the affinity of AP-4 for the human proenkephalin and SV40 enhancers (Mermod *et al.*, 1988). As shown in Figure 8, affinity purified AP-4 protects sequences located between −94 and −76 of the proenkephalin enhancer from DNase I digestion. This region includes ENKCRE-2 but is centered over sequences just downstream of the ENKCRE-2 element defined by SBS analysis, and indicates that AP-4 *in vitro* binds to a site within the human proenkephalin enhancer which overlaps the 3′ end of ENKCRE-2. The amount of AP-4 protein required to give complete DNase I protection of the human proenkephalin enhancer is comparable to the amount required to give complete protection of the SV40 enhancer (data not shown), indicating that AP-4 binds to both enhancers with similar affinity. The *in vivo* enhancer activity of several different ENKCRE-2 point mutants was compared with their ability to bind purified AP-4 *in vitro*. As shown in Figure 8, SBS mutations within ENKCRE-2 at positions −86 and −87 inhibit binding of AP-4, SBS mutations at −88 and −89 (data not shown) exhibit reduced binding, while SBS mutations at positions −90 and −91 (data not shown) have no effect on AP-4 binding as determined by DNase I footprinting. These results indicate that AP-4 makes important contacts with DNA at nucleotides −88 to −86 and it is therefore possible that binding of AP-4 to the enhancer is the critical regulatory event altered by the −87 and −86 enhancer mutations. Because our SBS analysis stopped at position −86 we have not yet assessed the effects of mutations within the 3′ half of the AP-4 binding site on *in vivo* inducible enhancer function and AP-4 binding.

The transcription factor AP-2 binds adjacent to ENKCRE-2

The transcription factor AP-2, affinity purified using the SV40 enhancer, has also been reported to bind to the human proenkephalin enhancer region (Mitchell *et al.*, 1987). Binding of affinity purified AP-2 to the proenkephalin enhancer region was compared with the DNase I protection observed in crude nuclear extracts. As shown in Figure 9, purified AP-2 protects sequences between −85 and −66 from DNase I cleavage, the identical region observed in crude nuclear extracts. Moreover, binding of AP-2 is not affected by the 3′ most SBS mutant −86 (data not shown)

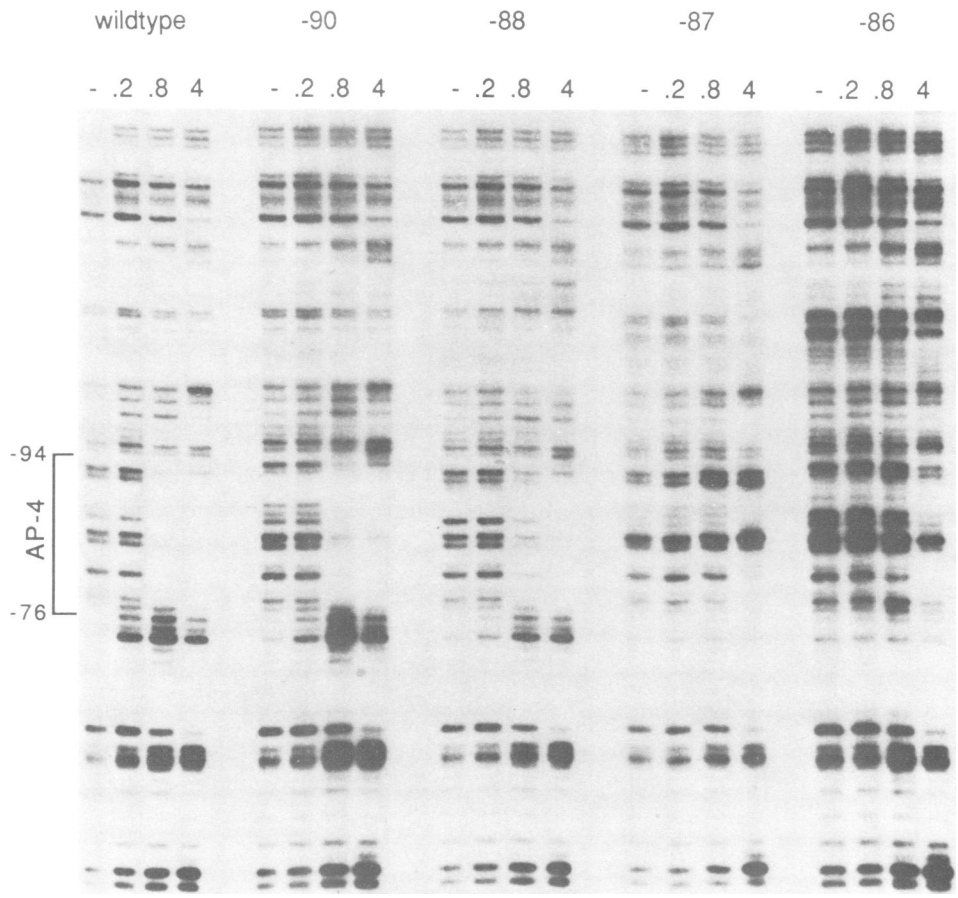

Fig. 8. Binding of affinity purified AP-4 to wild-type and single base substituted proenkephalin enhancers. Binding of affinity purified AP-4 to the proenkephalin enhancer and effects of point mutations within the ENKCRE-2 element on protection of the ENKCRE-2 region by AP-4. The indicated volumes of affinity purified AP-4 (−, 0.2, 0.8 and 4 μl) were incubated with end-labeled DNA probes (labeled on the coding strand at position +1) purified from the wild-type enhancer region and the indicated ENKCRE-2 single base substituted mutants. See Figure 3b and c for the *in vivo* effects of each SBS mutation on cAMP inducible enhancer function. Location of the AP-4 footprint within the proenkephalin enhancer is denoted to the left of the panel.

suggesting that its binding site is located within the C-rich region located between −72 and −78, similar to the proposed AP-2 consensus sequence (Imagawa *et al.*, 1987; Mitchell *et al.*, 1987).

Discussion

Two elements which function synergistically constitute the proenkephalin cAMP inducible enhancer

The identification of a short cAMP and TPA inducible enhancer, located within the 5′-flanking sequences of the human proenkephalin gene, has enabled us to define two functionally distinct elements located within the enhancer. SBS mutations located within either the promoter distal element, ENKCRE-1, or the promoter proximal element, ENKCRE-2, are consistent with the destruction of binding sites for positive-acting factors. Although destruction of the ENKCRE-1 element lowers the response to regulators at least 10-fold, both the Δ97 deletion mutant and various ENKCRE-1 point mutants give a small residual 2- to 3-fold induction in response to either cAMP or TPA. This suggests the presence of a second responsive element, either ENKCRE-2 or elements located downstream of ENKCRE-2. We conclude that both ENKCRE-1 and ENKCRE-2 elements are required for maximal cAMP and TPA transcriptional induction and each has a strong synergistic effect on gene expression. Whereas ENKCRE-1 in the absence of a functional ENKCRE-2 element appears to be incapable of inducible enhancer activity, ENKCRE-2 alone or in conjunction with other downstream elements is capable of augmenting transcription 2- to 3-fold in the absence of ENKCRE-1. Since the enhancer functions when placed upstream of either the POMC or TK promoters (Comb *et al.*, 1986) it seems likely that additional elements specific to the proenkephalin gene are not required, however we cannot rule out the possibility that these promoters share common essential elements.

The ENKCRE-1 and ENKCRE-2 elements are separated by one turn of the DNA helix, suggesting that proteins bound at each site would be located on the same side of the DNA helix, in a position favorable for protein−protein contacts. Experiments reported here indicate that the stereospecific positioning of these elements is an important requirement for inducible enhancer function. When the spacing between elements is increased by 14 bp, a factor bound at ENKCRE-1 is moved to the opposite side of the DNA helix relative to a protein bound at ENKCRE-2, and ENKCRE-1 function is completely eliminated. In contrast, insertion of 10 bp (1 helical turn) between ENKCRE-1 and ENKCRE-2 elements results in a functional enhancer. The effects on the positioning of ENKCRE-1 may be due either to loss of interactions between the ENKCRE-1 and the ENKCRE-2

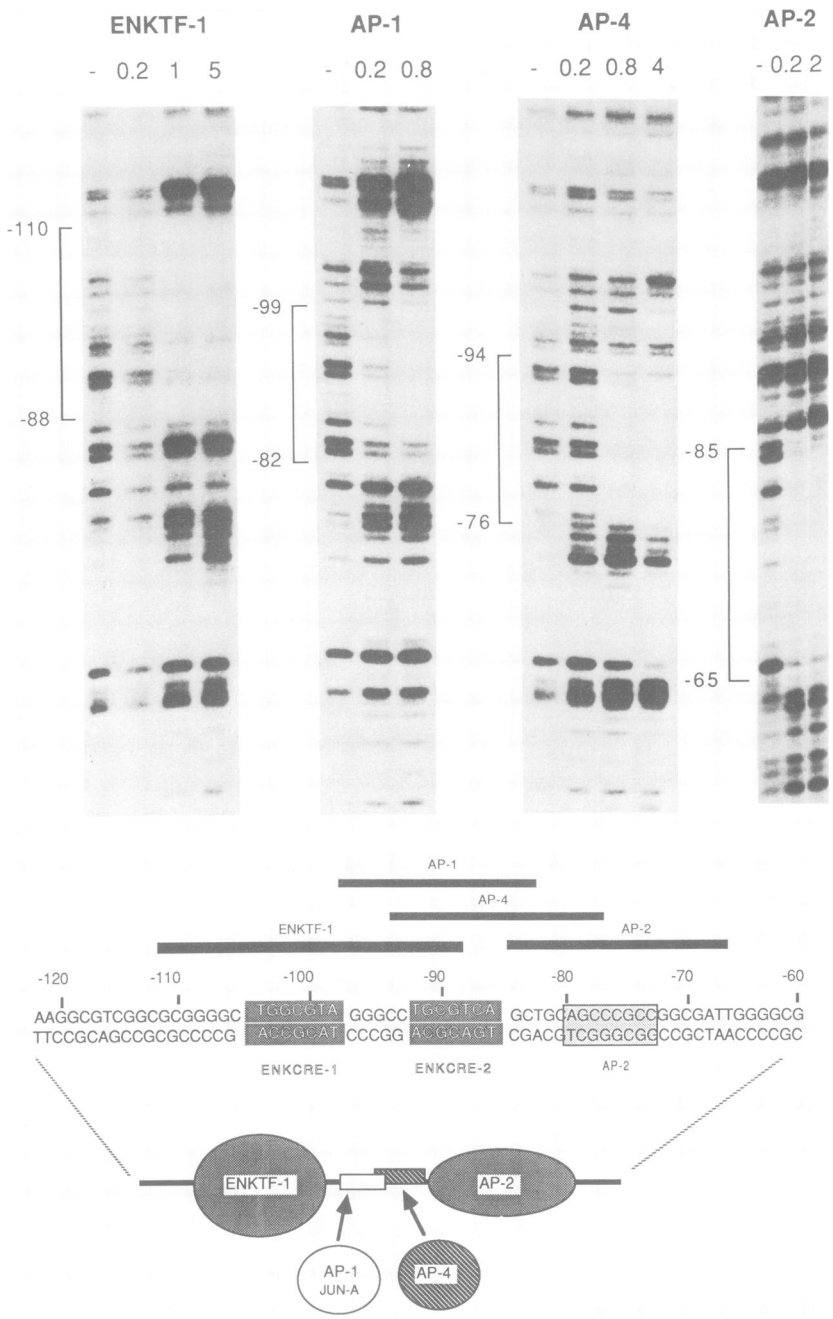

Fig. 9. A schematic illustration of the binding sites of affinity purified ENKTF-1, AP-1, AP-4 and AP-2 to the proenkephalin enhancer. DNase I footprints are shown above and the ENKCRE-1 and ENKCRE-2 elements defined by *in vivo* SBS analysis are illustrated as boxes below.

elements, or between ENKCRE-1 and elements downstream of ENKCRE-2. Similar stereospecific alignment requirements have been demonstrated between the SV40 enhancer and early promoter elements, between glucocorticoid receptor binding sites flanking the tyrosine aminotransferase gene (Jantzen *et al.*, 1987), and between a CACCC-box binding factor and a glucocorticoid receptor binding site of the tryptophan oxygenase gene (Schule *et al.*, 1988). The synergistic interactions between ENKCRE-1 and ENKCRE-2 elements may occur at the level of DNA binding or may be due to protein–protein interactions which enhance transcription but do not affect the affinity of either factor for DNA. The fact that an ENKTF-1 footprint can be observed in the absence of a footprint at the ENKCRE-2 site and AP-1 and AP-4 footprints are observed in the absence of ENKTF-1 footprints, and that binding at either site is not dramatically affected by oligonucleotide binding site competition at the adjacent site (data not shown) suggest that DNA binding is not grossly affected by proteins bound to the adjacent site. However, further analysis is required to establish the level at which these interactions occur.

Analysis of enhancers consisting of multiple tandem repeats also emphasize the importance of the spacing between elements in achieving inducible enhancer function. Multiple tandem copies of the 30 bp oligonucleotide containing both ENKCRE-1 and ENKCRE-2 function in an additive fashion while multiple tandem copies of a 97 bp fragment, which contains both elements flanked by additional DNA, cannot

function additively to enhance induced transcription. These data suggest that the magnitude of the transcriptional response of a given gene depends directly upon both the number of responsive elements and upon their stereospecific alignment. Taken together our *in vivo* analysis indicates that human proenkephalin cAMP and TPA inducible enhancer function involves the synergistic actions of positive acting factors bound at the ENKCRE-1 and ENKCRE-2 elements in the appropriate stereospecific configuration. The basis of the ENKCRE-1 and ENKCRE-2 synergism is not understood but may result from cooperative effects on DNA binding, cooperative protein−protein interactions, or from the recruitment by proteins bound at ENKCRE-1 and ENKCRE-2 of a third protein essential for transcriptional activation.

ENKTF-1 mediates inducible enhancer function

A novel factor, called ENKTF-1, has been affinity purified from HeLa cell nuclear extracts and binds to the promoter distal enhancer element ENKCRE-1. Two lines of evidence suggest that ENKTF-1 plays a critical role in inducible enhancer function. First, deletion and SBS analysis show that the ENKCRE-1 element is essential for inducible enhancer function. Second, point mutations which inactivate ENKCRE-1 function *in vivo* also inactivate binding of ENKTF-1 correlating the *in vitro* binding of this factor with inducible enhancer function.

DNase I footprint experiments provide strong evidence that ENKTF-1 is distinct from transcription factors previously described. First, footprint competition data (S.E. Hyman *et al.*, submitted) using crude HeLa cell nuclear extracts indicate that binding of this factor is not competed by the cAMP regulatory elements of the somatostatin (Montminy *et al.*, 1986), VIP (Tskuada *et al.*, 1987) or fos cAMP regulatory elements (Hyman *et al.*, 1988). Second, binding of this factor to the proenkephalin enhancer is not competed by ATF site located within the E4 promoter (Lee *et al.*, 1987). These experiments demonstrate that ENKTF-1 is not ATF/CREB. Third, purified AP-1 interacts with this sequence only at high protein concentrations, and AP-1 and ENKTF-1 give different DNase I footprints over ENKCRE-1. We therefore conclude that ENKTF-1 is distinct from previously described transcription factors responsive to cAMP or TPA. In addition to HeLa and C6-glioma extracts we have detected similar footprints over the ENKCRE-1 element using extracts prepared from the rat adrenal cell line PC-12 and also from the mouse pituitary AtT-20 cells. We conclude that ENKTF-1 is present in a wide variety of cell types indicating that its expression is not tissue or species specific.

Multiple factors interact in vitro with the ENKCRE-2 region

The sequence of ENKCRE-2 is similar, but not identical to both the proposed binding sites of a nuclear factor, CREB/ATF, implicated in transcriptional regulation of the somatostatin gene by cAMP (Montminy and Bilezikjian, 1987) and adenovirus early genes by E1a (Lee *et al.*, 1987), and to the binding site of the transcription factor AP-1 (Angel *et al.*, 1987; Lee *et al.*, 1987b). The fact that SBS mutations within ENKCRE-2 can either generate ATF/CREB or AP-1 consensus binding sites underscores the similarity of these elements. The CRE element of the VIP or somatostatin gene, which bind ATF/CREB (Montminy and Bilezikjian, 1987; Lin and Green, 1988), when co-transfected with ENKAT-12 into tissue culture cells, competes *in vivo* for positive *trans*-acting factors required for proenkephalin induction by cAMP (Hyman *et al.*, 1988). In support of these observations, an ENKCRE-2 oligonucleotide but not an ENKCRE-1 oligonucleotide competes *in vitro* for binding of a factor to the VIP CRE (Hyman *et al.*, 1988). These data suggest that the proenkephalin ENKCRE-2 element and the VIP CRE bind a common positive acting factor essential for transcriptional activation by cAMP.

Although AP-1 binds the ENKCRE-2 element with high affinity, our analysis suggests that the *in vitro* binding of AP-1 alone to ENKCRE-2 is not sufficient to account for the *in vivo* effects of several ENKCRE-2 SBS mutations. SBS mutations at positions −86, −87, −88 and −89 eliminate both inducible enhancer function and AP-1 binding. However, two other SBS mutations (−90 and −92) bind AP-1 *in vitro* with wild-type affinity, while *in vivo* both mutations strongly inhibit both basal and regulated enhancer function. Furthermore, AP-1 binds with wild-type affinity to the −91 mutant which generates an ATF/CREB consensus binding site, suggesting that both AP-1 and ATF/CREB can bind identical DNA sequences *in vitro*.

Binding of the recently described transcription factor, AP-4, to sequences which overlap ENKCRE-2 suggests that this factor may also play an important role in enhancer function. Location of the AP-4 DNase I footprint, the binding profile of AP-4 to point mutants within ENKCRE-2, and homologies with other AP-4 binding sites suggest that AP-4 binds to the sequence TCAGCTG found at the 3′ end of ENKCRE-2. The overlapping nature of the AP-1 and AP-4 binding sites suggest that these two proteins may not be able to bind simultaneously to the ENKCRE-2 element. Further experiments are necessary to determine the interactions of these two proteins at the ENKCRE-2 site.

AP-2 (Mitchell *et al.*, 1987), the fourth transcription factor interacting with the human proenkephalin enhancer, binds to a site directly adjacent to and downstream of ENKCRE-2. The role of AP-2 binding to the enhancer region was not explored *in vivo* in this paper, since AP-2 binding lies outside of the mutagenized region. However, a role for AP-2 binding in both cAMP and TPA regulation has been suggested by the recent report that AP-2 can mediate transcriptional responses to both cAMP and TPA (Imagawa *et al.*, 1987). Several observations indicate that the binding of AP-2 to the proenkephalin enhancer alone is not sufficient to confer regulated enhancer function. First, we have previously shown that sequences 5′ to the AP-2 binding site are capable of transferring cAMP and TPA regulation to both the POMC and TK promoter, although the possibility that AP-2 interacts with these promoters cannot be entirely ruled out. Second, the pENKAT-Δ84 deletion mutant contains an intact AP-2 binding site yet is unable to functionally respond to either cAMP or TPA. This indicates that if AP-2 binding contributes to cAMP or phorbol ester regulation it must do so in a fashion similar to ENKTF-1, by interacting synergistically with the ENKCRE-1 and ENKCRE-2 elements. Inactivation of AP-2 binding *in vivo* by mutagenesis will help to clarify the role of AP-2 in the response to cAMP and TPA.

At this time the identity and role of proteins interacting *in vivo* with the ENKCRE-2 site is unclear. AP-1 may play

an important role in basal enhancer activity, as it does for hMTII gene expression (Lee *et al.*, 1987a), or it may contribute to transcriptional regulation under circumstances different from those examined in our *in vivo* assay. It is possible that AP-1, *in vivo*, is tightly associated with other proteins that may directly alter the interaction of AP-1 with DNA in the −92 to −90 region, or may directly contact the DNA themselves in this region. These proteins may be lost during purification and hence may alter the binding specificity and affinity observed *in vitro*. Moreover, since the binding activity of AP-1 is modified by TPA (Angel *et al.*, 1987) it is possible that the DNA binding characteristics of AP-1 *in vivo* differ as a result of the state of protein modification. Finally, other factors distinct from AP-1 (Bohman *et al.*, 1987) may interact with the ENKCRE-2 element to mediate inducible enhancer function. The interaction of affinity purified ATF/CREB with wild-type and mutant ENKCRE-2 elements needs to be investigated and will further our understanding of the contribution of this factor to inducible expression.

The results presented here emphasize the need for a careful examination of the role of protein binding *in vitro* to the ENKCRE-2 site with *in vivo* enhancer function to establish a functional correlation between factor binding and inducible enhancer function. The recent observation that multiple distinct proteins can bind identical or very similar DNA recognition sequences (Dorn *et al.*, 1987) and the observation that many proteins which bind DNA regulatory elements *in vitro* are unable to do so *in vivo* (Becker *et al.*, 1987) further underscore the need for caution in the interpretation of *in vitro* DNA binding data.

Transcriptional regulation by second messenger dependent pathways

Transcriptional regulation of the proenkephalin cAMP and TPA inducible enhancer appears to occur by mechanisms different from those described for other genes regulated by cAMP or TPA. First, at least two different DNA elements, which must function in concert, are required for inducible transcription, whereas cAMP regulation of the somatostatin, VIP and glycoprotein alpha subunit genes appear to require only an ATF/CREB binding element. Second, the response of the proenkephalin gene to TPA requires the presence of phosphodiesterase inhibitors (Comb *et al.*, 1986) or simultaneous activation of the cAMP pathway, and hence is different from that of the collagenase or SV40 enhancers which show large transcriptional effects which appear to be independent of the cAMP pathway (Imbra and Karin, 1986; Angel *et al.*, 1987). The complex interactions between the cAMP and phorbol ester pathways on proenkephalin transcription may be due in part to the interdependence of transcriptional activation upon the ENKCRE-1 and ENKCRE-2 elements and the proteins with which they interact. Since inactivation of either ENKCRE-1 or ENKCRE-2 produce indistinguishable effects on either cAMP or TPA induced transcription, it appears that signals transduced through these pathways do not act through distinct cAMP and TPA inducible elements but rather appear to modify the activity of the enhancer as a whole. Clearly a major challenge is to define the pathways and molecular mechanisms interposed between protein kinase activation and inducible enhancer function. Identification of the DNA elements and transcription factors which receive and mediate signals transduced through these pathways will increase our knowledge of these mechanisms and will further our understanding of the long-term regulation of neurotransmitter signalling.

Materials and methods

Materials

All restriction and DNA modifying enzymes used in the construction of plasmids and S1 and footprinting probes were from New England Biolabs. [^{14}C]Chloramphenicol was from Amersham and Butyrl-CoA was from Pharmacia.

Cell culture

C6-glioma and CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). PC-12 cells were grown in DMEM supplemented with 10% FCS and 5% horse serum. HeLa cells were grown in spinner culture using Joklik modified Eagle's medium supplemented with 7.5% horse serum.

Transient and stable analysis of gene expression

Transfection of CV-1 cells was performed as previously described (Comb *et al.*, 1986). For transient expression CAT plasmids (5 μg) were co-transfected with 10 μg pRSVBGAL (Edlund *et al.*, 1985) and 10 μg pUC18. Following transfection with $CaPO_4$ precipitated DNA and glycerol shock, cells were incubated for 16 h in media containing 10% FCS. Cells were induced by incubation with forskolin (25 μM) and IBMX (0.5 mM) for 6 h, and harvested for protein extracts. CAT activities were measured by a modification of the standard assay (Seed and Shen, 1988). Butyrl-CoA (3 mM) was used instead of acetyl-CoA and reaction products were extracted with 400 μl of a 2:1 mixture of pristane/xylenes instead of TLC chromatography. Under these conditions butyrlated chloramphenicol was preferentially extracted into the organic phase and 300 μl was counted directly in scintillation fluid. β-Galatosidase activities were determined as previously described (Comb *et al.*, 1986) and used to normalize CAT activities within a given experiment. Stably expressing cell lines were produced by $CaPO_4$ co-transfecting 8×10^5 C6-glioma cells in a 10-cm dish with 4 μg pRSVNEO (Gorman *et al.*, 1983) and 20 μg indicator CAT plasmid. One day following transfection cells were transferred to selective media (500 μg/ml G418) and grown for 2 weeks. Under these conditions of co-transfection between 25 and 50% of the G418 resistant colonies express the non-selected CAT plasmid. Independent G418 resistant colonies (800−3000) from each transfection were treated with trypsin and pooled into two plates containing identical numbers of cells. Cells were induced by incubation with forskolin (25 μM) and IBMX (0.5 mM) for 2−4 h and harvested for analysis of RNA. Total cytoplasmic RNA was isolated by lysis of cells with 0.5% NP-40, removal of nuclei by centrifugation (13 000 r.p.m. in microfuge), multiple phenol/chloroform extractions and ethanol precipitation of RNA. Samples (40 μg) of RNA from control and cells treated with regulators were hybridized with a single-stranded DNA probe spanning hENK sequences −193 to +70, treated with S1 nuclease as previously described (Comb *et al.*, 1986), loaded onto 8% sequencing gels and exposed to film for 16−48 h in the presence of an intensifying screen.

Production and analysis of SBS mutants

Each of the four nucleotide solutions used in the synthesis of each single-stranded oligonucleotide contained 1.5% of the other three nucleotides, resulting in random SBS at a 1:20 base frequency. The double-stranded 34mer synthesized in such a fashion contained on the average 1.5 changes for each single strand or three substituted bases per double strand (Hutchison *et al.*, 1986). After cloning the random collection of mutant double-stranded oligonucleotides into the *Sac*I/*Pst*I sites of pENKAT-84, each amplified plasmid should contain 1.5 changes on average, close to what was found by DNA sequencing. Approximately 20 SBS mutants were identified by DNA sequencing. The remaining SBS mutants were synthesized as seven different double-stranded oligonucleotides each containing two randomly substituted adjacent bases. Each of these oligos was cloned into the −84 vector and SBS mutants were again identified by DNA sequencing. DNA from each SBS mutant was purified and transfected into CV-1 for transient CAT analysis, and transfected into C6-glioma cells for stable RNA analysis. The effect of SBS on basal and regulated transcription *in vivo* was determined by treating the transfected cells with forskolin and phorbol esters as described above.

Preparation of nuclear extracts and affinity purified proteins

Small scale nuclear extracts were prepared from 2−5 g of C6-glioma, HeLa,

PC-12 or AtT-20 cells as described by Dignam *et al.* (1983). Large scale nuclear extracts were prepared from HeLa S3 cells (Dignam *et al.*, 1983) grown in spinner culture in 7.5% horse serum. Purification of ENKTF-1 by affinity chromatography was carried out as described by Kadonaga and Tjian (1986) using a synthetic oligonucleotide spanning human proenkephalin sequences between −110 and −85. A detailed description of the purification of this protein will be described elsewhere. Affinity purification of AP-1 was as described by Lee *et al.* (1987) and affinity purification of AP-4 was carried out as described by Mermod *et al.* (1988).

DNase I footprinting

DNase I footprinting was carried out as described by Lee *et al.* (1987). Typically 10 fmol of ^{32}P-labeled DNA probe (end-labeled on the coding strand at position +1 by the 'filling in' reaction using [α-^{32}P]dCTP) was incubated on ice for 15 min with crude nuclear extract in the presence of 1 μg poly(dI−dC) or with purified proteins in the absence of competitor DNA. Samples were then treated with DNase I for 1 min at 25°C. Samples were phenol/chloroform extracted, ethanol precipitated and analyzed by 7% acrylamide−urea gel electrophoresis.

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