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NOTES

An AP-2 Element Acts Synergistically with the Cyclic AMP- and Phorbol Ester-Inducible Enhancer of the Human Proenkephalin Gene

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An enhancer with two DNA elements, one containing the sequence CGTCA, is required for cyclic AMP- and phorbol ester-inducible transcription of the human proenkephalin gene. We report that an AP-2 element located adjacent to the enhancer acts synergistically with it to confer maximal response to cyclic AMP and phorbol esters.

Cyclic AMP (cAMP) and diacylglycerol act as second messengers in important signal transduction pathways regulating many cellular functions, including gene expression (4). For example, expression of the gene encoding the endogenous opioid peptide precursor proenkephalin has been shown to be inducible by cAMP and phorbol esters (3). A cAMP- and phorbol ester-inducible enhancer has been identified within the 5′-flanking sequences of the human proenkephalin gene spanning nucleotides −114 to −84 with respect to the mRNA cap site (3; M. Comb, N. Mermond, S. E. Hyman, J. Pearlberg, M. E. Ross, and H. M. Goodman, EMBO J., in press). The enhancer contains two elements which bind distinct *trans*-acting factors (8; Comb et al., in press). An additional element extending from bases −78 to −70 binds AP-2, a 52-kilodalton protein which also binds enhancer regions of the simian virus 40 (SV40) and human metallothionein IIA (hMT-II_A) genes (13). It has recently been shown that five copies of a synthetic oligonucleotide encoding the hMT-II_A AP-2-binding sequence confer activation by both cAMP and phorbol esters on the human beta-globin gene (9). Since deletion of the proenkephalin gene 5′ to base −84 retains the AP-2 element but results in loss of both basal and induced transcriptional activity, this element alone is not sufficient to produce an active promoter (3).

We studied the functional properties of the proenkephalin AP-2 element by comparing basal and induced levels of chloramphenicol acetyltransferase (CAT) activity produced by pENKAT-Δ114, a plasmid containing wild-type proenkephalin sequences, with those produced by pENKAT-Δ114M, a plasmid in which the AP-2 element has been mutated (Fig. 1). pENKAT-Δ114 contains human proenkephalin gene sequences from bases −114 to +154 fused to CAT coding sequences. This includes the entire cAMP- and phorbol ester-inducible enhancer and the AP-2 element. When transfected into rat C6 glioma cells and monkey CV-1 cells, this plasmid is transcriptionally activated by cAMP agonists, such as forskolin. Active phorbol esters, such as 12-*O*-tetradecanoyl-phorbol-14 acetate (TPA), produce only small independent transcriptional effects but produce a strong response in the presence of agents which increase intracellular levels of cAMP, such as the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IMX), even at concentrations of IMX that produce no independent effect on transcription (Comb et al., in press; S. E. Hyman, M. Comb, J. Pearlberg, and H. M. Goodman, submitted for publication). In addition, TPA increases the response to forskolin and IMX by about twofold and can produce responses greater than can be obtained with maximally effective doses of forskolin and IMX alone (Hyman et al., submitted).

In pENKAT-Δ114M, six bases (−73 to −78) of the wild-type proenkephalin AP-2 element have been replaced by a *Bgl*II restriction site (Fig. 1). This disrupts the AP-2 recognition site without altering the position of the cAMP- and phorbol ester-inducible enhancer with respect to the TATA box (confirmed by DNA sequencing [2]). Plasmids were also constructed in which proenkephalin 5′-flanking sequences were transferred to the herpes virus thymidine kinase (HSV TK) promoter. By using linkers, the DNA spanning bases −114 to −62 of either pENKAT-Δ114 or pENKAT-Δ114M was cloned between the unique *Bam*HI and *Hin*dIII sites upstream of the HSV TK promoter in the CAT fusion plasmid pBLCAT-2 (gift of B. Luckow) (11). Plasmids were transfected into CV-1 cells by $CaPO_4$ precipitation (7). To control for transfection efficiency, CAT plasmids were cotransfected with RSV-βGal (6), which expresses β-galactosidase activity under the control of the uninducible Rous sarcoma virus promoter. Drug treatments were performed as previously described (8; Comb et al., in press). The oligonucleotide was synthesized on an Applied Biosystems DNA synthesizer; enzymes and linkers were purchased from New England Biolabs.

Mutation of the AP-2 element within pENKAT-Δ114 decreased both basal and induced CAT expression (Fig. 2). Basal expression was reduced to approximately 50% of wild-type basal expression (five separate experiments). cAMP (forskolin and IMX) response was diminished four-

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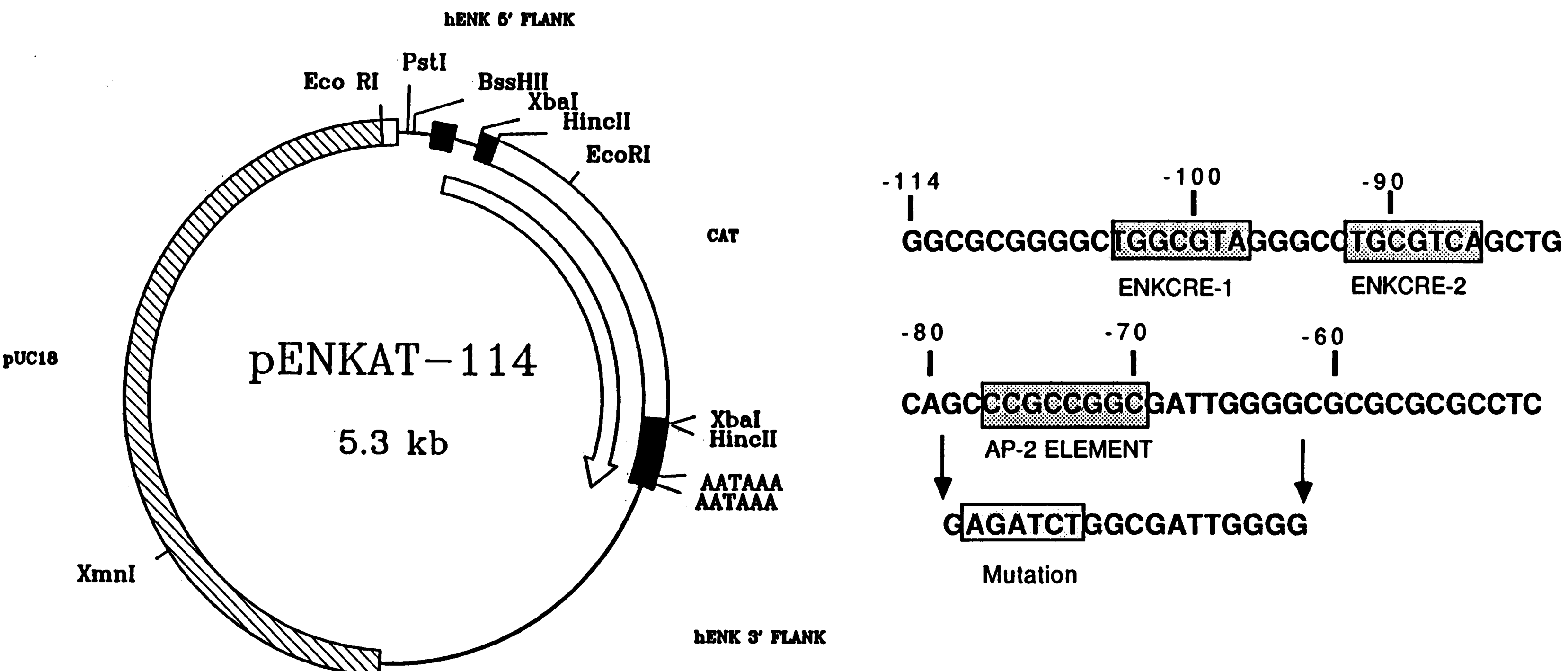

FIG. 1. (Left) Structure of pENKAT-Δ114. Thick black bars represent noncoding exons from the human proenkephalin gene; thin black lines represent regulatory sequences, introns, and 3'-flanking sequences from the proenkephalin gene; open boxes represent chloramphenicol acetyl transferase-coding sequences; diagonal lines represent pUC18 sequences. (Right) Human proenkephalin gene 5'-flanking sequences spanning bases −114 to −50, showing the two elements of the cAMP- and phorbol ester-inducible enhancers (ENKCRE-1 and ENKCRE-2) and the AP-2 element. In the mutant pENKATΔ-114M, the *Bss*HII- *Pst*I fragment which contains the AP-2 recognition sequence (boxed) was replaced with a synthetic oligonucleotide which disrupted the sequence (arrows).

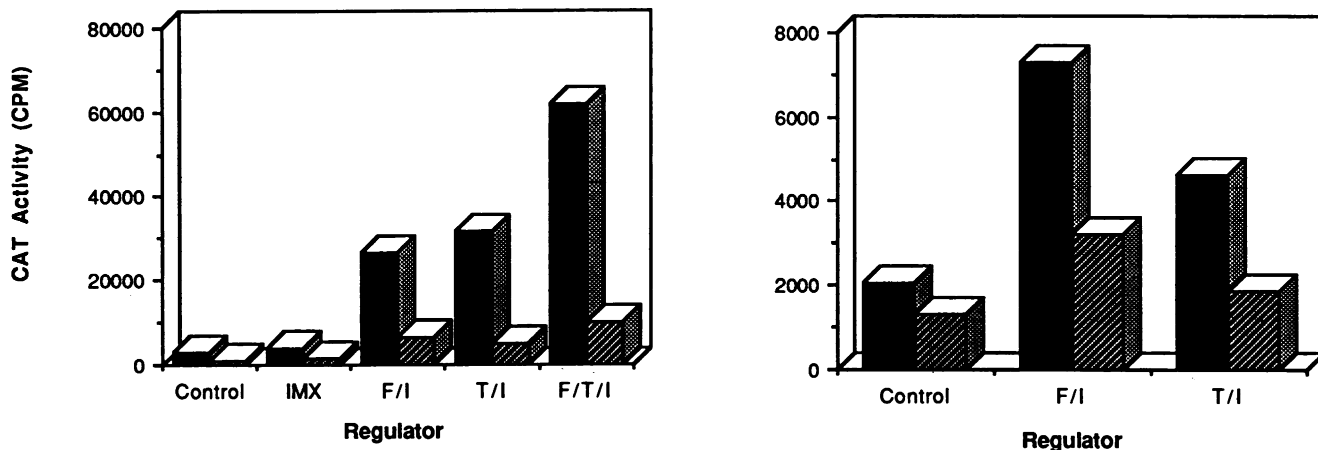

FIG. 2. (Left) Basal and induced expression of CAT activity by transient transfection analysis of pENKAT-Δ114 (solid bars) and pENKAT-Δ114M (hatched bars) in CV-1 cells. The data are normalized to β-galactosidase activity. Twenty-four hours after transfection, medium containing 10% fetal calf serum was replaced by medium containing 0.1% fetal calf serum and regulators were added. IMX (I) was used at 0.5 mM, forskolin (F) was used at 10 μM, and TPA (T) was used at 50 nM. After 6 h of incubation, cells were harvested, and CAT and β-galactosidase assays were performed as described previously (6, 17). (Right) Basal and induced expression of CAT activity by transient transfection analysis of proenkephalin-HSV TK promoter fusions in the expression vector pBLCAT-2 (11) in CV-1 cells. The plasmid with a wild-type AP-2 element (solid bars) is compared with a plasmid that has a mutated element (hatched bars). Regulator treatment and cell harvesting were done as described for the left panel. Note that the scale for CAT activity is 10-fold smaller in this panel.

fold; the effect of TPA and IMX was diminished fivefold; and the effect of all three regulators in combination was diminished nearly sixfold. The function of the AP-2 element was confirmed by transfer of the enhancer with either the wild-type or mutated AP-2 element to the cAMP-unresponsive HSV TK promoter (Fig. 2) within pBLCAT-2 (11). Without additional sequences, pBLCAT-2 gave no response to cAMP or phorbol esters. Transfer of the proenkephalin enhancer and wild-type AP-2 element (proenkephalin bases −114 to −62) to pBLCAT-2 gave a threefold greater response to forskolin and IMX and a fourfold greater response to TPA and IMX than transfer of the intact enhancer with the mutated AP-2 element. The wild-type proenkephalin fragment reproducibly conferred greater cAMP and phorbol ester responsiveness on its own promoter than on the TK promoter. This suggests either that the position of the proenkephalin sequences in the TK fusion (25 bases farther upstream of the TATA box than in the proenkephalin gene) diminishes their ability to function or that other elements exist downstream of base −62 within the proenkephalin promoter that further stimulate transcription. DNase I footprinting (5; Comb et al., in press) of fragments from pENKAT-Δ114 and pENKAT-Δ114M by affinity-purified AP-2 (10, 13) (kindly provided by Trevor Williams) confirmed that AP-2 protected the wild-type proenkephalin sequences between nucleotides −85 and −65, but not the mutated sequences (Fig. 3).

The mechanism by which cAMP and phorbol esters regulate transcription in eucaryotic cells now appears increasingly complex. The previously described cAMP- and phorbol ester-inducible enhancer within the proenkephalin gene contains two elements which bind distinct *trans*-acting factors (Fig. 1) (Comb et al., in press). The promoter-distal element ENKCRE-1 binds a factor which we have called ENKTF-1 (Comb et al., in press). The ENKCRE-2 element (Fig. 1), which contains the CGTCA sequence common to cAMP response elements in a variety of genes (8, 15, 18, 19), can bind multiple different factors, including AP-1 (which has previously been reported to confer phorbol ester responsiveness on the collagenase gene [1], AP-4 (Comb et al., in

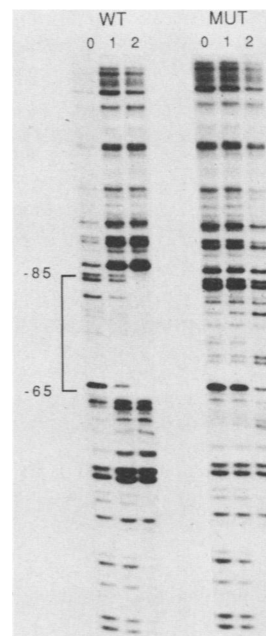

FIG. 3. DNase I footprinting of *AvaI-HaeII* fragments (extending from a site in the vector to proenkephalin base +1) of pENKAT-Δ114 and pENKAT-Δ114M. Fragments were labeled at the 5′ end and footprinted with purified AP-2. The amount of AP-2 used was none (lanes 1 and 4), 1 μl (lanes 2 and 5), or 2 μl (lanes 3 and 6). The probe in lanes 1–3 contained the wild-type AP-2 element and was protected by AP-2 from bases −85 to −65. The probe in lanes 4–6 contained the mutated element and was not protected by AP-2. A slight degree of protection seen at the bottom of the gel in the lanes with the highest concentration of protein (lanes 3 and 6) was not reproducible and appeared to represent a nonspecific interaction, perhaps due to interaction of the protein with the end of the probe. WT, Wild type; MUT, mutation.

press), and probably CREB (8, 14) (which was originally purified by its affinity for the somatostatin cAMP response element TGACGTCA [14]). It is not yet clear which of these factors, if any, is the positive activator of proenkephalin transcription. The present data demonstrate that the single AP-2 element within the proenkephalin gene acts in concert with these two other elements to produce maximal responsiveness to cAMP and phorbol esters. The ENKCRE-2 element is absolutely required for basal and regulated transcription of the proenkephalin gene. Lack of an intact ENKCRE-1 element decreases cAMP and phorbol ester response 10-fold (Comb et al., in press); it now appears that lack of an intact AP-2 element decreases response to cAMP fourfold and to phorbol esters plus IMX fivefold. Synergistic interactions have previously been reported for elements involved in SV40 late transcription (AP-1 and AP-4 elements) (12) and for a glucocorticoid receptor-binding site and a CACCC box in the rat tryptophan oxidase gene (16). Synergistic interactions may be the result of positive cooperativity in binding of proteins to the DNA, interactions of the non-DNA-binding domains of the proteins, or recruitment of additional proteins into the transcription complex.

In addition to demonstrating the synergistic interaction of three DNA elements within the human proenkephalin gene, these results also demonstrate that the cAMP and phorbol ester responses converge on all three elements, i.e., no one of these elements appears to account for the response to a single second messenger. The modular arrangement of various sequence elements permits enhancers to confer highly regulated programs of expression on the genes to which they are linked. This mechanism is well illustrated by elements which are involved in cAMP- and phorbol ester-stimulated gene expression.

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