Oncogenic Conversion Alters the Transcriptional Properties of ets

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Abstract
The v-Ets oncprotein and its progenitor cEts1(p68) belong to a growing family of transcription factors that are related by the conserved ets domain. We show here that the ets domain and adjacent COOH-terminal amino acids are required for DNA binding by cEts1(p68). v-Ets differs from cEts1(p68) in both the COOH-terminal sequence and an amino acid substitution in the ets domain. The change in the COOH-terminal sequence markedly decreases its affinity for specific DNA, and the ets domain mutation further diminishes binding. v-Ets does not trans-activate through the ets (PEA3) motif in vivo. Surprisingly, v-Ets still efficiently trans-activates the promoters of two genes, stromelysin and collagenase, that are found to be overexpressed in transformed cells. The AP1 motifs of both promoters are required for efficient activation. v-Ets does not bind to the AP1 motif, even in the presence of cJun and cFos. The DNA-binding domain of Ets1 is required for activation through the AP1 element. Activation is inhibited by the expression of the glucocorticoid and retinoic acid receptors, suggesting that activation by Ets does not involve reversal of negative regulators of AP1. We suggest that activation is by an indirect mechanism involving activation of endogenous genes. Our results show that v-Ets differs from its progenitor cEts1(p68) in its trans-activating properties. The findings suggest that activation of the Jun and Fos oncprotein pathway is important for transformation by Ets.

Introduction
Oncotranscription factors appear to be key mediators of cell transformation by a variety of nonnuclear oncogenes (reviewed in Ref. 1) since, for example, a trans-dominant mutation in the transcription factor Jun reverts ras-transformed cells (2). It is important to understand how these factors regulate gene expression in both normal and transformed cells. We have previously shown that Ets1, Ets2, Jun, and Fos mediate transcription activation by several nonnuclear oncogenes (3). We have dissected Ets to understand how its properties are altered by oncogenic conversion.

CEts1(p68)2 is the progenitor of v-Ets, which is expressed as a fusion protein with vMyb by the avian leukemia virus E26 (4, 5). The v-Ets oncogene transforms erythroblasts and fibroblasts and affects myeloid transformation by v-myb (6–8). It belongs to a gene family that includes ets1, ets2, erg, elk-1 and -2, pu1 (sp1), e74, iil-1, and gabpa (1, 9–12). The activities of Ets1 and Ets2 are probably regulated by both pre- and posttranslational mechanisms, since their synthesis is induced upon growth stimulation of mouse fibroblasts by serum (13), and they are phosphorylated in response to mitogenic signals (14, 15).

Transcription factors form a vital link between specific DNA motifs in promoters and the general transcription machinery. They are composed of several functional domains, involved in activation or DNA binding (16). Activation domains interact either directly with the general transcription machinery, or through bridging factors called adapters or coactivators (see, for example, Refs. 17–19). We have previously shown that there are two independent activation domains in cEts1(p68) and cEts2. Sandwiched between these domains, there are moderately conserved sequences that may regulate the activation domains (20). Various types of DNA-binding domains have been identified (21). The ets DNA-binding domain contains a distinct conserved “ets domain” and recognizes the purine-rich core motif A/CGGAA (1, 11).

We show that the ets domain is not sufficient for DNA binding by cEts1; COOH-terminal sequences are also important. The altered COOH-terminal sequence of v-Ets inhibits DNA binding. v-Ets does not trans-activate efficiently through the ets (PEA3) motif but retains the ability to stimulate through AP1 motifs by an indirect mechanism. These studies provide important insights into transcription activation by an oncogenic member of the ets gene family.

Results
The DNA-binding Domain of cEts1 Encompasses the ets Domain and COOH-terminal Nonconserved Sequences. Deletion mutants of cEts1(p68) were synthesized in rabbit reticulocyte lysates, and specific DNA binding activity was analyzed by band-shift (Fig. 1A) with a specific probe (PEA3) (22). The quality and quantity of protein were controlled by SDS-PAGE (Fig. 1B). DNA binding was abolished by the NH2-terminal deletion from amino acid 374 (N70) to 382 (N69) (Fig. 1A, Lanes 1–5; 1B, Lanes 1′–5′; and 1C for structures). It was greatly decreased by

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2 The abbreviations used are: cEts1(p68), chicken Ets1 (p68) (other proteins are similarly designated); h-2, human; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; N, nucleotide; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase.
COOH-terminal deletion to 478 (N70-AatII) and abolished by further deletion (Fig. 1A, Lanes 7–10; 1B, Lanes 7'–10'; and 1C). COOH-terminal deletion to 478 of full-length cEts1(p68) also reduced binding (data not shown). These results show that both the cts domain and COOH-terminal nonconserved sequences are required for specific DNA binding by cEts1.

The COOH-terminal Sequence of vEts Inhibits DNA Binding. The COOH-terminal sequence of vEts is different from cEts1(p68) (473–485 LHAMLVDKPDADE is changed to HSSASGLTSSMACSSF in vEts). This alteration greatly decreased complex formation between the DNA-binding domain of Ets and a specific probe (compare N70 to N70-vt, Fig. 2A, Lanes 1 and 2). An additional mutation, in a highly conserved amino acid of the cts domain (445, isoleucine to valine), decreased DNA binding even further (vN70, Fig. 2A, Lane 3; the N70-vt complex is visible on an autoradiogram exposed for a longer period). Consistent with these results, DNA binding by vEts (the cts part of the E26 gag-myb-ets fusion protein; see Fig. 2) was inhibited compared to cEts1(p68) (see Fig. 2B, Lanes 1 and 4, and Fig. 2C, Lanes 1 and 2) and was hardly detectable above background (Fig. 2B, Lanes 2–5). vEts is produced as a fusion protein with vMyb by the E26 virus (Refs. 4 and 5; see Fig. 2). vMyb-Ets did not form a detectable complex (Fig. 2B, Lanes 6 and 7); the upper complex is present in the reticulocyte lysate control, Lane 2. No additional bands compared with the control lane were observed in any part of the Myb-Ets band-shift. Similar results were obtained with two variants of the wild-type probe 5’-TGGACGAGAATGCAGTCA-3’ and 5’-TGGAGCCCGGAAGTGACGTCGA-3’). We cannot exclude that vMyb-Ets migrates with the endogenous activity. However, the intensity of this band does not detectably increase (compare Fig. 2, Lanes 2 and 6), excluding the possibility that DNA binding activity is restored to the level of cEts. Control experiments showed that the Ets proteins were efficiently synthesized in the reticulocyte lysates (data not shown). These results show that the fusion of vEts with vMyb does not restore binding to the PEA3 motif. Extracts from COS cells transfected with expression vectors for cEts1(p68), vEts, and vMyb-Ets gave similar results to reticulocyte lysates (data not shown). These data, together with the deletion analysis, show that sequences outside and COOH-terminal to the cts domain are important for DNA binding. They also show that vEts binds less efficiently than its progenitor cEts1(p68) to the PEA3 (cts) motif.

vEts Does Not Efficiently Activate Transcription through the ets-responsive Element of the Stromelysin Promoter. Transcription activation by vEts was studied in
HeLa cells by cotransfection of expression vectors with a reporter containing the PE3 motif from the stromelysin promoter (pBLCAT4-PAL) and the control pBLCAT4 lacking the PE3 motif (Fig. 3C; Ref. 22]). An internal control was included to correct for variations in transfection efficiency (see "Materials and Methods"). We consistently found that vEtS (see Fig. 2 for structure) was much less efficient than cEtS1(p68) at activating transcription (Fig. 3, Lanes 1–20), even when another reporter containing PE3 (ets) motif (pBLCAT4-B4) (3) is used (data not shown). Immunoprecipitation experiments confirmed that vEtS was efficiently synthesized (data not shown, and see below). These results show that vEtS is a poor activator of transcription through the PE3 (ets) motif, in keeping with its low affinity for this sequence.

**vEtS Activates the Stromelysin Promoter.** We investigated the possibility that vEtS retains an ability to regulate transcription, using promoters that are overexpressed in transformed cells such as stromelysin and collagenase.

**vEtS expression stimulated transcription from reporters containing 1100, 550, or 209 base pairs of the stromelysin promoter (Fig. 4A, Lanes 1–3; 4B; and 4C for structures of reporters, and results not shown). Point mutation of the ets-responsive element had little effect on activation by vEtS (compare Lanes 4–6 with Lanes 1–3) but decreased activation by cEtS1(p68) (Lanes 7–12), in keeping with the results described above. vEtS was as efficient as cEtS1(p68) at activating the mutated 209 reporter (209-MUT; compare Lanes 4–6 and Lanes 10–12). Indeed, in certain experiments with high amounts of expression vector, vEtS was even more efficient than cEtS1(p68) (see below). These results confirm that poor activation by vEtS through the PE3 motif is not simply due to inefficient synthesis.
The Stromelysin AP1 Motif Linked to the TATA Element Is Sufficient for Activation by vEts. We localized the vEts-responsive element using various mutants. Deleting the promoter to −121 or −84 did not significantly affect activation by vEts and cEtsl(p68) (data not shown). Similarly, reporters with the −89/−48, −79/−48, or −72/−48 sequences linked to the TATA element (−33/+8) were efficiently activated (data not shown), indicating that the deleted sequences, which resemble the ets core motif, could not account for the stimulation. The remaining 32-base pair element contains a consensus AP1 motif and a related sequence (indicated by continuous and broken arrows, respectively, in Fig. 5C). Mutation of the nonconsensus sequence did not affect activation (Fig. 5A, compare m6a, m7a, Lanes 1–6 and Lanes 13–16; see also Fig. 6, B and C). In contrast, mutation of the consensus motif reproducibly decreased activation [Fig. 5A, m8a; Fig. 6A, Lanes 7–9, 17, and 18 (the signal was detectable on longer exposure of the autoradiogram); Fig. 6B]. Note that with 10 μg of expression vector, but not with 1 μg, a residual low level of activation was still observed with m8a and the minimal promoter construct 5 (stromelysin sequences −33/+8; compare Fig. 5A, Lanes 17–20 with Lanes 7–12; see Fig. 6B). Titration experiments, in which the quantity of expression vector was varied between 1 and 10 μg, show that activation through the AP1 motif can be quite large in the absence of an effect on the TATA element (data not shown, and see below). These results indicate that the AP1 motif is required for activation by vEts and cEtsl(p68).

vEts Activates the Collagenase Promoter through the AP1 Motif. We extended our studies to a second pro-

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**Fig. 5.** A point mutation in the AP1 motif of the stromelysin promoter decreases activation by vEts. HeLa cells were transfected by the calcium phosphate technique with 1 or 10 μg of vectors (VEC) that express vEts (V) or cEtsl(p68) (C). **A, B,** CAT assays. **C,** bar graph. ACTIVITY, CAT activity, taking into account variations in the internal control, expressed in arbitrary units. C, structure of recombinants. Arrows, the AP1 motif; broken arrows, a similar sequence.

**Fig. 6.** The −73/−42 region of the collagenase promoter is required for efficient activation by vEts and cEtsl(p68). HeLa cells were transfected by the calcium phosphate technique with 2–8 μg (Lanes 2–5, 7–10, 12–15, and 17–20) of vectors (EXP.VEC.) that express vEts or cEtsl(p68), 5 μg internal control, and 5 μg reporters. A, CAT assays. **B,** bar graph. ACTIVITY, CAT activity, taking into account variations in the internal control, expressed in arbitrary units. C, structure of recombinants. The reporter contains the −73/−42 collagenase promoter (COL) sequence upstream from the thymidine kinase (TK) promoter of pBLCAT4 (CAT4).
moter that is activated by cell transformation. Initially, we found that vEts and cEts1(p68) expression efficiently stimulated reporters containing collagenase promoter sequences from −517 to +63 or −73 to +63, but not a similar recombinant lacking the promoter (data not shown). The −73/−42 sequence linked to the TK promoter was also efficiently stimulated (Fig. 6A, Lanes 1–5 and 11–15; 6B; and 6C). The TK promoter was not stimulated with the lower amounts of ets expression vector (see 2, 4, and 6 μg, Fig. 6A, Lanes 6–9 and 16–19; 6B; and 6C). However, with larger amounts of expression vector, we consistently observed a small effect on the control reporter (Fig. 6, Lanes 6 and 10; see below for cEts1) that was abolished by deleting the TK promoter. A similar effect was seen with the minimum stromelysin promoter (see above). The only homology between the −73/−42 collagenase and −72/−48 stromelysin sequences is the AP1 motif, indicating that it is required for efficient activation. Indeed, a reporter containing five copies of the collagenase AP1 motif was stimulated by Ets (data not shown).

vEts Does Not Bind to the AP1 Motif. vEts may have acquired an affinity for the AP1 motif. To investigate this possibility, we synthesized vEts in reticulocyte lysates and performed band-shift experiments with the AP1-containing sequence of the stromelysin promoter. vEts did not detectably bind to this sequence (compare Fig. 7, Lanes 2 and 3; control experiments showed that vEts was efficiently synthesized in the reticulocyte lysates). Similarly, vEts synthesized in COS cells from a transfected expression vector did not bind to this sequence, suggesting that the lack of binding was not due to an inhibitor specifically present in reticulocyte lysates (data not shown). As expected, cJun and cFos did form a complex with the stromelysin AP1 motif (Fig. 7, Lane 1). The mobility of this complex was not affected by the addition of vEts (Fig. 7, Lane 4), or vEts followed by Ets-specific antibodies (data not shown). No additional complexes were observed using lower concentration acrylamide gels, excluding the possibility that a very large complex formed that did not enter the gel. These results suggest that vEts does not form a complex with cFos and cJun.

The DNA-binding Domain of Ets Is Required for Activation through the AP1 Site. Activation by Ets through the AP1 element does not involve DNA binding and thus may not require the DNA-binding domain. 3′ deletion mutants of cEts1(p68) (Δ31, Δ32; see Fig. 8C for structure) that do not bind to the PEA3 element (data not shown, and see above) were found not to activate either 209-WT, containing both the ets-responsive palindrome and the AP1 site of the stromelysin promoter (Fig. 8A, Lanes 1–4; 8B), or m6a with only the AP1 element (Fig. 8A, Lanes 5–8). Control experiments showed that the deletion mutants were efficiently expressed (data not shown). These results show that the DNA-binding domain is required for activation through the AP1 motif.

Glucocorticoid and Retinoic Acid Receptors Repress Ets Activation through the AP1 Site. Ets may activate through the AP1 element by relieving repression, such as that mediated by the glucocorticoid and retinoic acid receptors (23–25). Previous studies (23) have shown that both the glucocorticoid receptor and the retinoic acid receptor α inhibit the stromelysin promoter through the AP1 site (Fig. 9, Lanes 1–3). Expression of these receptors efficiently repressed both vEts- and cEts1(p68)-mediated activation through the AP1 motif of m6a (Fig. 9, Lanes 4–9; and see Fig. 5 for the structure of m6a). As expected, they did not inhibit activation by cEts1(p68) through the ets-binding sites of the stromelysin promoter (data not shown). These results suggest that Ets does not activate through AP1 motifs by relieving repression.

Discussion

We have precisely delimited the specific DNA-binding domain of cEts. The NH2-terminal boundary is the beginning of the ets domain, which corresponds to a splice junction in hEts1 (amino acid 331) (26) as well as in another member of the ets gene family, PU1(amin acid 161) (27). However, the DNA-binding domain of hEts1 is encoded by two exons and that of PU1 is encoded by one, suggesting that exon organization is not simply related to this function. Surprisingly, COOH-terminal sequences beyond the ets domain are required for DNA binding. The COOH-terminal 27 amino acids are very similar in Ets1 and 2 (76% identity), consistent with their ability to bind tightly to the same DNA motif (3).* The

* C. Wasylyk and B. Wasylyk, unpublished results.
Transcriptional Properties of vEts and cEts

**Fig. 8.** Concurrent loss of activation through ets and AP1 motifs due to 3' deletion of cEts1(p68). HeLa cells were transfected by the calcium phosphate technique with 10 μg (Lanes 2-4 and 6-8) of vectors that express either cEts1(p68) (C), Δ31, or Δ32, 5 μg internal control, and 5 μg reporters (209-WT and m6a; see Figs. 4 and 5 for structures). A, CAT assays. B, bar graph. ACTIVITY, CAT activity, taking into account variations in the internal control, expressed in arbitrary units. C, structure of recombinants. Δ31 and Δ32 are 3' deletion mutants lacking ets sequences beyond amino acids 450 and 412, respectively. Black box, the ets domain (amino acids 379-458).

The glucocorticoid receptor (GR) and the retinoic acid receptor αRAR) repress ets-mediated activation through the AP1 motif of the stromelysin promoter. HeLa cells were transfected by the calcium phosphate technique with 10 μg of vectors that express either vEts (Lanes 4-6) or cEts1(p68) (Lanes 7-9), 1 μg of expression vectors for the retinoic acid receptor α (Lanes 2, 5, and 8) (43) or glucocorticoid receptor (Lanes 3, 6, and 9) (45); 5 μg internal control, and 5 μg m6a (see Fig. 5 for structure). After removing the calcium phosphate precipitate, 10-6 M retinoic acid or dexamethasone was added to the transfections containing the corresponding receptors. A, CAT assays. B, bar graph. ACTIVITY, CAT activity, taking into account variations in the internal control, expressed in arbitrary units.

vEts differs from cEts1 principally by sequence alterations in the DNA-binding domain [cEts1(p68) amino acids 445 (isoleucine to valine) and 473-485 (LHAMLDOVPD ADE to HSSASGLTSSMCASSF); but also 285 (alanine to valine) outside the DNA-binding domain]. The COOH-terminal alteration suffices to strongly inhibit complex formation, in keeping with a requirement for COOH-terminal sequences for DNA binding. The isoleucine to valine change in the ets domain further decreases DNA binding, indicating that this amino acid is important for interaction with DNA. An isoleucine is found in this position in all members of the ets gene family except GABP-α and PU1. The amino acid change at 285 does not appear to affect DNA binding, since sequences NH2-terminal to the ets domain are not required for DNA binding by cEts1, cEts2, or PU1 (see "Results").

**Fig. 9.** Length as well as truncated vEts lacking this amino acid bind much less efficiently than cEts1 to the PEA3 or similar sequences. Interestingly, loss of DNA binding activity has not been described previously as a mechanism of activation of an oncogene. vRel and v-ErbA bind to their cognate motifs and act as transdominant repressors. vMyb, vJun, and vFos have changes that alter their regulation by phosphorylation or repressors (reviewed in Refs. 28 and 29).

Loss of DNA binding activity is expected to inactivate a transcription factor. Indeed, vEts is much less efficient than cEts1(p68) at activating through PEA3 motifs in various reporters, including the ets-responsive palindrome of the stromelysin promoter and the oncogene-responsive unit of the Polyoma virus enhancer. These results suggest that activation through PEA3 motifs may not be important for transformation of cells by vEts.
Since the completion of this work, two reports have appeared addressing the DNA binding (30, 31) and trans-activating properties of vEts (31). In agreement with these studies, we find that vEts binding is less efficient than cEts1(p68) to the PEA3 motif (see Ref. 30) and that vEts is less efficient than cEts1(p68) at trans-activating through the PEA3 motif (see Ref. 31). Surprisingly, Lim et al. (31) found that the vEts binds to the PEA3 motif. We consistently observed poor binding to the PEA3 motif under our conditions, with vEts synthesized in both reticulocyte lysates and in COS cells, and LePrince et al. (30) reached similar conclusions with bacterially produced proteins. The reason for this discrepancy is unclear at present. Lim et al. (31) also reported that cEts1 does not bind to the cognate motif in vitro, although it does efficiently activate through this sequence in vivo, suggesting that their in vitro conditions do not reflect the in vivo situation. It appears that binding by Ets is very sensitive to an unknown factor.

We searched for altered transcription properties of vEts by studying promoters that are activated in transformed cells. vEts transforms fibroblasts in culture (32). Transformation of fibroblasts by various oncogenes increases the promoter activity of genes such as stromelysin and collagenase (33). We found that vEts can stimulate both promoters and that their AP1 motifs, associated with a minimum promoter element or a heterologous promoter, are sufficient for efficient activation. It is unlikely that our results could be explained by increased general levels of transcription, since the SV40 early and Rous sarcoma virus promoters are not activated by Ets expression (data not shown). Interestingly, the AP1 motifs of both of these promoters are also required for activation by a number of nonnuclear oncogenes, such as ras (22, 34, 35), suggesting that vEts can feed into this network of cell signaling. cEts1(p68) has a similar activity, indicating that vEts has retained one of the functions of cEts and that a second property, binding and activation through PEA3 motifs, may not be essential for cell transformation.

A number of mechanisms could account for activation through AP1 motifs. It appears unlikely that specific binding to sequences different from the PEA3 motif is involved. vEts does not bind or activate through any of the PEA3-like or related motifs we have tested (see "Results," and data not shown). cEts1 can activate through the same AP1 elements as vEts, suggesting that it should bind to the putative second sequence. However, a number of different studies have failed to identify such a binding site (see, for example, Ref. 36). Alternatively, vEts may activate without binding to DNA, by protein-protein interactions with other activators and the general transcription machinery. Weak interactions with the general transcription machinery may suffice for activation with high concentrations of Ets, but stronger interactions with activators may lead to activation with lower concentrations of Ets. Protein-protein interactions have been described between the ets domain of GABP-α and the protein GABP-β (12). In an extensive series of experiments using a variety of approaches, we have not been able to detect complex formation between Ets and cjun plus cFos. Perhaps Ets interacts with other factors and thereby stimulates transcription of genes whose products affect AP1 activity. Interestingly, Elk-1, another member of the ets gene family, does not bind efficiently to DNA unless associated with serum response factor (37). It remains to be seen whether Ets also interacts with serum response factor and thereby stimulates the fos promoter or with any other proteins involved in the multiple pathways that affect AP1 activity (reviewed in Refs. 28 and 38). We have shown that deletion of the COOH-terminal 35 amino acids of cEts prevents activation through the AP1 motif, showing that sequences that are required for DNA binding are necessary for activation through AP1. However, we cannot exclude the possibility that these amino acids are also required for interaction with unknown proteins, in analogy with GABP-α (12). Glucocorticoid and retinoic acid receptors have been shown to inhibit AP1 activity, possibly through direct protein-protein interactions (25). We have shown that both the glucocorticoid and retinoic acid receptors inhibit Ets activation through the AP1 motif, but not through the ets motif. These results suggest that Ets does not reverse repression of AP1 by receptor-like molecules, but rather than the critical event is upstream from repression. The most likely mechanism is that Ets activates cellular genes, perhaps through protein-protein interactions between the ets domain and unknown factors. It will be interesting to identify both Ets-inducible genes and interacting factors.

vEts transforms cells in vitro, but fusion to vMyb is required for leukemogenicity in animals. Variant viruses containing fusion proteins with only amino acids 239–488 of vEts are fully active (39). These sequences contain the crippled DNA binding domain and a weak activation domain. It remains to be seen how fusion with vMyb, which contains an additional activation domain and a fully functional DNA-binding domain, generates a leukemogenic transcription factor.

At least three members of the ets gene family, vEts, Flt1, and Spi1/PU1, share the property of transforming erythroid cells. Surprisingly, they have characteristics which suggest that they do so by different mechanisms. They transform different cells of the erythroid lineage (reviewed in Ref. 40). Flt1 and PU1 retain their DNA binding activities, whereas vEts has a mutated DNA-binding domain. PU1 has a DNA binding specificity which is different from that of cEts1, and vEts does not bind to the PU1 motif. It remains to be seen which genes are trans-activated by these members of the ets gene family and how they contribute to the transformed phenotype. Our work shows that vEts does not efficiently activate through PEA3 (ets)-like elements. We suggest that the important property of vEts for transformation is its ability to activate AP1.

Materials and Methods

Construction of Recombinants

**NH3-terminal Deletion Mutants of cEts1(p68).** c-ets1 complementary DNA in pSG5 was digested with Bal31 from the 5′ end, ligated to the 5′al site of KOZ1, and,

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1. N. Yancheva and B. Wasylyk, manuscript in preparation.
2. C. Wasylyk, unpublished results.
after sequencing of the junction, transferred if necessary into either KOZ2 or KOZ3. KOZ1, 2, and 3 are derived from pSG5 (41) and contain Smal sites in either reading frame following a consensus AUG initiation codon and unique restriction sites for KpnI and XhoI. The sequences from the EcoRI to the BamHI sites are:

KOZ1: GAATTCCCGGCCACATGGCTGATGACCCGGTTAGATTC
KOZ2: GAATTCCCGGCCACATGGCTGAGGTACC
KOZ3: GAATTCCGGCGGCACATGGCTGAGGTACC

The first nucleotides of the deletion mutants were: N45, 1118; N37, 1157; N70, 1088; N69, 1212; N72, 1214 (GGCETSA1 numbering, EMBL data bank).

vEts Mutants. vEts expression vector: E26 sequences from Hpal to EcoRI (753–2461; Ref. 5) instead of equivalent fragment of the cEts1(p68) vector. Myb-Ets vector: E26 SstI fragment in same site of pT7L1. N70-nt and nV70: PstI-EcoRI (1928–2461, REAEV1 EMBL data bank) and HindIII-EcoRI (1766–2461) instead of equivalent fragments of N70, respectively.

ΔA31, ΔA32. cEts-1(p68) complementary DNA in the pSG5 expression vector was digested with Ba31 from the 3′ BamHI site, Klenow repaired, transferred into a pSG5 derivative containing stop codons in all three phases, and sequenced to determine the deletion endpoint. ΔA31 and ΔA32 code for amino acids 1–450 and 1–412, respectively.

Reporters. pBLCAT4–P4: HindIII-XbaI fragment from pG1PAL containing the ets-responsive sequences from the stromelysin promoter (22) between the equivalent sites of pBLCAT4 (35). For 209-WT and 209-MUT, see Ref. 22; for m6a, m7a, and m8a, see Ref. 23; for –517 C, –73 C, –73–42 and pBLCAT5, see Ref. 42; for TREx5 and pBLCAT4, see Ref. 35.

Transfection of Cells and Analysis

HeLa cells in Dulbecco medium containing 2.5% fetal calf serum and 2.5% calf serum were transfected by the calcium phosphate technique with 20 μg of DNA composed of 5 μg reporter, 5 μg internal control (Rous sarcoma virus-β-galactosidase, CH110 or pX40), and expression vectors, made up to 20 μg with pEMBL18. After 20 h, the cells were washed and incubated in fresh medium for an additional 24 h. Cell extracts were prepared, and both protein concentration and β-galactosidase activity were measured (43). CAT activity was quantified by counting the amount of [14C]chloramphenicol converted to the monooacylated forms. Similar conclusions were reached whether the CAT activities were normalized for β-galactosidase or protein. Representative experiments are shown in the figures. Transfections were repeated at the very least four times with two preparations of DNA.

In Vitro Analysis

RNA was synthesized from linearized pSG5-derived expression vectors with T7 RNA polymerase. RNA was quantitated by incorporating radioactive precursors, and its integrity was verified by gel electrophoresis. Optimized amounts of RNA were used to synthesize proteins in rabbit reticulocyte lysates according to the manufac-

turer’s instructions (Amersham or Promega). Efficient synthesis of full-length protein was systematically checked both by measuring incorporation of radioactive precursors ([35S]methionine) and by SDS-PAGE. Band shifts were as described previously (3).

Antibodies and Immunoprecipitation

Antibodies were raised by injecting peptides (PA93: CDEVARRWGRKRNHPK, DNA-binding domain hEtS1; PA94: CEELHAWLQKPADE, COOH-terminal tail hEtS1) coupled to ovalbumin with m-maleimidobenzoyl-N-hydroxysuccinimide ester into New Zealand rabbits. The sera were used for immunoprecipitation (44). Specificity was verified by blocking immunoprecipitation with the corresponding peptides and with preimmune serum.

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