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Repression of AP-1-stimulated Transcription by c-Ets-1*

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The transcriptional activities of c-Ets-1 and v-Ets and their functional interaction with the AP-1 factor c-Jun were investigated. Several recombinant Ets proteins were produced and purified either from bacteria or from insect cells. Plasmid DNAs that contained the polyoma virus enhancer Ets/AP-1 element were used as templates for *in vitro* transcription assays in the presence of HeLa nuclear extract and various combinations of the Jun and Ets proteins. Under these conditions full-length c-Ets-1 on its own does not markedly influence transcription but abolishes the strong transcriptional stimulation normally elicited by Jun. This repression depends on the Ets-binding site and on specific features of c-Ets-1 structure, as both v-Ets and a natural splicing variant c-Ets-1 (ΔVII) fail to inhibit Jun activity. These findings suggest that c-Ets may act both as a transcriptional repressor or activator depending on promoter context and splicing pattern.

The c-*ets-1* gene was discovered as the cellular progenitor of v-*ets*, a transforming sequence found fused with v-*myb* in the genome of avian leukemia virus E26 (Leprince *et al.*, 1983; Nunn *et al.*, 1983). Like other protooncogenes, c-*ets-1* is highly conserved among vertebrates; its major translation product is a nuclear protein (c-Ets-1(p54) in chicken) that is expressed in diverse cell types but is especially abundant in T and B lymphoid cells (Chen, 1985; Ghysdael *et al.*, 1986). High expression of c-*ets-1* also occurs in endothelial cells during blood vessel formation (Wernert *et al.*, 1992). c-Ets-1 is now recognized as the prototype of a large protein family implicated in the control of various developmental, physiological, and oncogenic processes (for review, see Macleod *et al.* (1992)).

Recent work indicates that c-Ets-1, as well as other Ets family members (*e.g.* Ets-2, Elk-1, GABP, PEA-3, and PU-1/Spi-1) act as promoter-specific transcription regulators (Bosselut *et al.*, 1990; Gunther *et al.*, 1990; Ho *et al.*, 1990; Klemsz *et al.*, 1990; Urness and Thummel, 1990; Wasylyk *et al.*, 1990; Hipskind *et al.*, 1991; LaMarco *et al.*, 1991; Dalton and Treisman, 1992; Rao and Reddy, 1992; Xin *et al.*, 1992). First, c-Ets-1 binds *in vitro* to a number of promoters and enhancers at DNA sites that are required for their function *in vivo*. In particular, in several cases c-Ets-1 sites are necessary for conferring optimal promoter responsiveness to extracellular signals such as phorbol esters. Second, in cotransfection experiments, reporter genes that carry c-Ets-1 binding elements can, under certain conditions, be activated following transient overexpression of c-Ets-1. c-Ets-1-responsive elements include sequences from the polyoma virus enhancer (Wasylyk *et al.*, 1990), the HTLV-I[1] enhancer (Bosselut *et al.*, 1990), and the stromelysin promoter (Wasylyk *et al.*, 1991). c-Ets-1-responsive elements analyzed so far display a mosaic structure, with binding sites for other factors located in close proximity to the Ets sites. There is evidence that binding of accessory proteins to these neighboring sites is required for trans-activation by c-Ets-1; similar requirements for cofactors seem to be a general feature of Ets proteins. In the polyoma enhancer Ets-responsive element, the Ets site (also called PEA-3 site) is juxtaposed to a binding site for transcription factors of the AP-1 family. Coexpression of the minor, avian-specific variant c-Ets-1 (p68) with AP-1 components c-Jun and c-Fos appears to activate the combined Ets/AP-1 element in a synergistic fashion (Wasylyk *et al.*, 1990). In the HTLV-I enhancer, the ubiquitous factor Sp1 cooperates with c-Ets-1 for DNA binding *in vitro* and for trans-activation *in vivo*, and integrity of the Sp1 site is necessary for transcriptional enhancement by c-Ets-1 (Gégonne *et al.*, 1993). Based on these results, c-Ets-1 seems to act partly by modifying the activity of neighboring DNA-bound factors.

As with other transcription factors, distinct functional modules have been defined within the c-Ets-1 molecule. At the carboxyl terminus lies a domain that is conserved between all members of the Ets family (hence named "ETS domain") and that mediates nuclear localization and specific DNA binding (Boulukos *et al.*, 1989; Gégonne *et al.*, 1992; Lim *et al.*, 1992; Nye *et al.*, 1992; Wang *et al.*, 1992; Wasylyk *et al.*, 1992). Deletion analysis and experiments with LexA-Ets-1 and GAL4-Ets-1 fusions have delineated transcription-activating regions at the amino terminus (Gégonne *et al.*, 1992; Schneikert *et al.*, 1992). Finally, a negative regulatory region, located just upstream of the DNA-binding domain, appears to interact directly with the latter, resulting in a significant inhibition of DNA binding activity (Lim *et al.*, 1992; Nye *et al.*, 1992; Wasylyk *et al.*, 1992). In addition, a second inhibitory sequence has been localized to the carboxyl-terminal end (Hagman and Grosschedl, 1992; Lim *et al.*, 1992).

An alternatively spliced c-*ets-1* mRNA that lacks exon VII, *i.e.* the sequence encoding the upstream negative regulatory domain mentioned above, has been described by Reddy and Rao (1988). In addition to abundant full-size c-Ets-1, primary thymocytes and T cell lines from mouse and human express a smaller c-Ets-1 isoform that is very likely the translation product of this alternatively spliced mRNA (Koizumi *et al.*, 1990; Pognonec *et al.*, 1990). Another effect of the loss of exon VII is an alteration, as compared with full-size c-Ets-1, in the phosphorylation of the protein in response to extracellular signals. Full-size c-Ets-1 contains multiple potential phosphorylation

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[1] The abbreviations used are: HTLV, human T cell lymphotropic virus; TCR, T cell receptor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CAT, chloramphenicol acetyltransferase; M-CAT, metallothionein promoter-chloramphenicol acetyl transferase gene.

sites and in T cells becomes rapidly hyperphosphorylated upon mitogenic or antigenic stimulation; calcium influx is required for this process (Pognonec *et al.*, 1988; Koizumi *et al.*, 1990; Pognonec *et al.*, 1990). Hyperphosphorylation correlates with a loss of DNA affinity (Pognonec *et al.*, 1989). In contrast, in c-Ets-1 (ΔVII), phosphorylation is minimal or absent, and T cell mitogens do not affect DNA binding (Pognonec *et al.*, 1990). c-Ets-1 (ΔVII), therefore, seems to be uncoupled from a signal-dependent pathway that controls the activity of full-size c-Ets-1. Although the domain encoded by exon VII evidently mediates some form of biological regulation, its role in transcriptional control is still obscure. In cotransfection experiments using a HTLV-I-derived reporter, c-Ets-1 and c-Ets-1 (ΔVII) displayed about the same activity (Gégonne *et al.*, 1992).

The idea that the physiological role of c-Ets-1 is always that of a trans-activator is not entirely born out by the data currently available. The mouse sarcoma virus enhancer, T cell receptor (TCR) α enhancer, and TCR β enhancer bind c-Ets-1 *in vitro* at sites that are required for their activity in T cells (Gunther *et al.*, 1990; Ho *et al.*, 1990; Prosser *et al.*, 1992). Yet, the mouse sarcoma virus and TCR α enhancers apparently fail to respond to c-Ets-1 expression (Gunther *et al.*, 1990; Ho *et al.*, 1990; Leiden, 1992). Furthermore, a reporter carrying the TCR β site was actually repressed by exogenously expressed c-Ets-1, whereas it was not affected by Ets-2 and was stimulated by phorbol ester (TPA); TPA stimulation was abolished in the presence of cotransfected c-Ets-1 (Prosser *et al.*, 1992). Moreover, taken in isolation, the Tα2 binding element of the TCR α enhancer, which binds c-Ets-1 *in vitro*, functions *in vivo* as a T cell-specific silencer (Ho and Leiden, 1990). The fact that the trans-acting function of c-Ets-1 depends on the target DNA sequence might be explained by the observation, mentioned above, that additional, sequence-specific DNA binding factors participate in the trans-activation process. This process may also be complicated by the presence of multiple Ets proteins with similar DNA binding specificity. For instance, the polyoma Ets/AP-1 element can bind several members of the Ets family, including PEA-3 (Martin *et al.*, 1988; Xin *et al.*, 1992) and Ets-2 (Wasylyk *et al.*, 1990) in addition to c-Ets-1.

In view of this complexity, we decided to investigate the transcription-modulating potential of c-Ets-1 directly in *in vitro* transcription assays performed in the presence of purified c-Ets-1.

EXPERIMENTAL PROCEDURES

Plasmid Constructions

Bacterial Ets Expression Vectors—Using standard polymerase chain reaction technique, a DNA sequence encoding MASHHHHHHSR (single-letter amino acid code) was introduced immediately upstream of codon Glu^{30} in c-Ets-1 (p54) or at the homologous position in v-Ets. The modified cDNAs were then transferred into the T7-based vector pAR3040, yielding plasmids pMT93 (encoding $(His)_6$-c-Ets-1) and pMT92 (encoding $(His)_6$-v-Ets). $(His)_6$-ΔVII was obtained by polymerase chain reaction-mediated deletion of the internal sequence in $(His)_6$-c-Ets-1, corresponding to residues 244–332 in c-Ets-1 (p54). The resulting plasmid is pMT94.

Eukaryotic Ets Expression Vectors—Using polymerase chain reaction, a DNA sequence encoding FHHHHHH(stop) was introduced after codon Glu^{441} at the 3′ end of *c-ets-1*. The 5′ end of the cDNA was modified by the addition of a translation initiation sequence (Kozak, 1989). The modified cDNA was inserted between the human cytomegalovirus promoter/enhancer and the SV40 polyadenylation sequence. The resulting plasmid, pMT43, encodes c-Ets-1-$(His)_6$, *i.e.* full-size c-Ets-1 (p54) bearing a carboxyl-terminal hexahistidine tag. For v-Ets expression, the same cDNA as that of pMT92 (encoding $(His)_6$-v-Ets) was inserted in the cytomegalovirus-based vector yielding plasmid pMT51. The empty vector used as a transfection control was equivalent to pMT43 devoid of cDNA. All constructs were verified by sequencing.

Construction of M-CAT-derived Reporters—The following oligonucleotides were synthesized.

5′ GATCCAGCAGGAAGTGACTAACTGA
GTCGTCCTTCACTGATTGACTCTAG

OLIGONUCLEOTIDE EA

5′ GATCCAGCA**AAA**AGTGACTAACTGA
GTCGT**TTT**TCACTGATTGACTCTAG

OLIGONUCLEOTIDE eA

The EA oligonucleotide contains both the Ets and AP-1 sites; the eA oligonucleotide cannot bind Ets (mutations in boldface) but still binds AP-1 (Wasylyk *et al.*, 1990). EA and eA were phosphorylated with polynucleotide kinase and multimerized with T4 DNA ligase. Head-to-tail multimers were selected by digestion with *Bam*HI. Multimers of the desired sizes were isolated by gel electrophoresis and cloned into the unique *Bgl*II site of pM-CAT (Bohmann and Tjian, 1989). Constructs were verified by sequencing.

Purification of Bacterially Expressed Ets Proteins

Escherichia coli BL-21 (DE3) cells were transformed with the relevant plasmids. Overnight cultures of freshly transformed bacteria were diluted into 500 ml of Luria broth, grown up to A_{600} = 0.3, and induced for 90 min with 1 mM isopropyl-1-thio-β-D-galactopyranoside (Boehringer Mannheim). The bacteria were then pelleted (7,000 rpm, 10 min), washed in ice-cold isotonic buffer (50 mM glucose, 0.25 mM Tris-HCl (pH 8), 10 mM EDTA), and pelleted again. Each pellet was resuspended in 12 ml of lysis buffer (6 M guanidine HCl, 100 mM Na_2HPO_4/NaH_2PO_4, 1 mM β-mercaptoethanol (pH 8.0)) and incubated for 30 min at room temperature. The bacterial lysate was then cleared by centrifugation (10,000 rpm, 4 °C) and sonicated on ice with a Branson microtipped sonifier at setting 1.5 for 3 × 10 s in order to reduce viscosity. The lysate was next incubated overnight with 1 ml (packed volume) of Ni^{2+}-chelate-agarose (nitrilotriacetic acid resin, Diagen GmbH) at 4 °C on a rotator. The resin suspension was poured into a Bio-Rad Polyprep column, and the resin was washed at room temperature with 5 ml of lysis buffer and then with 25–30 ml of wash buffer (6 M guanidine HCl, 100 mM Na_2HPO_4/NaH_2PO_4, 1 mM β-mercaptoethanol (pH 5.9)). The subsequent operations were performed at 4 °C. The column was washed once again with 5 ml of lysis buffer, and then the adsorbed Ets protein was renatured *in situ* by progressively decreasing the guanidine HCl concentration while increasing that of native buffer (20 mM Hepes-KOH (pH 7.8), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 1 mM β-mercaptoethanol). To this effect, the column was successively washed with 4 ml of each of the following mixtures (volume of lysis buffer: volume of native buffer), 2:1, 1:2, 1:5, and 1:11, and then with 4 ml of pure native buffer. Finally, the column was washed with 4 ml of native buffer supplemented with 20 mM imidazole, and the Ets protein was eluted in 2 × 1 ml of native buffer supplemented with 100 mM imidazole, 0.1% lauryldimethylamine oxide (Calbiochem), 0.2 mM phenylmethylsulfonyl fluoride, and 10 μM leupeptin. The imidazole was removed by dialysis for 4 h against two changes of native buffer. The preparation was aliquoted, frozen in liquid nitrogen, and stored at −70 °C. Roughly equivalent amounts of $(His)_6$-c-Ets-1, $(His)_6$-v-Ets, and $(His)_6$-ΔVII were recovered. Comparing the DNA binding capacities of equal amounts of bacterial $(His)_6$-c-Ets-1 and c-Ets-1 purified using a recombinant baculovirus and assuming all of the baculoviral protein was in a native state, it could be estimated that ≈25% of the bacterial molecules were renatured, at least in the DNA-binding domain.

In Vitro Transcription Assays

HeLa cell nuclear extracts were prepared as previously described (Bohmann and Tjian, 1989) using the method of Dignam *et al.* (1983) with the modification introduced by Wildeman *et al.* (1984). The reporter plasmids were prepared under standard conditions (Sambrook *et al.*, 1989) and purified by two cycles of centrifugation on CsCl/ethidium bromide density gradients. M-CAT-derived plasmids were linearized by digestion with *Eco*RI. The pM34 adenovirus template was linearized with *Sph*I (Miyamoto *et al.*, 1984). After digestion, the templates were extracted once with phenol and twice with chloroform and then precipitated with ethanol/LiCl and resuspended in water at 50 ng/μl. Transcription reactions were carried out in 20 μl (final volume) of 20 mM Hepes-KOH (pH 7.8); 10% glycerol; 35 mM KCl; 4 mM $MgCl_2$; 4 mM spermidine; 0.1 mM EDTA; 0.5 mM dithiothreitol; 0.5 mM each of ATP, GTP, and CTP; 0.01 mM UTP (final concentrations) supplemented with 5 μCi of [α-^{32}P]UTP (Amersham Corp.) per reaction. The reaction mixtures were typically assembled by successively adding Ets and/or Jun protein (amounts are indicated in the figure legends), 4 μl of nuclear extract, 7 μl of concentrated reaction buffer, and 4 μl (200 ng) of linear-

ized DNA template. The mixtures were incubated for 1 h at 25 °C. Reactions were stopped by adding 200 µl of phenol/chloroform (1:3, v/v) followed by 80 µl of 3.125 M ammonium acetate, 0.25 mg/ml yeast total RNA (Boehringer Mannheim). After extraction, nucleic acids were precipitated (240 µl of ethanol/80 µl of extracted reaction mixture), pelleted, washed once with 70% ethanol, taken up in 95% formamide, briefly heated to 80 °C, and analyzed on a 6% polyacrylamide/urea sequencing gel. The gel was dried and exposed at −70 °C to Kodak X-AR 5 film with an intensifying screen. Transcription was quantitated by scanning suitable autoradiographs with an LKB laser densitometer, integrating the specific absorbance peaks on an on-line computer, and expressing the resulting values as ratios between M-CAT and internal control transcripts. Transcription experiments were repeated at least 3 times with essentially the same results.

Electrophoretic Mobility Shift Assays

Oligonucleotides EA or eA (see plasmid constructions) were labeled by filling-in with Klenow enzyme and [α-^{32}P]dCTP (800 Ci/mmol, Amersham Corp.) and purified by polyacrylamide gel electrophoresis. 10–20,000 Cerenkov cpm (10–20 fmol) of the probe was incubated with 0.2–2 µl (5–50 ng) of purified recombinant protein in a total volume of 20 µl containing 0.8 µg pf poly(dI·dC) in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 10% glycerol. After 20 min at room temperature, the reaction mixture was loaded onto a 6% polyacrylamide gel and subjected to electrophoresis in 0.25 × TBE for 90 min at room temperature (1 × TBE is 90 mM Tris borate (pH 8), 2 mM EDTA). The gel was dried and exposed at −70 °C under Kodak X-AR5 film with an intensifying screen.

Transient Expression and CAT Assays

HeLa tk$^-$ cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine, penicillin, and streptomycin at 37 °C in a 95% air, 5% CO_2 atmosphere. The cytomegalovirus-based Ets expression vectors and the M-CAT-derived reporters described above were used throughout these experiments. HeLa tk$^-$ cultures that had been divided the previous day were seeded onto 60-mm dishes (4 × 10^5 cells/dish). The next day, the cells were transfected by the calcium phosphate technique with a mixture of 0.5 µg of c-Ets-1 or v-Ets or empty expression vector, 1 µg of reporter plasmid, and 9.5 µg of carrier (pUC) DNA/dish. The cells were incubated overnight with the DNA precipitate and then washed and incubated for 24 h in Dulbecco's modified Eagle's medium containing 0.5% fetal calf serum. TPA was added (100 ng/ml medium) for the indicated durations. The cells were scraped in phosphate-buffered saline, washed, resuspended in 0.25 M Tris-HCl (pH 7.8), and lysed by three freeze-thaw cycles. CAT activity was assayed essentially as described (Gorman *et al.*, 1982). Thin layer chromatography plates were exposed to Kodak X-AR5 film. The reactions were quantitated by excising the relevant portions of the thin layer chromatography plates and counting in a scintillation counter. Transfection experiments were repeated 3 times with essentially the same results.

RESULTS

Production of Purified Ets and Jun Proteins—Histidine-tagged c-Ets-1, v-Ets, and c-Ets-1 (ΔVII) proteins (Fig. 1*A*) were expressed in bacteria and purified to approximately 95% homogeneity by nickel-chelate affinity chromatography, as described under "Experimental Procedures" (Fig. 1*B*).

Recombinant c-Jun protein was expressed and purified from bacteria as previously described (Bohmann and Tjian, 1989). In most experiments, we used a constitutively active version of c-Jun called HJ30. This molecule lacks the amino-terminal conditionally inhibitory domain (δ) found in full-size c-Jun; as a result, its transcriptional activity is strongly enhanced (Bohmann and Tjian, 1989; Baichwal and Tjian, 1990). Results obtained with HJ30 were confirmed by assaying bacterially expressed full-size c-Jun (called HJ40) or AP-1 protein highly purified from human cells by sequence-specific DNA affinity chromatography.

c-Ets-1 but Not v-Ets Represses Transcription in Vitro—In order to evaluate the effect of c-Ets-1 on promoter-specific transcription directly, the protein was added to standard *in vitro* transcription mixtures containing a DNA template, a radiolabeled nucleotide mix, and a HeLa cell nuclear extract as a source of RNA polymerase II and general transcription factors. As templates, we used reporter plasmids containing one copy (construct 1xEA) or four copies (construct 4xEA) of the polyoma virus Ets/AP-1 element upstream of the metallothionein IIa minimal promoter (Fig. 1*C*). In many experiments, we also included a control template containing the adenovirus major late promoter (plasmid pM34) (Miyamoto *et al.*, 1984).

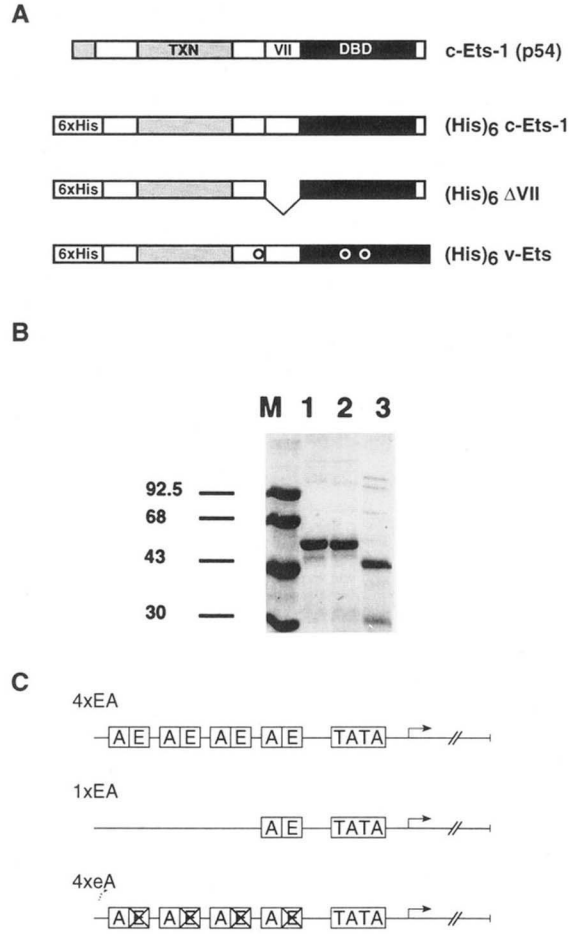

FIG. 1. *Panel A*, structure of the Ets proteins utilized in this study. *c-Ets-1 (p54)*, normal, full-size chicken protein expressed from a baculovirus vector (Bosselut *et al.*, 1990); *gray box*, alternatively spliced region (specific for c-Ets-1 (p54) and different in c-Ets-1 (p68)); *TXN*, transcription-activating region (Gégonne *et al.*, 1992); *VII*, exon VII-encoded sequence; *DBD*, DNA-binding domain. In order to fuse the $(His)_6$ affinity tag (*e.g.* see Papavassiliou *et al.* (1992)) to the amino terminus of c-Ets-1, truncation of the c-*ets-1* cDNA were carried out; as shown, the truncation removed the sequence that differs between c-Ets-1 (p54) and c-Ets-1 (p68) but did not affect the transcription-activating region that has been delineated in c-Ets-1 (p54) (Gégonne *et al.*, 1992). An identical truncation was used to construct both $(His)_6$-ΔVII and $(His)_6$-v-Ets. As a result, the differences between $(His)_6$-c-Ets-1 and $(His)_6$-v-Ets consist of the three point mutations (*circles* in $(His)_6$-v-Ets) and a carboxyl-terminal substitution (*black box*) (Leprince *et al.*, 1988). *Panel B*, production of purified bacterial proteins. Preparations of $(His)_6$-c-Ets-1 (*lane 1*), $(His)_6$-v-Ets (*lane 2*), and $(His)_6$-ΔVII (*lane 3*) were analyzed on a 10% SDS-polyacrylamide gel and stained with Coomassie blue. *M*, molecular mass markers (sizes are in kilodaltons). *Panel C*, structure of the M-CAT-derived transcription templates. M-CAT contains a minimal promoter obtained from the human metallothionein IIa gene linked to the chloramphenicol acetyltransferase coding sequence (Bohmann and Tjian, 1989). *E*, polyoma enhancer Ets-binding site; *A*, AP-1 site; *arrow*, transcription start site.

Fig. 2*A* shows the result of a typical experiment, performed with the 4xEA and 1xEA reporters. The polyoma-derived reporters and the control template yielded run-off transcripts of the expected sizes (*S* and *C*, respectively; *lanes 1–3*). $(His)_6$-c-Ets-1 and $(His)_6$-v-Ets affected transcription efficiency only very moderately (*lanes 4* and *5*). In contrast, c-Jun (HJ30)

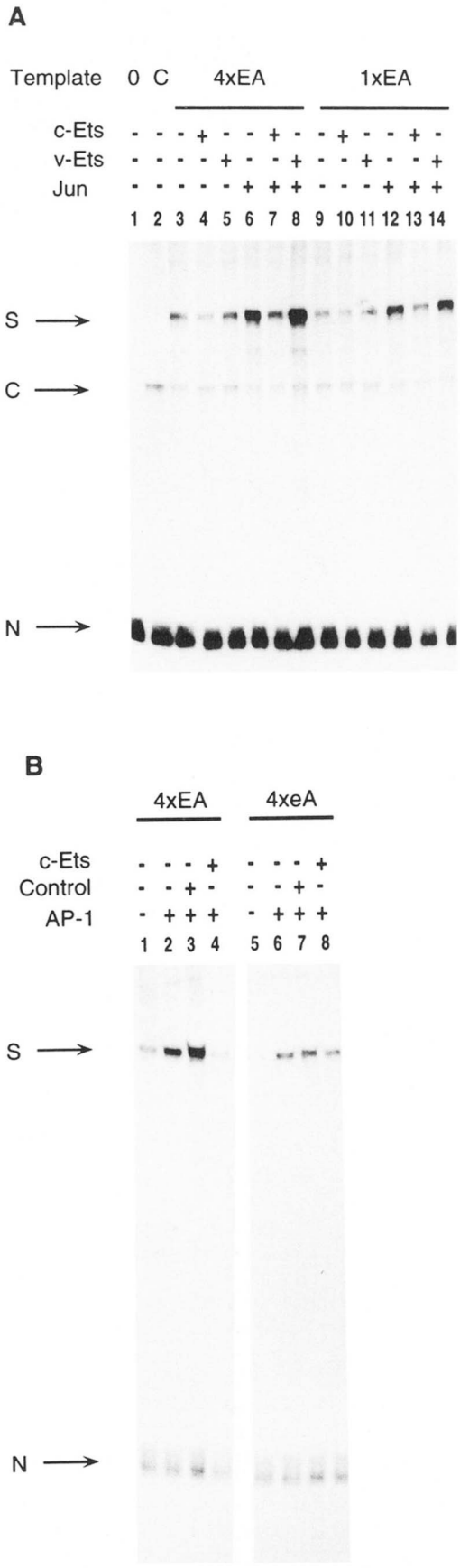

FIG. 2. *Panel A*, effects of Jun, $(His)_6$-c-Ets-1, and (His)6-v-Ets on *in vitro* transcription. Transcription reactions were performed by incubation of linearized DNA templates (depicted in Fig. 1*C*) in the presence of a labeled nucleotide mix, HeLa nuclear extract, and various combinations of purified bacterial Ets and Jun proteins, as indicated at the *top* of the figure. The transcript of the adeno major late control template (220 bp) is indicated with an *arrow* (*C*). The reporter-specific transcript (289 bp) is indicated with an *arrow* (*S*). Where indicated, 50 ng of Jun (HJ30), 250 ng of $(His)_6$-c-Ets-1, or 250 ng of $(His)_6$-v-Ets were added to the reaction. *N*, nonspecific reaction product. *Panel B*, effects of human AP-1 and baculovirus-expressed c-Ets-1 on *in vitro* transcription. Transcription reactions were carried out in the same manner as described in *Panel A*. Where indicated, 20 ng of AP-1 protein (purified from HeLa cells by sequence-specific chromatography) was added. Reactions in *lanes 4* and *8* contained 0.4 μl of extract from Sf9 cells infected with a recombinant baculovirus expressing the *c-ets-1* cDNA (1 μg of total protein, containing ≈10 ng of c-Ets-1). Control reactions contained an equivalent amount of extract prepared from noninfected Sf9 cells.

protein caused a large (up to 12-fold) increase in specific transcription (*lane 6*), as expected based on both cotransfection experiments (Wasylyk *et al.*, 1990) and *in vitro* studies of Jun (Bohmann and Tjian, 1989). $(His)_6$-c-Ets-1 and $(His)_6$-v-Ets influenced this Jun-dependent activation in a strikingly different way. In the presence of $(His)_6$-c-Ets-1, stimulation by c-Jun (HJ30) was reduced to about 2-fold (*lane 7*), whereas $(His)_6$-v-Ets mediated a moderate (up to 15%) additional enhancement on top of the c-Jun-induced increase (*lane 8*). The adenovirus internal control was not significantly influenced by the Ets or Jun proteins, demonstrating that modulation of transcription by these proteins was promoter-specific. Qualitatively, the same results were obtained using both orientations of a monomeric instead of a tetrameric Ets/AP-1 module in the reporter (*lanes 9–14* and data not shown). $(His)_6$-c-Ets-1, but not $(His)_6$-v-Ets, also suppressed the weak transcriptional stimulation brought about by full-size c-Jun (HJ40) (data not shown). We occasionally detected a weak transcriptional repression by c-Ets-1 in the absence of added c-Jun (*lane 2*). As we used nuclear extracts that had not been depleted of endogenous AP-1 protein, inhibition of the endogenous AP-1 activity might account for this observation.

Repression by $(His)_6$-c-Ets-1 was dose-dependent, being clearly detectable at Ets/DNA ratios of around 8 (about 2 pmol of total Ets protein/0.24 pmol of binding sites in a standard reaction) (Fig. 3, *lanes 1–7*). Equivalent or higher amounts of $(His)_6$-v-Ets appeared slightly to stimulate transcription (*lanes 8–12*).

In order to verify that repression by $(His)_6$-c-Ets-1 was not due to some artifact associated with our expression/purification system, we substituted the bacterial protein with nontagged c-Ets-1 derived from insect Sf9 cells infected with a suitable baculovirus vector (Bosselut *et al.*, 1990). In transcription assays similar to the ones described above, we used either crude extracts from the baculovirus-infected cells (comparing them with noninfected controls) or baculoviral c-Ets-1 purified to 50% homogeneity by a nondenaturing procedure involving sequence-specific DNA affinity chromatography (data not shown). These c-Ets-1 preparations repressed c-Jun (HJ30)-induced transcription at least as efficiently as the bacterial product; repression displayed the same dose dependence as with $(His)_6$-c-Ets-1 (data not shown). Furthermore, repression was also observed when AP-1 protein purified from human cells (Bohmann and Tjian, 1989) was employed instead of bacterial c-Jun (Fig. 2*B*, *lanes 1–4*). We conclude that repressor activity is very likely intrinsic to the c-Ets-1 protein and cannot be explained by the addition of the amino-terminal histidine tag, by some modification introduced by the bacterial environment, or by the denaturation/renaturation cycle applied during purification.

Repression Depends on Ets-binding Site—To determine whether repression of transcription by c-Ets-1 depends on binding to the cognate sites of the test template, we studied transcription of 4xeA, a mutated version of the 4xEA template (see

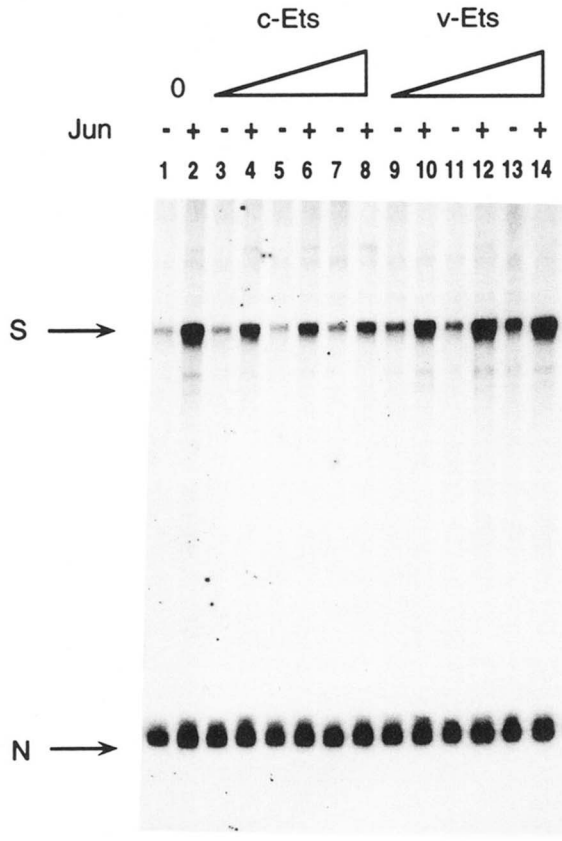

FIG. 3. **Titration of $(His)_6$-c-Ets-1 and $(His)_6$-v-Ets transcriptional activities.** Transcription reactions (see Fig. 2, *panel A*) were performed with 4xEA template in the absence (*odd-numbered lanes*) or the presence (*even-numbered lanes*) of 50 ng of bacterial Jun (HJ30). *Lanes 1* and *2*, no Ets protein present. Amounts of $(His)_6$-c-Ets-1 added: *lanes 3* and *4*, 100 ng; *lanes 5* and *6*, 250 ng, *lanes 7* and *8*, 500 ng. Amounts of $(His)_6$-v-Ets added: *lanes 9* and *10*, 100 ng; *lanes 11* and *12*, 250 ng; *lanes 13* and *14*, 500 ng. *S*, specific transcript; *N*, nonspecific product.

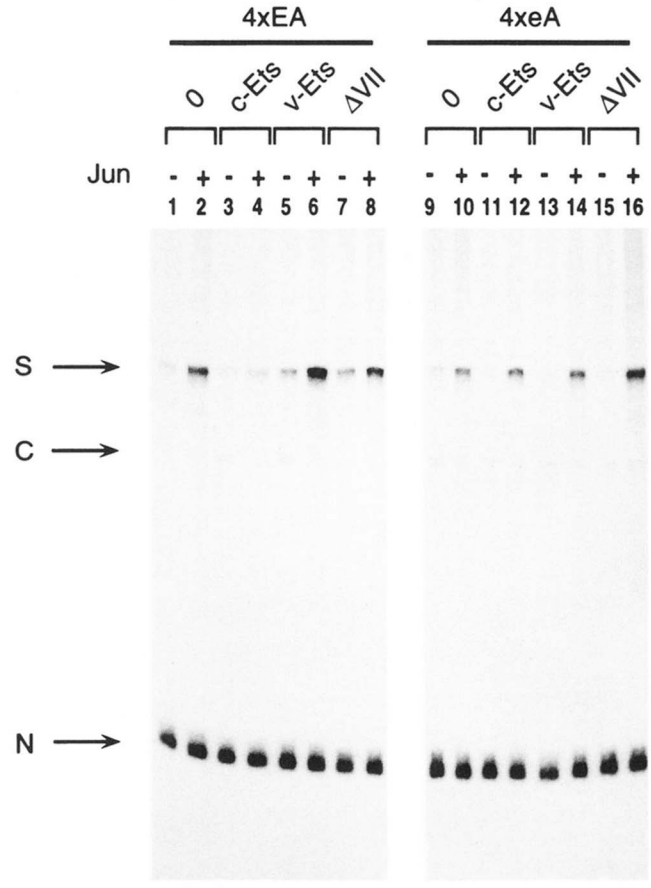

FIG. 4. **The transcriptional effect of the Ets proteins is binding site- and exon VII-dependent.** Transcription reactions were performed with either the 4xEA template or the 4xeA template, which carries a double point mutation in the Ets-binding site. Reactions were performed in the absence (*odd-numbered lanes*) or the presence (*even-numbered lanes*) of 50 ng of Jun (HJ30). Reaction mixtures contained either no Ets protein (*lanes 1*, *2*, *9*, and *10*) or 250 ng of $(His)_6$-c-Ets-1 (*lanes 3*, *4*, *11*, and *12*), $(His)_6$-v-Ets (*lanes 5*, *6*, *13*, and *14*), or $(His)_6$-ΔVII (*lanes 7*, *8*, *15*, and *16*). *S*, specific transcript; *C*, control transcript; *N*, nonspecific product.

Fig. 1*C*), in which the Ets-binding site had been inactivated by a two-base pair substitution. As can be seen in Fig. 4, *lanes 9–14*, transcription of 4xeA is still strongly (9-fold) stimulated by c-Jun (HJ30) alone, but this stimulation is now insensitive to the addition of $(His)_6$-c-Ets-1 (compare *lanes 1–4* with *lanes 9–12*). Similar results were obtained using human AP-1 and baculoviral c-Ets-1 (Fig. 2*B*, *lanes 5–8*). We conclude that repression by c-Ets-1 depends on its binding to DNA. In line with this, the moderate enhancement caused by $(His)_6$-v-Ets-l (*lanes 5* and *6*) cannot be detected in the absence of the Ets-binding site (Fig. 4, *lanes 13* and *14*).

c-Ets-1 (ΔVII), a Natural c-Ets-1 Splice Variant, Fails to Efficiently Repress AP-1 Activity in Vitro—As noted in the Introduction, the activity of the alternative splice product c-Ets-1 (ΔVII) appears to differ in some respects from that of full-size c-Ets-1; c-Ets-1 (ΔVII) exhibits a constitutively higher affinity for the Ets-binding sites of the polyoma (Wasylyk *et al.*, 1992; this study) and HTLV-I (Gégonne *et al.*, 1992) enhancers, and binding of c-Ets-1 (ΔVII) to both nonspecific (Pognonec *et al.*, 1990) and specific[2] DNA is unresponsive to calcium-dependent signals. It was, therefore, of interest to assess the activity of $(His)_6$-ΔVII in our *in vitro* transcription assay. In the absence of Jun, $(His)_6$-ΔVII did not inhibit transcription. Although $(His)_6$-ΔVII somewhat inhibited the normalized Jun-induced increase (reducing it from 9- to 6-fold), this inhibition was far weaker than that (down to 2-fold) caused by $(His)_6$-c-Ets-1 (Fig. 4, compare *lanes 7* and *8* with *lanes 3* and *4*). The transcriptional activity of c-Ets-1 (ΔVII) thus differs from that of the full-size protein. This result suggests that regulation through the exon VII-encoded domain modulates the mode of interaction of c-Ets-1 with Jun and the transcription machinery.

c-Ets-1 and Jun Can Bind Simultaneously to Polyoma Enhancer—Since repression by c-Ets-1 is binding site-dependent, it cannot easily be explained by a "squelching" type of mechanism in which excess unbound trans-activator would sequester general transcription factors or Jun co-activators present in limiting amounts. The fact that neither $(His)_6$-v-Ets nor $(His)_6$-ΔVII efficiently represses also argues against such a mechanism. As the Ets and Jun sites are partially overlapping in the polyoma Ets/AP-1 element, one different possibility is that of steric inhibition of Jun binding by c-Ets-1. This possibility was examined in electrophoresis mobility shift assays (Fig. 5). A polyoma-derived Ets/AP-1 oligonucleotide probe was preloaded with excess $(His)_6$-c-Ets-1 or $(His)_6$-v-Ets. The resulting complexes were further incubated with either no addition (*lanes 1* and *2*) or with c-Jun (HJ30) (*lanes 4* and *5*). A control reaction was performed with HJ30 alone (*lane 3*). Fig. 5 shows that addition of HJ30 together with $(His)_6$-c-Ets-1 or $(His)_6$-v-Ets gave rise to a complex (C3) whose mobility was slightly lower than that of the pure HJ30-DNA complex (C2) and much lower than that of the Ets-DNA complex (C1). This suggests formation of a complex harboring both c-Ets-1 and c-Jun, bound

[2] B. Rabault and J. Ghysdael, manuscript in preparation.

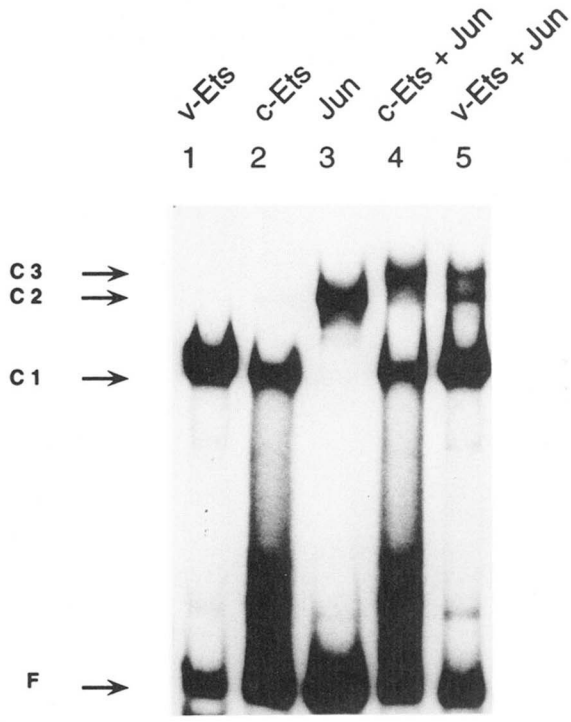

FIG. 5. **Simultaneous binding of Ets and Jun to the polyoma enhancer.** An oligonucleotide probe (probe EA), containing both the Ets and AP-1 sites of the polyoma virus TPA-responsive unit, was incubated with purified bacterial proteins in the presence of poly(dI·dC) as a nonspecific competitor. The proteins added were as follows: *lane 1*, $(His)_6$-v-Ets alone; *lane 2*, $(His)_6$-c-Ets-1 alone; *lane 3*, Jun (HJ30) alone; *lane 4*, $(His)_6$-c-Ets-1 followed by Jun (HJ30); *lane 5*, $(His)_6$-v-Ets followed by Jun (HJ30). *F*, free probe; *C1*, Ets-DNA complex; *C2*, Jun-DNA complex; *C3*, Ets-Jun-DNA complex. The dark smear at the bottom of *lanes 2* and *4* is very likely caused by dissociation of the $(His)_6$-c-Ets-1-containing complex during electrophoresis.

to the same DNA molecule. The amount of complexes containing c-Jun only (C2) was strongly reduced in the presence of Ets proteins, but this reduction occurred to the same extent with c-Ets-1 and v-Ets (compare *lanes 4* and *5*) as well as with c-Ets-1 (ΔVII) (data not shown). Therefore, repression cannot be attributed simply to a direct effect of c-Ets-1 on Jun binding. c-Ets-1 appears to bind DNA with a slightly higher off-rate than v-Ets, which is apparent by a smear of dissociated probe in *lanes 2* and *4*. We regard it unlikely that this small difference accounts for binding site-dependent negative effect of c-Ets-1 *versus* v-Ets in our experimental system, especially because c-Ets-1 is the weaker binder.

c-Ets but Not v-Ets Inhibits TPA-induced Activation of Polyoma Enhancer in HeLa tk⁻ Cells—The *in vitro* data presented above suggest the possibility that, at least under certain conditions, c-Ets-1 might inhibit AP-1 *in vivo*. In an attempt to define such conditions, we made use of the fact that the polyoma Ets/AP-1 element is highly sensitive to TPA-induced AP-1 activity (Imler *et al.*, 1988). HeLa tk⁻ cells (known to possess TPA-stimulatable AP-1 activity) (Angel *et al.*, 1987) were transfected with the 4xEA polyoma reporter together with expression vectors for chicken c-Ets-1 or v-Ets (see "Experimental Procedures"). The transfected cells were incubated for 24 h in low serum and then stimulated with TPA for increasing amounts of time, after which the cells were lysed, and CAT activity was measured. A typical experiment is shown in Fig. 6. In cells transfected with empty expression vector, the reporter gene was highly inducible by TPA (*lanes 1*, *4*, *7*, and *10*), as expected (Imler *et al.*, 1988). In contrast, in cells that were transfected with c-Ets-1-expressing plasmid, TPA induction was significantly attenuated (*lanes 2*, *5*, *8*, and *11*). On the

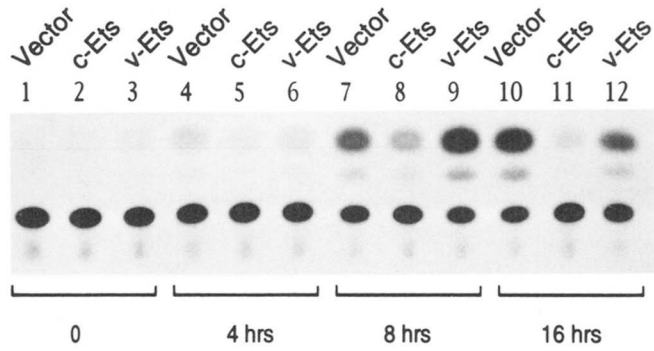

FIG. 6. **Effect of transiently expressed c-Ets-1 and v-Ets on TPA activation of the polyoma Ets/AP-1 element.** HeLa tk⁻ cells were transfected with 1 μg of the 4xEA reporter together with 0.5 μg of empty expression vector (*lanes 1*, *4*, *7*, and *10*), c-Ets-1 vector (pMT43) (*lanes 2*, *5*, *8*, and *11*), or v-Ets vector (pMT51) (*lanes 3*, *6*, *9*, and *12*). The cells were next rendered quiescent by a 24-h incubation in low serum medium (0.5% fetal calf serum) and then stimulated with TPA (100 ng/ml) for the indicated times. Cells were lysed, and CAT activity was assayed as described. *Lower spot*, nonacetylated substrate; *upper spot*, reaction product. After a 16-h TPA treatment, CAT activity (expressed in percent conversion to the acetylated form) was stimulated 43-fold in the presence of empty vector, 4.6-fold in the presence of c-Ets-1 vector, and 23-fold in the presence of v-Ets vector, as compared with quiescent cells.

other hand, in cells transfected with v-Ets, TPA induction was similar to that in control cells (*lanes 3*, *6*, *9*, and *12*). It thus appears that c-Ets-1 can inhibit AP-1-mediated stimulation of the polyoma enhancer *in vivo* in a fashion similar to the repression seen *in vitro*.

DISCUSSION

c-Ets-1 Can Function as a Sequence-specific Transcriptional Repressor and Activator—The data presented here demonstrate that c-Ets-1 can repress transcriptional activation by AP-1 *in vitro*. Several conclusions can be drawn concerning the nature and specificity of this effect. First, the repression observed does not appear to be a consequence of nonspecific aberrations in the purified c-Ets-1 or AP-1. c-Ets-1 and AP-1 proteins prepared by different methods displayed qualitatively identical activities. The effect of the highly purified bacterial $(His)_6$-c-Ets-1 makes it unlikely that repression would be caused by an undefined contaminant. $(His)_6$-c-Ets-1 does differ from normal c-Ets-1 by two features: deletion of the variable amino terminus and amino-terminal fusion to the hexahistidine tag. Yet neither of these features seems responsible for the repressor activity since nontagged, nontruncated c-Ets-1 expressed in the baculovirus system acted exactly like $(His)_6$-c-Ets-1. Moreover, a bacterial protein carrying the hexahistidine sequence at the carboxyl terminus with no deletion, c-Ets-1-$(His)_6$, also repressed transcription (data not shown). Because the baculovirus-expressed product did not undergo denaturing treatment, its activity indicates that misfolding of the protein does not account for repression. Second, repression is binding site-dependent; this rules out a general toxic effect of the c-Ets-1 preparations on transcription, as well as a squelching mechanism in which a general transcription factor would be titrated by excess c-Ets-1. Third, Ets and Jun proteins can bind to the same DNA molecule in an electrophoresis mobility shift assay. While at the same time c-Ets-1 reduces the amount of pure Jun-DNA complexes, v-Ets causes an equal reduction. Therefore, repression cannot be simply explained by steric exclusion of Jun binding. It is possible, however, that c-Ets-1 directly occludes the transcription activating regions of Jun. Fourth, repression cannot be observed with v-Ets, and it is quite weak with c-Ets-1 (ΔVII); yet these two structural variants leave DNA binding intact. This result shows that mere occupancy of the Ets-binding site by bacterial proteins about

the same size as c-Ets-1 is, in itself, not sufficient to hinder Jun access to the transcription machinery. Fifth, a qualitatively comparable result to that obtained *in vitro* was observed *in vivo* after cotransfection experiments; c-Ets-1 but not v-Ets repressed the activity of an indicator gene driven by the composite Ets/AP-1 binding site. Hence, repression appears as a specific activity of the c-Ets-1 molecule.

Several examples of transcription factors have been reported that can function both as repressors and as activators (Sauer and Jäckle, 1991; Weintraub *et al.*, 1992). The evidence presented here indicates that Ets-1 factors (at least in the context of an Ets/AP-1 promoter element) can operate in such a bifunctional manner. The negative or positive transcriptional effects described here are determined by alternative splicing. In addition, the minor structural differences between c-Ets-1 and v-Ets turn the latter from an inhibitor of AP-1 transcriptional function into a weak co-activator. In many instances the conversion of a protooncogene into an oncogene "freezes" a previously signal-dependent regulatory factor in a permanently active state. It is tempting to speculate that the observed differences in the activity between c-Ets-1 and v-Ets are a reflection of such an effect (*i.e.* c-Ets-1 might need to be modified to switch from repressor to activator mode, whereas v-Ets is permanently active). Such a modification might, for example, be a signal-dependent phosphorylation or dephosphorylation that is inefficiently carried out or not carried out at all in the HeLa-derived *in vitro* system employed here. Indeed, such a scenario would help to explain observations made in transfection experiments where c-Ets-1-binding sites have been reported to act as positive or negative transcription control elements (Gunther *et al.*, 1990; Ho *et al.*, 1990; Leiden, 1992). Conceivably the differences in activity described in a number of contexts may depend on several parameters such as cell type, growth state, and promoter context. This view is supported by the results of the transfection studies presented here. An inhibitory effect of c-Ets-1 is seen after cotransfection into HeLa cells that have been stimulated by exposure to the protein kinase agonist TPA. Such cells should physiologically be quite comparable with the logarithmically growing HeLa cells used for the preparation of the transcription extracts employed in the *in vitro* experiments, consistent with the similar transcriptional effect of c-Ets-1 in both assays. In quiescent fibroblast cells, however, which were used for transfections in a previous report (Wasylyk *et al.*, 1990), a positive, synergistic effect of AP-1 and c-Ets-1 was found. Emphasizing the caveat that such experiments are often difficult to interpret due to the interference with other Ets-related factors that are present in most tissue culture cells, one might suppose an influence of the growth state of a cell on the positive or negative effect of c-Ets-1 at least on composite AP-1/Ets promoter elements. It is important to point out that interactions of c-Ets-1 (or its derivatives) with transcription factors other than AP-1 might have quite different regulatory consequences than the ones reported above. It has, for example, recently been shown that c-Ets-1 and the housekeeping transcription factor Sp1 synergistically activate transcription through the HTLV-I enhancer (Gégonne *et al.*, 1993). Using HTLV-I-reporter constructions we could never observe the negative transcriptional effect of c-Ets-1 seen on the polyoma-derived constructs (data not shown). It is conceivable that even the interaction between AP-1 and c-Ets-1 might vary depending on the juxtaposition. It would be interesting to test this idea by analyzing a panel of artificial spacing mutants.

Possible Biological Role of Repressor Activity—A specific repressor function for c-Ets-1 might explain some features of gene regulation during T cell activation. In that process, along with stimulation of the protein kinase C pathway, an increase in intracellular calcium is required for activation of several cytokine promoters such as interleukin-2, interleukin-4, interleukin-5, or granulocyte-macrophage colony-stimulating factor (Ullman *et al.*, 1990). Some of the calcium/TPA-response elements that have been located in these promoters bear a striking similarity to the Ets/AP-1-binding site of the polyoma enhancer (see Miyatake *et al.* (1991)). In fact, the granulocyte-macrophage colony-stimulating factor promoter element ("CLE-0" in Miyatake *et al.* (1991)) can bind both recombinant c-Ets-1 and c-Jun *in vitro*.[3] c-Ets-1 is known to be abundant in peripheral, quiescent T cells (Bhat *et al.*, 1990).[4] In the absence of a calcium signal, c-Ets-1 might bind and prevent activation of granulocyte-macrophage colony-stimulating factor-type promoters by AP-1 or other activators; following calcium influx, c-Ets-1 would become hyperphosphorylated resulting in a change of its transcriptional mode of action and/or detachment from the promoter, perhaps to be replaced by another member of the Ets family. If endogenous c-Ets-1 acts as a calcium-regulated repressor of Ets/AP-1 sites in T cells, one would predict that in these cells, calcium influx would be required for trans-activation of the polyoma Ets/AP-1 element. In line with this expectation, in the mouse T cell line EL-4, TPA alone cannot activate our 4xEA reporter, but like with cytokine promoter elements, trans-activation of 4xEA can be obtained by treating the cells with a combination of TPA and calcium ionophore.[3]

In this view, the alternative splice product c-Ets-1 (ΔVII) may function to counteract the repressor activity of c-Ets-1. Indeed, the ratio of c-Ets-1 (ΔVII) to c-Ets-1 may be markedly low in normal quiescent T cells (Bhat *et al.*, 1990; Pognonec *et al.*, 1990). Interestingly, in what might be a similar case, a novel splice variant of the Max protein was reported to stimulate rather than antagonize transformation by the Myc nuclear oncoprotein (Makela *et al.*, 1992); splice variants with opposing transcriptional effects have also been described in the Fos and FosΔ oncoproteins (Wisdom *et al.*, 1992) and in the liver-specific transcription factors LIP and LAP (Descombes and Schibler, 1992). As yet, however, no data are available regarding the possible effect of c-Ets-1 (ΔVII) on cell growth.

Finally, if one aspect of the physiological role of c-Ets-1 is repression of AP-1 activity at those sites where Ets and AP-1 bind in close proximity, the disappearance of this repressor function in v-Ets may contribute to the transforming properties of that protein. Stimulation of AP-1 is often involved in normal or malignant growth. By competing with a c-Ets-1-like protein for binding to DNA, v-Ets may increase AP-1-driven transcription of only a subset of the AP-1 target genes, namely those that contain dual Ets/AP-1 elements of the polyoma enhancer type. Such a subset might include genes that can only contribute to proliferation in a certain cellular background, for example multipotential erythroid progenitors (Graf *et al.*, 1992). This would stand in contrast with the global enhancement of growth by AP-1 activity brought about by Fos, Jun, and many nonnuclear oncoproteins. It might thus help explain the narrow spectrum of cell types that are susceptible to v-Ets transformation and some of their unique properties (Metz and Graf, 1991; Graf *et al.*, 1992).

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[3] Y. Goldberg and D. Bohmann, unpublished observations.

[4] R. Bosselut and J. Ghysdael, unpublished data.

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