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cis-Acting Sequences Required for Inducible Interleukin-2 Enhancer Function Bind a Novel Ets-Related Protein, Elf-1

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The recent definition of a consensus DNA binding sequence for the Ets family of transcription factors has allowed the identification of potential Ets binding sites in the promoters and enhancers of many inducible T-cell genes. In the studies described in this report, we have identified two potential Ets binding sites, EBS1 and EBS2, which are conserved in both the human and murine interleukin-2 enhancers. Within the human enhancer, these two sites are located within the previously defined DNase I footprints, NFAT-1 and NFIL-2B, respectively. Electrophoretic mobility shift and methylation interference analyses demonstrated that EBS1 and EBS2 are essential for the formation of the NFAT-1 and NFIL-2B nuclear protein complexes. Furthermore, in vitro mutagenesis experiments demonstrated that inducible interleukin-2 enhancer function requires the presence of either EBS1 or EBS2. Two well-characterized Ets family members, Ets-1 and Ets-2, are reciprocally expressed during T-cell activation. Surprisingly, however, neither of these proteins bound in vitro to EBS1 or EBS2. We therefore screened a T-cell cDNA library under low-stringency conditions with a probe from the DNA binding domain of Ets-1 and isolated a novel Ets family member, Elf-1. Elf-1 contains a DNA binding domain that is nearly identical to that of E74, the ecdysone-inducible *Drosophila* transcription factor required for metamorphosis (hence the name Elf-1, for E74-like factor 1). Elf-1 bound specifically to both EBS1 and EBS2 in electrophoretic mobility shift assays. It also bound to the purine-rich CD3R element from the human immunodeficiency virus type 2 long terminal repeat, which is required for inducible virus expression in response to signalling through the T-cell receptor. Taken together, these results demonstrate that multiple Ets family members with apparently distinct DNA binding specificities regulate differential gene expression in resting and activated T cells.

Members of the *ets* proto-oncogene family have recently been shown to encode transcription factors that interact in a sequence-specific manner with purine-rich motifs in the promoters and enhancers of several viral and cellular genes, including the polyomavirus enhancer (55), the Moloney sarcoma virus (MSV) long terminal repeat (LTR) (21), the human T-cell receptor (TCR) α-chain gene enhancer (23), and the class II major histocompatibility complex promoter (32). Comparisons of the DNA binding sites of *ets-1* and two Ets-related genes, murine PU.1 and *Drosophila* E74, have led to the derivation of a consensus sequence for the Ets DNA binding site, C/AGGAA/T (30). By using this consensus sequence, we have identified potential Ets binding sites in the promoter/enhancer regions of a variety of T-cell-specific genes, including those for the lymphokines interleukin-2 (IL-2), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-3, the cell surface receptor molecules CD2, CD3, and TCR α, β, and δ, and a variety of T-cell-tropic viruses, including human immunodeficiency virus type 2 (HIV-2), human T-cell leukemia virus type I (HTLV-I), and MSV. To test the validity of this approach for identifying Ets binding sites and to better understand the functional importance of these Ets binding sites in regulating inducible gene expression in T cells, we have investigated the properties of the two predicted Ets binding sites within the IL-2 enhancer.

The IL-2 gene serves as a model system for studies of the molecular mechanisms underlying inducible gene expression in eukaryotic cells (7, 9). Quiescent peripheral blood T cells can be activated by TCR binding of an antigenic peptide presented in association with a self major histocompatibility complex molecule (1, 10). Previous studies have demonstrated that the IL-2 gene is not expressed in resting peripheral blood T cells but is transcriptionally induced within 1 h of T-cell activation (12, 14, 34, 53). Induction of IL-2 by cross-linking of the TCR is thought to involve at least two signals and can be mimicked by the simultaneous activation of protein kinase C and increases in intracellular calcium levels (45, 57, 59). IL-2 gene expression is regulated by a 320-bp T-cell-specific transcriptional enhancer that is located in the 5′ flanking region of the gene (9, 11, 15, 16, 18, 26, 50). This enhancer contains a number of distinct nuclear protein binding sites that are important in regulating IL-2 transcription in activated T cells (5, 11, 15, 46).

Two of these previously defined nuclear protein binding sites, NFAT-1 and NFIL-2B, contain purine-rich sequence motifs that correspond to the consensus Ets binding site; we have therefore called these sites Ets binding site 1 (EBS1) and Ets binding site 2 (EBS2), respectively. In the studies described in this report, we have demonstrated that EBS1 and EBS2 are involved in the binding of the NFAT-1 and NFIL-2B T cell nuclear protein complexes and are required for inducible IL-2 gene expression. Two Ets family members, Ets-1 and Ets-2, were known to be expressed in T cells. However, neither of these proteins bound to the IL-2 en-

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hancer in vitro. We therefore used low-stringency hybridization with an Ets-1 cDNA probe to screen an activated T-cell cDNA library for additional Ets-related family members. One of the novel clones identified in this screen, Elf-1, was found to bind specifically to both of the Ets binding sites in the IL-2 enhancer but not to the previously described Ets-1 binding site in the TCR α enhancer. In addition, Elf-1 was shown to bind to an identical sequence motif within the HIV-2 enhancer that is required for inducible HIV-2 transcription in response to stimulation via the TCR. These results suggest that Ets proteins may regulate the expression of multiple T-cell genes and that different Ets family members may display different DNA binding specificities and thereby differentially regulate gene expression during both T-cell development and activation.

MATERIALS AND METHODS

Cells and cell lines. Human peripheral blood mononuclear cells were isolated from buffy coats obtained by leukophoresis of healthy donors age 21 to 31 years by using density gradient centrifugation. Purified T cells were then isolated from peripheral blood mononuclear cells by negative selection using immunoabsorption as described previously (28). Cell purification was routinely monitored by flow cytometry and histochemistry. Monocytes, B cells, and large granular lymphocytes were not detectable by immunofluorescence analysis. Viability was greater than 99% as measured by trypan blue exclusion. T cells were cultured at a concentration of 2×10^6/ml in RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 2 mM L-glutamine, 100 U of penicillin G per ml, 100 μg of streptomycin per ml, and 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4) (GIBCO). Freshly isolated T cells were activated by culture on plates coated with a saturating quantity of the anti-CD3 monoclonal antibody (MAb) G19-4 in the presence or absence of the anti-CD28 MAb 9.3 (1 μg/ml) for 6 to 8 h as described previously (28) or by treatment for 6 to 8 h with phorbol myristate acetate (PMA) (10 ng/ml), ionomycin (0.4 μg/ml), or both. For transfection experiments, murine EL4 T-cell tumor cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and penicillin-streptomycin (GIBCO) as described previously (24).

Plasmids. The pSPCAT reporter plasmid containing the minimal simian virus 40 (SV40) promoter immediately 5′ to the bacterial chloramphenicol acetyltransferase (CAT) gene has been described previously (37). Plasmid pIL2CAT, which contains the IL-2 promoter/enhancer (−585 to +18) immediately 5′ to the CAT gene, has been described previously (3). Three copies of a wild-type NFAT-1 oligonucleotide (AGAAAGGAGGAAAAACTGTTTCATACAGAAG GCGTT) or a mutant NFAT-1 oligonucleotide (AGAAA CGTCCTAAAACTGTTTCATACAGAAGGCGTT) containing *Bam*HI-*Bgl*II overhanging ends were cloned into the *Sma*I site of pSPCAT immediately upstream of the SV40 promoter to create plasmids pSPNFATCAT and pSPm NFATCAT. Mutations were introduced into the EBS1, EBS2, and 3′NFAT sites of pIL2CAT by gapped heteroduplex oligonucleotide-mediated site-directed mutagenesis as described previously (31) with the following oligonucleotides: mEBS1 (GAAGATCTTCGTCACTGTTTC; −282 to −261), mEBS2 (ATCAGACGTCCTAAA; −137 to −124), and m3′NFAT (AAATGTGCAGTCTGCAGCGGCGTT AAT; −271 to −245). All mutations were confirmed by dideoxy DNA sequence analysis. The pRSVβgal reference plasmid in which the bacterial β-galactosidase gene is under the control of the Rous sarcoma virus LTR has been described previously (24). A truncated version of the human Ets-1 cDNA (bp 864 to 1347) containing a consensus eukaryotic initiation codon at the 5′ end was prepared by the polymerase chain reaction, using 5′ and 3′ synthetic oligonucleotide primers with the sequences CCAAGCTTG GATCCCACCATGGAGGACTATCCGGCTGCCCTGCCC and CCAAGCTTTCCCCAGCCCCTTCAGTGC, respectively. This truncated cDNA was cloned into the *Hin*dIII site of pCDM7 for use in in vitro transcription and translation reactions. A truncated version of the Elf-1 cDNA (bp 312 to 1926) was prepared by the polymerase chain reaction with 5′ and 3′ synthetic oligonucleotide primers consisting of the sequences GGGATATCCCACCATGGCTGCTGTTGTC CAACAGAAC and GGGATATCTCACCACAGGTGCAG TAGCACTGAAG, respectively. This truncated cDNA was cloned into the *Eco*RV site of pcDNA1/neo (InVitrogen, San Diego, Calif.) for use in in vitro transcription and translation reactions. Plasmid DNA for use in transfections was prepared by cesium chloride density gradient centrifugation as described previously (20).

Preparation of nuclear extracts. Nuclei were isolated from cultured T cells by centrifugation at 14,000 × *g* for 2 min following cell lysis with 40 mM KCl–10 mM HEPES (pH 7.0)–3 mM $MgCl_2$–1 mM dithiothreitol (DTT)–5% glycerol–8 μg of aprotinin per ml–2 μg of leupeptin per ml–0.5 mM phenylmethylsulfonyl fluoride (PMSF)–0.2% (vol/vol) Nonidet P-40. Nuclei were resuspended in 20 mM HEPES (pH 7.9)–0.42 M KCl–1.5 mM $MgCl_2$–0.2 mM EDTA, 0.5 mM DTT–0.5 mM PMSF–25% (vol/vol) glycerol for 30 min at 4°C. Extracts were cleared by centrifugation at 14,000 × *g* for 10 min at 4°C. The resulting supernatants were dialyzed for 4 h at 4°C against buffer containing 20 mM HEPES (pH 7.9), 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 20% (vol/vol) glycerol (buffer D) and frozen in aliquots at −70°C. Protein concentrations were determined by using a commercially available kit (Bio-Rad, Richmond, Calif.).

Electrophoretic mobility shift assays (EMSAs). The following double-stranded oligonucleotides containing overhanging *Bam*HI-*Bgl*II ends were synthesized on an Applied Biosystems model 380B DNA synthesizer and labelled with ^{32}P-nucleotides by fill-in with the Klenow fragment of DNA polymerase I prior to use in EMSAs:

NFAT:	AGAAAGGAGGAAAAACTGTTTCATACAGAAGGCGTT
mNFAT:	AGAAACGTCCTAAAACTGTTTCATACAGAAGGCGTT
IL-2B:	AAAGAGTCATCAGAAGAGGAAAAATGAAGGT
mIL-2B:	AAAGAGTCATCAGAAGTCCTAAAATGAAGGT
MSV LTR:	TCGGAGAGCGGAAGCGCGC
Tα2:	CCTCTTCTTTCCAGAGGATGTGGCTTCTGCGA
Tα3:	AGAGATAGCATCGCCCCAGGCCACGTGCCGAGGG
HIV-2 LTR:	CCATTTAGTTAAAGACAGGAACAGCTAT

Binding reactions using T-cell nuclear extracts contained 1 to 1.5 μg of T-cell nuclear protein, 20,000 dpm (0.1 to 0.5 ng) of radiolabelled oligonucleotide probe, 250 ng of poly(dI-dC) in 50 mM KCl, 10 mM Tris (pH 7.5), 10 mM HEPES, 1.25 mM DTT, 1.1 mM EDTA, and 15% (vol/vol) glycerol in a final reaction volume of 20 μl. Following incubation at room temperature for 30 min, DNA-protein complexes were fractionated by electrophoresis on nondenaturing 4% polyacrylamide gels at 100 V for 2.5 h at room temperature in TGE buffer (0.05 M Tris, 0.5 M glycine, 0.5 mM EDTA) with 47 μM β-mercaptoethanol. Binding reactions using 1 μl of bacterially produced Elf-1 protein from purified inclusion

bodies were performed identically to those using T-cell nuclear extracts with the exception that the gels were run at 110 V for 4 h at 4°C. Binding reactions using in vitro-transcribed and -translated Elf-1 and Ets-1 proteins contained 3 μl of in vitro-translated protein, 20,000 dpm of radiolabelled oligonucleotide probe, 250 ng of poly(dI-dC) in 75 mM KCl, 10 mM Tris (pH 7.5), 1 mM DTT, 1 mM EDTA, and 4% Ficoll. Following incubation for 30 min at room temperature, DNA-protein complexes were fractionated by electrophoresis in 4% nondenaturing polyacrylamide gels which were run in 0.25× Tris-borate-EDTA buffer at 110 V for 4 h at 4°C. All gels were dried and subjected to autoradiography using intensifying screens as described previously (24).

Methylation interference and UV cross-linking. For methylation interference experiments, an NFAT-1 oligonucleotide labelled with [^{32}P]ATP by using T4 polynucleotide kinase was incubated with dimethylsulfate at a final concentration of 0.05 M for 5 min at room temperature. The resulting oligonucleotide was used in an EMSA binding reaction (see above) that contained 2×10^5 dpm of radiolabelled methylated oligonucleotide, 10 μg of T-cell nuclear protein, and 1 μg of poly(dI-dC) in a final reaction volume of 50 μl. The products were separated by electrophoresis in a 4% nondenaturing polyacrylamide gel. Following autoradiography, the bound and free probes were excised and eluted from the gel. The recovered DNA was cleaved with 1 M piperidine at 90°C for 30 min prior to polyacrylamide gel electrophoresis (PAGE) on a standard 12% polyacrylamide DNA sequencing gel. An equal number of counts was loaded into each lane of the gel.

For UV cross-linking experiments, 5 μg of T-cell nuclear extract was incubated with 10^5 dpm of radiolabelled NFAT-1 oligonucleotide with bromodeoxyuridine (BrdU) incorporated on the antisense strand in an EMSA binding reaction mixture containing 1 μg of poly(dI-dC) as described above. The resulting complexes were separated by electrophoresis in a 4% nondenaturing polyacrylamide gel. Following electrophoresis, the gel was subjected to UV irradiation (250 mJ/cm^2), using a Stratalinker model 1800 apparatus (Stratagene, La Jolla, Calif.) containing a 254-nm source. Following autoradiography, the NFAT-1 band was excised and the DNA-protein complexes were fractionated directly from the gel slice by electrophoresis in a 10% denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel (1.5 h at 20 mA plus 2.5 h at 30 mA at room temperature).

Transfections and CAT assays. Exponentially growing cultures containing 10^7 EL4 T cells were transfected with 10 μg of reporter plasmid and 2.5 μg of the pRSVβgal reference plasmid, using DEAE-dextran as described previously (25). At 36 h after transfection, cells were treated with medium alone or PMA (50 ng/ml) plus ionomycin (1.4 μg/ml) for 12 h prior to harvesting. Transfected cell extracts were normalized for protein content by using a commercially available kit (Bio-Rad) and assayed for CAT and β-galactosidase activities as described previously (24).

Isolation of Elf-1 cDNA clones. Elf-1 cDNA clones were isolated by screening a λgt11 cDNA library from PMA-plus-ionomycin-activated Jurkat T cells (23) by a low-stringency hybridization with a radiolabelled 188-bp *Bal*I-*Aat*II cDNA fragment containing the basic domain of human Ets-1 (bp 1113 to 1301). Final washing condition were 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–0.1% SDS at 50°C for 60 min. Elf-1 cDNAs were subcloned into Bluescript KS for restriction enzyme and DNA sequence analysis. Dideoxy DNA sequencing reactions were performed by using a commercially available kit (U.S. Biochemicals, Cleveland, Ohio). All sequence was obtained on both strands. To obtain overlapping sequences, deletional clones were made by using a commercially available kit (Stratagene). DNA sequence analyses were performed with the DNASTAR (Madison, Wis.) software package.

In vitro transcription and translation reactions. In vitro transcription reactions were carried out by using a commercially available kit (InVitrogen) according the manufacturer's instructions. In vitro translation reactions were performed by using a commercially available rabbit reticulocyte system (Promega, Madison, Wis.) according to the manufacturer's instructions as described previously (23).

Production of recombinant Elf-1 protein. The truncated Elf-1 cDNA (see above) was excised from pcDNA1/neo by digestion with *Eco*RV and cloned into the *Bam*HI site of the pET-3b vector (52) following blunting with the Klenow fragment of polymerase I. The resulting plasmid was transformed into competent *Escherichia coli* BL21(DE3), and a single colony was grown in M9ZB medium (52) containing carbenicillin (100 μg/ml) to an optical density at 600 nm of 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 2 mM, and cells were grown at 37°C for 2.5 h with shaking. Cells were pelleted by centrifugation at 4,000 rpm in a microcentrifuge (Eppendorf 5415) and lysed in 50 mM Tris (pH 8.0)–1 mM EDTA–1 mM DTT–1 mM PMSF–260 μg of lysozyme per ml. Inclusion bodies were pelleted by centrifugation at 4,000 rpm in a microcentrifuge (Eppendorf 5415). Proteins were denatured and solubilized in 6 M urea and then renatured by dialysis against buffer D overnight at 4°C prior to freezing in aliquots at −70°C. Control extracts were prepared from *E. coli* BL21(DE3) containing plasmid pET-3b without a cDNA insert.

Nucleotide sequence accession number. The complete nucleotide sequence of Elf-1 is available through GenBank, accession number M82882.

RESULTS

Two Ets binding sites in the IL-2 enhancer. The recent demonstration of the sequence-specific binding of three Ets family members, Ets-1, PU.1, and E74, has allowed the derivation of a consensus Ets binding sequence (23, 30). An examination of known T-cell promoters and enhancers for this sequence revealed the presence of potential Ets binding sites in a large number of T-cell-associated genes, including those for the lymphokines IL-2, IL-3, and GM-CSF, the cell surface receptors CD2, CD3, and TCR α, β, and δ, and a variety of T-cell-tropic viruses, including HIV-2, HTLV-I, and MSV (Fig. 1). Interestingly, the predicted Ets binding sites in the HTLV-I LTR have recently been shown to bind Ets-1 (4). To further understand the role of Ets family members in regulating inducible gene expression in T cells, we investigated the properties of the Ets binding sites in the human IL-2 enhancer. Analysis of the 320-bp IL-2 enhancer revealed two potential Ets binding sites which we will refer to hereafter as EBS1 and EBS2 (Fig. 2). EBS1 is located at the 5′ end of the NFAT-1 DNase I footprint (47), whereas EBS2 is contained within the previously described NFIL-2B binding site (11). Previous studies of NFAT-1 have suggested that this site binds a T-cell-specific nuclear protein complex that is induced in T-cell tumor lines following stimulation with phytohemagglutinin and PMA (11, 43, 47). In an initial set of experiments, we demonstrated that nuclear extracts from normal human peripheral blood T cells

A

IL-2 Enh:	AGGAGGAAAAA	(NFAT-1)
	AAGAGGAAAAA	(IL-2B)
GM-CSF Pr:	CAGAGGAAATG	
IL-3 Pr:	GGGAGGAAGTA	
TCR α Enh:	CAGAGGATGTG	(Tα2)
TCR β Enh:	AACAGGATGTG	(Tβ3)
CD3 δ Enh:	TTGAGGATGAG	
CD3 ε Enh:	CGCAGGATGTG	
TCR δ Enh:	GAAAGGATTAG	
CD2 Enh:	CAGAGGAAAAA	

B

MSV LTR:	GAGCGGAAGCG	
HTLV-1 LTR:	GGGAGGAAATG	
HIV-2 LTR:	GACAGGAACAG	(CD3R)

FIG. 1. Potential Ets binding sites in T-cell genes. (A) Ets binding sites in the transcriptional regulatory elements of genes known to be expressed in lymphoid cells. The IL-2 enhancer sequence is from Durand et al. (11), the GM-CSF sequence is from Miyatake et al. (40), the IL-3 sequence is from Miyatake et al. (41), the TCR α enhancer sequence is from Ho et al. (23), the TCR β enhancer sequence is from Gottschalk and Leiden (20), the CD3δ enhancer sequence is from Georgopoulos et al. (19), the CD3ε enhancer sequence is from Clevers et al. (8), the TCR δ enhancer sequence is from Redondo et al. (44), and the CD2 enhancer is from Lake et al. (35). (B) Ets binding sites in the LTRs of T-cell-tropic retroviruses. The MSV LTR sequence is from Gunther et al. (21), the HTLV-I LTR sequence is from Bosselut et al. (4), and the HIV-2 LTR sequence is from Guyader et al. (22) and Markovitz et al. (38).

activated by cross-linking of the TCR-CD3 complex also contained a protein complex that bound to the NFAT-1 oligonucleotide probe by EMSA (Fig. 3). This binding activity was not present in nuclear extracts from resting peripheral blood T cells, was suppressed by treatment with cyclosporin A, was enhanced by costimulation with CD28, and was also induced by treatment of resting T cells with PMA plus ionomycin but not with either PMA or ionomycin alone (Fig. 3).

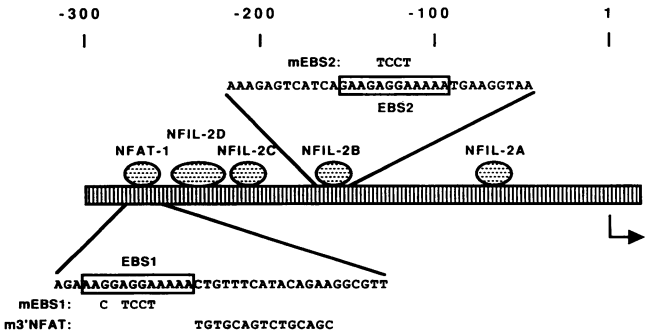


FIG. 2. Schematic representation of the human IL-2 enhancer. The IL-2 enhancer is shown as a hatched bar. Nuclear protein complexes that bind to the enhancer are from Durand et al. (11). The sequences of the wild-type NFAT-1 and NFIL-2B sites are shown below and above the map, respectively (11, 47). Potential Ets binding sites (EBS1 and EBS2) are boxed and labelled. Nucleotide substitutions incorporated into mutant NFAT-1 (mEBS1 and m3'NFAT) and NFIL-2B (mEBS2) oligonucleotides are shown below and above the wild-type sequences, respectively. The heavy arrow represents the transcriptional start site (9, 16, 50). Nucleotides are numbered relative to the transcriptional start site and are shown above the map.

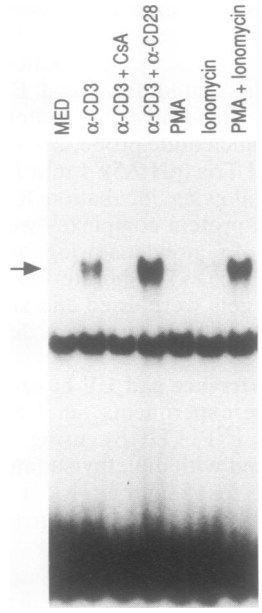


FIG. 3. EMSAs of NFAT-1 with nuclear extracts from normal human T cells. Purified human peripheral blood T cells were cultured for 7 h in medium alone (MED) or in tissue culture dishes coated with an anti-CD3 MAb (α-CD3). Cyclosporin A (CsA) or the anti-CD28 MAb 9.3 (α-CD28) was added to some cultures. Duplicate cultures of cells were treated for 7 h with PMA, ionomycin, or PMA plus ionomycin as indicated. Nuclear extracts prepared from these cultures were used in EMSAs with a radiolabelled NFAT-1 oligonucleotide (see Materials and Methods). The arrow denotes the band of altered mobility corresponding to the previously described NFAT-1 complex from human Jurkat T cells.

To determine whether EBS1, the potential Ets binding site in NFAT-1, was involved in the binding of the NFAT-1 nuclear protein complex, we performed methylation interference experiments using nuclear extracts from anti-CD3-stimulated normal human T cells. As shown in Fig. 4, methylation of two G residues at the core of EBS1 prevented binding of the NFAT-1 complex. Methylation of these same core G residues has been shown previously to prevent binding of Ets-1 to the MSV LTR (21). In addition, it should be noted that methylation of at least one G residue 3′ of EBS1 but within NFAT-1 also inhibited binding of the NFAT-1 complex (Fig. 4).

Further support for the hypothesis that NFAT-1 contains a functional Ets binding site was obtained from EMSAs using wild-type and mutated NFAT-1 oligonucleotides (Fig. 5). These experiments demonstrated that NFAT-1 binding could be inhibited efficiently by unlabelled wild-type NFAT-1 competitor oligonucleotides but not by a mutant NFAT-1 competitor (mNFAT-1) containing nucleotide substitutions within the EBS1 core sequence (Fig. 5A) (see Fig. 2 for mutations). The binding of NFAT-1 could also be competed for efficiently by an unlabelled IL-2B competitor oligonucleotide but not by a mutant IL-2B competitor (mIL-2B) in which the core of EBS2 (AGGA) was inverted (Fig. 5). The low-level competition seen with the mIL-2B competitor, which contains the sequence TTAGGACT on the antisense strand, suggests that more than the conserved AGGA in the ETS binding site core is necessary for binding

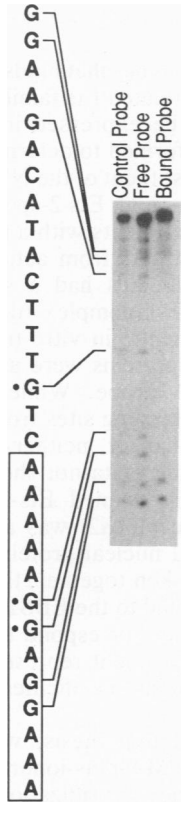


FIG. 4. Methylation interference analysis of the NFAT-1 nuclear protein complex. Partially methylated, radiolabelled NFAT-1 oligonucleotide was incubated in the absence (control probe) or presence of nuclear extract from anti-CD3 MAb-treated normal human peripheral blood T cells. Bound and free probes were separated by electrophoresis on a 4% nondenaturing polyacrylamide gel and cleaved with piperidine. The sequence of the NFAT-1 oligonucleotide is shown at the left (the 5′ end of the sequence is at the bottom). The putative Ets binding site (EBS1) is boxed. G residues protected from cleavage by NFAT-1 binding are dotted.

of the NFAT-1 complex. More extensive mutations of EBS2 abolished the ability of the oligonucleotide to compete for NFAT-1 binding (data not shown). NFAT-1 binding was also not inhibited by the unrelated Tα3 competitor oligonucleotide. Taken together, these results suggested that both EBS1 and EBS2 represent nuclear protein binding sites and that EBS1 is required for the binding of the NFAT-1 nuclear protein complex.

NFAT-1 contains a binding site for a second non-Ets nuclear protein. The methylation interference experiments shown in Fig. 4 suggested that G residues 3′ of EBS1 also play an important role in the binding of the NFAT-1 complex. This result raised the possibility that NFAT-1 contains binding sites for two distinct nuclear proteins. To examine this possibility more directly, we performed UV cross-linking of the NFAT-1 nuclear protein complex to a BrdU-substituted NFAT-1 oligonucleotide probe and analyzed the resulting products by SDS-PAGE (Fig. 6). These experiments revealed the presence of two major cross-linked bands with sizes of approximately 98 and 69 kDa. In addition, a minor band of 82 kDa was observed in some experiments. Analysis of UV cross-linking of the BrdU-substituted NFAT-1 oligonucleotide probe in the nonspecific DNA complex from the same gel failed to demonstrate cross-linking to any proteins in the size range of 69 to 98 kDa (data not shown). Taken together, these data are consistent with the hypothesis that the NFAT-1 nuclear protein complex contains at least two proteins, one of which is an Ets family member that binds to EBS1 and the second of which binds to the 3′NFAT sequence located immediately 3′ of EBS1.

Functional significance of Ets binding sites in the IL-2 enhancer. Previous studies have demonstrated that NFAT-1 oligonucleotides can confer T-cell-activating properties on a reporter gene when oligomerized upstream of a minimal promoter (11, 47). To determine the importance of EBS1 for NFAT-1 function, we produced CAT reporter plasmids in which three copies of a wild-type NFAT-1 oligonucleotide or a mutant NFAT-1 oligonucleotide (mNFAT-1) containing nucleotide substitutions within EBS1 were cloned upstream of a minimal SV40 promoter/CAT transcription unit (Fig. 7). These plasmids were transfected into murine EL4 T cells. The transfected cultures were divided into two portions; one was left unstimulated, and the second was activated by

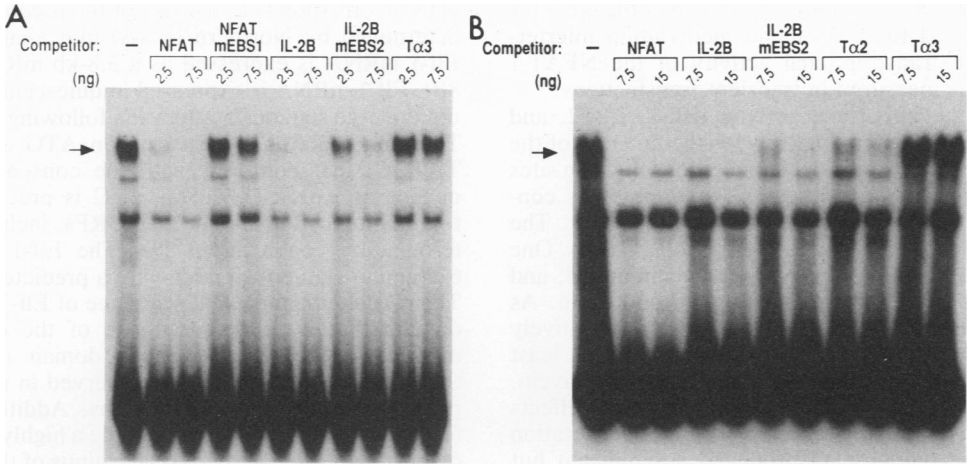


FIG. 5. EMSAs of NFAT-1. EMSAs were performed with a radiolabelled NFAT-1 oligonucleotide probe (see Materials and Methods) and nuclear extracts from anti-CD3 MAb-treated normal human T cells in the absence or presence of the specified amounts of cold-competitor oligonucleotides. The specific NFAT-1 complex is shown with an arrow.

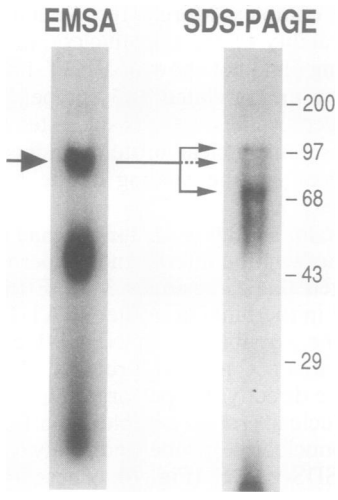


FIG. 6. UV cross-linking of the NFAT-1 complex. A radiolabelled, BrdU-substituted NFAT-1 probe was incubated with nuclear extract from anti-CD3 MAb-treated human T cells, and the resulting complexes were separated by electrophoresis in a 4% nondenaturing polyacrylamide gel (EMSA). Following UV cross-linking, the NFAT-1 band (heavy arrow) was excised from the EMSA gel and the DNA-protein complexes were fractionated directly from the gel slice by electrophoresis in a 10% denaturing SDS-polyacrylamide gel (SDS-PAGE). Solid arrows on the left of the SDS-PAGE autoradiogram correspond to the major UV cross-linked bands of 98 and 69 kDa. The dotted arrow represents a faint band of 82 kDa that was seen in some experiments. Size markers in kilodaltons are shown at the right.

treatment with PMA plus ionomycin. Transfections of EL4 with a control plasmid containing the SV40 promoter/CAT cassette but lacking the NFAT-1 oligonucleotides produced low levels of CAT activity that were not increased by treatment with PMA plus ionomycin (data not shown). The inclusion of three copies of the wild-type NFAT-1 oligonucleotide upstream of the CAT transcription unit had no effect on basal levels of CAT expression but did lead to an eightfold induction following stimulation of the transfected cells with PMA plus ionomycin (Fig. 7A). In contrast, mutation of the EBS1 sites completely abolished the inducibility of the plasmid during T-cell activation (Fig. 7A). Thus, the EBS1 site is required both for the binding of the NFAT-1 complex, as assayed by EMSA and methylation interference, and for the transcriptional activity of the NFAT-1 oligonucleotide, as measured in transient transfections.

To determine the importance of the EBS1, EBS2, and 3'NFAT nuclear protein binding sites for the function of the native IL-2 enhancer, we introduced mutations of these sites into a reporter plasmid in which CAT expression is controlled by the intact 585-bp IL-2 enhancer/promoter. The resultant plasmids were then transfected into EL4 cells. One half of each transfected culture was left unstimulated, and the other half was treated with PMA plus ionomycin. As shown in Fig. 7B, the wild-type enhancer was relatively inactive in untreated EL4 cells but was induced at least 100-fold following treatment with PMA plus ionomycin. Mutation of either EBS1 or EBS2 had relatively small effects on inducibility (35 or 53% reduction, respectively). Mutation of the 3'NFAT site alone also produced a significant but incomplete decrease in the inducibility of the enhancer. In contrast, mutation of both EBS1 and EBS2 completely abolished the inducibility of the enhancer. Thus, the inducible activity of the IL-2 enhancer requires either EBS1 or EBS2 but not both.

A novel Ets family member that binds specifically to the IL-2 enhancer. Two highly related Ets family members, Ets-1 and Ets-2, are known to be expressed in T cells (2). Several experiments were performed to determine whether either of these proteins is a constituent of the NFAT-1 nuclear protein complex. First, Ets-1- and Ets-2-specific antibodies were used in shift-shift experiments with a radiolabelled NFAT-1 probe and nuclear extracts from activated human T cells. Neither of these antibodies had a specific effect on the mobility of the NFAT-1 complex (data not shown). In a second set of experiments, in vitro-transcribed and -translated Ets-1 and Ets-2 proteins were used in EMSAs with a radiolabelled NFAT-1 probe. While both proteins were shown to bind to Ets binding sites from the MSV LTR and the human TCR α enhancer, neither protein bound to the NFAT-1 oligonucleotide (data not shown; see also Fig. 9). Finally, the previously defined Ets-1 binding site in the human TCR α enhancer (Tα2) was a poor competitor for binding of the NFAT-1 nuclear protein complex to the IL-2 enhancer (Fig. 5B). Taken together, these results suggested that the proteins that bind to the EBS1 and EBS2 sites in the IL-2 enhancer might not correspond to Ets proteins. Alternatively, these proteins might represent novel members of the Ets family with binding specificities that are distinct from those of Ets-1 and Ets-2.

To test this second hypothesis, we screened a cDNA library prepared from PMA-plus-ionomycin-activated Jurkat T cells by low-stringency hybridization with a cDNA probe from the COOH-terminal basic domain of Ets-1, a region that is highly conserved in all Ets family members (30). These experiments resulted in the isolation of a cDNA clone whose Ets domain shared only 45 of 86 amino acids with the Ets domain of either Ets-1 or Ets-2. In contrast, the Ets domain of this clone was nearly identical to the inducible *Drosophila* transcription factor E74, a member of the Ets family whose binding specificity was used to arrive at a consensus Ets family binding site (Fig. 8). We named this clone Elf-1, for E74-like factor 1. DNA sequence analysis of a 3.6-kb Elf-1 cDNA revealed a 315-bp 5' untranslated region, a single long open reading frame (ORF) of 1,857 bp, and a 1,392-bp 3' untranslated region. There is a consensus polyadenylation signal (AATAAA) beginning at bp 3548, 16 bp before the 3' end of the clone. In addition, the size of this cDNA corresponds to the size of the predominant mRNA as determined by Northern (RNA) blot analyses. In T cells, Elf-1 mRNA is expressed as a 3.8-kb mRNA species (Fig. 8B). Elf-1 mRNA is expressed in quiescent T cells and does not undergo significant alteration following T-cell activation. The Elf-1 ORF is initiated by an ATG at bp 316 (TTAT TATGG) that conforms with the consensus initiation sequence of Kozak (33). This ATG is preceded by multiple termination codons in all three ORFs, including an in-frame termination codon at bp 298. The Elf-1 ORF encodes a 619-amino-acid polypeptide with a predicted size of 68 kDa. The predicted amino acid sequence of Elf-1 contains a basic domain that is related to those of the other Ets family members as well as an α-helical domain immediately 5' of the basic region that is also conserved in most of the other previously described Ets proteins. Additional noteworthy features of the Elf-1 protein include a highly acidic region (20 of 46 amino acids) at the NH_2 terminus of the protein (amino acids 55 to 100). Such acidic domains have been shown to function as transcriptional activator domains in other transcription factors (39). Elf-1 also contains a serine/threonine-

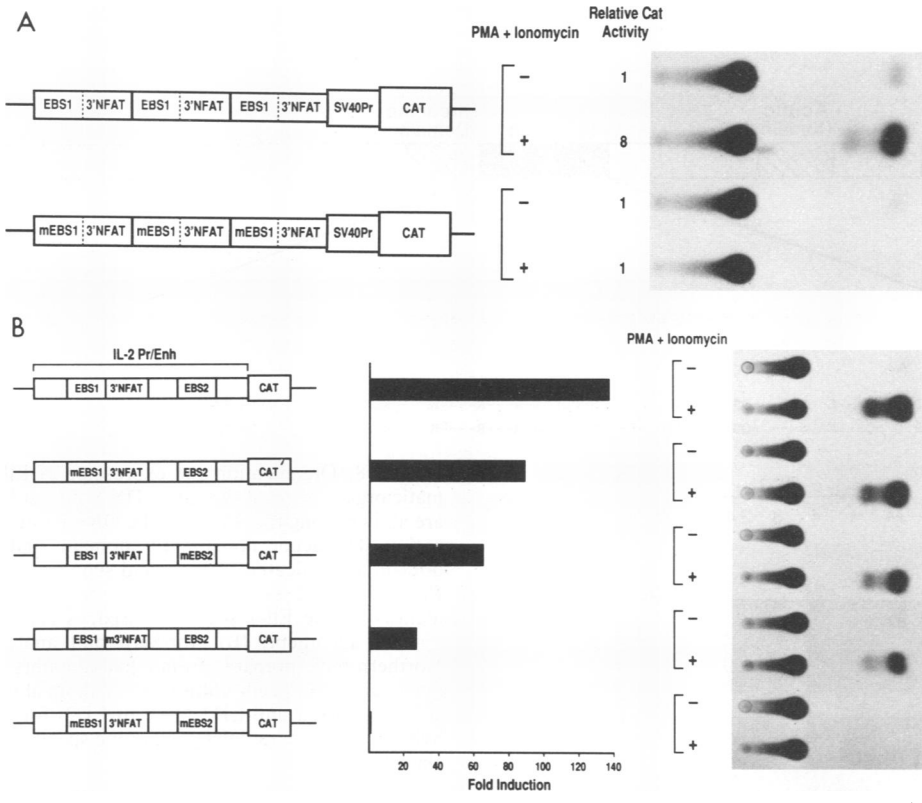
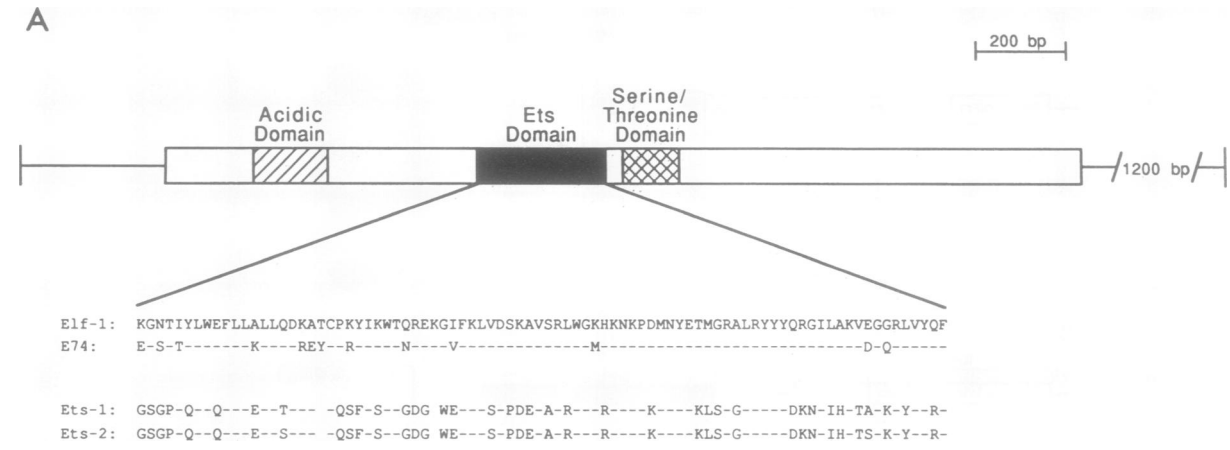


FIG. 7. Functional significance of nuclear protein binding sites in the human IL-2 enhancer. (A) Three copies of the wild-type NFAT-1 oligonucleotide or a mutant NFAT-1 oligonucleotide containing nucleotide substitutions in EBS1 (mEBS1; see Materials and Methods) were cloned directly upstream of the minimal SV40 promoter (SV40Pr) and the bacterial CAT gene in pSPCAT as shown schematically at the left. Following transfection into EL4 T cells, one half of the transfected cultures was incubated in medium alone (−) and the other half was treated with PMA plus ionomycin (+) for 12 h. To correct for differences in transfection efficiencies, all transfection mixtures also contained 2.5 μg of the pRSVβ-gal reference plasmid. Cell extracts were assayed for CAT and β-galactosidase activities. (B) EL4 T cells were transfected as described for panel A with plasmid pIL-2CAT, in which transcription of the CAT gene is under the control of the 585-bp wild-type IL-2 promoter and enhancer (IL-2Pr/Enh) or equal amounts of plasmids containing mutations in the EBS1 (mEBS1), EBS2 (mEBS2), or 3'NFAT (m3'NFAT) sequence. As described for panel A, all transfection mixtures also contained 2.5 μg of the pRSVβ-gal reference plasmid. Portions of the transfected cultures were treated for 12 h with medium alone (−) or with PMA plus ionomycin (+), and cell extracts were assayed for CAT and β-galactosidase activities. Fold induction represents CAT activity from the PMA-plus-ionomycin-treated culture/CAT activity from the untreated culture following corrections for differences in transfection efficiencies.

rich region immediately COOH terminal to the basic domain. Ets-1 and Ets-2 have both been shown to be phosphorylated during the process of normal T-cell activation (17, 42). Therefore, it is tempting to speculate that this domain of Elf-1 might also undergo phosphorylation in response to specific extracellular signals in T cells.

Elf-1 binds to the EBS elements in NFAT-1 and IL-2B. We reasoned that the high level of sequence identity between the basic domains of Elf-1 and E74 might result in similar DNA binding specificities. Urness and Thummel have recently shown that E74 can bind the sequence ACGAATCAGG AAAACTG, which contains a core sequence that is identical to those of EBS1 and EBS2 (54). Therefore, we tested Elf-1 for the ability to bind to EBS1 or EBS2 in the IL-2 enhancer. We performed a series of EMSAs using in vitro-transcribed and -translated and recombinant Elf-1 protein and a radiolabelled NFAT-1 oligonucleotide probe (Fig. 9). In vitro-transcribed and -translated and recombinant Elf-1 proteins bound to the NFAT-1, IL-2B, and MSV LTR probes (Fig. 9). The binding of Elf-1 to NFAT-1 was specific for the EBS1 sequence because Elf-1 failed to bind to a mutant NFAT-1 (mNFAT) oligonucleotide containing nucleotide substitutions within EBS1 or to the unrelated Tα3 oligonucleotide. Similarly, Elf-1 bound poorly to a radiolabelled mutant IL-2B competitor. The two major specific bands observed with the recombinant Elf-1 protein (Fig. 9B, arrows) reflected the presence of several forms of the Elf-1 protein in the crude bacterial lysate as assessed by SDS-PAGE (data not shown). These different-sized Elf-1 proteins may have arisen via the use of multiple initiation codons or by proteolytic breakdown of the full-length Elf-1 protein. In control experiments, both in vitro-translated truncated Ets-1 protein (containing amino acids 290 to 441 of human Ets-1) and full-length bacterially produced Ets-1 protein bound to radiolabelled MSV LTR but failed to bind to an NFAT-1 oligonucleotide (Fig. 9A and data not shown). Together, these results suggest that the fine specificities of DNA binding of the Ets-1 and Elf-1 proteins are different and may be determined by differences in the sequences that flank the Ets core motif, C/AGGAA/T.

As shown in Fig. 1, potential Ets binding sites can be identified in multiple T-cell genes. Thus, for example, the CD3R site in the HIV-2 enhancer shared significant sequence identity with the IL-2, EBS1, and EBS2 sites. CD3R



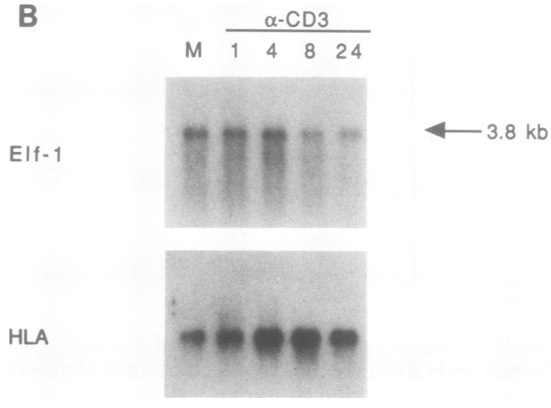


FIG. 8. Organization and expression of Elf-1 mRNA. (A) Schematic organization of the Elf-1 cDNA. 5′ and 3′ untranslated regions are shown along the thin line; the ORF is represented by the boxed region. The acidic domain, Ets domain, and serine/threonine-rich domain are indicated. Amino acid sequences of the Ets domains of Elf-1, E74 (6), Ets-1 (56), and Ets-2 (56) are shown. Dashes indicate identity to the Elf-1 sequence. Spaces are introduced to achieve optimal alignment. (B) Northern blot analysis of Elf-1 mRNA. Northern blots prepared from equal amounts of total cellular RNA from quiescent T cells cultured in medium alone (M) or activated by an immobilized anti-CD3 MAb (α-CD3) for 1, 4, 8, or 24 h were hybridized with a radiolabelled probe specific for Elf-1 (top) or HLA class I (bottom).

is required for the inducibility of HIV-2 transcription in response to cross-linking of the TCR-CD3 complex (38). Like EBS1 and EBS2, the CD3R site was also shown to bind Elf-1 protein preferentially. As shown in Fig. 9A, the CD3R site binds in vitro-transcribed and -translated Elf-1 protein with high affinity but binds poorly to the truncated Ets-1 protein. Thus, Elf-1 but not Ets-1 binds to multiple sites that are important in mediating inducible gene expression during T-cell activation.

DISCUSSION

The studies described in this report have demonstrated that the two predicted Ets binding sites, EBS1 and EBS2, within the human IL-2 enhancer are required for the formation of the previously described NFAT-1 and NFIL-2B nuclear protein complexes in vitro. More importantly, our in vitro mutagenesis studies demonstrated that at least one Ets binding site is necessary for IL-2 enhancer function. The presence of two distinct Ets binding sites in the IL-2 enhancer is consistent with previous reports that the IL-2 enhancer can be divided into discrete and redundant subdomains, one of which contains NFAT-1 (EBS1) and the other of which contains NFIL-2B (EBS2) (11, 58). Mutation of both EBS1 and EBS2 within the context of the intact IL-2 enhancer results in the complete loss of transcriptional induction during T-cell activation. In contrast, our work and that of others have suggested that mutations of other individual nuclear protein binding sites within the IL-2 enhancer, including 3′NFAT (this report), NF-κB, AP-1, Oct-1/2, and AP-3 (13, 27, 29, 49), each result in only a partial decrease in activationally induced enhancer function. Finally, despite the fact that Ets binding sites appear to be essential for inducible IL-2 enhancer function, multimerized EBS1 or CD3R Elf-1 binding sites do not function as inducible transcriptional enhancers, and overexpression of Elf-1 does not by itself result in the transactivation of either the IL-2 or HIV-2 enhancer (data not shown). Together, these data suggest that cooperation between an Ets family member and a variety of inducible DNA-binding proteins is required for the activation of IL-2 and HIV-2 enhancer function.

Several types of data support the hypothesis that Ets family members function to regulate the IL-2 enhancer (and other enhancers) by interacting with other inducible transcription factors bound to adjacent sites on the DNA. First, both EBS1 and EBS2 are contained within DNase I-footprinted sites that appear to bind multiple nuclear proteins. Thus, EBS2 is contained within the previously described NFIL-2B DNase I footprint, which also contains an AP-1 site (5, 11), and EBS1 is contained within the NFAT-1 footprint, which also contains a binding site for a second nuclear protein (this report). Thus, the NFIL-2B footprint could be the result of the inducible binding of a nuclear protein complex which contains AP-1 as well as an Ets family member. Wasylyk and colleagues (55) have shown recently that Ets-1 and AP-1 (Fos/Jun) cooperatively interact in such a complex by binding to a 14-bp region in the polyomavirus enhancer. Similarly, we have found that Ets-1 also binds to the TCR α enhancer in concert with a second non-Ets protein (23). Interestingly, as is the case for EBS1 and EBS2, the Ets binding sites in both the TCR α and polyomavirus enhancers are required for enhancer activity, yet these sites by themselves do not display enhancer function, nor does overexpression of Ets-1 alone activate these enhancers (55).

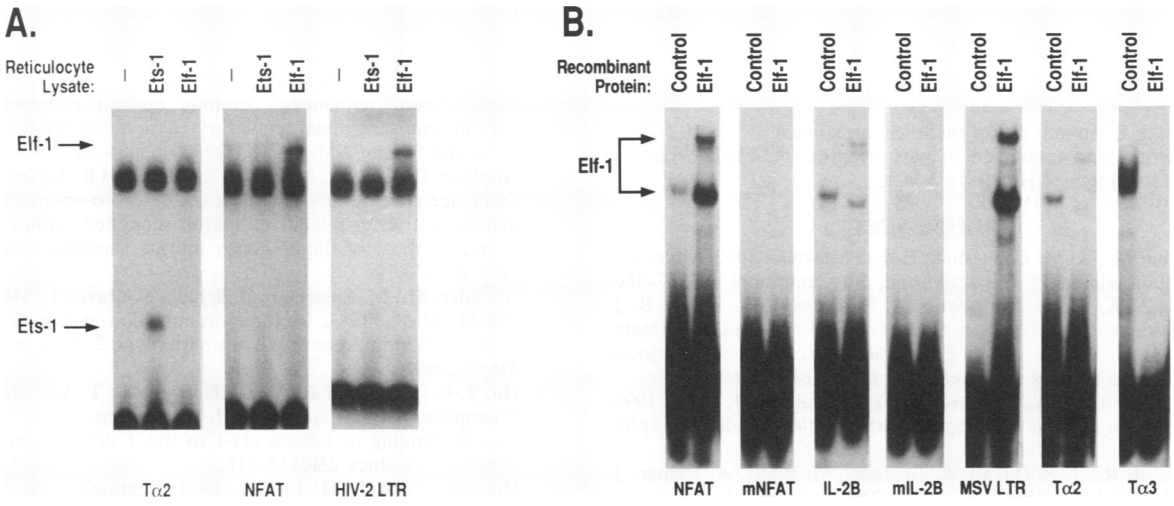


FIG. 9. EMSA analysis of the DNA binding specificities of Ets-1 and Elf-1 proteins. (A) EMSAs using in vitro-transcribed and -translated Ets-1 and Elf-1 proteins. Individual radiolabelled probes are indicated at the bottom (see Materials and Methods). Control lysates (−) were translated in the absence of exogenous RNA. Bands of altered mobility corresponding to binding of the in vitro-translated Ets-1 and Elf-1 proteins are shown with arrows and labelled at the left. (B) EMSAs using bacterially produced recombinant Elf-1 protein. Individual radiolabelled probes are indicated at the bottom. Control binding reaction mixtures contained bacterial protein prepared from cells containing the pET-3b expression vector without the Elf-1 cDNA. Bands of altered mobility corresponding to binding of the recombinant Elf-1 protein are shown with arrows and labelled.

EBS1 also appears to be a component of a larger, more complex nuclear binding site referred to as NFAT-1 (47). In addition to containing EBS1, NFAT-1 appears to contain the recognition sequence for a second nuclear protein that we have called 3′NFAT. The ability of multimerized NFAT-1 oligonucleotides to confer T-cell-activating properties on a heterologous promoter requires both a functional EBS1 site and a functional 3′NFAT-1 site (Fig. 7A and data not shown), again consistent with the idea that Ets proteins function as transcriptional cofactors. It is also important to emphasize that the 3′NFAT site is not the only site that can cooperate with EBS1 and/or EBS2 to activate the IL-2 enhancer. The IL-2 enhancer containing a mutated 3′NFAT site was still induced by 27-fold in response to PMA plus ionomycin. In addition, enhancer truncations lacking the entire NFAT-1 site but still containing the AP1, NF-κB, AP-3, and EBS2 sites retain a significant degree of inducibility (58). Because the IL-2 enhancer contains functional binding sites for AP-1, AP-3, OCT-1/2, and NF-κB (5, 13, 27, 29, 36, 48), Ets proteins could potentially interact with a number of different inducible transcription factors to regulate IL-2 transcription.

Although the studies presented in this report have demonstrated that Ets binding sites are required for the transcriptional activation of the IL-2 and HIV-2 enhancers, the identities of these Ets proteins remain unresolved. None of the proteins that can be UV cross-linked to a BrdU-substituted NFAT-1 probe correspond in size to Ets-1 (51/48 kDa) or Ets-2 (56 kDa). In addition, Ets-1 and Ets-2 fail to bind to the IL-2 enhancer and are therefore not candidates for the IL-2 regulatory proteins. In contrast, the Elf-1 gene reported here does bind to both EBS1 and EBS2 in the IL-2 enhancer and to the CD3R site in the HIV-2 enhancer and could, therefore, play a role in regulating both IL-2 and HIV-2 transcription. In addition, the predicted size of Elf-1 (68 kDa) corresponds closely to the size of one of the proteins that can be cross-linked to a BrdU-substituted NFAT-1 probe. Previous studies using T-cell tumor cells have demonstrated that the NFAT-1 DNA binding activity is T-cell specific and is observed only following T-cell activation (11, 47). In addition, NFAT-1 binding activity is blocked by inhibitors of protein synthesis such as cycloheximide (47). We have confirmed these results in normal human T cells. Northern blot analyses have shown that Elf-1 mRNA (3.8 kb) is expressed in mouse thymus, spleen, and kidney. In addition Elf-1 mRNA is expressed both in B cells and in resting as well as activated normal human T cells (Fig. 8B). Thus, levels of NFAT-1 binding activity do not correlate with Elf-1 mRNA levels. However, it remains possible that Elf-1 DNA binding activity might be regulated in vivo at the posttranscriptional or posttranslational level. Alternatively, because EBS1 and 3′NFAT both appear to be necessary for the binding of the NFAT-1 complex to DNA, it is possible that the proteins that bind to the 3′NFAT site are T-cell specific and activationally induced. In this model, the inducibility and T-cell specificity of the full IL-2 enhancer would be regulated by 3′NFAT, AP-1, NF-κB, and/or AP-3, whereas Elf-1 would be an essential cofactor which functions to integrate the combined effects of these other inducible transcription factors. Finally, it is possible that it is an Elf-1-related protein, and not Elf-1 itself, that regulates IL-2 and HIV-2 enhancer activities in vivo. It may be quite difficult to resolve these possibilities because it is now clear that many Ets proteins are expressed in T cells and that many anti-Ets antibodies cross-react with multiple Ets proteins. Thus, the unequivocal identification of the Ets protein(s) that regulates the IL-2 and HIV-2 enhancers in vivo will probably require the characterization of additional Ets family members expressed in activated T cells, the generation of antibody reagents that are specific for each of these related Ets family members, and antisense and gene knockout experiments in both cell lines and animals. In summary, it now appears that multiple Ets family members are expressed in T cells and that these proteins display distinct patterns of expression and DNA binding specificities. Such heterogeneity may help explain how Ets gene products

contribute to the regulated expression of different sets of genes during both T-cell development and activation.

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