Evolutionarily Conserved Ets Family Members Display Distinct DNA Binding Specificities

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Summary

Members of the Ets family of proto-oncogenes encode sequence-specific transcription factors that bind to a purine-rich motif centered around a conserved GGA trinucleotide. Ets binding sites have been identified in the transcriptional regulatory regions of multiple T cell genes including the T cell receptor α and β (TCR-α and -β) enhancers and the IL-2 enhancer, as well as in the enhancers of several T cell–trophic viruses including Maloney sarcoma virus, human leukemia virus type 1, and human immunodeficiency virus-2. T cells express multiple members of the Ets gene family including Ets-1, Ets-2, GABPα, Elf-1, and Fli-1. The different patterns of expression and protein–protein interactions of these different Ets family members undoubtedly contribute to their ability to specifically regulate distinct sets of T cell genes. However, previous studies have suggested that different Ets family members might also display distinct DNA binding specificities. In this report, we have examined the DNA binding characteristics of two Ets family members, Ets-1 and Elf-1, that are highly expressed in T cells. The results demonstrate that the minimal DNA binding domain of these proteins consists of adjacent basic and putative α-helical regions that are conserved in all of the known Ets family members. Both regions are required for DNA binding activity. In vitro binding studies demonstrated that Ets-1 and Elf-1 display distinct DNA binding specificities, and, thereby interact preferentially with different naturally occurring Ets binding sites. A comparison of known Ets binding sites identified three nucleotides at the 3′ end of these sequences that control the differential binding of the Ets-1 and Elf-1 proteins. These results are consistent with a model in which different Ets family members regulate the expression of different T cell genes by binding preferentially to purine-rich sequences that share a GGA core motif, but contain distinct flanking sequences.

The coordinate transcriptional regulation of sets of genes represents one of the important mechanisms that enable eukaryotic cells to respond to diverse developmental and environmental signals. Thus, for example, resting T lymphocytes express a set of tissue-specific genes that are important for their specialized functions, including the TCR/CD3 genes, and the genes encoding accessory molecules such as the CD4, CD8, and CD28 cell-surface antigens. Activation of such resting peripheral blood T cells after binding of antigen/MHC determinants by the TCR results in a complex pattern of de novo gene expression that includes the transcriptional induction of genes encoding multiple lymphokines and cell-surface antigens. The molecular mechanisms underlying tissue-specific gene expression in resting T cells and coordinate transcriptional induction after T cell activation have been the subject of intense scrutiny over the past several years (1).

Recent studies from several laboratories have demonstrated that members of the Ets proto-oncogene family encode transcription factors that recognize a purine-rich sequence:

$$\begin{matrix} \text{AAGA} \\ \text{GGCC} \end{matrix}\ \text{GGA}\ \begin{matrix} \text{AAAA} \\ \text{TGTG} \\ \text{C} \end{matrix}$$

This sequence is present in the transcriptional regulatory regions of several viral and cellular genes that are preferentially expressed in T cells (2–6). Thus, for example, Ets-1 binding sites in the human TCR-α gene enhancer (4), as well as the Maloney sarcoma virus (MSV)[1] (3) and human leukemia virus type 1 (HTLV-1) (2) enhancers appear to play critical roles in regulating the expression of these genes. Similar

[1] *Abbreviations used in this paper:* aa, amino acid; dpm, disintegrations per minute; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; HTLV-1, human leukemia virus type 1; MSV, Maloney sarcoma virus.

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purine-rich sequences are also present in the transcriptional regulatory regions of a number of additional T cell–specific genes including the TCR-β enhancer (7), the IL-2 (8, 9), IL-3 (10), and GM-CSF (11) promoter/enhancers, and the human immunodeficiency virus type 2 (HIV-2) enhancer (12) (see Fig. 1). The large number of potential Ets binding sites in these genes that are known to be expressed at distinct developmental and activational stages in T cells raised the question of how these genes could all be regulated by a common set of Ets proteins. Recent studies of T cell Ets proteins have suggested a solution to this apparent paradox. First, it is now clear that multiple Ets proteins are present in resting and activated human T cells. These include Ets-1 (13), Ets-2 (13), Elf-1 (9), Fli-1 (14), and GABPα (15). Second, two purine-rich sequences in the previously described NFAT-1 and NF-IL2B footprints of the IL-2 enhancer (16) were shown to bind to a novel Ets family member, Elf-1, but not to Ets-1 (9). These results suggested that different Ets family members might display distinct DNA binding specificities, and, thereby bind to, and regulate distinct sets of genes in resting and activated T cells. In this report, we provide experimental evidence that proves this hypothesis and elucidates the molecular basis for the distinct DNA binding specificities of two different Ets family members.

Using deletion and mutation analyses we have localized the DNA binding domain of Ets-1 to a 116-amino acid polypeptide that contains adjacent basic and putative α-helical domains, and that is conserved in all of the known Ets family members. Comparisons of the structures of the DNA binding domains of the different Ets family members, as well as their DNA binding specificities in vitro demonstrated that there are sub-families of Ets proteins that contain evolutionarily-conserved DNA binding domains. Members of these different sub-families display distinct DNA binding specificities. Thus, for example human Ets-1, which contains a DNA binding domain that is nearly identical to those of human and Drosophila Ets-2, binds preferentially to purine-rich sites within the TCR-α and -β enhancers, but not to two Ets binding sites in the IL-2 enhancer. Conversely, Elf-1, which contains a DNA binding domain that is nearly identical to that of the Drosophila transcription factor, E74, binds preferentially to the IL-2 and HIV-2 enhancers, but not to the Ets binding sites in the TCR-α and -β enhancers. Finally, a comparison of the known Ets binding sites in different T cell genes allowed the identification of three nucleotides at the 3′ end of the binding sites that play an important role in controlling the fine specificity of DNA binding by Ets-1 and Elf-1. Taken together, these findings help to explain how different Ets proteins regulate T cell transcription in response to multiple developmental and activational signals.

Materials and Methods

Plasmids. Truncated versions of the human Ets-1 cDNA containing a consensus eukaryotic initiation codon at the 5′ end were prepared by the PCR using the following synthetic oligonucleotide primers:

(tEts-$1_{325-441}$)

5′ Primer: CGAAGCTTCCACCATGGCCCTAGCTGGCTACACAGGCAGTG-GACCAATC

3′ Primer: GCGATATCACTCGTCGGCATCTGGCTTGACGTCCAGCATGGC

(tEts-$1_{372-441}$)

5′ Primer: CCAAGCTTCCACCATGGCCAGGAGATGGGGAAAGAGGAAAAAC

3′ Primer: GCGATATCACTCGTCGGCATCTGGCTTGACGTCCAGCATGGC

(tEts-$1_{325-392}$)

5′ Primer: CGAAGCTTCCACCATGGCCCTAGCTGGCTACACAGGCAGTG-GACCAATC

3′ Primer: GCGGATCCTCAGCCACGGCTCAGTTTCTCATAATTCATCTT-AGG

These truncated cDNAs were cloned into the HindIII and EcoRV sites of pcDNA1/NEO (Invitrogen, San Diego, CA) for use in in vitro transcription and translation reactions. The sequence of the full-length Elf-1 cDNA is available from Genbank, accession number M82882. A truncated version of the Elf-1 cDNA (Elf-$1_{108-304}$) was prepared by PCR with the following synthetic oligonucleotide primers:

5′ Primer: GGGATATCCCACCATGGATTCCCCTGGCCCTATGCTGGATG

3′ Primer: GCCTCGAGCTAAAAAGAGTTGGGTTCCAGCAGTTCGTTTTG

This truncated cDNA was cloned into the EcoRV and XhoI sites of pcDNA1/NEO (Invitrogen) for use in in vitro transcription and translation reactions. The α-helix, basic domain, and W2 and W3 mutants of Ets-1 were constructed by the overlap extension method of PCR (17) with the following sets of PCR primers:

(α-helix mutant)

5′ Primer 1: CAGCCTATCCAGAATCCCGCTATACCTCGG

3′ Primer 1: CAAGTCCTGGCTTTCCTTTCCCAACTGCGC

5′ Primer 2: AGATCTCAGGTTCATCTGGAATTACTCACTGATAAATCCT-GTCAG

3′ Primer 2: GAGTAATTCCAGATGAACCTGAGATCTCTGGATTGGTCCA-CTGCCTGTGTAGCC

(Basic domain mutant)

5′ Primer 1: CAGCCTATCCAGAATCCCGCTATACCTCGG

3′ Primer 1: CAAGTCCTGGCTTTCCTTTCCCAACTGCGC

5′ Primer 2: GTAGGCAACTCTTCCGACAAAAACATCATCCACAAGACAG-CGGGG

3′ Primer 2: GATGTTTTTGTCGGAAGAGTTGCCTACGCCACGGCTCAGT-TTCTCATAATTCATCTTAGG

(W2 mutant)

5′ Primer 1: CAGCCTATCCAGAATCCCGCTATACCTCGG

3′ Primer 1: CAAGTCCTGGCTTTCCTTTCCCAACTGCGC

5′ Primer 2: AGCTTGACAGGAGATGGCTGGGAATTCAAACTTTCTGAC

3′ Primer 2: CCCAGCCATCTCCTGTCAAGCTGATAAAAGACTGACAGGAT-TTATCAGTGAG

(W3 mutant)

5′ Primer 1: CAGCCTATCCAGAATCCCGCTATACCTCGG

3′ Primer 1: CAAGTCCTGGCTTTCCTTTCCCAACTGCGC

5′ Primer 2: AGATCCGGAAAGAGGAAAAACAAACCTAAGATGAATTATGAG

3′ Primer 2: AGGTTTGTTTTTCCTCTTTCCGGATCTCCTGGCCACCTCAT-CTGGGTCAAAAAC

For the α-helix and basic domain mutants, the products of the second PCR reaction were digested with SphI and AatII, and the resulting fragment containing the mutation was ligated into SphI/AatII-digested pcDNA1/NEO plasmid containing the full-length Ets-1 cDNA. For the W2 and W3 mutants, the products of the second PCR reaction were subjected to repeat PCR using the (Ets-$1_{325-441}$) primers (see above) before cloning into the HindIII and EcoRV sites of pcDNA1/NEO. The sequence of each mutant was confirmed by dideoxy DNA sequence analysis. Plasmid

DNA was prepared by cesium chloride density gradient centrifugation as previously described (7).

In Vitro Transcription and Translation Reactions. In vitro transcription reactions were carried out using a commercially available kit (Invitrogen) according to the manufacturer's instructions. In vitro translation reactions were performed using a commercially available rabbit reticulocyte system (Promega Corp., Madison, WI) according to the manufacturer's instructions, as described previously (18).

Electrophoretic Mobility Shift Assays (EMSAs). The following double-stranded oligonucleotides containing overhanging BamHI/BglII ends were synthesized on a model 380B DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) and labeled with ^{32}P-nucleotides by fill-in with the Klenow fragment of DNA polymerase I before use in EMSAs:

NFAT:	AGAAAGGAGGAAAAACTGTTTCATACAGAAGGCGTT
MSV LTR:	TCGGAGAGCGGAAGCGCGC
Tα2:	CCTCTTCTTTCCAGAGGATGTGGCTTCTGCGA
HIV-2 LTR:	CCATTTAGTTAAAGACAGGAACAGCTAT

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 radiolabeled oligonucleotide probe, 250 ng of polydI:dC, in 75 mM KCl, 10 mM Tris (pH 7.5), 1 mM dithiothreitol (DTT), 1 mM EDTA, and 4% Ficoll. After incubation for 30 min at room temperature, DNA protein complexes were fractionated by electrophoresis in 4% nondenaturing polyacrylamide gels that were run in 0.25 × TBE at 110 V for 3 h at 4°C. All gels were dried and subjected to autoradiography using intensifying screens as described previously (4).

Results

Definition of the DNA Binding Domain of the Ets-1 and Elf-1 Proteins. A comparison of the amino acid sequences of the known Ets family members has allowed the identification of an 82 amino acid (aa) ETS domain that is conserved in all Drosophila, avian, and mammalian Ets proteins (19). This ETS domain is, in turn, composed of a 42–43 aa basic region and a 14 aa NH_2-terminal domain that is predicted to adopt an α-helical conformation in computer analyses using both the Garnier-Robson and Kyte algorithms of DNAStar software (Madison, WI) (Fig. 2). Previous deletional analyses have suggested that the basic domain of Ets-1 is required for

TCR α Enh:	CAGAGGATGTG*	(Tα2)
TCR β Enh:	AACAGGATGTG*	(Tβ3)
CD3 δ Enh:	TTGAGGATGAG	
IL-2 Enh:	AGGAGGAAAAA*	(NFAT-1)
	AAGAGGAAAAA*	(IL-2B)
GM-CSF Pr:	CAGAGGAAATG*	
	CACAGGAACAT*	
IL-3 Pr:	GGGAGGAAGTA	
MSV LTR:	GAGCGGAAGCG*	
Igκ 3' Enh:	TTCAGGAACTG*	
HIV-2 LTR:	GACAGGAACAG*	(CD3R)
Consensus:	AGGAGGAAATG	
	GACC TGAA	
	C	

Figure 1. Potential Ets binding sites in lymphoid genes. Sequences present in the transcriptional regulatory regions of lymphoid genes that correspond to the consensus Ets binding site are shown. Previously described names for these nuclear protein binding sites are shown in parentheses at the right of the binding sites. (*) Sites that have been shown to bind Ets proteins. The human TCR α enhancer (Enh) sequence (4), human TCR-β enhancer sequence (7), and CD3δ enhancer binding site (36) have been described previously. The human IL-2 enhancer sequences are from Fujita et al. (37). The GM-CSF and IL-3 promoter sequences are from Miyatake et al. (11) and Miyatake et al. (10), respectively. The MSV LTR sequence is from Gunther et al. (3). The Igκ 3' enhancer sequence is from Meyer and Neuberger (38). The HIV-2 LTR sequence is from Markovitz et al. (12).

the ability of this protein to bind to whole calf thymus DNA (20). However, the precise localization of the minimal sequence-specific DNA binding domain of the Ets proteins remained unclear. To address this question we asked whether a 116 aa truncated form of Ets-1 (tEts-$1_{325-441}$) containing the basic domain and adjacent α-helical regions of the molecule was able to bind in an EMSA to the Ets-1 binding site from the MSV LTR. As shown in Fig. 3 *A*, in vitro transcribed and translated tEts-$1_{325-441}$ bound at least as well, if not better than, full-length Ets-1 to the MSV LTR. Similar results were obtained using a truncated form of Elf-1 (tElf-1 108–304) that also contained the α-helical region and basic domains

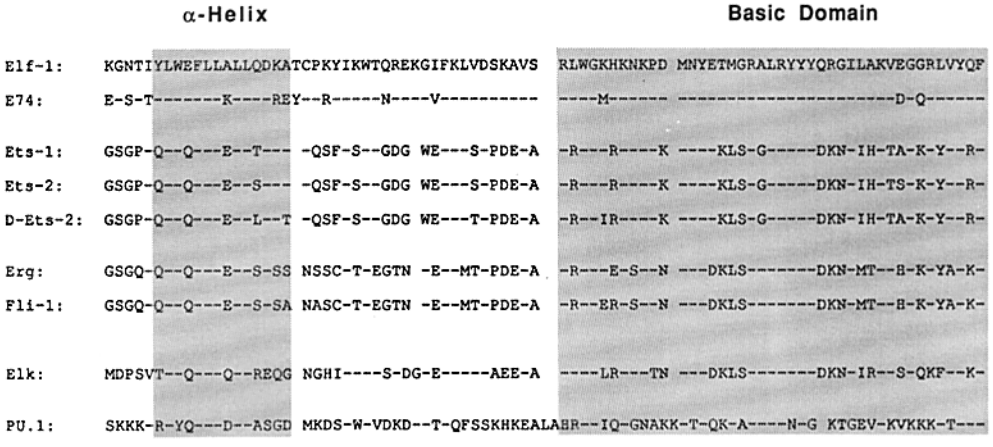
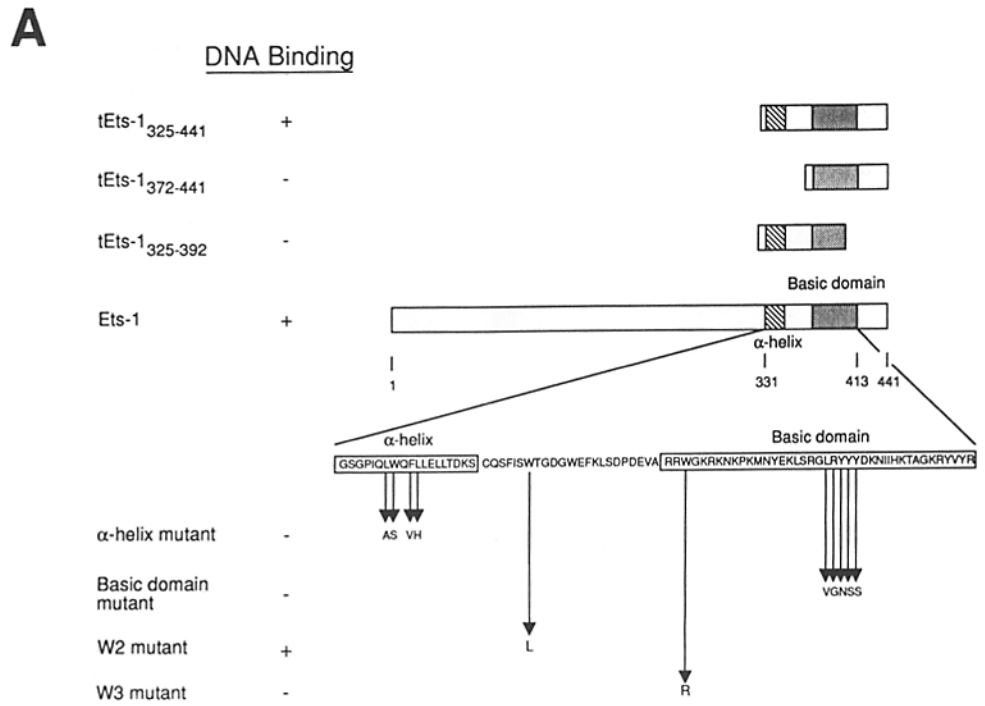
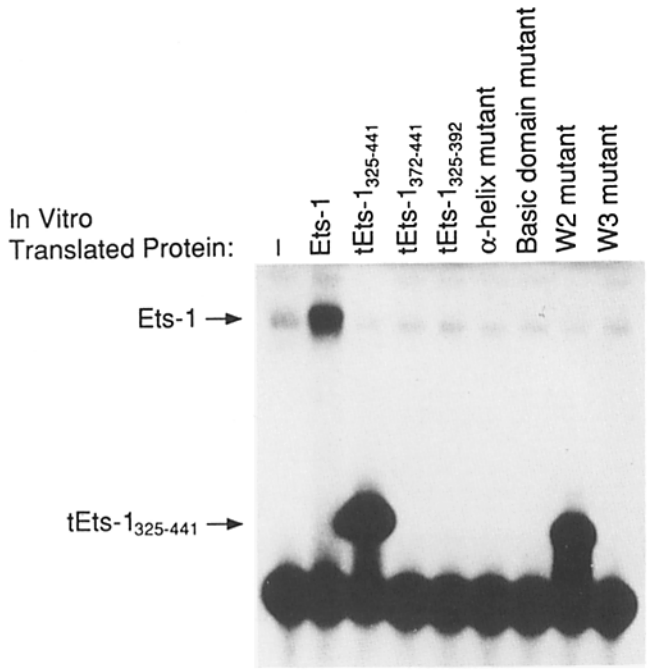
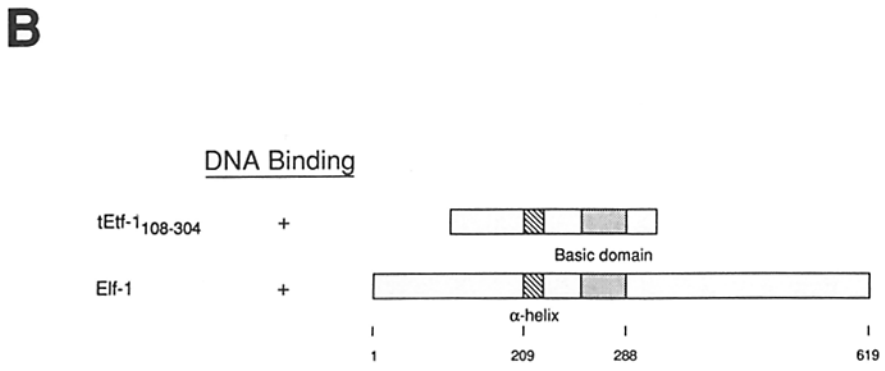


Figure 2. Structural comparison of the DNA binding domains of known Ets proteins. The amino acid sequences of the DNA binding domains of human Elf-1 (9), Drosophila E74 (22), human Ets-1 (23), human Ets-2 (23), Drosophila Ets-2 (D-Ets-2) (24), human Erg (25), human Fli-1 (14), human Elk (39), and human PU.1 (40) were aligned using the ALIGN program of DNASTAR Inc. software (Madison, WI). Spaces represent gaps introduced to produce optimal alignment. Dashes represent amino acids identical to those of human Elf-1. Ets family members with highly similar DNA binding domains are grouped together. The α-helical and basic domains conserved in all Ets family members are shaded and labeled.


A
DNA Binding
tEts-1325-441 +
tEts-1372-441 -
tEts-1325-392 -
Ets-1 +
α-helix mutant -
Basic domain mutant -
W2 mutant +
W3 mutant -
Basic domain
α-helix
1
331
413 441
α-helix
Basic domain
GSGPIQLWQFLLELLTDKS CQSFISWTGDGWEFKLSDPDEVA RRWGKRKNKPKMNYEKLSRGLRYYYDKNIIHKTAGKRYVYR
AS VH
L
R
VGNSS


In Vitro Translated Protein:
-
Ets-1
tEts-1325-441
tEts-1372-441
tEts-1325-392
α-helix mutant
Basic domain mutant
W2 mutant
W3 mutant
Ets-1
tEts-1325-441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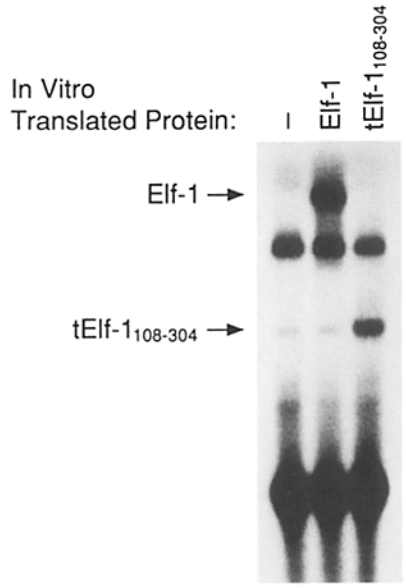
Elf-1 +
Basic domain
α-helix
1
209
288
619


In Vitro Translated Protein:
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Elf-1
tElf-1108-304
Elf-1
tElf-1108-304

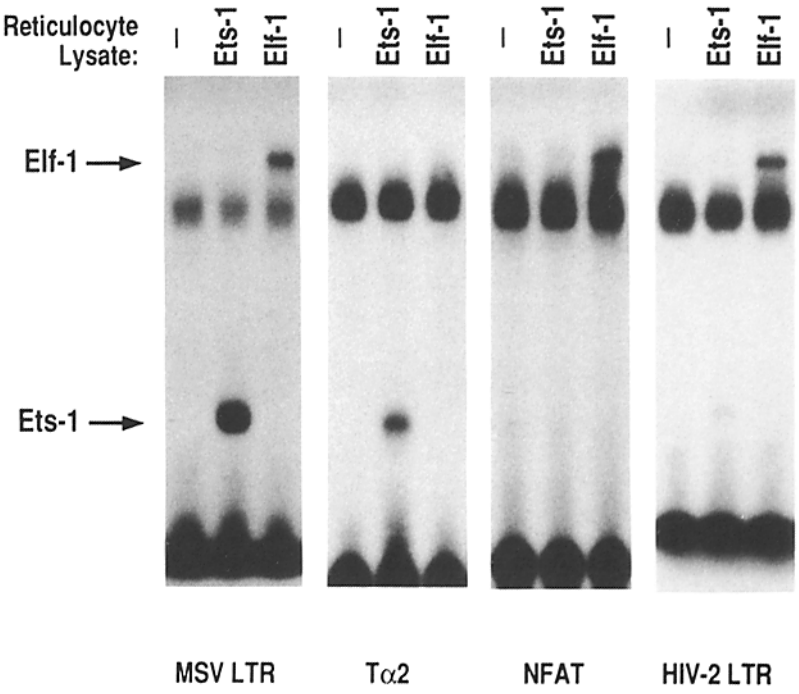

Figure 4. Electrophoretic mobility shift analysis of the DNA binding specificities of Ets-1 and Elf-1 proteins. EMSAs using in vitro transcribed and translated tEts-$1_{325-441}$ and Elf-1 proteins. (*Bottom*) Individual radiolabeled probes (see Materials and Methods). Control lysates (−) were translated in the absence of exogenous RNA. (→) Bands of altered mobility corresponding to binding of the in vitro translated tEts-$1_{325-441}$ (Ets-1) and Elf-1 proteins.

of that protein (Fig. 3 *B*). In contrast, truncated forms of Ets containing deletions of either the α-helical region or the basic domain (tEts-$1_{372-441}$ and tEts-$1_{325-392}$) failed to bind to this same probe (Fig. 3 *A*). To better assess the importance of the basic domain and α-helical regions for DNA binding we introduced amino acid substitutions separately into conserved regions of these two domains of Ets-1, and determined the effects of these mutations on DNA binding activity by EMSA (Fig. 3 *A*). Mutation of either the basic domain or α-helical region abolished the DNA binding activity of both the full-length and truncated forms of Ets-1 (Fig. 3 *A*). Thus, both the basic and α-helical domains are required for DNA binding by Ets-1.

All of the known ETS domains contain a conserved repeat of three tryptophans separated by 17–18 aa (19). Similar tryptophan repeats are present in the DNA binding domain of the c-myb protein (21). It has been hypothesized that these tryptophan residues may play an important role in the DNA binding activities of both the Myb and Ets proteins (19). To assess the role of the tryptophan repeats in the DNA binding activity of Ets-1, each of the tryptophans was mutated in the context of the tEts-$1_{325-441}$ protein (Fig. 3 *A*). Mutation of W3 abolished DNA binding. In contrast, mutation of W2 decreased binding only minimally. Finally, mutations of W1 as part of the α-helix mutant also abolished DNA binding. However, because this mutant contained three additional amino acid substitutions in the α-helical domain, the importance of W1 alone could not be assessed from this experiment. In summary, these results suggested that the tryptophans present in the α-helix and basic domains (W1 and W3) play an important role in DNA binding. In contrast, the conserved tryptophan in the spacer region between the α-helix and the basic domain (W2) is not required for the DNA binding activity of Ets-1. It should be emphasized that the observed differences in binding between the mutant and wild-type forms of the Ets-1 protein were not simply the result of differences in the efficiencies of in vitro transcription or translation because equal amounts of in vitro translated Ets proteins as determined by SDS-PAGE were used in each of the binding reactions shown in Fig. 3 *A*.

Evolutionarily Conserved Ets Proteins with Distinct DNA Binding Specificities. A comparison of the DNA binding do-

Figure 3. The DNA binding domains of Ets-1 and Elf-1. (*Middle*) Schematic illustrations of the full length (Ets-1, Elf-1) and truncated (tEts-1, tElf-1) forms of the human Ets-1, and Elf-1 proteins. Amino acids are numbered below the maps. (▨) α-helix. (■) Basic domain. Amino acid sequences of the wild-type and mutant forms of Ets-1 are shown below the Ets-1 schematic. (*A*) An EMSA using a radiolabeled MSV LTR oligonucleotide probe (see Materials and Methods) and in vitro transcribed and -translated Ets-1 proteins is shown at right. Equal amounts of in vitro translated protein as assayed by SDS-PAGE were used in each binding reaction. (→) Positions of Ets-1 and tEts-1 bands. (*B*) An EMSA using a radiolabeled MSV LTR oligonucleotide probe and in vitro transcribed and translated Elf-1 proteins is shown at right. Equal amounts of in vitro translated proteins as assayed by SDS-PAGE were used in each binding assay. (→) positions of the Elf-1 and tElf-1 bands. (*Left*) DNA binding activities of the different Ets-1 and Elf-1 proteins are summarized schematically.

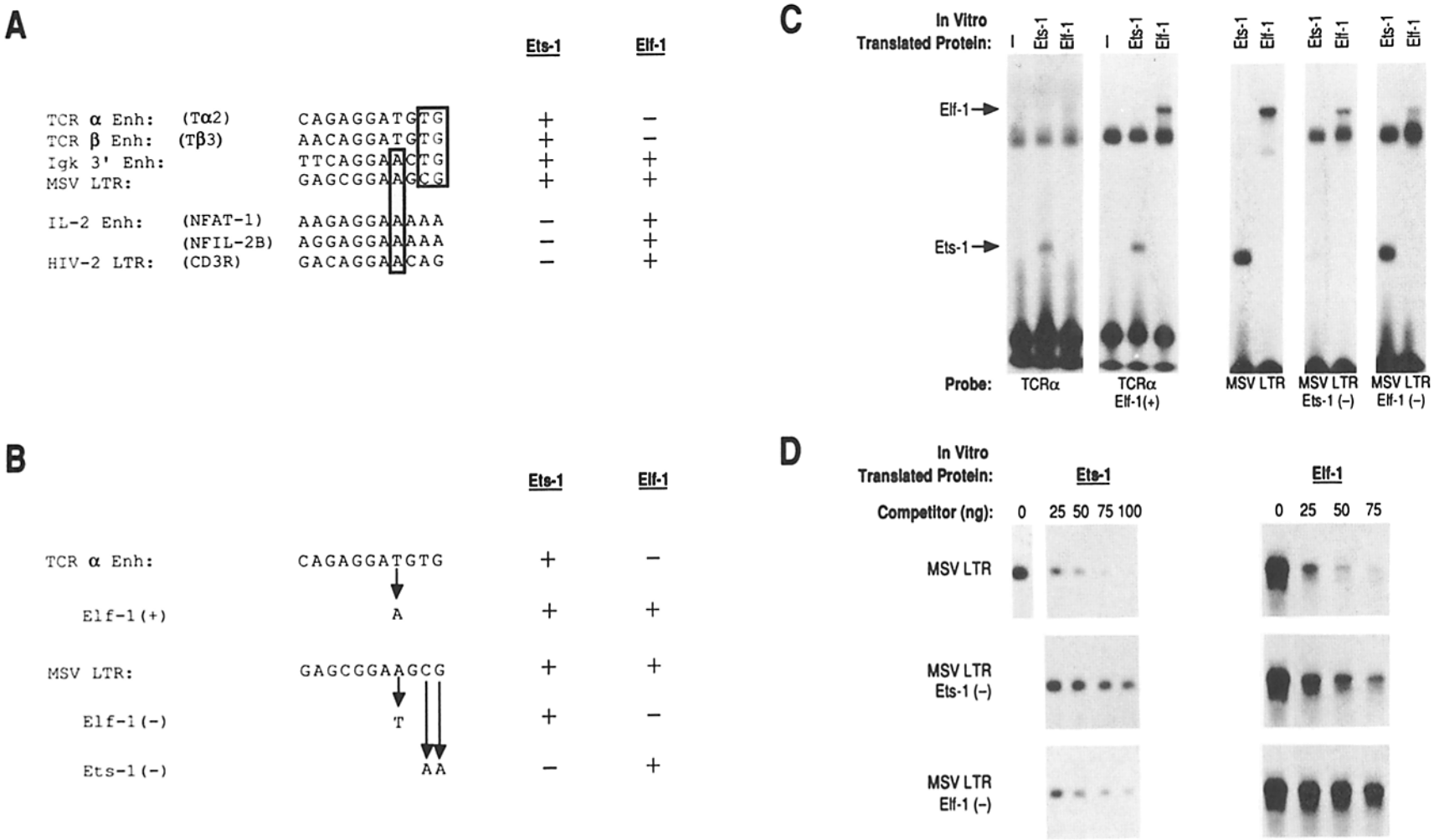

Figure 5. The molecular basis of the different DNA binding specificities of Ets-1 and Elf-1. (*A*) Comparison of the Ets binding sites in different lymphoid promoters and enhancers. (*Right*) Ets-1 and Elf-1 binding activities of each site are summarized. (*B*) Mutant oligonucleotide probes with altered Ets-1 and Elf-1 binding activities. The wild-type TCR-α and MSV LTR Ets binding sites are shown in their entirety. Nucleotide substitutions are shown below the arrows. (*Right*) DNA binding activities of the wild-type and mutant oligonucleotides are summarized. (*C*) EMSAs using the wild-type and mutant Ets binding sites. In vitro-transcribed and -translated Elf-1 or tEts-$1_{325-441}$ (Ets-1) proteins were used in EMSAs with the probes shown below each panel (see *B*). Control translations (−) were programmed with water instead of RNA. (→) positions of the Elf-1 and tEts-$1_{325-441}$ (Ets-1) containing bands. (*D*) Cold competition experiments using wild-type and mutant Ets-1 oligonucleotides. (*Left*) In vitro-transcribed and -translated Ets-1 or (*right*) Elf-1 proteins were used in EMSAs with a radiolabeled MSV LTR oligonucleotide probe. Increasing amounts of unlabeled wild-type or mutant MSV LTR competitor oligonucleotides (see *B*), shown to the left of the autoradiograms, were added to the binding reactions. All of the binding reactions with each in vitro translated protein were electrophoresed on a single gel and identical autoradiographic exposures are shown.

mains of the known Ets proteins revealed that they can be divided into several subsets based upon the structures of their basic and α-helical regions (Fig. 2). For example, the basic domain of Elf-1 (9) is almost identical to that of Drosophila E74 (22) (39 of 42 amino acids are identical). Similarly, the basic domains of mammalian Ets-1 and Ets-2 (23) are highly related to each other and to those of D-Ets-2 (24) (40 of 42 amino acids are identical), but significantly different from those of Elf-1 and E74. Finally, the basic domain of Erg (25) is almost identical to that of Fli-1 (14) (40 of 42 amino acids are identical). The remarkable similarities between the Drosophila and human proteins demonstrated that these subfamilies have been conserved over at least 600 million years of evolution.

The differences in the structures of the DNA binding domains between the different sub-families of Ets proteins suggested that these proteins might display distinct DNA binding specificities. We have reported previously that the Elf-1 protein binds to two purine-rich sequences (EBS1 and EBS2) in the IL-2 enhancer, but not to the previously defined Ets-1 binding site in the human TCR-α enhancer (9). To examine this question more systematically, we compared the binding activities of in vitro translated Ets-1 and Elf-1 proteins to four different naturally occurring Ets-1 binding sites, those from the MSV LTR, the TCR-α enhancer (Tα2), the IL-2 enhancer (NFAT), and the HIV-2 LTR (Fig. 4). Both Ets-1 and Elf-1 bound well to the MSV LTR. In contrast, only Ets-1 bound to the TCR-α enhancer, while only Elf-1 bound well to NFAT and the HIV-2 LTR. Thus, as predicted from the structural analysis of their DNA binding domains, members of the different sub-families of Ets proteins display subtly different DNA binding specificities.

The Molecular Basis of the Distinct DNA Binding Specificities of Ets-1 and Elf-1. We reasoned that it might be possible to identify specific nucleotides within the naturally occurring Ets binding sites that determine the affinities of these sites for different Ets proteins. A comparison of the sequences of several naturally occurring Ets binding sites that are known to display different affinities for the Ets-1 and Elf-1 proteins identified three nucleotides at the 3′ ends of the binding sites that correlated with Ets-1 or Elf-1 binding activity (Fig. 5 *A*). All of the sites that bind the Elf-1 protein contain an A at nucleotide 8 of the binding site. In contrast, the two sites that fail to bind Elf-1 contain a T at this position. Similarly, all of the sites that bind Ets-1 contain a CG or TG at positions 10 and 11 of the binding site, whereas those that fail to bind Ets-1 contain an AA or an AG at these positions. These observations are consistent with the finding that certain sites, such as that from the MSV LTR which contains both an A at position 8 and a CG at positions 10 and 11, are capable of binding both Ets-1 and Elf-1 (Fig. 4).

To more directly test the importance of nucleotides 8, 10, and 11 for Elf-1 and Ets-1 binding, respectively, we synthesized synthetic oligonucleotides with specific nucleotide substitutions at these sites (Fig. 5 *B*), and determined the effects of these substitutions on the affinities of these sites for the Ets-1 and Elf-1 proteins (Fig. 5, *C* and *D*). As predicted by the model, changing the T at position 8 in the TCR-α enhancer Ets-1 binding site to an A enabled this oligonucleotide to bind Elf-1 in addition to Ets-1 (Fig. 5 *C*). Conversely, changing the A at position 8 to a T in the MSV LTR significantly reduced the ability of this site to bind Elf-1, while having little or no effect on Ets-1 binding (Fig. 5, *C* and *D*). Altering the CG at positions 11 and 12 in the MSV LTR binding site to an AA abolished the ability of this site to bind Ets-1 with little or no effect on Elf-1 binding (Fig. 5, *C* and *D*). Finally, altering the AA at positions 11 and 12 in NFAT to a TG conferred the ability to bind Ets-1 on the NFAT site without significantly altering the ability of NFAT to bind Elf-1 (data not shown).

To confirm the differences in DNA binding affinities conferred by these mutations, we tested the ability of the mutated oligonucleotides to compete for binding by EMSA (Fig. 5 *D*). The Ets-1(−) mutant of the MSV LTR did not compete well for Ets-1 binding to the wild-type radiolabeled MSV LTR site, but competed quite well for Elf-1 binding to this same radiolabeled probe (Fig. 5 *D*). Conversely, the Elf-1 (−) mutant of this site competed poorly for Elf-1 binding to the MSV LTR, but competed well for binding of Ets-1 to the same probe (Fig. 5 *D*). Taken together, these experiments suggested that an A at nucleotide 8 of the Ets binding site plays an important role in the binding of Elf-1, while a T at this position abolishes binding. Similarly, a CG or TG at positions 11 and 12 in the binding site allows binding of Ets-1, while an AA or AG at this position greatly reduces or abolishes binding.

Discussion

Many mammalian transcription factors belong to families that contain multiple members which bind to highly related or identical DNA sequence motifs. Thus, for example there are at least eight CREB/ATF proteins that bind to a consensus octanucleotide, TGACGTCA (26), and at least three GATA proteins that bind to the hexanucleotide WGATAR (27). Similarly, the family of mammalian Ets proteins that bind to a purine-rich consensus sequence with a GGA core, contains at least eight members (9, 14, 15, 17). This multiplicity of related transcription factors raised the question of how these large families of DNA binding proteins can differentially regulate gene expression in different cell types and in response to distinct extracellular signals. In some cases, it is clear that different factors with apparently identical DNA binding specificities are expressed in different cell lineages. Thus, for example, GATA-1 is expressed in erythroid cells, megakaryocytes, mast cells, and their common progenitors (28, 29), while GATA-3 expression in hematopoietic cells is restricted to T lymphocytes (18). In other cases, protein–protein interactions alter the DNA binding specificities of specific transcription factors. Thus, for example, heterodimerization with c-jun is required for the DNA binding activity of c-fos (30–34). In the studies described in this report, we have demonstrated that subtle differences in DNA binding specificities between different members of the large family of related Ets

transcription factors can also provide a mechanism whereby multiple family members can regulate the expression of distinct genes in the same cell.

The divergence in the DNA binding specificities of the different Ets family members appears to have occurred quite early in evolution as evidenced by the remarkable similarity between the human Elf-1 and Drosophila E74 proteins, and the human Ets-1/Ets-2 and the Drosophila Ets-2 proteins. These differences in protein structure also appear to be reflected in DNA binding specificities as both Elf-1 and E74 bind the consensus sequence A/C G G A A A/G (5, this report). Finally, the high degree of structural conservation between the Drosophila E74 and human Elf-1 proteins is also paralleled by interesting similarities in the presumed functions of the two proteins. The preponderance of evidence suggests that E74 plays a critical role in activating coordinate changes in gene expression during Drosophila development in response to an extracellular hormonal signal (ecdysone) (5, 21). Similarly, Elf-1 binds to sequences within the IL-2 and HIV-2 enhancers that have been shown previously to play essential roles in activating gene expression in response to extracellular signals mediated through the TCR during the process of T cell activation (9, 12).

The experiments presented in this report have demonstrated that specific nucleotides at the 3′ end of the Ets binding sites can determine the fine specificity of DNA binding of different Ets family members. Thus, sites with an A at position 8 of the binding site bind Elf-1, while those with a T at this position do not. Similarly, sites with a CG or TG at positions 11 and 12 of the binding site bind Ets-1, while those with an AA or AG at these positions do not. An examination of several known Ets binding sites in T cell genes suggests that this mechanism may at least in part, allow for the coordinate expression of specific sets of T cell genes in resting and activated T cells. Thus, for example, the TCR-α and -β genes are coexpressed in resting T cells and the Ets binding sites in the TCR-α and -β enhancers bind Ets-1, but not Elf-1 (T at position 8, and CG or TG at positions 11 and 12). Conversely, the IL-2, IL-3, and GM-CSF genes are only expressed after T cell activation, and Ets binding sites in the IL-2 enhancer, the GM-CSF promoter (first site only), and the IL-3 promoter would be predicted to bind Elf-1 but not Ets-1. Although the differences in the DNA binding specificities of the Ets-1 and Elf-1 proteins are likely to be important in controlling differential gene expression in resting and activated T cells, it remains possible that differences in the patterns of expression or posttranslational processing of the different Ets family members also play a role in differentially regulating gene expression in T cells. Thus, for example, recent studies have demonstrated that Ets-1 is expressed in resting T cells but is downregulated after T cell activation (35). Finally, although our data suggests that both the α-helical region and the basic domain of Ets proteins are important for DNA binding, a precise understanding of the role of each of these domains in contacting specific nucleotides in the Ets binding site awaits mutagenesis and domain swapping experiments between the different Ets family members and known Ets binding sites.

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