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Elf-1 Binds to a Critical Element in a Second CD4 Enhancer

ANDREA L. WURSTER,[1] GERALD SIU,[2] JEFFREY M. LEIDEN,[1] AND STEPHEN M. HEDRICK[1]*

Department of Biology and Cancer Center, University of California, San Diego, La Jolla, California 92093-0063,[1] and Departments of Medicine and Pathology, University of Chicago, Chicago, Illinois 60637[2]

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The coordinated expression of CD4 and CD8 during T-cell development is tightly coupled with the maturation state of the T cell. Additionally, the mutually exclusive expression of these receptors in mature T cells is representative of the functional T-cell subclasses ($CD4^+$ helper T cells versus $CD8^+$ cytotoxic T cells). We have studied the regulation CD4 gene transcription during T-cell development in an attempt to gain an understanding of the molecular mechanisms involved in T-cell development and differentiation. Here we present the identification of a second transcriptional enhancer in the murine CD4 locus 24 kb upstream of the CD4 promoter. This enhancer is active in mature T cells and is especially active in $CD4^+$ helper T cells. A number of nuclear proteins bind to elements in the minimal CD4 enhancer that includes consensus sites for AP-1, Sp1, Gata, and Ets transcription factor families. We find that the Ets consensus site is crucial for enhancer activity and that the recently identified Ets factor, Elf-1, which is expressed at high levels in T cells and involved in the regulation of several other T-cell-specific genes, is a dominant protein in T-cell nuclear extracts that binds to this site.

T-cell development involves the differentiation of functionally immature pre-T cells into mature, antigen-reactive effector cells (reviewed in reference 61). This process includes selection events in which the maturing T-cell population is depleted of self-reactive clones and enriched for those that can recognize foreign peptides bound by self-major histocompatibility complex (MHC). The development and selection of T-cell precursors occur in the thymus and are characterized by a coordinated expression of tissue-specific genes. Some of these genes encode cell surface molecules that directly promote T-cell differentiation and selection events. Understanding the molecular mechanisms responsible for the expression of these T-cell-specific genes is a window on the process of T-cell development.

The CD4 glycoprotein is one of the surface molecules that is important as a receptor in the development and function of T cells (reviewed in references 39 and 46). In mature antigen-specific T cells, CD4 is expressed only on cells that have a T-cell receptor (TCR) specific for antigen associated with class II MHC molecules, while CD8, a cell surface protein functionally similar to CD4, is expressed only on T cells that recognize antigen bound to MHC class I molecules (56). The mutually exclusive expression of CD4 and CD8 on mature T cells also corresponds to T-cell function; CD4 is expressed primarily on helper T cells, while CD8 is expressed on cytotoxic T cells. CD4 participates in antigen recognition by binding to nonpolymorphic regions of class II MHC molecules, while the T-cell antigen receptor recognizes a specific peptide antigen that is bound by the MHC molecule (7). The CD4-MHC interaction serves to increase the avidity of the T cell to the antigen-presenting cell but also induces an intracellular signaling event through the CD4-associated tyrosine kinase, $p56^{lck}$ (8, 59). Therefore, CD4 plays a critical role in the antigen recognition required for T-cell activation and helper T-cell functions.

Expression of CD4 during T-cell development is tightly regulated with the expression of CD8 (11, 61). T cells enter the thymus lacking expression of CD4 and CD8. After a transient low-level expression of either CD4 or CD8, the maturing thymocytes express both CD4 and CD8 at intermediate levels. This transition appears to be coregulated with the rearrangement and productive expression of the TCR β chain. The TCR^+ $CD4^+$ $CD8^+$ cells then undergo selection processes that eliminate overtly self-reactive T cells and select for MHC class I- and class II-responsive cells. This latter process appears to be responsible for the coordination of MHC class I specificity with CD8 expression and MHC class II specificity with CD4 expression. Therefore, during residence in the thymus, T cells not only develop and gain the ability to recognize foreign antigen but also differentiate into functional subclasses. The important role of CD4 in these developmental processes can be illustrated by the interference of thymic selection events by in vivo administration of antibodies against CD4 (12, 41, 69). Additionally, analyses of mice deficient in CD4 or MHC class II molecules show that the CD4-MHC interaction is required for proper T-cell development as well as for normal helper T-cell functions (16, 20, 31, 47).

Because of the important role of CD4 in T-cell function and its highly regulated expression pattern during T-cell development and thymic selection events, we were interested in studying the developmental regulation of murine CD4 gene expression. Nuclear run-on studies indicate that the primary control of CD4 expression is at the level of transcription (53), although some posttranscriptional effects on CD4 expression have also been described (45, 57). Previous studies by our laboratory and others on CD4 gene regulation have identified and characterized CD4 promoter and enhancer elements in the mouse and human CD4 loci that are capable of stimulating transcription of reporter genes in transient transfections of cultured cells as well as in transgenic mice (2, 49, 51, 54). To date, however, reconstitution of the developmental pattern and T-cell subclass-specific expression of CD4 has been accomplished only through the use of large cosmid constructs that contain many kilobases of genomic DNA sequences within and around the human CD4 locus (2, 14, 31). Clearly other elements in the CD4 locus are required in addition to the promoter and enhancer elements already identified. Here we

* Corresponding author. Phone: (619) 534-6269. Fax: (619) 534-5792.

† Present address: Department of Microbiology, Columbia University, College of Physicians and Surgeons, New York, NY 10032.

describe the identification of a second, distal transcriptional enhancer in the murine CD4 locus that resides 24 kb upstream of the promoter region. Using reporter gene assays in transient transfections of various $CD4^+$ and $CD4^-$ cell lines, we find that the enhancer is active in mature T cells and is especially active in T helper cells. Using DNase I footprinting and electrophoretic mobility shift assays (EMSAs), we find that numerous nuclear factors are able to bind the enhancer in vitro, although mutagenesis studies indicate that enhancer activity is predominantly mediated by an Ets family consensus site. We also determined that an Ets family transcription factor, Elf-1, is a dominant protein in T-cell nuclear extracts that binds to this site in vitro. Elf-1 has previously been shown to be required for the activation induced transcription of the T-cell-specific interleukin-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) genes as well as in the regulation of the T-cell-specific human immunodeficiency virus type 2 (HIV-2) and human T-cell leukemia virus type I (HTLV-I) enhancers (4, 17, 37, 64). Therefore, the involvement of Elf-1 in the regulation of the CD4 gene follows a previously established pattern for T-cell-specific gene regulation.

MATERIALS AND METHODS

Cosmids and subcloning. The J4 cosmid clone containing the murine CD4 gene was described previously (54). The J31 and J32 cosmid clones were generously provided by Rick Barth, Rochester, N.Y. The 2J3 cosmid clone was isolated by screening a cosmid library derived from the G8 T-cell clone (5) with a single-copy probe from the 5′ end of the J4 cosmid clone. The cosmid clones were mapped with restriction enzymes by using partial restriction enzyme digests.

Restriction fragments for the enhancer trap analysis were subcloned into the upstream *Bam*HI site of either pVOP or pVOPΔ-22 (54), which includes approximately 1 kb or 22 bp, respectively, of CD4 5′ flanking sequences driving the luciferase reporter gene. Alternatively, the restriction fragments were subcloned into the downstream polylinker sites of J21 (42), using the minimal c-*fos* promoter. The restriction fragments used in the enhancer trap experiments are depicted in Fig. 1A and are listed in Table 1.

The restriction fragment 2J3G11, which contains the CD4 enhancer activity, was subcloned into both the J21 and pVOPΔ-22 constructs as described above. The 2J3G11 enhancer deletion fragments were isolated as illustrated in Fig. 2A. The TCR β-chain enhancer used in these experiments is a 1.0-kb *Bgl*II-*Nco*I restriction fragment derived from the TCR β-chain gene (33).

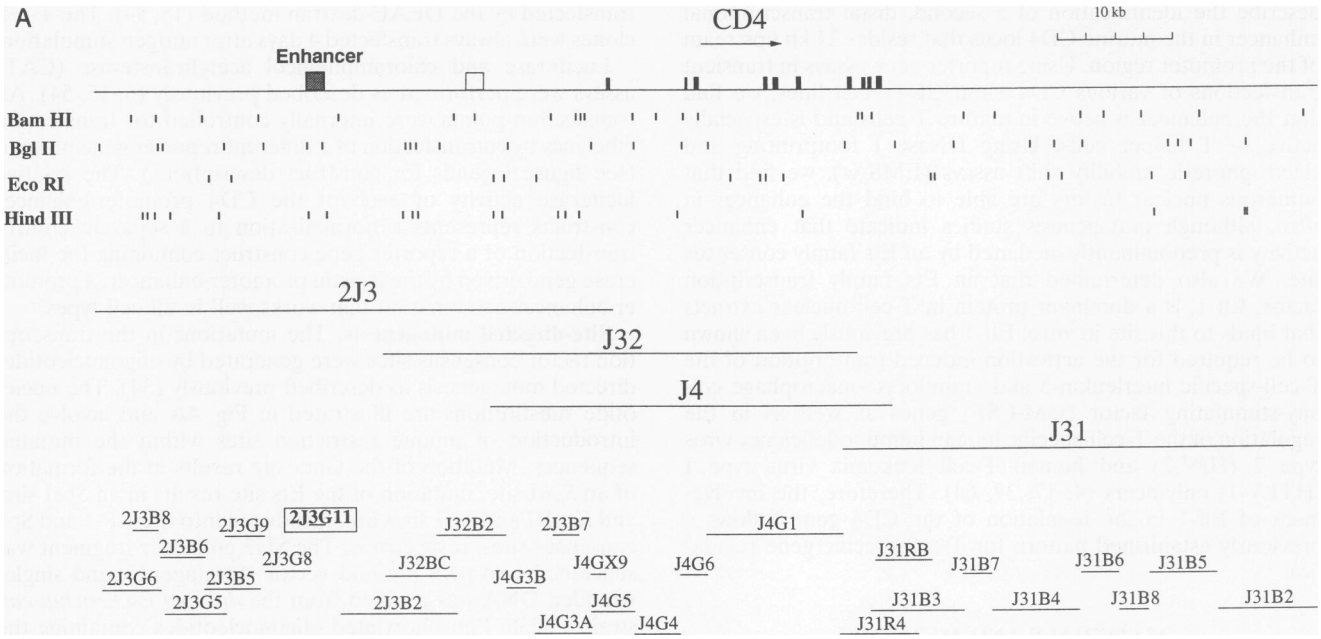
Cell culture and transient transfections. All T-cell clones and cell lines were grown in EHAA medium (Click's medium) supplemented with 10% fetal calf serum, 1 mM glutamine, 50 μM β-mercaptoethanol, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. For representatives of immature T cells, we used AKR1G1, a $CD4^+$ $CD8^+$ $CD3^-$ $J11d^-$ thymoma, and S49, a $CD4^-$ $CD8^-$ $CD3^+$ $J11d^+$ thymoma (25, 48). Non-T cells used in this study include the murine B-cell hybridoma LK 35.2 (29), the human B-cell line Namalwa, and the human epithelial cell line HeLa. For mature TCR^+ T cells, we used the human T-cell line Jurkat and the antigen-specific T-cell clones D10 and L3 (30, 40). D10 is mature $CD4^+$ $CD8^-$ helper T clone whose TCR recognizes conalbumin presented on $I\text{-}A^k$ MHC. L3 is a mature $CD4^-$ $CD8^+$ T-cell cytotoxic clone that has an alloreactivity to $H\text{-}2^d$ MHC. The D10 and L3 T cells were stimulated with antigen every 14 days as described previously (54). All of the cell lines and clones were transiently transfected by the DEAE-dextran method (18, 54). The T-cell clones were always transfected 4 days after antigen stimulation.

Luciferase and chloramphenicol acetyltransferase (CAT) assays were performed as described previously (6, 15, 54). All transfection points were internally controlled for transfection efficiency by cotransfection of a different reporter gene plasmid (see figure legends for construct descriptions). The relative luciferase activity of each of the CD4 promoter/enhancer constructs represents a normalization to a separate control transfection of a reporter gene construct containing the luciferase gene driven by the β-actin promoter/enhancer, a promoter/enhancer combination that works well in all cell types.

Site-directed mutagenesis. The mutations in the transcription factor consensus sites were generated by oligonucleotide-directed mutagenesis as described previously (34). The nucleotide substitutions are illustrated in Fig. 4A and involve the introduction of unique restriction sites within the mutated sequences. Mutation of the Gata site results in the formation of an *Sph*I site, mutation of the Ets site results in an *Spe*I site, and *Eco*RI and *Nsi*I sites are introduced into the AP-1 and Sp1 consensus sites, respectively. The SH3 enhancer fragment was subcloned into pKS plasmid vector (Stratagene), and single-stranded DNA was purified from the *dut ung Escherichia coli* strain CJ236. Phosphorylated oligonucleotides containing the altered consensus site sequences were annealed to the uracil-containing template, and the second strand was synthesized by using T7 DNA polymerase and T4 DNA ligase. The extended products were then transformed into the dut^+ ung^+ *E. coli* strain DH1. The resulting mutants were initially screened for the presence of new restriction sites introduced at the sites of mutation and then confirmed by DNA sequencing. The new mutant enhancer fragments were then subcloned into the pVOPΔ-22 luciferase expression vector and transfected into D10 T cells as described above.

DNase I footprinting. Nuclear extracts from Jurkat cells for footprinting and EMSA studies were prepared by the method of Waterman and Jones (66) as described by Siu et al. (54) with the exception that the extract was dialyzed at 4°C overnight against TM buffer (50 mM Tris-HCl [pH 7.9], 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.1 M KCl) instead of by desalting on a gel filtration column. The Namalwa and HeLa nuclear extracts were kindly provided by Eric Sinn, Rockefeller University, New York, N.Y.

DNase 1 footprinting was performed as described before (26). An end-labeled DNA probe was prepared by digesting 20 μg of plasmid DNA with a restriction enzyme recognizing a polylinker site outside of the VH3 enhancer fragment. The DNA was labeled by filling in the recessed 3′ ends with [α-^{32}P]CTP and Klenow fragment. The insert was then isolated from the plasmid by digestion with a second restriction enzyme on the other side of the insert. The end-labeled probe (7,000 cpm) was mixed with 1 μg of poly(dI-dC) and 10 μl of 10% polyvinyl alcohol in a total volume of 25 μl. This DNA mixture was then incubated on ice for 15 min with 40 μg of nuclear extract in a total volume of 50 μl of TM buffer. Fifty microliters of 10 mM $MgCl_2$–5 mM $CaCl_2$ was then added to the protein-DNA mix; the mix was left at room temperature for 1 min and then subjected to a 1-min DNase I digestion (the concentration of DNase I was determined by a titration experiment for each probe). The reaction was stopped by the addition of 90 μl of 20 mM EDTA–1% sodium dodecyl sulfate (SDS)–0.2 M NaCl–325 μg of carrier RNA per ml. The digested DNA was phenol-chloroform extracted twice, chloroform extracted once, and ethanol precipitated. Half of the reaction was analyzed on a sequencing gel after boiling in 80% formamide–10 mM NaOH–1 mM EDTA–0.1% xylene cyanol–



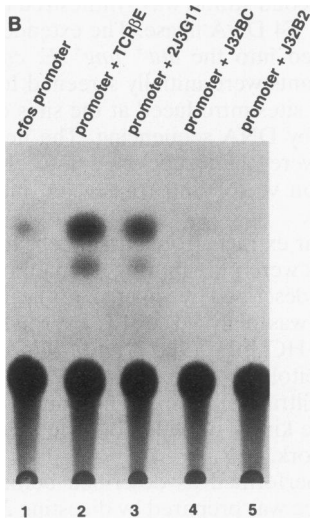


FIG. 1. A second transcriptional enhancer is located 24 kb 5′ of the CD4 gene. (A) Genomic map and subcloning of the murine CD4 gene. Black boxes indicate exons of the CD4 gene. The stippled and white boxes represent the distal and proximal enhancers, respectively. The overlapping, genomic cosmid clones are indicated under the restriction map. The restriction fragments subcloned and assayed for enhancer activity are diagrammed at the bottom and described in Table 1. All restriction fragments were tested for enhancer activity in both D10 $CD4^+$ $CD8^-$ T cells and AKR1G1 $CD4^+$ $CD8^+$ T cells two to five times (data not shown). The 2J3G11 (boxed) fragment containing the distal enhancer is a 1.2-kb *Bgl*II fragment derived from the 2J3 cosmid. (B) Enhancer activity is observed in the 2J3G11 restriction fragment in D10 $CD4^+$ $CD8^-$ T cells. Representative CAT assays compared enhancer activities seen in the 2J3G11 fragment (J21.2J3G11; lane 3), the TCR β-chain enhancer (J21.TCRβE; lane 2), and the c-*fos* minimal promoter alone (J21; lane 1). Two additional subclones from the CD4 locus are also presented for comparison and are described in Table 1. The transfections are internally controlled for transfection efficiency by cotransfection of a luciferase reporter gene construct under the control of the human β-actin promoter/enhancer (pHβAprlux). These transfections were repeated three to five times.

0.1% bromphenol blue and compared with DNA ladders generated by DNase I digestion without prior incubation with nuclear extract proteins. The locations of the footprints within the probe were determined by comparing the DNase I-digested probe with G ladders generated by piperidine cleavage of a dimethyl sulfate-treated probe (43).

EMSA and Western blotting (immunoblotting). The EMSA was performed as described previously (42, 54). An end-labeled DNA probe (2×10^4 cpm) prepared as described above was incubated with 0.4 μg of salmon sperm DNA, 2.5 μg of Jurkat nuclear extract, and 4 μl of 5× G/B I buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-KOH [pH 7.9], 250 mM NaCl, 25 mM Tris-HCl [pH 7.5], 75 mM EDTA, 5 mM dithiothreitol, 50% glycerol) in a total volume of 20 μl. The proteins were allowed to bind to the DNA probe for 20 min at room temperature. The protein-DNA complexes were then resolved on a 4% nondenaturing polyacrylamide gel run in a glycine buffer (190 mM glycine, 25 mM Tris-HCl [pH 8.5], 1 mM EDTA) for 2 h at 150 V. The gel was then dried and exposed to X-ray film. In EMSA reactions that included cold (unlabeled) oligonucleotide competitors, the nuclear extracts was allowed to incubate with the cold oligonucleotides for 10 min at room temperature before the addition of the labeled DNA probe. The double-stranded oligonucleotides used as probes and cold competitors in the EMSAs were CD4-Ets (5′AAACAGGAAGTC CTGCCCCC3′), CD4-EtsM (5′AAACAACTAGTCCTGCCC CC3′), CD4-Gata (5′GCTGAACCAGGCAGATAGAA3′), Ets-1-Tα2 (5′CTTTCCAGAGGATGTGGCTT3′), and Elf-1-HIV (5′TTAAAGACAGGAACAGCTAT3′). In EMSA reactions that included antibodies, 1 μl of the antiserum specific for Elf-1 or Ets-1 or normal rabbit serum was incubated with the nuclear extract for 15 min at 4°C before the subsequent addition of the labeled DNA probe. The Elf-1-specific antiserum is a rabbit antiserum raised against recombinant human Elf-1 protein (37). The Elf-1-specific monoclonal antibody, 5A3, was raised against residues 433 to 619 of the human Elf-1 protein. The

TABLE 1. Restriction fragments used in enhancer trap analysis

Subclone	Restriction fragment (5′-3′)	Fragment size (kb)	Derived from cosmid:
2J3B8	*Bam*HI-*Bam*HI	2.0	2J3
2J3G6	*Bgl*II-*Bgl*II	4.0	2J3
2J3B6	*Bam*HI-*Bam*HI	3.5	2J3
2J3G5	*Bgl*II-*Bgl*II	5.0	2J3
2J3B5	*Bam*HI-*Bam*HI	4.5	2J3
2J3G8	*Bam*HI-*Bam*HI	3.5	2J3
2J3G11	*Bgl*II-*Bgl*II	1.2	2J3
2J3B2	*Bam*HI-*Bam*HI	10.0	2J3
J32BC	5′ cosmid-*Bam*HI	6.0	J32
J32B2	*Bgl*II-*Eco*RI	6.0	J32
J4G3A	*Eco*RI-*Bgl*II	5.0	J4
2J3B7	*Bgl*II-*Bgl*II	3.5	2J3
J4BX9	*Bgl*II-*Xho*I	1.0	J4
J4G5	*Bgl*II-*Bgl*II	3.0	J4
J4G4	*Bgl*II-*Bgl*II	4.0	J4
J4G6	*Bgl*II-*Bgl*II	2.0	J4
J4G1	*Bgl*II-*Bgl*II	12.0	J4
J31R4	5′ cosmid-*Eco*RI	6.5	J31
J31RB	*Bam*HI-*Eco*RI	4.5	J31
J31B3	*Bam*HI-*Bam*HI	8.0	J31
J31B7	*Bam*HI-*Bam*HI	3.0	J31
J31B4	*Bam*HI-*Bam*HI	7.0	J31
J31B6	*Bam*HI-*Bam*HI	3.5	J31
J31B8	*Bam*HI-*Bam*HI	3.0	J31
J31B5	*Bam*HI-*Bam*HI	5.0	J31
J31B2	*Bam*HI-*Bam*HI	9.0	J31

Ets-1-specific antibody is a rabbit polyclonal antibody against human Ets-1 that was obtained from Santa Cruz Biotechnology. In EMSA reactions that included in vitro-translated Elf-1, 3 μl of either the control lysate or the Elf-1-containing lysate was used instead of the nuclear extract.

The Western blot on the EMSA complexes was performed as follows. An EMSA reaction scaled up 10-fold was carried out and run on a 4% nondenaturing acrylamide gel. The wet gel was then exposed to X-ray film overnight, and the complexes of interest were excised from the gel. The gel slice was then directly loaded on an SDS–10% polyacrylamide gel and run for 2 h at 35 mM in SDS-Tris-glycine buffer, and the proteins in the gel were transferred to nitrocellulose. After blocking of the membrane in 5% milk in Western buffer (20 mM Tris [pH 7.5], 500 mM NaCl), the blot was incubated with a 1:2,000 dilution of the Elf-1-specific antiserum in Western buffer for 1 h. The proteins were then visualized by using the ECL (enhanced chemiluminescence) Western blotting system (Amersham). Biotinylated protein markers were also supplied by Amersham and detected by using the ECL Western blotting system.

RESULTS

Identification of a second T-cell-specific enhancer in the CD4 gene locus. To identify enhancer elements in the murine CD4 locus, we used a standard enhancer trap strategy. We previously reported the isolation of a cosmid clone, J4, that contained the murine CD4 gene (54). Using single-copy probes from the 5′ and 3′ ends of the cosmid clone, we obtained additional, overlapping cosmid clones that span a total of 100 kb of the murine CD4 locus. Restriction fragments from this entire locus region were subcloned into either a CAT reporter gene construct under the control of the minimal c-*fos* promoter or a luciferase reporter construct with the CD4 promoter (Fig. 1A). These constructs were then transiently transfected into D10, a mature $CD4^+$ T-cell clone, and AKR1G1, a T-cell thymoma with the immature $CD4^+$ $CD8^+$ phenotype. The transfections were analyzed for an increase in reporter gene activity over that observed with the promoter alone. The only restriction fragment to demonstrate significant enhancer activity was a 1.2-kb *Bgl*II fragment from the 2J3 cosmid, 2J3G11, located 24 kb 5′ of the CD4 promoter region (Fig. 1). This activity was observed only in the D10 T-cell clone; no enhancer activity was seen in the AKR1G1 thymoma (Fig. 1B and data not shown). The enhancer activity was similar to that of the TCR β-chain enhancer and exerted its effect on the heterologous c-*fos* promoter when cloned downstream of the CAT reporter gene and over 2 kb away from the promoter (J21.2J3G11). We were unable to detect enhancer activity in large restriction fragments that contain the previously identified, proximal enhancer when assayed in D10, AKR1G1, or Jurkat cells (Fig. 1B and data not shown) (51). Although we find this result puzzling, we did not use the precise 800-bp *Nde*I-*Hinf*II restriction fragment reported by Sawada and Littman (51) in our enhancer trap experiments (Table 1 and Fig. 1), and it is possible that this enhancer activity can be observed in transient transfections only when assayed on a small DNA fragment. Subsequent transgenic experiments have clearly shown that this enhancer can function in vivo (2, 22, 31).

To locate the enhancer activity of the distal enhancer, we used restriction fragments derived from the 2J3G11 fragment and generated a series of enhancer deletion constructs by using the minimal CD4 promoter and the luciferase reporter gene (Fig. 2A). These constructs were transiently transfected into the D10 $CD4^+$ T-cell clone and L3, a $CD8^+$ T-cell clone (Fig. 2A). Similar to results for the J21.2J3G11 CAT construct (c-*fos* promoter), considerable enhancer activity was observed with the 1.2-kb 2J3G11 enhancer fragment when combined with the CD4 promoter in D10 T cells. In this case, the activity of the CD4 promoter/distal enhancer combination exceeded by threefold the activity of the human β-actin promoter/enhancer (21), a powerful transcription unit that works well in most cell types. The 2J3G11 fragment, when combined with the CD4 promoter, was also considerably stronger than the TCR β-chain enhancer driving the minimal CD4 promoter (Fig. 2A). Full enhancer activity (35-fold stimulation over promoter alone) was still observed when 2J3G11 was truncated to the 700-bp *Hin*dIII-*Pst*I SH3 fragment. Significant but reduced enhancer activity was still detected with the 247-bp *Hin*dIII-*Pvu*II VH3 fragment. No enhancer activity was seen with either the SH2 or VH2 fragment derived from the 3′ end of 2J3G11. Therefore, the enhancer activity was determined to reside in the 5′ end of the 2J3G11 fragment. A small amount of enhancer activity was seen in the mature $CD4^-$ $CD8^+$ T-cell clone, L3, using the full-length 2J3G11 fragment (Fig. 2A). On the other hand, when the SH3 fragment is used instead in L3 transfections, significant enhancer activity is observed, comparable to that seen with the human β-actin promoter/enhancer combination. Although this finding implies the existence of a potential negative regulatory element in the 3′ end of the 2J3G11 fragment, in more recent transgenic studies we have found that the 2J3G11 fragment does not contain a dominant-negative control element in cell types other than $CD4^+$ T cells (53).

One of these CD4 enhancer constructs (SH3) was also transfected into other $CD4^+$ and $CD4^-$ T cells as well as non-T cells. The CD4 promoter/distal enhancer combination was inactive in the $CD4^-$ $CD8^-$ S49 and $CD4^+$ $CD8^+$ AKR1G1 T-cell thymomas that represent immature, developing T cells (Fig. 2B). Enhancer activity was also tested in

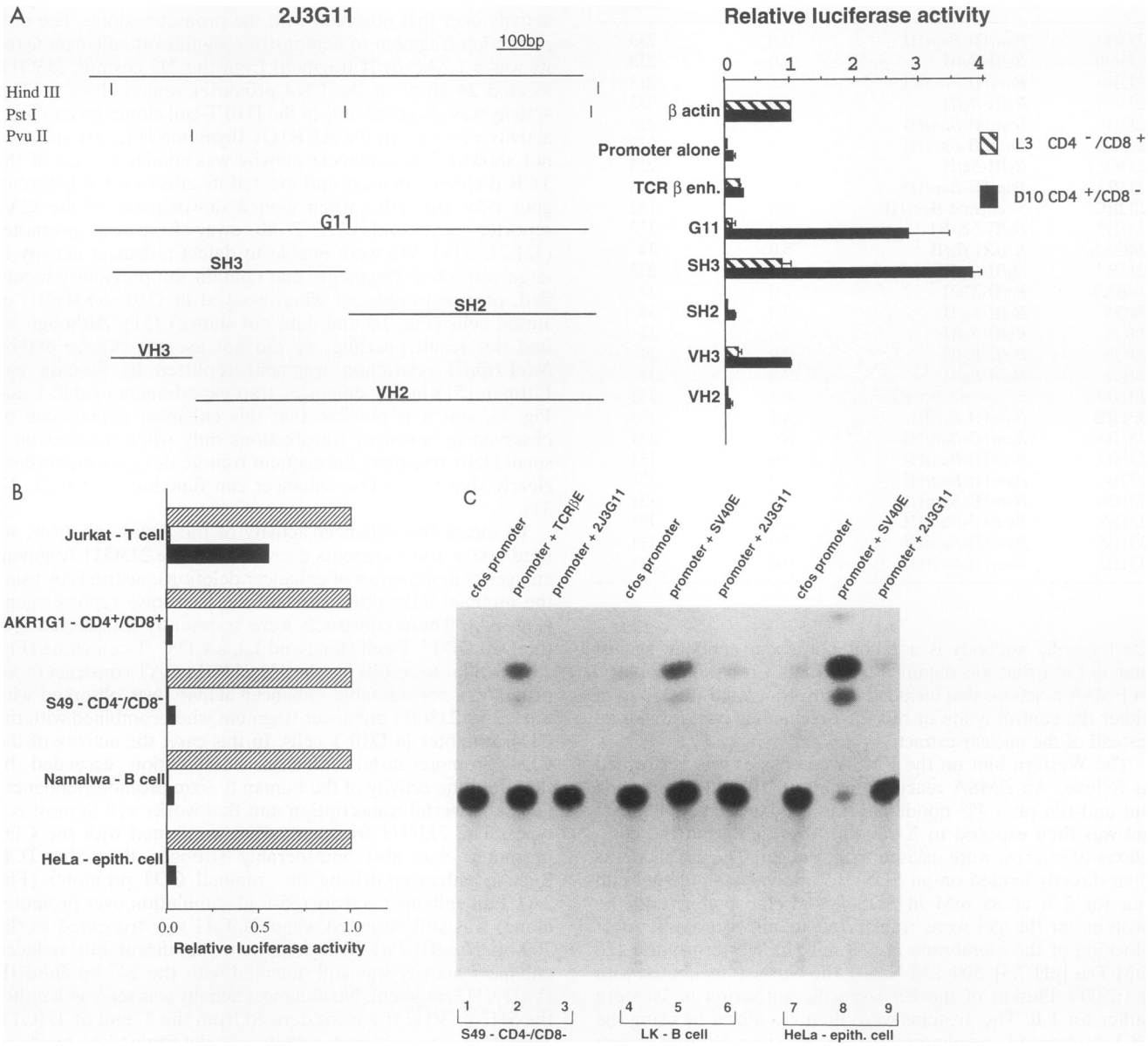


FIG. 2. Functional activities of CD4 distal enhancer deletions in $CD4^+$ and $CD4^-$ cells. Restriction fragments derived from the 2J3G11 subclone were subcloned upstream of the minimal CD4 promoter (described in Materials and Methods) driving the expression of the luciferase reporter gene. The relative enhancer activity reflects the normalization to the control plasmid that is driven by the human β-actin promoter/enhancer, a promoter/enhancer combination that works well in all cell types tested. The approximate average light units produced for each cell type by this control plasmid are as follows: 35,000 for D10, 20,000 for L3, 40,000 for AKR1G1, 10,000 for S49, 65,000 for HeLa, 50,000 for Namalwa, and 15,000 for Jurkat. All transfections were controlled for transfection efficiency by cotransfection of a construct containing the CAT gene under the control of the thymidine kinase promoter or the c-*fos* promoter/TCR β-chain enhancer. All transfections were repeated at least three times. (A) The restriction map the of the 2J3G11 enhancer fragment is illustrated on the left. The indicated restriction fragments were tested for enhancer activity when combined with the CD4 minimal promoter by transient transfection into the D10 $CD4^+$ $CD8^-$ T-cell clone and L3 $CD4^-$ $CD8^+$ T-cell clone. The activity of the TCR β-chain enhancer combined with the CD4 minimal promoter was also tested for comparison. (B) The SH3 enhancer fragment was assayed for enhancer activity in the indicated T cells and non-T cells. Similar data were also obtained for the B-cell hybridoma LK 35.2 (data not shown). Reporter construct: ▨, β-actin promoter plus enhancer; ▨, CD4 promoter alone; ■, CD4 promoter plus CD4 enhancer. epith., epithelial. (C) The 2J3G11 enhancer fragment combined with the c-*fos* promoter was assayed for enhancer activity in the indicated T cells and non-T cells (lanes 3, 6, and 9). The c-*fos* promoter alone (lanes 1, 4, and 7) and the c-*fos* promoter with the indicated positive control enhancers (the TCR β-chain enhancer in lane 2 and the simian virus 40 enhancer [SV40E] in lanes 5 and 8) are shown for comparison. Transfections were performed and controlled as described for Fig. 1B. Similar data were also obtained for Namalwa B cells and the AKR1G1 thymoma cells (data not shown).

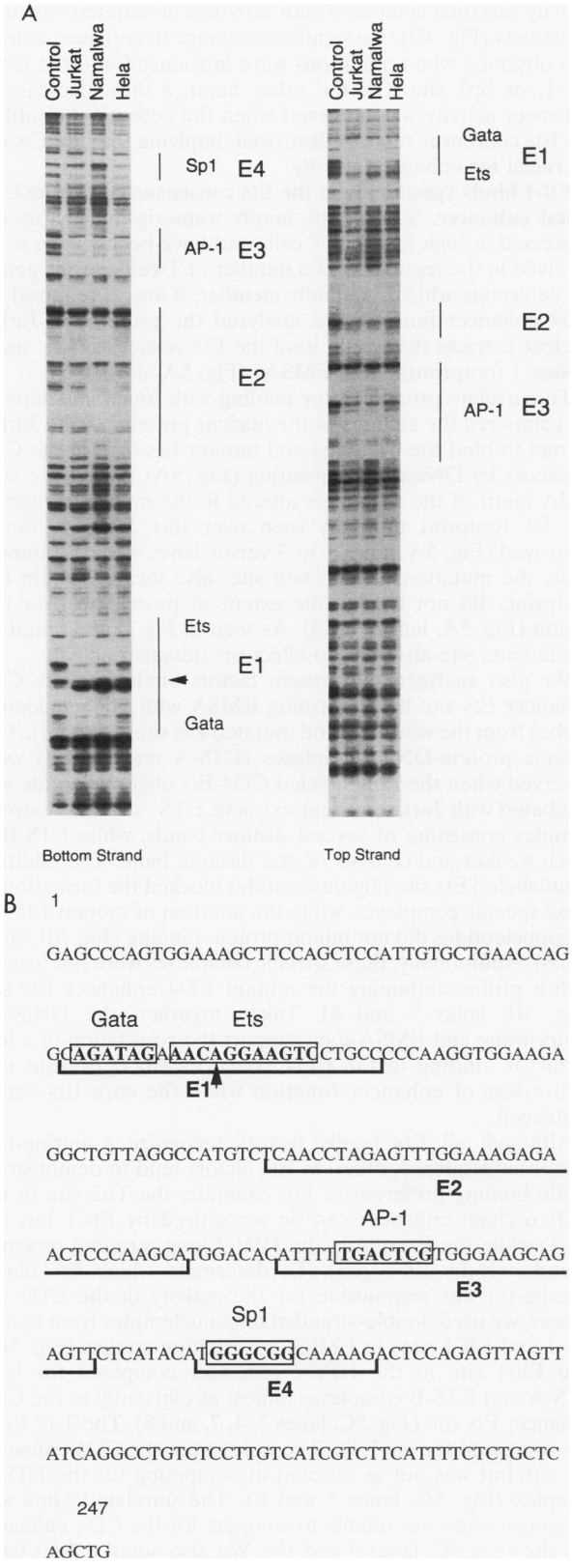


FIG. 3. Multiple nuclear factors can bind to the CD4 distal enhancer. (A) DNase I footprinting of the VH3 enhancer fragment, using nuclear extracts from T (Jurkat), B (Namalwa), and epithelial (HeLa) cells. DNase I digestion of the probes in the absence of nuclear proteins is indicated in the control lanes. Footprinted regions are indicated by vertical lines and are labeled E1 to E4 for both DNA strands. A DNase I-hypersensitive site seen within footprint E1 is indicated by an arrow. Transcription factor consensus sites within the footprinted regions are indicated. Mapping of the DNase I-footprinted regions was determined by DNA sequencing ladders and G ladders generated by piperidine cleavage of a dimethyl sulfate-treated probe (data not shown and Fig. 5A). (B) CD4 enhancer sequence. Footprinted regions determined in panel A are indicated by brackets. The transcription factor consensus sites found within the footprinted regions are boxed and labeled. A DNase I-hypersensitive site seen within the E1 footprint is indicated by an arrow.

several human tumor cell lines. The CD4 promoter and enhancer show significant activity in the T-cell tumor cell line Jurkat, while minimal activity was observed with the B-cell line Namalwa and the epithelial cell line HeLa (Fig. 2B). Because the activity of the CD4 promoter alone was close to background levels in the immature T cells and non-T cells, there was significant enhancement in all four cell types: AKR1G1, 30-fold; S49, 8-fold; Namalwa, 7.5-fold; and HeLa, 6.7-fold (Fig. 2B). To investigate the specificity of the enhancer itself, we also transfected S49, B-cell hybridoma LK 35.2, and HeLa cells with the construct that includes the distal CD4 enhancer and the c-*fos* promoter. No significant enhancer activity was evident over the c-*fos* promoter alone, a promoter that is active in these cell lines (Fig. 2C). Therefore, this CD4 enhancer activity is specific for mature T cells, particularly $CD4^+$ T cells.

Multiple nuclear factors bind to the distal CD4 enhancer. To determine which transcription factors could be responsible for the enhancer's activity, we performed DNase I footprint analysis on the 247-bp VH3 region, using nuclear extracts prepared from Jurkat T cells, Namalwa B cells, and HeLa epithelial cells. Four regions (E1 to E4) were protected from DNase I cleavage (Fig. 3A). Sequence analysis of the minimal CD4 distal enhancer revealed transcription factor consensus sequences within several of the DNase I footprints (Fig. 3). Footprint E1 encompasses potential binding sites for Ets and Gata family transcription factors. Several Ets family members (Ets-1, Ets-2, and Elf-1) (1, 58) and a Gata family member (Gata-3) are expressed at high levels in T cells (24, 27, 32). In Jurkat extracts, the region around these two sequences is well protected and includes the presence of a hypersensitive site. Interestingly, the position of this hypersensitive site within the Ets binding site is indicative of footprints generated by proteins with Ets DNA binding domains (44). Limited protection is observed in this region with the B-cell extract, and even less is observed with the HeLa extract. The sequences within footprints E3 and E4 correspond to binding sites for the ubiquitously expressed transcription factor families AP-1 (36) and Sp1 (10, 28), and in fact, these regions were protected from DNase I cleavage by all extracts tested. The E2 footprint was consistently observed on the bottom strand with the B-cell nuclear extract, was variably observed with the T-cell extract, and was absent with the HeLa extract. Protection in the E2 footprint was seen with all extracts on the top strand. The sequences within the E2 footprint do not correspond to any known transcription factor recognition sites. The factor(s) binding to this region could represent a novel protein(s) yet to be identified.

Functional analysis of nuclear factor binding sites in the CD4 distal enhancer: mutation of the Ets consensus sequence destroys enhancer function. To determine the importance of the nuclear factor consensus sites within the DNase I footprints present in the CD4 distal enhancer, a series of mutant enhancer constructs was generated and tested for enhancer

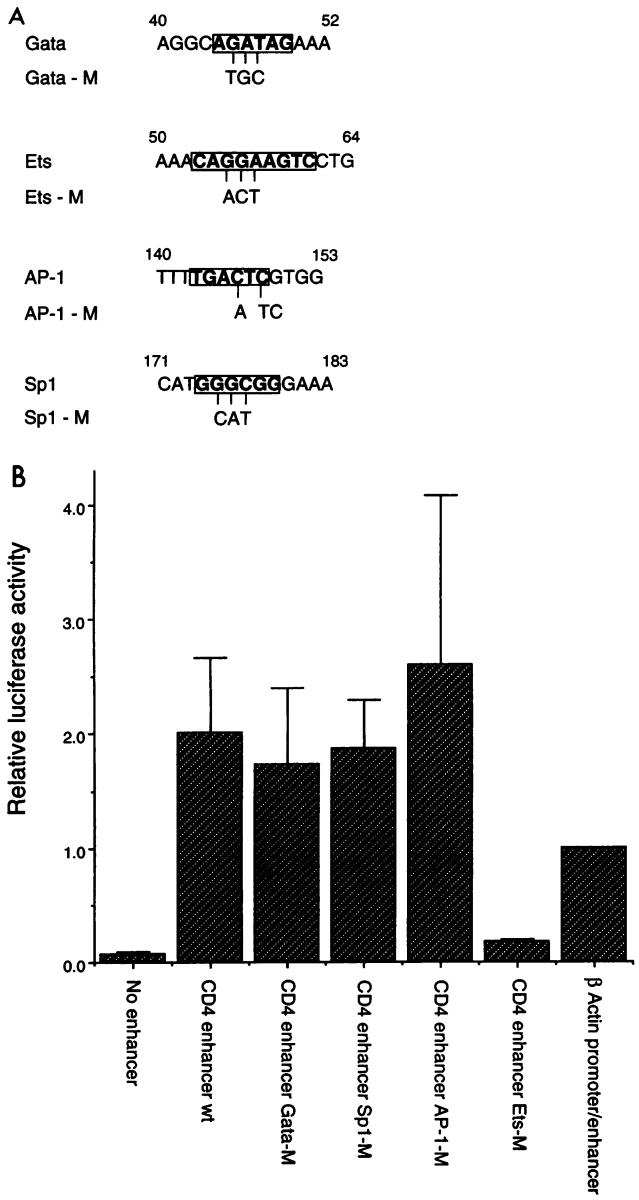


FIG. 4. Functional analysis of CD4 distal enhancer mutant constructs. (A) Nucleotide substitutions introduced into the SH3 enhancer fragment by site-directed mutagenesis within the footprinted regions. The transcription factor consensus sites are boxed, and the nucleotide positions within the CD4 enhancer are shown. M, mutant. (B) Transfections of unmutated and mutated enhancer constructs into the D10 $CD4^+$ T-cell clone. The mutations were introduced into the SH3 enhancer fragment and subcloned upstream of the minimal CD4 promoter driving the luciferase reporter gene. The transfections were analyzed and controlled as described for Fig. 2.

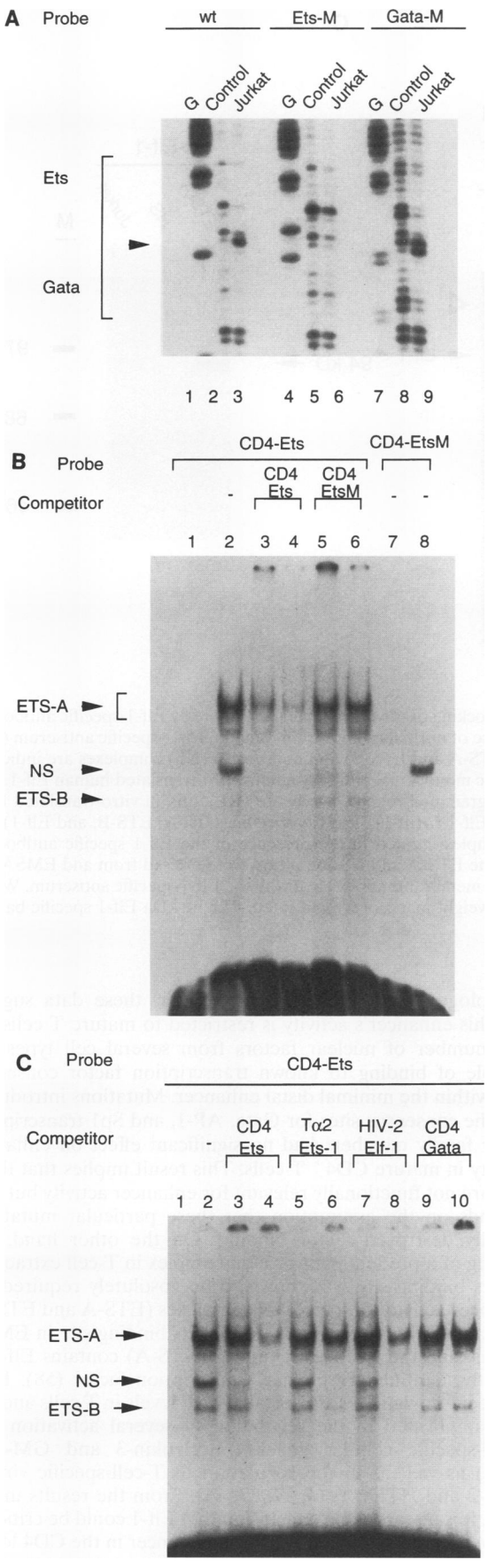
activity in transiently transfected D10 cells. By using site-directed mutagenesis, specific nucleotides were altered in the AP-1, Sp1, Ets, and Gata consensus sites of the SH3 fragment (Fig. 4A). The choices for nucleotide substitutions were based on previous studies in which either factor binding or functional activity of the particular binding site was destroyed (3, 23, 24, 38). These mutant enhancer fragments were subcloned into the luciferase expression vector with the minimal CD4 promoter and transiently transfected into D10 T cells, and the enhancer activity was then compared with activities unmutated enhancer constructs (Fig. 4B). No significant change in enhancer activity was observed when mutations were introduced into the Gata, AP-1, or Sp1 site. On the other hand, a 90% decrease in enhancer activity was observed when the core GGA motif of the Ets consensus site was destroyed, implying that the Ets site is crucial for enhancer activity.

Elf-1 binds specifically to the Ets consensus site of the CD4 distal enhancer. Several Ets family transcription factors are expressed at high levels in T cells and have been shown to be involved in the regulation of a number of T-cell-specific genes. To determine which Ets family member, if any, is required for CD4 enhancer function, we analyzed the proteins in Jurkat nuclear extracts that could bind the Ets consensus site, using DNase I footprinting and EMSAs (Fig. 5A and B).

To correlate protein factor binding with functional activity, we compared the abilities of the nuclear proteins in the Jurkat extract to bind the wild-type and mutant Ets sites in the CD4 enhancer by DNase I footprinting (Fig. 5A). When the core GGA motif of the Ets site is altered in the mutant construct, the E1 footprint normally seen over this 24-bp region is destroyed (Fig. 5A, lanes 1 to 3 versus lanes 4 to 6). Interestingly, the mutation in the Gata site, also located within this footprint, did not change the extent of protection over this region (Fig. 5A, lanes 7 to 9). As seen in Fig. 4, the mutation in the Gata site also had no effect on enhancer activity.

We also analyzed the protein factors binding to the CD4 enhancer Ets site by performing EMSA with oligonucleotide probes from the wild-type and mutated Ets sites (Fig. 5B). Two specific protein-DNA complexes (ETS-A and ETS-B) were observed when the radiolabeled CD4-Ets oligonucleotide was incubated with Jurkat nuclear extracts. ETS-A is a very strong complex consisting of several distinct bands, while ETS-B is much weaker and consists of one discrete band. The addition of unlabeled Ets site oligonucleotides blocked the formation of these specific complexes, while the addition of mutant Ets site oligonucleotides did not inhibit protein binding (Fig. 5B, lanes 2 to 6). Additionally, these specific complexes were not formed with a probe containing the mutant CD4 enhancer Ets site (Fig. 5B, lanes 7 and 8). Taken together, the DNase I footprinting and EMSA data support the correlation of a loss of factor binding to the CD4 enhancer Ets consensus site with a loss of enhancer function when the core Ets site is destroyed.

Although all Ets family factors recognize a purine-rich consensus sequence, different Ets factors tend to demonstrate subtle binding preferences. For example, the Tα2 site in the TCR α-chain enhancer can be recognized by Ets-1 but not Elf-1, while the Ets site in the HIV-2 long terminal repeat is bound only by Elf-1 (63). To determine which Ets family member(s) was responsible for the activity in the CD4 enhancer, we used double-stranded oligonucleotides from known Ets-1 and Elf-1 sites in EMSA competition studies (Fig. 5C). The Elf-1 site in the HIV-2 enhancer competed for both ETS-A and ETS-B complexes almost as efficiently as the CD4 enhancer Ets site (Fig. 5C, lanes 3, 4, 7, and 8). The Tα2 Ets-1 site competed for the ETS-B complex from the CD4 enhancer Ets site but was not as efficient at competing for the ETS-A complex (Fig. 5C, lanes 5 and 6). The unrelated Gata site oligonucleotide was unable to compete for the CD4 enhancer Ets site (Fig. 5C, lanes 9 and 10). We also noted a faint band of slower mobility that did not compete for the Ets-specific oligonucleotides, and we did not characterize this complex further. These data indicate that a known Elf-1 site has a higher affinity for the proteins bound specifically to the CD4 enhancer in the ETS-A complex than a known Ets-1 site.



We next used antibodies raised to Ets transcription factors in EMSA-antibody assays (Fig. 6A). A polyclonal rabbit antiserum raised against Elf-1 has been shown previously to specifically block the binding of recombinant Elf-1 as well as Elf-1 in crude nuclear extracts to the HIV-2 enhancer (37). This same antiserum also blocked the formation of at least one band in the ETS-A complex, while normal rabbit serum did not (Fig. 6A, lanes 1, 2, and 4). Interestingly, a second band in the ETS-A complex is revealed by the pretreatment with Elf-1 antiserum, and its mobility is apparently unaffected by the antibody. The Elf-1-specific antiserum also had not effect on the mobility of the ETS-B complex (Fig. 6A, lane 2). A polyclonal antibody specific for Ets-1 had no effect on the migration of any of the Ets-specific complexes (Fig. 6A, lane 3). Monoclonal antibodies specific for Ets-1 as well as for Ets-2 were also tested in these EMSA-antibody experiments, and no change in mobility of ETS-A or ETS-B was observed (data not shown). A monoclonal antibody specific for human Elf-1 was also able to supershift a band in the ETS-A complex (Fig. 6B). Additionally, in vitro-translated Elf-1 ran at the same mobility as the ETS-A complex in EMSA analysis and was also similarly reactive to the Elf-1-specific monoclonal antibody (Fig. 6B). We concluded from these experiments that Elf-1 is a dominant protein binding the CD4 Ets site in T-cell nuclear extracts and is a candidate for the transcription factor required for CD4 enhancer function in vivo.

To strengthen this evidence, we analyzed the protein recognized by the Elf-1-specific antibody on a denaturing polyacrylamide gel (Fig. 6C). The protein-DNA complexes were first resolved on a nondenaturing EMSA gel, and the wet gel was exposed to X-ray film. After autoradiography, the ETS-A complex was excised from the gel and loaded directly onto an SDS–10% polyacrylamide gel. The proteins were then transferred to nitrocellulose by Western transfer, and the presence of Elf-1 in the complex was visualized with the Elf-1-specific antiserum. A single band was observed in the proteins eluted from the ETS-A complex and in whole nuclear extract but was

FIG. 5. Nuclear factors binding to distal enhancer mutants. (A) DNase I footprinting of wild-type (wt; lanes 1 to 3), Ets site mutant (Ets-M; lanes 4 to 6), and Gata site mutant (Gata-M; lanes 7 to 9) enhancer fragments. The E1 footprinted region is contained within the bracket, and the locations of the Ets and Gata binding sites within the footprint are indicated. The hypersensitive site within the E1 footprint is identified with an arrow. Piperidine-cleaved G ladders of each probe are shown in lanes 1, 4, and 7. DNase I-cleaved probes in the presence of Jurkat nuclear extract (lanes 3, 6, and 9) are compared with control digests without added nuclear extract (lanes 2, 5, and 8). The nucleotide substitutions made within the enhancer sequence are the same as shown in Fig. 4. (B) EMSA analysis of the CD4 enhancer Ets site and mutated Ets site. The indicated end-labeled, double-stranded oligonucleotide probes were incubated with Jurkat T-cell nuclear extract (lanes 2 to 6 and 8) and without nuclear extract (lanes 1 and 7). Two specific DNA-protein complexes (ETS-A and ETS-B) are indicated. NS refers to a nonspecific complex. Oligonucleotide competition was done with the indicated cold competitors at 25-fold (lanes 3 and 5) and 200-fold (lanes 4 and 6) molar excess. The sequences of the double-stranded oligonucleotide probes and competitors are given in Materials and Methods. (C) Comparison by EMSA of the CD4 enhancer Ets site with known Ets-1 and Elf-1 binding sites. The CD4 Ets probe was incubated with (lanes 2 to 10) and without (lane 1) Jurkat T-cell nuclear extract. The specific (ETS-A and ETS-B) and nonspecific (NS) complexes are indicated by arrows. The cold oligonucleotide competitors were included at 25-fold (lanes 3, 5, 7, and 9) and 200-fold (lanes 4, 6, 8, and 10) molar excess. The sequences of the double-stranded oligonucleotide probes and competitors are given in Materials and Methods.

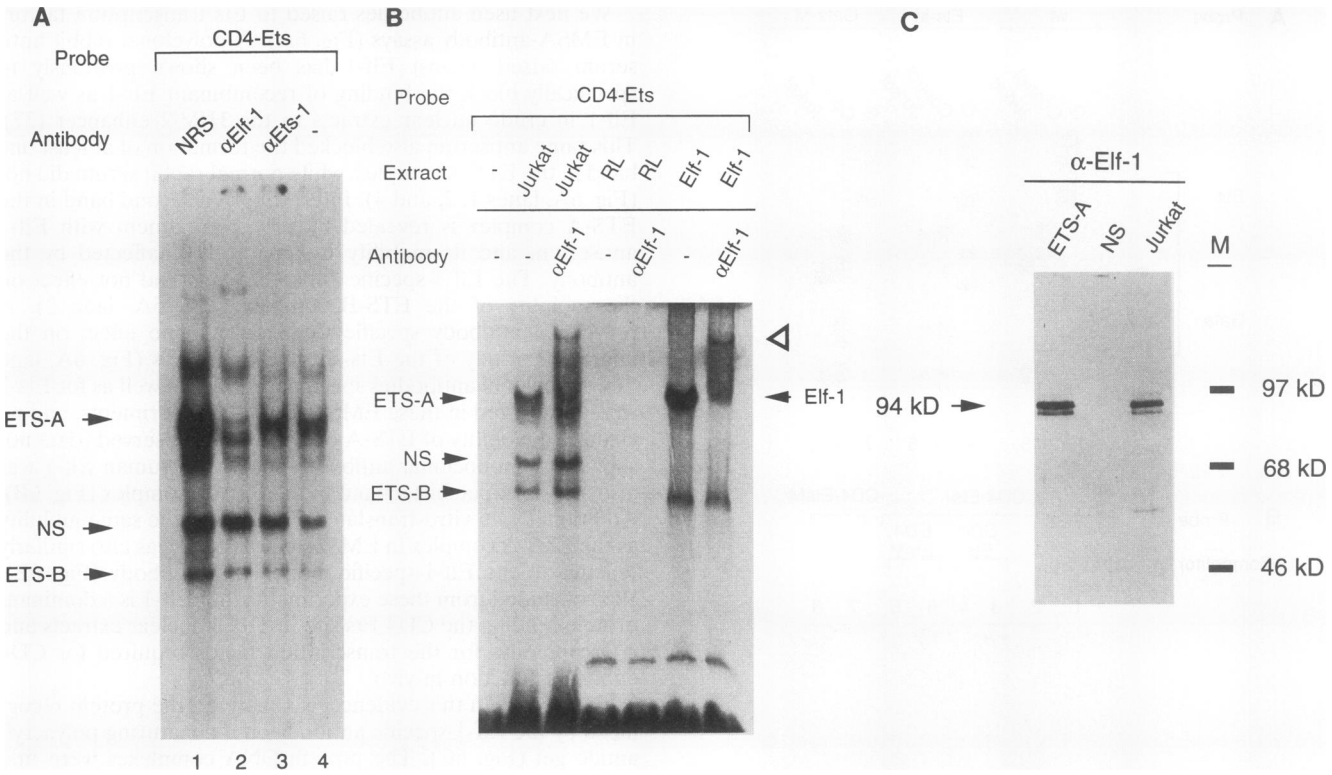


FIG. 6. Elf-1 binds to the CD4 distal enhancer Ets site. (A) EMSA-antibody blocking of the CD4 enhancer Ets site by Elf-1-specific antibodies. The CD4 Ets probe was incubated with Jurkat T-cell nuclear extract in the presence of normal rabbit serum (lane 1), Elf-1-specific antiserum (lane 2), Ets-1-specific antiserum (lane 3), or no antibody (lane 4). The Ets-specific (ETS-A and ETS-B) and nonspecific (NS) complexes are indicated by arrows. (B) EMSA analysis of the CD4 enhancer Ets site, using an Elf-1-specific monoclonal antibody and in vitro-translated human Elf-1. The CD4 Ets probe was incubated with Jurkat T-cell nuclear extract (Jurkat), unprogrammed reticulocyte lysate (RL), and in vitro-translated Elf-1 (Elf-1) in the presence or absence of a monoclonal antibody specific for human Elf-1 (αElf-1). The Ets-specific (ETS-A, ETS-B, and Elf-1) and nonspecific (NS) complexes are indicated by closed arrows. A supershifted complex created in the presence of the Elf-1 specific antibody is indicated by the open arrow. (C) Western blot analysis of the ETS-A complex. The ETS-A and NS complexes were excised from and EMSA gel, separated by SDS-polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane, and probed with the Elf-1-specific antiserum. Whole Jurkat nuclear extract (2.5 μg) was included for comparison. Protein molecular weight markers are indicated. The 94-kDa Elf-1 specific band is indicated by the arrow.

not present in proteins from the nonspecific complex also excised from the EMSA gel (Fig. 6C). The protein had a molecular mass of ~94 kDa, which is similar to the previously reported molecular mass for Elf-1 (64). Identical experiments were also conducted with an antibody specific for Ets-1 and Ets-2, and the presence of these proteins in the ETS-A complex was not detected even though they were readily detected in the whole Jurkat extract (data not shown). These data support the conclusion that Elf-1 binds to the CD4 enhancer Ets site.

DISCUSSION

In this study, we have identified a second transcriptional enhancer in the murine CD4 locus 24 kb upstream of the CD4 promoter. This distal enhancer is capable of activating transcription of reporter gene constructs when combined with a heterologous promoter and synergizes with the CD4 promoter. Enhancer activity was observed in a T-cell tumor and T-cell clones and is especially high in the $CD4^+$ antigen-specific T-cell clone, D10. Minimal activity was observed by the CD4 promoter/distal enhancer combination in immature thymomas and non-T cells, and no detectable activity was observed in these cell types when the enhancer was combined with a heterologous promoter. Taken together, these data suggest that this enhancer's activity is restricted to mature T cells.

A number of nuclear factors from several cell types are capable of binding to known transcription factor consensus sites within the minimal distal enhancer. Mutations introduced into the consensus sites for Gata, AP-1, and Sp1 transcription factor family members had no significant effect on enhancer activity in mature $CD4^+$ T cells. This result implies that these sites are not functionally relevant for enhancer activity but also depends on the assumption that these particular mutations actually destroyed factor binding. On the other hand, the binding of a predominant protein complex in T-cell extracts to an Ets binding site was shown to be absolutely required for enhancer activity. Two protein complexes (ETS-A and ETS-B) were able to bind specifically to the Ets binding site in EMSA studies, and the strongest complex (ETS-A) contains Elf-1, a recently identified Ets family transcription factor (58). Elf-1 has been shown to be expressed at high levels in T cells and has been implicated in the regulation of several activation and T-cell-specific cellular genes (interleukin-3 and GM-CSF genes) as well as in the regulation of T-cell-specific viruses (HIV-2 and HTLV-I) (4, 17, 37, 64). From the results in our study, it is a reasonable conclusion that Elf-1 could be critically involved in the activity of the distal enhancer in the CD4 locus.

The identity of the factor(s) in the ETS-B complex has not been determined. So far it has not reacted with antibodies to Ets-1, Ets-2, or Elf-1 (Fig. 6 and data not shown). Similarly, at least one additional protein-DNA complex is present in ETS-A that is Ets site specific but does not react with any of the Ets-specific antibodies tested so far. These proteins could represent Ets family transcription factors that we have not assayed for yet, or they could be different factors capable of specifically recognizing a similar binding site. We cannot conclude from these studies if Elf-1 or the other Ets site-specific proteins in ETS-A and ETS-B are responsible for enhancer function in vivo, but we are proposing that they are all candidates for this activity. Interestingly, a recent report on the human CD4 promoter has also shown that an Ets-like protein in $CD4^+$ cells can bind to a crucial element in the core promoter region (49). Although the specific role of Elf-1 was not investigated in that study, the finding does support the notion of the importance of Ets family transcription factors in CD4 gene regulation.

Ets factors have been shown to be able to interact cooperatively in binding and functional studies with a variety of other transcription factors that include AP-1, Sp1, core-binding factor, and Myb (9, 13, 62, 65). We are interested in determining what factor(s) could be interacting with Elf-1 or other Ets factors binding the distal CD4 enhancer. Elf-1, in particular, has been reported to interact with AP-1 transcription factors in the inducible expression of the T-cell-specific interleukin-3 and GM-CSF genes (17, 62). In both of these cases, the AP-1 factors and Elf-1 bind to sites that are directly adjacent to each other, mutation of either recognition sites reduces enhancer activity, and in the case of the GM-CSF promoter, the bindings by AP-1 and Elf-1 are cooperative (17, 62). Even though an AP-1 recognition site is present in the CD4 enhancer and protected in DNase I footprinting experiments, the Ets and AP-1 recognition sites were separated by over 80 bp and mutation of the AP-1 recognition site had no significant effect on enhancer activity. Additionally, EMSA experiments using an antibody to c-Jun did not detect AP-1 transcription factors binding to the minimal CD4 enhancer (data not shown). These results suggest that in the case of the CD4 enhancer, Elf-1 is not interacting with AP-1 transcription factors. We were also intrigued by the Gata site, which directly abuts the Ets site within the E1 footprint. We suspected that Gata-3, a Gata family member that is expressed at high levels in T cells and involved in the regulation of several T-cell-specific genes, would also be a prime candidate for an Ets/Elf-1 binding partner (24, 32, 35, 42, 68). However, experiments presented here involving mutation of the Gata site indicate that Gata-3 is probably not involved in the activity of the distal CD4 enhancer. Additional studies need to be performed to rule out this possibility, since we have observed recombinantly produced Gata-3 binding to the CD4 enhancer Gata site in EMSA experiments (data not shown). It is also reasonable to propose that a transcription factor other than Gata-3 could be cooperating with Elf-1 in the activity of the CD4 enhancer. The mutations made thus far in footprint E1 have been limited to the known consensus sites within the footprint. Additional mutants for this region need to be constructed and assayed for both loss of enhancer function and loss of factor binding so that we can carefully map protein-DNA contact sites and subsequently determine what other protein factors are binding there. This will allow us to identify the potential binding partner of Elf-1 and determine the combinatorial effects of the factors on enhancer specificity and activity.

Several *cis*-acting elements in the murine and human CD4 loci have already been described, but these elements alone cannot account for CD4 expression at all stages of T-cell development and subset differentiation (2, 49, 51, 52, 54). In vitro transfection studies in our laboratory on the murine CD4 promoter and elsewhere on the human CD4 promoter show that these elements themselves are remarkably T cell specific and, in the case of the murine CD4 promoter, T-cell subset specific (49, 54). Transient transfection experiments indicate, however, that the promoter itself requires additional enhancer elements in order to drive gene expression at all stages of T-cell development (54). A proximal enhancer, located 13 kb 5′ of the murine CD4 promoter, was also shown to be T cell specific in transient transfection assays, but the enhancer activity was observed in $CD4^+$ as well as in $CD4^-$ T-cell subsets, even when combined with the CD4 promoter (51, 52). Additionally, several groups have also reported in vivo studies of transgene constructs that include the proximal enhancer driving the expression of reporter cDNA genes (2, 31). Although these transgenes were expressed on T cells, transgene expression failed to be silenced in B cells or $CD4^-$ T cells. Clearly, additional regulatory elements outside of the CD4 promoter and proximal enhancer are required for the complete reconstitution of the developmentally regulated expression of the CD4 gene.

Several groups have also examined CD4 gene regulation in vivo by analyzing transgenic animals with large, genomic constructs containing the human CD4 gene, flanking sequences, and the proximal enhancer. These experiments allow the analysis of promoter and proximal enhancer activity in the context of the genomic sequences in the human CD4 locus. Although expression of these transgenes is, for the most part, restricted to the $CD4^+$ T-cell subsets (2, 14, 22, 31), significant differences in expression patterns are also apparent. Gillespie et al. and Hanna et al. presented very similar transgene constructs that included 5′ and intronic genomic sequences (14, 22). Gillespie et al. found that their transgene was expressed exclusively on $CD4^+$ cells in peripheral blood mononuclear cells, although no other cell surface marker or lymphoid tissue was examined (14). Hanna et al. found that expression of their transgene was expressed on most $CD4^+$ cells from mesenteric lymph nodes, but the transgene was also expressed on 47% of $CD8^+$ T cells (22). Killeen et al. and Blum et al. also published human CD4 genomic transgene constructs (2, 31). These constructs included either the human or murine proximal enhancer linked to a human CD4 clone containing intronic and flanking genomic sequences. Once again, expression of these transgenes was primarily restricted to $CD4^+$ T cells, although Blum et al. also reported limited transgene expression in B cells and $CD8^+$ T cells (2). Additionally, penetrance of transgene expression in the $CD4^+$ T-cell subset was incomplete in T cells from the thymus and spleen (2). These results indicate that additional regulatory sequences even outside of these genomic constructs may be required to drive expression of the transgene in all of the appropriate $CD4^+$ T cells.

We do not know whether the human homolog of the distal enhancer described in this report is included in the transgenes described above. We have examined in our laboratory the expression of minilocus transgene constructs that include the distal and proximal enhancers and a number of other nuclease-hypersensitive sites in the murine CD4 locus (53). The expression of these constructs indicates that the distal and proximal enhancers are not dominant-negative elements for restricting CD4 expression from $CD4^-$ T cells and B cells. Instead, an additional negative regulatory element in the CD4 locus is responsible for silencing CD4 expression in these cell types. On the basis of transient transfection studies presented here, we

propose that the distal enhancer is important for the maintenance of CD4 expression in mature helper T cells and, in conjunction with additional regulatory elements in the CD4 locus, contributes to the $CD4^+$ T-cell subset-specific expression.

At this point, we also cannot exclude the possibility that the distal enhancer is involved in the regulation of other lymphoid cell-specific transcripts in the murine CD4 locus. The coordinated regulation of several genes by a distal regulatory element has been illustrated before with the β-globin locus control region (19). An analysis of the α-globin locus has also revealed the presence of many linked transcripts (60). Future experiments in our laboratory will address this question and could potentially reveal a locus of coordinately transcribed genes.

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