A Potential Role for Elf-1 in CD4 Promoter Function*

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The control of CD4 gene expression is believed to be linked directly to the signaling events that mediate T cell development and is directly dependent on the CD4 promoter. We have previously determined that this promoter contains four factor-binding sites important for its function. One of these sites, referred to as the P4 site, contains an Ets consensus recognition sequence. Using functional and biochemical analyses, we determine that Elf-1 binds to this site and specifically activates the CD4 promoter, indicating that Elf-1 is playing an important role in CD4 promoter function. In addition, a second nuclear factor binds to this region. Although there are consensus recognition sites for other factors, we demonstrate that none of these factors binds to the P4 site, nor do other known members of the Ets family. Thus, a novel transcription factor may bind to the CD4 promoter and help mediate its function.

CD4 is a cell surface molecule that is essential for T cell development and function (1–3). During antigen recognition, CD4 binds to a nonpolymorphic region of the MHC class II molecule, thereby increasing the avidity of the T cell for its target. Both CD4 and CD8, a similar coreceptor molecule, also contribute to T cell antigen receptor signaling by recruiting the tyrosine kinase Lck to the T cell antigen receptor/CD3 complex and thus are critical for the activation of the mature T cell (4). The decision to express CD4 or CD8 on mature T cells correlates with both its MHC restriction and its functional specialization and occurs during development in the thymus (5, 6). Precursor T cells in the thymus undergo a “double-positive” stage in the thymus in which the thymocyte expresses CD4, CD8, and T cell antigen receptor on its surface. The selection processes that determine both the antigen and MHC class specific identity of each T cell occurs at this stage (5, 7–11). During this process, a decision is made to turn off either CD4 or CD8 so that the T cell will either become a CD4+CD8- helper T cell (T(H)) that recognizes antigen bound to MHC class II or a CD4-CD8+ cytotoxic T cell (T(C)) that recognizes antigen bound to MHC class I (12–16). Thus the control of expression of the CD4 gene is directly linked to T cell development (17–23).

CD4 expression during development is controlled primarily by transcriptional mechanisms (24). At least five transcriptional control elements have been identified in the CD4 locus: a distal enhancer (25), a proximal enhancer (26, 27), the promoter (28–30), a transcriptional silencer (24, 31, 32), and a novel thymocyte-specific enhancer (33). Each of these elements contributes in different ways to the specificity of CD4 gene expression. Thus, there are many signaling events and complex interactions between transcription factors that are necessary to mediate the complexities of tissue- and developmental stage-specific CD4 expression. To address these issues, we study the structural and factor binding requirements of each regulatory element and their contribution to the control of CD4 gene expression. We have determined that HES-1 and the novel transcription factor SAF bind to functional sites in the CD4 silencer and help mediate its function (34).2 In addition, work by Sawada and Littman (26, 27) has shown that a heterodimer of the basic-helix-loop-helix proteins HER and E12 bind to a functional site in the proximal enhancer. However, all of these factors are expressed in many different hematopoietic cells, and thus their expression pattern cannot explain the specificity of CD4 gene expression.

To understand how subclass-specific expression of CD4 is controlled, it is important to understand the functional interaction of the different CD4 transcriptional control elements. The CD4 promoter is an important central regulatory element in the control of CD4 gene expression; consequently, it is important to understand the factors that bind to its functional sites and how they interact with transcription factors that bind to the other elements. We have therefore conducted an extensive analysis of the CD4 promoter (28, 30). There are four factor-binding sites, denoted P1, P2, P3, and P4, that are important for full promoter function (28). The transcription factors c-Myb and Maz bind to sites P1 and P2, respectively, implicating these factors in CD4 promoter function (30). The P4 region contains consensus initiator (36) and Ets (37) sequences. Previous work by Klatzmann and colleagues demonstrated that exogenously expressed Ets-1 is capable of binding and transactivating the human CD4 promoter, consistent with these observations (29). Here, we determine that the Ets consensus sequence in the P4 site is important for regulation of transcription of the CD4 gene. Using functional and biochemical analyses, we determine that Elf-1 and not Ets-1 or Ets-2 binds to the Ets site and mediates CD4 promoter function. In addition, we determine that a novel factor also binds the P4 site. Taken together, these data support the hypothesis that Elf-1 acts as a positive regulator at the CD4 promoter and, together with a novel factor, plays a complex role in the induction of CD4 gene expression.

MATERIALS AND METHODS

Cell Transfection and Maintenance—The CD4+CD8- T(H)2 clone D10 and the CD4+CD8+ T(C) clones L3 and B18 (38) were maintained in Chic’s medium (EHAA) supplemented with 10% fetal calf serum, penicillin/streptomycin (0.1 mg/ml), l-glutamine (2 mm), 2-mercaptoethanol.

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In short, the gel and all membranes were placed in transfer buffer (39 and DE81 paper, using a semidry transfer apparatus as described (45). Blotted simultaneously onto two different membranes, nitrocellulose 30–ml reaction volume. The reactions were then run at 250 V on a 4% SDS-PAGE gel, and subjected to electrophoresis (44).

The core TCC sequence is essential for Ets family factor DNA-binding domain that shares a common structural motif. The core TCC sequence, the consensus sequence, the DNA-binding domain that shares a common structural motif.

RESULTS

An Elf-1 Recognition Site in the P4 Region—Using linker scanning mutations and transient transfections, we have identified four functional sites in the CD4 promoter, which we refer to as P1, P2, P3, and P4 (30) (Fig. 1A). The P4 site encompasses both the initiation point of transcription for the CD4 gene and important 5’ functional regions. An alignment of P4 with the consensus binding sequences of several Ets family members are aligned with the broad family consensus sequence.

Elf-1 in CD4 Promoter Function

![Fig. 1. The P4 site of the CD4 promoter contains an Ets consensus sequence. A, schematic representation of the minimal CD4 promoter and its cis- and trans-regulatory elements; see text for details. The sequence around the P4 site is shown in detail, with the Ets core consensus sequence underlined. Consensus binding sequences for several Ets family members (C) are aligned with the Ets core consensus sequence, the CD4 distal enhancer Elf-1 binding sequence and the CD4 promoter P4 site (B). The Ets core consensus is outlined. The sequence degeneracy around the core consensus determines the specificity of binding in vivo for different members of the family.](image-url)
defining the Elf-1 DNA binding domain. Thus, the identity of the Ets family member binding to a given site can be determined by studying the effects of mutations in these regions on factor binding. Experiments by several groups have demonstrated that Elf-1 is the sole member of the Ets family that recognizes the core sequence TTCC but cannot bind to the sequence ATCC; all other members of the Ets family, including Ets-1, Ets-2, Pu.1, PEA, Fli-1, Erg, and Elk-1, can bind to the ATCC core Ets recognition sequence (46–50). This difference in DNA binding specificity is due to a sequence difference in the Elf-1 DNA binding domain in comparison with the other Ets family members (49, 50). The pSS031 mutation is a point mutation that changes the first thymidine in the core TTCC sequence in the P4 site to an adenine (Fig. 2); thus, our functional data suggest that the factor binding to P4 and mediating CD4 promoter function cannot recognize the ATCC variant of the Ets consensus sequence. This observation is consistent with the hypothesis that the endogenous factor mediating promoter function at the P4 Ets site contains an Elf DNA-binding domain.

Elf-1 and Not Ets-1 or Ets-2 Binds Specifically to P4—To address this issue biochemically, we conducted a series of EMSAs to determine whether Elf-1 binds the P4 site. Three major (A, B, and C) and two minor protein-DNA complexes form when the P4 probe is used in an EMSA reaction with nuclear extracts purified from CD4 SP T11 cell clone D10. Addition of excess nonradioactive P4 oligonucleotide inhibits formation of all complexes, whereas nonspecific DNA did not, indicating that all of the factors that form these complexes bind specifically to the P4 probe. Similarly, the addition of large molar excesses of an oligonucleotide containing the consensus Elf-1 recognition site from the CD4 distal enhancer also inhibits complex formation. Addition of excess nonradioactive oligonucleotide that contains a large mutation in the Ets site (P4mETS; see Figs. 2 and 3A) does not inhibit the formation of complexes A and B, suggesting that these two complexes represent nuclear factors binding to a region surrounding the Ets consensus site in P4. Formation of complex C is inhibited effectively with the P4mETS oligonucleotide, suggesting that this complex represents a factor binding to P4 outside of the consensus Ets region.

As discussed above, the pSS031 mutation, representing a change in the core Ets recognition site from TTCC to ATCC, can be recognized by all members of the Ets family except Elf-1 (49, 50). This change results in the loss of Elf-1 binding and subsequent transcriptional control element function. Our functional data presented above suggest that the factor binding to P4 cannot recognize the ATCC variant of the Ets consensus site, implying that the P4-binding factor has a Elf DNA binding domain. Thus, should endogenous Elf-1 bind to P4, we predict that a mutant oligonucleotide with a single base change that alters the TTCC to ATCC should neither be able to inhibit complex formation with the unmuted P4 probe in an EMSA experiment nor be able to form complexes A and B when used as a probe. We tested these predictions directly using additional EMSA experiments. As can be seen in Fig. 3A, with large molar excesses of the 0031 oligonucleotide we could successfully inhibit the formation of complexes C, but not complexes A and B, satisfying our first prediction. Fig. 3B demonstrates that the 031 probe, like the P4 probe, is able to form complex C, but unlike the P4 probe, it is unable to form complexes A and B when incubated with D10(CD4SP) cell extract. As a control, we
showed that an oligo with an extensive mutation of the Ets core consensus, P4mEts, also fails in complex formation and so does oligo 058, which has a similar mutation to 031 (see Fig. 2A). In addition, we demonstrated that the complexes formed by the 031 probe are specific, because they can be competed away with the P4mEts and 031 oligos, but not with the linker oligo (Fig. 3B). Thus, we satisfy our second prediction and demonstrate that a single base pair change, previously proven to abrogate the P4mEts and 031 oligos, but not with the linker oligo (Fig. 3B). Thus, we satisfy our second prediction and demonstrate that a single base pair change, previously proven to abrogate Elf-1 complex formation (49), abrogates formation of complexes A and B.

To demonstrate conclusively that Elf-1 is binding to P4, we conducted EMSA supershift experiments with an antiseraum specific for Elf-1 (Fig. 4). We could completely ablate complex B with the addition of an anti Elf-1 antiserum and detected a novel slower-mobility complex (Fig. 4). The thin arrow indicates a third major specific complex binding to P4 outside of the Ets consensus (C). Three minor specific complexes were also present (see text for details). B, EMSA using D10(CD4SP) cell extracts and four different probes, as indicated above the lanes. Probe sequences are shown in Fig. 2A. Cold competition of the complexes, formed with the 031 probe, are shown; conditions are as in Fig. 3A.

The Complex A Does Not Contain Elf-1—Interestingly, we could not supershift complex A (Figs. 3 and 4). It is possible that this complex represents Elf-1 either in a different conformation or bound in a multifactor complex, the formation of which blocks the epitope recognized by the antiserum. Alternatively, this complex could represent a novel transcription factor. To distinguish between these possibilities, we conducted multiple biochemical experiments using the anti-Elf-1 antibody to characterize the complex A. Should Elf-1 be present in complex A and the antiserum-reactive epitopes be blocked by protein conformation or by a bound cofactor, we would predict that denaturing the complex would expose the epitope and thus enable the antiserum to identify the Elf-1 component. To test this, we conducted a large-scale EMSA reaction with the P4 probe, resolved the reaction on a nondenaturing polyacrylamide gel as for a normal EMSA experiment, and simultaneously transferred the protein products in the gel under denaturing conditions to nitrocellulose and the radioactive DNA probe to DE81 paper. The nitrocellulose membrane was then probed with the anti-Elf-1 antiserum, and the DE81 paper was exposed to x-ray film. Thus, two superimposable images were formed; proteins identified by Western blotting can be assigned to complexes formed in the EMSA experiment by aligning the images. Should complex A contain Elf-1, the epitope of which is blocked by protein conformation or by a bound cofactor, we would predict that we would be able to detect Elf-1 in complex A in this experiment because the protein is denatured during the transfer to the membrane. As seen in Figs. 4 and 5, using nuclear extracts from D10(CD4 SP), AKR1G1(DP), and S49(DN) T cells, we could detect multiple complexes binding to the P4 probe (Fig. 5, EMSA panels, A, left lane; B and C, left and middle lanes). As expected, we can detect Elf-1 binding to the probe (Fig. 5, α-Elf-1 blot panels). This band does not appear in the lanes containing EMSA reactions supershifted with the anti-Elf-1 antisera, because its mobility has been slowed significantly by the anti-Elf-1 antibody. These data strongly support our previous data, suggesting that this complex represents endogenous Elf-1 binding to P4. Interestingly, we were not able to detect Elf-1 in complex A on Western analysis under any conditions (Fig. 5, α-Elf-1 blot panels; arrow labeled A). This is especially clear in the supershift lanes, in which signal from the Elf-1 complex cannot obscure a fainter signal from the A complex. These data are also consistent with our EMSA-supershift data described above and suggest that complex A does not contain Elf-1.

It is possible that the sensitivity of the shift-Western assay is insufficient to detect Elf-1 in the A complex. To address this, we immunodepleted the nuclear extracts with the anti-Elf-1 antiserum and used these extracts in EMSA reactions with the P4 probe. Although complex A is not recognized by the antisera, should it contain Elf-1 in a different conformation, complete immunodepletion of all Elf-1 in the cell may shift the overall equilibrium of the conformation or binding reaction, thus leading to a diminution of intensity of complex A on EMSA. We immunoprecipitated Elf-1 out of S49(DN) extracts and tested the efficiency of the immunodepletion by Western analysis (Fig. 6A). The presence of Elf-1 is easily detectable in the immunoprecipitate (Fig. 6A, α-Elf-1 IP), although it is not detectable in the supernatant as compared with an equal amount of untreated extract (Fig. 6A, post IP sup.). We could detect no differences between immunodepleted and untreated extracts in EMSA experiments with non-Elf site-containing probes, indi-
cating that the depleted extracts are essentially intact. As shown in Fig. 6B, after the immunodepletion of S49 nuclear extracts with anti-Elf-1 antibody, the Elf-1 complex did not form, whereas all other complexes remained unaffected. Because complex A remained unaffected by immunodepletion with anti-Elf-1 antibody, this experiment further supports the notion that complex A does not contain Elf-1.

Finally, we transfected 293T cells with an Elf-1 expression vector, purified nuclear extracts, and used these extracts in EMSAs. The 293T cell line does not express endogenous Elf-1 (Fig. 6A); should complex A represent Elf-1 in a different protein conformation or Elf-1 bound to a cofactor, we would predict that we would be able to detect both complexes on EMSA in the transfected 293T cells but not the untransfected 293T cells. Immunodepletion of these extracts led to the complete depletion of both of these protein species, suggesting that both represent Elf-1 (Fig. 6A). The reason for the difference between the two species is unknown, but these species may represent different stages of phosphorylation of Elf-1. EMSA with nuclear extracts purified from the Elf-1-transfected 293T cells revealed a single new complex that comigrates with the Elf-1 complex in the T cell nuclear extracts; however, we could not detect complex A (Fig. 6B). This novel complex in the nuclear extracts from Elf-1-transfected 293T cells could be detected on the Western blot of a shift-Western experiment (data not shown) and disappeared upon immunodepletion with anti-Elf-1 antibody (Fig. 6A). Thus, we can conclude that the full-length Elf-1 product binds specifically to the Ets sequence of the P4 probe and that complex A does not form in the presence of Elf-1 in 293T cells. Taken together, the above experiments support the hypothesis that complex A does not contain Elf-1.
Characterization of the Second P4-binding Factor—Although complex A does not contain Elf-1, it is possible that this complex represents one of the other two members of the Elf-1 family (51–53). One of these factors, MEF, is myeloid-specific; as complex A is clearly present in all T cells, MEF is unlikely to be a component of this complex (53). The second member of the Elf-1 family, NERF (51), has a DNA-binding domain that is almost identical to that of Elf-1 and has a broad tissue distribution; it is therefore possible that the Elf-1-like factor binding to P4 in our EMSA experiments is NERF. To address this issue directly, we conducted additional biochemical studies using an antiserum generated against human NERF. We first determined the expression pattern of NERF using Western analysis on human and murine cell lines. We could detect NERF in HEla and 293T cells, but not in Jurkat or Namalwa cells (Fig. 7A), suggesting that NERF is not expressed in the lymphoid lineage. In addition, 293T cells expressed NERF but did not form either complex A or B in our EMSA experiments (Fig. 6B). We could detect Elf-1 in all lymphocyte lines, as well as in Hela, but not the 293T cells, consistent with the expression pattern of Elf-1 (Fig. 7B). These data suggest that NERF is not likely to be a factor that we detect binding to P4 as complex A. Interestingly, we could not detect NERF expression in any of the murine cell lines, including the nonlymphoid L929 cell line. It is possible that the anti-NERF antibody does not recognize NERF of murine origin; alternatively, a murine homologue of NERF may not exist. However, our data presented above from the human cell lines exclude NERF as a possible P4-binding protein. Another possibility is that this second factor could be recognizing a different recognition sequence in the P4 region. An extensive data bank search showed that there were weak consensus Ikaros and TFII-I recognition sites within the P4 region (35, 64). However, using EMSA competition as well as antibody supershift/ablation experiments, we were unable to demonstrate that these factors are binding to the P4 probe, suggesting that complex A is not likely to be either of these factors (data not shown).

To characterize the second P4-binding factor further, we conducted UV cross-link experiments to estimate its molecular weight. A preparative EMSA reaction was resolved on a non-denaturing polyacrylamide gel and analyzed by Western blotting with the anti-Elf-1 antibody. The position of the Elf-1 protein is indicated by an arrow. The extracts are indicated above each lane. The second of the two S49 lanes represents treatment with twice as much antibody. The left panel shows the immunoprecipitated Elf-1 protein from all extracts, analyzed by Western blot. The right panel shows the SDS-PAGE analysis of the post-IP supernatants (indicated by sup after the cell line name). As a control, nonimmunoprecipitated extracts from S49(DN) and 293T cells are run on the same gel. Equal amounts of total protein were loaded on each lane as estimated by the Bradford assay and confirmed by Ponceau S staining of the membrane. B, EMSA of immunodepleted and untreated extracts from S49, 293T, and Elf-1-transfected 293T cells with the radioactively labeled P4 probe. Extracts and their treatment are indicated above each lane. The triangles indicate increasing amount of extract added for the 293T- and Elf-1-transfected 293T lanes; for the S49 lanes, it represents increasing amount of antibody used for the immunodepletion. Complexes A and B are indicated by filled and empty arrows, respectively.
Fig. 8. Determining the molecular mass of the P4-binding complexes. Preparative EMSA binding reactions were resolved on a nondenaturing PAGE gel; the gel was exposed to UV radiation, as described under "Materials and Methods". Complex A, the Elf-1 complex, and the intervening space were excised; and the protein-DNA products were eluted and resolved on a denaturing PAGE gel. The migration position of the complexes is indicated both on the SDS-PAGE and EMSA gels. Molecular mass markers are indicated.

Our data support the above conclusions that the second P4-binding factor is not a known member of these families. The lower P4 protein-DNA complex is approximately 110 kDa (Fig. 8, right lane), giving an apparent molecular mass for the lower P4-binding factor of 95 kDa, which is the size of Elf-1 when analyzed on an SDS-PAGE gel. Thus these results are consistent with our antibody experiments and support our hypothesis that the factor forming complex B contains Elf-1.

Elf-1 Activates the CD4 Promoter—Our data suggest that Elf-1 binds specifically to the CD4 promoter at P4. Thus, we predicted that we would be able to activate transcription from a reporter construct containing the CD4 promoter in a CD4+ T cell by overexpressing Elf-1. To test this, we conducted cotransfection experiments (Fig. 9). In this experiment, we transfected the pSS006 reporter construct into different T cell lines with increasing amounts of an expression construct containing the murine Elf-1 gene under the transcriptional control of the CMV LTR. The pSS006 construct contains the intact −101 minimal CD4 promoter driving the firefly luciferase gene; should Elf-1 bind to the P4 site, we would predict that we would be able to transactivate the CD4 promoter by overexpressing Elf-1. As a specificity control, we conducted similar experiments using the pSS031 and pSS054 reporter constructs, which contains a site-specific mutations in the Elf-1 recognition site (Fig. 2). As can be seen in Fig. 9, we detected a dose-dependent increase in CD4 promoter function in the CD4+ CD8+ T cells using the pSS006 construct and increasing amounts of the CMV-Elf-1 expression plasmid; addition of comparable amounts of CMV plasmid alone does not lead to promoter induction. This induction is also dependent upon the presence of the Elf-1 recognition site in P4; addition of increasing amounts of CMV-Elf-1 plasmid to the pSS054 transfection leads to only a slight increase in promoter activity. These data indicate that Elf-1 is capable of binding to P4 and inducing promoter function, supporting the hypothesis that endogenous Elf-1 is in fact mediating CD4 promoter function in vivo at the promoter. To test whether Elf-1 can transactivate the CD4 promoter in T cells that do not express CD4, we conducted transactivation experiments with the CMV-Elf-1 expression and the pSS006 reporter vectors in the L3 and B18 CD8 SP Tc clones (Fig. 9). Interestingly, although we could transactivate the CD4 promoter in the CD4+ CD8+ T cell line, we could not do so in either CD8 SP Tc clone, even with the addition of large amounts of CMV-Elf-1 expression plasmid. We obtained similar results with the CD4+ CD8+ T cell line S49 (data not shown). These data suggest that Elf-1 is not capable of transactivating the CD4 promoter in cell types that do not express CD4.

DISCUSSION

Elf-1 Binds to the P4 Site and Activates CD4 Promoter Function—Our data indicate that endogenous Elf-1, a member of the Ets family, binds to the P4 functional site of the murine CD4 promoter and activates its function. We draw this conclusion on the basis of results we have obtained from seven different experimental approaches. First, we demonstrate that a subset of the T cell nuclear factors binding to the P4 region recognize the Ets consensus sequence (Fig. 3). Second, we took advantage of the fact that Elf-1, alone of all the Ets family members, cannot bind to the Ets core sequence variant ATCC. Using biochemical experiments we demonstrate that the nuclear factors binding to P4 also cannot recognize a ATCC variant of the P4 sequence (Figs. 2 and 3). In addition, the single base mutation of the P4 site to the ATCC sequence abrogates CD4 promoter function (Fig. 2). Third, we were able to supershift a major P4-binding complex using antisera directed against Elf-1, but not with normal rabbit serum or antisera directed against Ets-1, Ets-2, or the other Ets family members, indicating that it is unlikely that these other Ets family members are binding to P4 (Fig. 4 and data not shown). Fourth, we could also detect the presence of Elf-1 in a major P4 protein-DNA complex by shift-Western experiments (Fig. 5). Fifth, Elf-1-depleted T cell extracts failed to form the specific P4 complex on EMSA (Fig. 6). Sixth, nuclear extracts purified from 293T cells transfected with an Elf-1 expression construct formed a novel complex with the P4 probe on EMSA that can be depleted with the anti Elf-1 antisera; this complex migrated in the exact same manner as the putative Elf-1-containing complex in T cell nuclear extracts (Fig. 6). Finally, we could transactivate the CD4 promoter by overexpressing Elf-1 in a CD4+ T cell line; this transactivation is dependent on the intact Elf-1 site (Fig. 9).

The observation that Elf-1 is important in CD4 gene expression is especially interesting in light of recent work on the role of the Ets transcription factors in lymphocyte development (47). The control of CD4 gene expression is intimately linked to
many different aspects of T cell development and function, and thus the factors that bind to the CD4 transcriptional control elements and mediate their function are believed to be linked to these processes. Elf-1 may be playing multiple roles in the control of CD4 gene expression during these events. It is interesting to speculate that Elf-1 may be playing a role in linking signaling from the T cell antigen receptor to the CD4 gene during T cell development via the mitogen-activated protein kinase (MAPK) pathway, which has been described as being important in thymic selection (54, 55). In Drosophila, several Ets transcription factors are targets of the MAPK pathway (see below for discussion); by analogy, Elf-1 may be a target of the mammalian MAPK pathway. One attractive hypothesis is that signaling from the MAPK pathway affects Elf-1 function, thus inducing or repressing the expression of CD4. Along these lines, it is interesting to note that the overexpression of Elf-1 cannot transactivate the CD4 promoter in CD4+ T cells but can do so in CD4- T cells, indicating that there may be functional differences in Elf-1 between T cells of different developmental phenotypes.

Other Factors Bind to the P4 Region—Interestingly, we have also shown that a novel factor also binds to the P4 region. The role of this novel protein in the control of CD4 gene expression is unknown. It is possible that this novel protein mediates the effect of Elf-1 on CD4 promoter function. There are several examples in which two factors bind to the same site and have different effects on the control of gene expression during development. The proper development of the R7 photoreceptor in Drosophila melanogaster requires signaling from the transmembrane receptor protein tyrosine kinase Sevenless (Sev) (56). Signals through Sev are transmitted through the MEK-1 and MAPK homologues DSOR1 and Rolled to two transcription factors: Pointed P2 and Yan (57). Both Pointed and Yan are homologues of mammalian Ets/Elf family transcription factors and play important roles in the control of developmental gene expression. Pointed is a positive transcription factor, whereas Yan represses transcription; whether or not the target gene is transcribed is dependent on which of the two factors predominates. It is interesting to speculate that Elf-1 and the novel factor are functioning in a similar manner at the CD4 promoter. However, additional experiments are necessary to demonstrate this point conclusively.

We can also identify other protein-DNA complexes in our EMSA experiments with the P4 probe (Fig. 3). The P4 site is only two bases 5' of the initiation site of transcription of the CD4 gene containing a consensus initiator (Inr) sequence. The close proximity of the Elf-1 and Inr sites in the CD4 promoter may indicate that Elf-1 and/or the Elf-like factor interact(s) directly with Inr-binding proteins to mediate promoter function. Ets proteins have been demonstrated to be weak activators by themselves, and their diversity of cell type expression is believed to be mediated at least in part through the association with cofactors. For example, Ets-1 binds to a functional site in the T cell antigen receptor α chain gene enhancer and is believed to function only in conjunction with LEF-1, the recognition site of which is directly adjacent to the Ets consensus (58). As the initiator sequence is immediately 3' of the Elf-1 recognition site, it is possible that an initiator factor may be interacting with Elf-1 at different stages of development to convey promoter specificity.

The Elf-1-containing P4-binding Complex Also Binds to the CD4 Locus Distal Enhancer—We have previously shown that the distal enhancer contains multiple factor-binding sites, including an Elf-1 recognition site. Interestingly, the Elf-1 probe from the CD4 locus distal enhancer is capable of competing away formation of all of the CD4 promoter P4-binding complexes (Fig. 3), indicating that the P4-binding factors have the same sequence specificity as the distal enhancer-binding factors. In addition, EMSAs with the Elf-1 site from the distal enhancer as a radioactive probe reveal the same complexes as EMSAs with the P4 probe (data not shown). These data indicate that similar Elf-1-Inr protein complexes may be binding both the promoter and the distal enhancer. Most promoter-binding factors can function equally well as enhancer-binding factors; therefore, the presence of similar complexes binding to both an enhancer and a promoter of a gene is not unprecedented. However, these experiments predict that the specificity of function of the promoter and the distal enhancer would be very similar. Our previous experiments, as well as those published here, support this hypothesis (25, 28). The similarity of function of these two elements is most likely the result of similar transcription factor complexes binding to critical functional sites in both elements.

Although the distal enhancer was first described as functioning on the CD4 promoter in transient transfection studies, its functional relevance for in vivo CD4 gene expression has not been demonstrated (25). Transgenic experiments demonstrate that the CD4 distal enhancer is not necessary for the correct developmental expression of the CD4 gene and indeed cannot enhance the CD4 promoter in vivo, providing evidence that it is irrelevant for the control of CD4 gene expression.2 It is possible that the distal enhancer may be a control element for a different gene located in the CD4 locus. There are at least two genes located in the CD4 locus adjacent to the distal enhancer: LAG-3, a gene with structural similarity to CD4 located immediately downstream of the distal enhancer; and the parathyroid hormone gene (59, 60). It is possible that the CD4 locus distal enhancer serves as a regulatory element for one of these other genes rather than the CD4 gene. Should this be the case, the requirement for similar Elf-1 protein complexes binding to these two control elements indicates that the expression of these genes may be coordinately controlled. This is at least partly the case for the LAG-3 gene, which is expressed at high levels in activated T cells (61–63). However, to address this issue in detail, it will be necessary to generate mice that are transgenic for constructs that contain different combinations of the CD4 promoter and enhancer elements to determine how these elements function independently of each other. We are currently conducting experiments to test these hypotheses further.

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