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A Myc-Associated Zinc Finger Protein Binding Site Is One of Four Important Functional Regions in the CD4 Promoter

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The CD4 promoter plays an important role in the developmental control of CD4 transcription. In this report, we show that the minimal CD4 promoter has four factor binding sites, each of which is required for full function. Using biochemical and mutagenesis analyses, we determined that multiple nuclear factors bind to these independent sites. We determined that an initiator-like sequence present at the cap site and an Ets consensus sequence are required for full promoter function. We also demonstrate that the Myc-associated zinc finger protein (MAZ) appears to be the predominant factor binding to one of these sites. This last site closely resembles the ME1a1 $G_3AG_4AG_3$ motif previously shown to be a critical element in the P2 promoter of the c-*myc* gene. We therefore believe that the MAZ transcription factor is also likely to play an important role in the control of developmental expression of the CD4 gene.

The CD4 cell surface glycoprotein is an important molecule in T-cell development and activation (21, 26, 38). T cells recognize antigens as oligopeptides bound to molecules encoded by the major histocompatibility complex (11, 42). CD4 and CD8, similar accessory molecules, bind to nonpolymorphic regions of major histocompatibility complex class II and class I molecules, respectively, thus contributing to the avidity of interaction between T cells and antigen-presenting cells (38). In addition, the expression of CD4 and CD8 in mature T cells correlates to T-cell function. $CD4^+$ $CD8^-$ T cells consist of primarily helper T cells, which regulate the immune response by producing cytokines, whereas $CD4^-$ $CD8^+$ T cells consist of cytotoxic T cells, which kill virus-infected cells and tumor cells (11, 42). The expression of CD4 or CD8 is coordinated with T-cell antigen receptor major histocompatibility complex class specificity during T-cell development in the thymus. During this process, immature T cells that migrate to the thymus express low levels of surface CD4 only (48). This population first downregulates CD4 expression and then expresses both CD4 and CD8 as well as increasing amounts of cell surface T-cell antigen receptor (10). This $CD4^+$ $CD8^+$ (double-positive) population then undergoes the positive and negative selection processes that determine both antigen and major histocompatibility complex specificity and also loses expression of either CD4 or CD8; only those T cells that express CD4 with a class II-restricted T-cell antigen receptor or CD8 with a class I-restricted T-cell antigen receptor survive thymic selection (24, 43). Thus, the expression of CD4 and CD8 is linked with the developmental program of developing T cells as they mature in the thymus (6, 7, 29, 44). The mechanism by which expression of CD4 and CD8 is regulated in T-cell development and linked with T-cell function remains unclear (44).

The control of CD4 gene expression during T-cell activation and development has been a topic of much interest recently. Nuclear run-on experiments show that CD4 gene expression is controlled primarily at the transcriptional level during T-cell development and activation (25, 35, 39). Using both transient transfection and transgenic approaches, we and others have identified multiple transcriptional regulatory elements in the CD4 locus (4, 13, 14, 19, 30–33, 35, 36). These regions include a distal enhancer located 24 kb 5′ of the first CD4 exon and a proximal enhancer located 13 kb 5′ of the first exon (31, 32, 49); both of these enhancers function primarily in T cells. We and others have identified a transcriptional silencer in the first intron; this silencer element is required for full tissue- and cell-type-specific expression of CD4 (33, 35). In addition, we and others have characterized the CD4 promoter region (30, 36). Using transient transfection analysis, we have determined that the murine CD4 promoter functions at high levels in $CD4^+$ $CD8^-$ T_H cells and at lower levels in $CD4^-$ $CD8^+$ T_C cells (36). In order to understand the contribution of the promoter to the developmental and tissue-specific regulation of CD4 expression, we have sought to define all the essential recognition sequences within the promoter. Using deletion analysis, we determined previously that 101 bp 5′ of the initiation point of transcription is the minimal required promoter sequence necessary for full function and that a Myb recognition site is important for promoter activity. In this study, using linker-scanning mutational analyses we have defined four regions within the CD4 minimal promoter that are required for full promoter function. We demonstrate that the Myc-associated zinc finger protein (MAZ) transcription factor (5, 18, 28) binds to one of these functionally important sites in the CD4 promoter and therefore is likely to play an important role in the control of CD4 gene expression.

MATERIALS AND METHODS

Cell transfection and maintenance. The $CD4^+$ $CD8^-$ T-cell clone D10 and the $CD4^+$ $CD8^-$ T-cell lymphoma Jurkat were maintained and transfected as described previously (36). The relative activity of each promoter mutation presented in Fig. 1 reflects normalization to a control transfection of a construct containing the luciferase gene under the transcriptional control of the human

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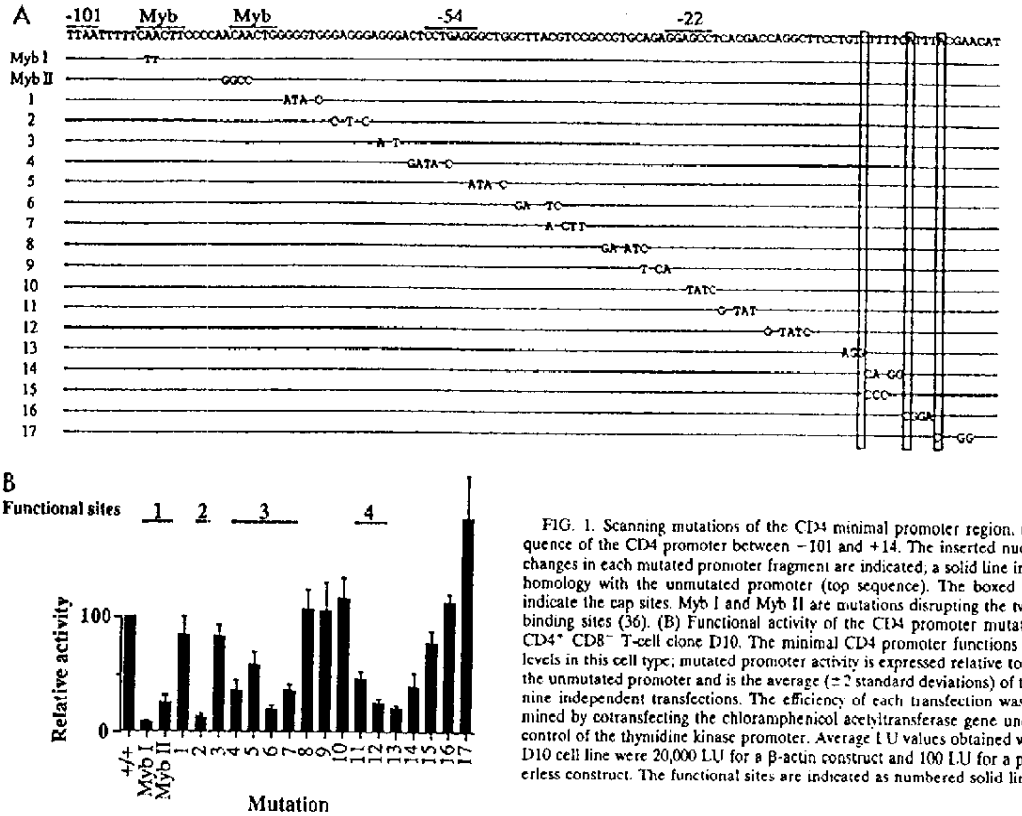

FIG. 1. Scanning mutations of the CD4 minimal promoter region. (A) Sequence of the CD4 promoter between −101 and +14. The inserted nucleotide changes in each mutated promoter fragment are indicated; a solid line indicates homology with the unmutated promoter (top sequence). The boxed regions indicate the cap sites. Myb I and Myb II are mutations disrupting the two Myb binding sites (36). (B) Functional activity of the CD4 promoter mutations in $CD4^+$ $CD8^-$ T-cell clone D10. The minimal CD4 promoter functions at high levels in this cell type; mutated promoter activity is expressed relative to that of the unmutated promoter and is the average (± 2 standard deviations) of three to nine independent transfections. The efficiency of each transfection was determined by cotransfecting the chloramphenicol acetyltransferase gene under the control of the thymidine kinase promoter. Average LU values obtained with the D10 cell line were 20,000 LU for a β-actin construct and 100 LU for a promoterless construct. The functional sites are indicated as numbered solid lines.

β-actin promoter and enhancer, which functions at high levels in this cell type (36). Average light unit (LU) values obtained with the D10 T_H1 clone were 20,000 LU for the β-actin construct and 100 LU for a promoterless construct. Conditions for each datum point were internally controlled for transfection efficiency by cotransfecting a construct containing the chloramphenicol acetyltransferase gene under the control of the thymidine kinase promoter.

Generation of promoter mutations. An *MseI-XhoI* restriction enzyme fragment that contained the minimal CD4 promoter between positions −101 and +71 was mutated by oligonucleotide-directed mutagenesis as described earlier (20, 36). Each oligonucleotide was designed to alter a specific 3- to 6-bp sequence within the promoter region; all other sequences within the region were unaltered, as determined by DNA sequencing. The mutated promoter fragments were cloned into the pSVOALΔ5′ luciferase reporter vector (8) and transfected into the D10 $CD4^+$ $CD8^-$ T_H clone in order to determine the effects of the mutations on promoter function.

DNase footprinting analysis. Nuclear extracts were purified from mouse T-cell line D10 and human leukemic T-cell lines CCRF-CEM and Jurkat as described earlier (36, 46). DNase I footprinting was performed essentially as described earlier (16). A mixture of end-labelled promoter fragment (positions −101 to +71), 1 μg of poly(dI-dC), and 10 μl of 10% polyvinyl alcohol was incubated with 40 μg of the T-cell nuclear extracts for 15 min. A 50-μl volume of 10 mM $MgCl_2$ and 5 mM $CaCl_2$ was added before the addition of DNase I after 1 min more. The reaction was stopped by adding 90 μl of 20 mM EDTA–1% sodium dodecyl sulfate (SDS)–0.2 M NaCl–325 μg of carrier RNA per ml, and the product was then phenol-chloroform extracted, ethanol precipitated, and analyzed on a sequencing gel.

EMSAs. Each of the four functionally defined regions was subcloned into the plasmid vector pKS (Stratagene Cloning Systems, La Jolla, Calif.): site 1, consisting of the sequence from −101 to −78 relative to the cap site of the promoter region; site 2, from −78 to −54; site 3, from −55 to −31; and site 4, from −20 to +4. Probes were generated from these subclones, labelled, and incubated at room temperature with nuclear extracts plus 1 μg of poly(dI-dC) (Pharmacia Biotech, Inc., Piscataway, N.J.) in a solution of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) KOH (pH 7.9), 50 mM NaCl, 5 mM Tris-HCl (pH 7.5), 25 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. After 20 min, the reaction mixtures were loaded onto a 4% nondenaturing polyacrylamide gel and run at 22°C in glycine buffer (190 mM glycine, 25 mM Tris-HCl [pH 8.5], 1 mM EDTA). For electrophoretic mobility shift assays (EMSAs), 1 to 2 μg of nuclear extract from the D10 or Jurkat T-cell line or of a bacterial lysate containing the MAZ-MBP fusion protein (5) was incubated with 1.5×10^4 cpm of the labelled probe. The monoclonal antibody treatment of the site 2 EMSA was carried out with monoclonal mouse anti-MAZ antibody 80, the generation and characterization of which will be described elsewhere (37). The reaction procedure consisted of a 30-min preincubation at room temperature of 2 μg of D10 nuclear extract, 1 μg of poly(dI-dC), and 10 μl of antibody in reaction buffer followed by a 15-min incubation with labelled site 2 probe. Reaction mixtures were loaded onto a 4% nondenaturing polyacrylamide gel and run at 4°C in glycine buffer.

Southwestern blot analyses. Twenty micrograms of Jurkat nuclear extract was resolved on an SDS–10% polyacrylamide gel and transferred onto a nitrocellulose membrane. Southwestern (DNA-protein) analyses were performed as described previously (28). Blotted proteins were denatured by incubation at 4°C for 5 min in binding buffer (20 mM HEPES [pH 7.9], 3 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol) containing 6 M guanidine hydrochloride. This solution was serially diluted with equal volumes of binding buffer in a series of four 5-min incubations to renature the blotted proteins. The filters were prehybridized in binding buffer–5% Carnation nonfat dry milk for 30 min before overnight hybridization at 4°C in binding buffer–0.25% dry milk with 10^6 cpm of ^{32}P-labelled site 2 probe, mutated site 2 oligonucleotide, or a probe consisting of the *XbaI-XhoI* fragment of the pKS multiple cloning site per ml. The filters were washed in 0.25% Carnation nonfat dry milk in binding buffer at 4°C and exposed to X-ray film. Approximate molecular weights were determined with prestained standards (Bio-Rad Laboratories, Hercules, Calif.).

RESULTS

Four functional regions within the CD4 promoter bind T-cell nuclear factors. Using deletional analysis, we have determined that 101 bp of the CD4 promoter is sufficient to generate high levels of promoter activity upon transient transfection

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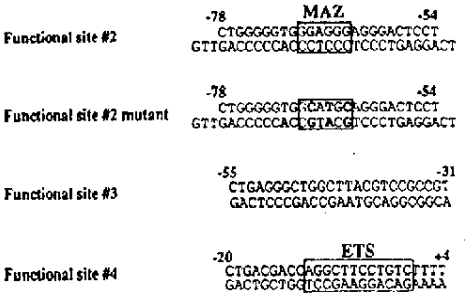

FIG. 2. CD4 promoter functional sites. Nucleotide sequences of the functional-site oligonucleotides used in the EMSA experiments. Homologies to known factor recognition sequences in site 2 and site 4 (boxed regions) and bases changed in the site 2 mutant (boldface letters) are indicated.

into activated CD4$^+$ CD8$^-$ T cells. In order to identify the *cis*-acting sequences within this region that are required for promoter function, we generated a panel of mutations that span the complete minimal promoter region by oligonucleotide-directed mutagenesis (Fig. 1A). Each promoter mutation was cloned into the pSVOALΔ5′ luciferase reporter vector and transfected into the CD4$^-$ CD8$^-$ T-cell clone as described above. The results depicted in Fig. 1B represent the activity of each construct relative to that of the unmutated minimal promoter, which functions at high levels in this cell type (36). Mutations in any of four regions reduced promoter activity by more than 60%. We previously demonstrated that a Myb family member binds to site 1 (36); sequence analysis revealed homology of site 2 to the ME1a1 site of the Myc promoter (2) and an Ets family consensus sequence within site 4 (30). A search of the National Center for Biotechnology Information transcription factor database revealed that site 3 is similar in sequence to an NF1/CTF recognition site (12). Oligonucleotides encompassing each of the four sites were used as radioactive probes in EMSAs with nuclear extracts isolated from the D10 CD4$^+$ CD8$^-$ T_H clone (Fig. 2 and 3). A single complex was observed with each probe (Fig. 3), indicating that the functional sites define factor binding sites. Each of the complexes was inhibited specifically by the unlabelled probe but not by the *Xba*I-*Xho*I fragment of the pKS multiple cloning site (Fig. 3) or by unlabelled probes from the other functional sites of the promoter used for cross-competition (data not shown).

Sequence requirements for transcription initiation of the CD4 gene. The CD4 promoter does not contain a consensus TATA recognition sequence. Genes without TATA sequences are often controlled by initiator (Inr) sequences present at the cap site. An Inr site consists primarily of a core YYANTYY sequence flanked by pyrimidines (15). The sequence encompassing the CD4 cap site (TTCCTGTCTTTTCATTTA) contains two consensus Inr sites: a downstream site surrounding the +7 start site (TCATTTA) and an upstream site at the +1 start site (TCTTTTC). Both of these sites have a 1-base mismatch from the ideal consensus sequence; for the downstream site, the +5 pyrimidine is an adenine, whereas for the upstream site the +1 site is a pyrimidine. This sequence is also similar to a consensus lymphoid enhancer factor 1 (LEF-1) recognition site (CTNTG [46]), and we were able to footprint this region by using bacterially produced LEF-1 protein (34). It is therefore possible that either Inr proteins or LEF-1 itself is important in the control of CD4 transcription. In order to determine if the recognition site at the CD4 cap site serves functionally as an Inr site or an LEF-1 site, we generated mutations of this region and tested each mutation for its effects on promoter

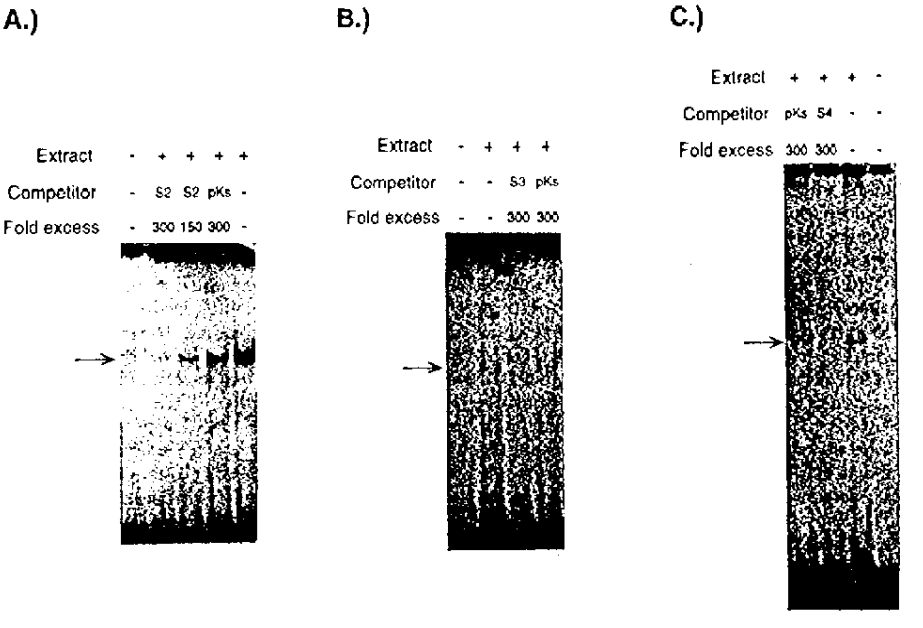

FIG. 3. EMSA analysis of functionally defined sites within the CD4 minimal promoter. EMSA analyses with D10 nuclear extract and with the site 2 (A), site 3 (B), and site 4 (C) oligonucleotides used as probes. The position of the specific complex formed with each probe (arrows) is indicated. Cold competitions with the site 2 (S2), site 3 (S3), and site 4 (S4) oligonucleotides and competition with the *Xba*I-*Xho*I fragment of the pKS multiple cloning site (pKS) are indicated. For each, the EMSA reaction consisted of a 20-min preincubation of nuclear extract with a 150- to 300-fold excess of an unlabelled probe followed by an additional 20-min incubation with a labelled specific probe. A single specific complex was observed with each of the subcloned sites.

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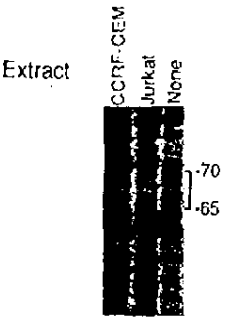

FIG. 4. Footprint analysis of functional site 2. The free probe is shown in the right lane (None); the probes incubated with nuclear extracts purified from Jurkat and CCRF-CEM T-cell clones are shown on the left. The precise position of the footprint and its position within the CD4 promoter are indicated by the bracket and sequence position numbers.

FIG. 5. Nuclear factor binding site 2 sequence specificity similarity to MAZ. Unmutated (+/+) and mutated (M) site 2 probes were incubated with and without (None) 1 μg of nuclear extract of D10 or a bacterial lysate containing the MAZ-MBP fusion protein (MAZ).

function (Fig. 1). The murine CD4 gene utilizes three closely clustered initiation points of transcription. Mutation 13 alters the +1 cap site and the upstream consensus initiator sequence. As can be seen from the data in Fig. 1, this change resulted in an 81% decrease in promoter activity, indicating that the mutation of this region is critical for promoter function. A mutation that disrupts the consensus initiator and LEF-1 sites resulted in a significant (61%) decrease in promoter activity (mutation 14), whereas a mutation at the same site that disrupts the LEF-1 site but not the consensus initiator sites (mutation 15) resulted in only a 20% decrease (mutation 15). These data suggest that despite the presence of a consensus site, LEF-1 is not essential for CD4 promoter function. Interestingly, mutations of the +7 and +11 cap sites and surrounding sequences did not lead to significant decreases in promoter activity and in fact led to slight increases in activity (mutations 16 and 17). These data suggest that the +1 site is the critical site for CD4 transcript initiation.

A T-cell nuclear factor binds to functional site 2 and is critical for CD4 promoter function. Using linker-scanning analysis, we determined that the sequences between positions −69 and −64 are required for full CD4 promoter function (Fig. 1). A mutation placed within this site (mutation 2) resulted in an 85% decrease in promoter activity, whereas mutations in directly adjacent regions (mutations 1 and 3) resulted in 15 to 17% decreases (Fig. 1). These data would predict that a transcription factor in T cells binds to this site and contributes to CD4 promoter function. To study this idea, we conducted DNase footprinting analysis on the CD4 promoter in this region with nuclear extracts purified from the CCRF-CEM and Jurkat $CD4^+$ leukemic T-cell lines and with the D10 $CD4^+$ $CD8^-$ T_H clone (Fig. 4). We detected a footprint extending from −70 to −65 of the promoter (TGGGAGGG). These data indicate that site 2 serves as a recognition site for a nuclear factor that may be important for overall promoter function. We also detected a specific complex when we used site 2 as a radioactive probe in EMSA analyses (Fig. 3). This complex migrates in the EMSA gel at the same position as the NF-4C factor complex that we identified previously as binding to the minimal CD4 promoter (36) (data not shown). When a radioactive probe containing mutation 2 was used in an EMSA, this complex was no longer detectable (Fig. 5), thus indicating that the recognition site is indeed site 2. Since this same mutation resulted in a significant decrease in CD4 promoter activity (Fig. 1), these data indicate that the NF-4C nuclear factor binds specifically to functional site 2 and is required for full CD4 promoter function. In some EMSAs, we detected a fainter complex that migrated just below the NF-4C complex (as discussed below); the intensity of this complex was not affected when the mutant site 2 probe was used. This result indicates that the factor that forms this fainter complex does not bind to functional site 2; since no other mutations within this region affect CD4 promoter function (Fig. 1), the significance of this fainter complex is not clear.

The MAZ transcription factor binds to site 2. To determine if the NF-4C factor has been identified previously, we have compared known consensus sequences with the site 2 sequence (Fig. 2). We were able to match functional site 2 with the consensus recognition site for transcription factor MAZ (2, 5). In order to determine if MAZ can recognize functional site 2, we conducted an EMSA, using a bacterially produced MAZ-MBP fusion protein with the functional site 2 and mutant site 2 radioactive probes (Fig. 5). The MAZ fusion protein formed a complex with the functional site 2 probe; in addition, introduction of the same mutation that abrogated NF-4C binding also abrogated MAZ binding. The mobility difference between the endogenous MAZ and the recombinant MBP-MAZ fusion protein is probably due to the molecular weight differences between these two proteins. These data indicate that MAZ is capable of binding to the CD4 promoter at the same site as NF-4C and exhibits sequence specificity in binding similar to that of NF-4C. As described above, this mutation caused a significant decrease in CD4 promoter function; these data are thus consistent with the hypothesis that MAZ binding to this region of the CD4 promoter is required for CD4 promoter function.

In order to determine the molecular weight of NF-4C, Southwestern analysis of a nuclear extract from the $CD4^+$ $CD8^-$ T-cell line Jurkat was done with the site 2 probe. As shown in Fig. 6 (left lane), we detected two proteins with the site 2 probe; no signal was seen in a parallel blot probed with the mutated MAZ site-CD4 promoter probe (right lane) or

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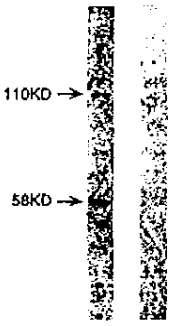

FIG. 6. Southwestern analysis of site 2. Twenty micrograms of Jurkat nuclear extract was resolved by SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane and hybridized with labelled site 2 sequence (left lane) or a labelled control probe consisting of either the mutated MAZ CD4 promoter site (right lane) or the *Xba*I-*Xho*I fragment of the pKS multiple cloning site (data not shown). Molecular masses were determined with both prestained and unstained markers. No signal was seen with either the mutated MAZ CD4 promoter site (right panel) or the plasmid linker control probe (data not shown). The two proteins detected are indicated (arrows).

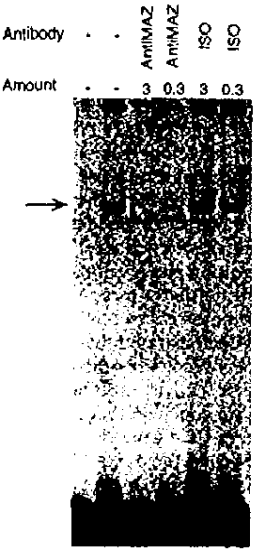

FIG. 7. Anti-MAZ monoclonal antibody inhibits factor binding to site 2. Gel shift reaction mixtures with site 2 and D10 nuclear extract probes were preincubated with a 3- or 0.3-μl volume (amount) of the anti-MAZ monoclonal antibody 80 culture supernatant (AntiMAZ) or isotype-matched control antibody (ISO) for 30 min prior to the addition of the labelled site 2 probe. The leftmost lane contained a free probe, and the next lane contained the EMSA binding reaction mixture with the site 2 probe and D10 nuclear extract without the addition of antibodies. The arrow indicates the position of the specific complex.

with the *Xba*I-*Xho*I fragment of the pKS multiple cloning site (data not shown). The smaller complex is of approximately 58 kDa, which corresponds closely to the estimated molecular mass of MAZ (60 kDa). Thus, these data are consistent with the theory that MAZ binds to site 2. The larger labelled protein had a molecular mass similar to that of Sp1, a 106-kDa transcription factor which recognizes the core sequence GGGCGG (17). Although site 2 does not contain a high-affinity recognition site for Sp1, Sp1 is capable of binding at low affinities to a consensus sequence similar to site 2 (17). However, the alteration of the central cytosine in the Sp1 recognition sequence has been shown to decrease Sp1 binding significantly (37); in our investigations, it has been difficult to show consistent Sp1 binding to site 2 (Fig. 3 and 5 and data not shown). Nonetheless, it is possible that Sp1 binds to functional site 2 as well and contributes to CD4 promoter function.

To determine if MAZ in the T-cell nuclear extracts binds to the CD4 promoter, we conducted antibody ablation-supershift experiments using D10 nuclear extracts and a monoclonal antibody directed against the MAZ transcription factor (37). This antibody, referred to as antibody 80, does not recognize the zinc finger domain of MAZ and does not cross-react with Sp1 (26a). As can be seen from Fig. 7, the specific complex observed in the EMSA analyses of site 2 was completely ablated by the anti-MAZ monoclonal antibody but not by the control antibody. The fainter complex migrating below the NF-4C complex was unaffected by the antibody treatment; together with the data showing that this complex forms on the functionally inactive mutated site 2, this result suggests that this complex is nonspecific and irrelevant to promoter function. We have used the ME1a1 site from the *c-myc* promoter in the same assay; this site is a known MAZ site and has been used extensively in the characterization of this transcription factor. In this experiment, we also detected the ablation of the specific MAZ complex (data not shown). In both assays, the MAZ complexes were completely ablated, indicating that the complexes formed consist entirely of MAZ binding to the probe (Fig. 7 and data not shown).

DISCUSSION

At least four distinct transcription factors are required for the activity of the CD4 minimal promoter. Our mutation analyses show that four sites within the CD4 minimal promoter, each serving as a recognition site for a nuclear factor, are required for full activity. We have previously shown that the Myb recognition sequences at site 1 are essential for CD4 promoter activity (36); in addition, we have determined that c-Myb can bind to this site (data not shown). Recently, Badiani et al. generated transgenic mice by using constructs that contained dominant-negative forms of the Myb transcription factor (3). These transgenic mice show significantly decreased populations of $CD4^+$ $CD8^-$ T cells both in the thymus and in peripheral lymph organs; $CD4^-$ $CD8^+$ T cells were less affected. These data are consistent with our data showing that the two Myb binding sites in the CD4 promoter are required for full promoter function in mature $CD4^+$ $CD8^-$ T cells. There is an Ets family consensus recognition sequence at functional site 4. Our data indicate that this sequence is in fact critical for CD4 promoter function and thus are supportive of the hypothesis that an Ets transcription factor is important for full promoter function (30). Using UV cross-linking, we have determined that multiple factors can bind this region, all with molecular weights similar to those of Ets transcription factors. However, we have been unable to demonstrate serologically that either Ets-1 or Ets-2 binds to the CD4 promoter in this region (data not shown). It is therefore possible that either another member of the Ets family or a novel transcription factor with the same recognition site binds site 4 and contributes to CD4 promoter function. We are currently conducting additional experiments to address these issues.

The CD4 promoter does not contain a functional TATA recognition sequence; in TATA-less promoters, the TFIID complex is still recruited to the template and plays an important role in transcription initiation (27). This role is believed

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to be accomplished by protein-protein interactions between TFIID and other promoter-bound transcription factors instead of by direct protein-DNA interactions with the TATA box. The TFIID complex is still believed to bind to the promoter, in approximately the same position relative to the promoter as in TATA-containing promoters (27). Although functional site 3 is 5′ to this region, it may nonetheless represent the interaction site of the TFIID complex and the CD4 promoter. Alternatively, this region could represent the binding sites of transcription factors that have yet to be identified. We are currently conducting experiments to address these questions.

The MAZ recognition site is critical for CD4 promoter function. We demonstrate that a recognition site for MAZ in the minimal CD4 promoter is essential for promoter activity. In addition, we have demonstrated that MAZ in $CD4^+$ $CD8^-$ T_H cell nuclear extracts recognizes this site and a mutation that abrogates promoter activity also abrogates MAZ binding. From these data, we conclude that it is likely that MAZ plays an important role in CD4 gene transcription. The MAZ transcription factor binds to the c-*myc* P2 promoter at the ME1a1 site, which is essential for the control of transcriptional initiation and elongation (2, 22, 47). Our data indicate that CD4 expression is also likely controlled by this transcription factor, and thus MAZ can also participate in the control of transcription of genes that are differentially expressed in lymphoid development.

Our EMSA analysis under the conditions described clearly demonstrates that one predominant complex is formed by using the site 2 probe and crude nuclear extract from $CD4^+$ $CD8^-$ T_H D10 cells. However, other transcription factors have been shown to bind to MAZ recognition sequences. As mentioned above, Sp1 can also bind to the ME1a1 site under certain EMSA conditions. Recently, Dufort and Nepveu (9) demonstrated that a human homolog of the *Drosophila* Cut homeodomain protein also binds to this site and represses c-*myc* promoter function. Our data indicate that the MAZ transcription factor is clearly the major factor binding to the CD4 promoter at site 2. It is possible, however, that these other ME1a1-site-binding transcription factors bind to the CD4 promoter region under different contexts. We and others have previously demonstrated that the CD4 promoter is functional at high levels in activated mature $CD4^+$ $CD8^-$ T cells (30, 36). The biochemical nature of this specificity is unknown; however, it is also possible that MAZ and these other transcription factors play a role. For example, it is possible that the mammalian homolog of the Cut homeodomain protein also binds to site 2 and that its expression in $CD4^-$ $CD8^+$ T_C cells provides a mechanism for this subclass specificity. We are currently conducting experiments to address these issues.

Potential roles of MAZ in CD4 promoter function. We have demonstrated that the MAZ transcription factor binds specifically to the CD4 promoter at a site that is critical for function. We therefore think it likely that MAZ plays an important role in CD4 gene transcription. Other groups have reported that MAZ can transactivate the insulin promoter in both insulinoma and HeLa cells (18). However, we have been unable to demonstrate similar transactivation in T cells by using the CD4 promoter. There are several possible explanations for these results. First, it is possible that a factor other than MAZ is binding to the CD4 promoter and is required for promoter activity. Although this is a formal possibility, it should be noted that we have been unable to detect other factors binding to the CD4 promoter at this site under a variety of conditions in EMSAs and we therefore consider this unlikely. Second, by Northern (RNA) analysis MAZ is expressed at high levels in T cells (data not shown). It is thus possible either that MAZ is not limiting in CD4 promoter function or that the high levels of MAZ make it difficult to observe transactivation in these transient transfection experiments. A third possibility, however, is that MAZ is not acting directly as a transcriptional activator but rather induces a bend in the DNA helix, facilitating interactions between other promoter-binding factors. Recently, it has been reported that MAZ is capable of inducing a 72°C bend in the DNA helix upon binding to its recognition site, which is consistent with this last theory (1). Several transcription factors that induce bending of DNA to affect promoter/enhancer function have been identified, including YY1 (23) and HMG I/(Y) (40). A well-studied example of this type of transcription factor is LEF-1 (41, 45, 46), which binds to the T-cell antigen receptor α-chain enhancer and generates a 130° bend in the DNA helix. Multimerized LEF-1 binding sites are not themselves sufficient to stimulate the basal activity of a heterologous *fos* or *tk* promoter, indicating that LEF-1 is dependent upon other transcriptional regulators to generate a biological effect. Finally, changes in the spacing distance between factor binding sites in the α-chain enhancer decrease enhancer function significantly, indicating that the spacing distance and the corresponding helical position of the LEF-1 site with respect to the other factor binding sites are critical for enhancer function. It is interesting that although there are multiple MAZ consensus recognition sites adjacent to each other in the CD4 promoter, only one of the sites appears to be required for CD4 promoter function. In addition to the core MAZ site described above at positions −71 to −65, there is a consensus MAZ site from −77 to −69 and one at −67 to −61. Despite the sequence similarity, neither of these last two sites appears to function as an MAZ recognition site in that the mutation of either site does not appear to affect promoter function. Thus, either the −71 to −65 MAZ recognition site has sequence-specific requirements that are not evident in other MAZ sites or, as in the LEF-1 site in the α-chain enhancer, the position of this site relative to the other factor binding sites is critical for function. This last hypothesis is consistent with the theory that MAZ is playing a structural role in CD4 promoter function. Further experiments will more directly address this issue.

A potential initiator recognition sequence at the CD4 cap site. Sequence analysis indicates that there are potential initiator and LEF-1 consensus recognition sites at the CD4 initiation point of transcription. The results of our mutagenesis analyses are consistent with the hypothesis that an initiator factor binds to the CD4 cap site and is responsible in part for CD4 transcript initiation. Using the TdT promoter as a model system, Javahery et al. determined that a consensus recognition sequence of YYANTYY around the initiation point of transcription was critical for Inr function (15). In the CD4 promoter, the best consensus Inr recognition sequence has the central adenine base at position +7 instead of the customary +1; however, previous analyses have indicated that Inr is functional even though the recognition is shifted slightly from the actual start site. However, the disruption of this site does not lead to decreased promoter function, whereas the mutation of an upstream putative Inr site surrounding the +1 cap site (TCTTTTC) results in a significant decrease in the level of promoter activity. These data indicate that only the upstream site functions as an in vivo Inr recognition site. However, it is necessary to conduct a more thorough analysis of the start sites of each individual mutant before this function is conclusively demonstrated. We are also currently conducting experiments to characterize the factors that recognize these putative Inr sites and contribute to CD4 promoter function.

Role of the promoter in control of CD4 gene expression. In addition to the promoter, at least two enhancers and a tran-

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scriptional silencer participate in the control of CD4 gene expression (36). From transfection studies and transgenic data, we have developed a hierarchy model of the functional relation among these three regulatory components of the CD4 locus. As mentioned above, promoter activity is restricted to activated $CD4^+$ T cells and is sufficient to generate high levels of activity (3, 14, 36). The enhancers extend expression to other T-cell compartments (mature $CD4^-$ $CD8^+$ T cells, immature $CD4^+$ $CD8^+$ T cells, and immature $CD4^-$ $CD8^-$ T cells) and potentially to other hematopoietic cells (4, 31, 35, 49). Thus, the net effect of the two enhancers is to overrule the specificity of the promoter and extend expression to other hematopoietic cells. Finally, the silencer suppresses enhancer activity and inhibits expression of CD4 in inappropriate cell types. This model proposes that the silencer is active in $CD4^-$ T cells and non-T cells, but inactive in $CD4^+$ $CD8^+$ T cells, whose expression is a function of enhancer-promoter interactions. Our analysis of the CD4 promoter and the experiments using c-Myb dominant-negative transgenic mice (3) permit us to elaborate on the hierarchy model. Because the promoter is the critical controlling element for CD4 expression in mature $CD4^+$ $CD8^-$ T cells, the inhibition of function of a promoter-binding factor such as c-Myb or MAZ will disable CD4 expression in these cells despite the presence of the enhancers. In mice transgenic for the dominant-negative c-Myb construct, the $CD4^+$ $CD8^-$ T-cell populations were affected far more severely than the other T-cell populations. In addition, T-cell activation was profoundly affected. Thus, although the dominant-negative form of Myb inhibits CD4 expression and subsequent T-cell development in all classes of T cells, the effect is most pronounced in the $CD4^+$ $CD8^-$ mature T-cell populations, which is consistent with the hierarchy model. Despite the ability of the enhancers and the silencer to alter the specificity of the promoter, these data clearly demonstrate that the promoter is still a critical element in the control of CD4 gene expression. We are currently conducting additional studies to determine the contributions of the promoter, enhancers, and silencer to the control of CD4 during T-cell development.

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