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Regulation of the T-Cell Receptor δ Enhancer by Functional Cooperation between c-Myb and Core-Binding Factors

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A T-cell-specific transcriptional enhancer lies within the $J_\delta 3$-C_δ intron of the human T-cell receptor (TCR) δ gene. The 30-bp minimal enhancer element denoted δE3 carries a core sequence (TGTGGTTT) that binds a T-cell-specific factor, and that is necessary but not sufficient for transcriptional activation. Here we demonstrate that the transcription factor c-Myb regulates TCR δ enhancer activity through a binding site in δE3 that is adjacent to the core site. Both v-Myb and c-Myb bind specifically to δE3. The Myb site is necessary for enhancer activity, because a mutation that eliminates Myb binding abolishes transcriptional activation by the δE3 element and by the 370-bp TCR δ enhancer. Transfection of cells with a c-Myb expression construct upregulates δE3 enhancer activity, whereas treatment of cells with an antisense c-*myb* oligonucleotide inhibits δE3 enhancer activity. Since intact Myb and core sites are both required for δE3 function, our data argue that c-Myb and core binding factors must cooperate to mediate transcriptional activation through δE3. Efficient cooperation depends on the relative positioning of the Myb and core sites, since only one of two overlapping Myb sites within δE3 is functional and alterations of the distance between this site and the core site disrupt enhancer activity. Cooperative regulation by c-Myb and core-binding factors is likely to play an important role in the control of gene expression during T-cell development.

The genes encoding the polypeptide chains of T-cell receptor (TCR) αβ and TCR γδ consist of discrete variable (V), diversity (D), joining (J), and constant (C) gene segments that rearrange during T-cell ontogeny to generate functional receptors on αβ and γδ T lymphocytes (14). The rearrangement and expression of these genes are controlled in a time- and lineage-dependent fashion during T-cell development (21, 59, 72). For example, the TCR δ, γ, and β genes are expressed prior to the TCR α gene, and TCR γδ lymphocytes arise prior to TCR αβ lymphocytes during thymic ontogeny. Further, TCR γδ lymphocytes that use distinct V_γ and V_δ gene segments arise in a defined temporal pattern during development. A detailed analysis of the elements that control transcription of TCR genes may shed light on the molecular basis for the developmental regulation of their expression.

Previous studies have defined transcriptional enhancers required for the expression of each of the TCR genes and have also identified some of the *trans*-acting factors that interact with regulatory sites within the enhancers (38). We identified a T-cell-specific transcriptional enhancer within the $J_\delta 3$-C_δ intron of the human TCR δ gene (60) and showed that a 30-bp element within this enhancer, denoted δE3, functions as a minimal enhancer (62). Further, we demonstrated that the binding of a T-cell-specific factor (NF-δE3A) to a core sequence (TGTGGTTT) in the 5′ end of δE3 is necessary but not sufficient for transcriptional activation by δE3 or by the 370-bp enhancer (61, 62). This core-binding factor (now known as CBF or PEBP2) (54, 55, 79) is apparently identical to previously characterized factors that interact with similar motifs in the enhancers of murine leukemia viruses (7, 32, 61, 75, 78) and is probably an important regulator of gene expression in T lymphocytes.

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Myb proteins have been shown to play important roles in the differentiation and proliferation of hematopoietic cells (42, 66). The Myb family includes the products of the cellular proto-oncogene c-*myb* and related cellular genes A-*myb* and B-*myb* (34, 35, 52, 64). Further, this family includes the v-*myb* oncogenes, which are truncated versions of c-*myb* transduced by avian myoblastosis virus and avian acute leukemia virus E26 (34, 35). The E26 virus, which carries a v-*myb* gene that is fused to the oncogene v-*ets*, transforms erythroid precursor cells and induces erythroleukemia in chickens (27, 53, 58).

Constitutive high-level expression of the c-*myb* gene is detected in immature hematopoietic cells, and this expression declines as a function of cell differentiation (17, 24, 28, 29, 39, 65, 82). Forced high-level expression of v-Myb or c-Myb protein has been shown to inhibit erythroid differentiation (4, 11, 44, 45, 76) and to have a dedifferentiating effect on myeloid cells (4, 5, 18). On the other hand, inhibition of c-Myb function by expression of a truncated, dominant-negative c-Myb protein has been shown to enhance erythroleukemia cell differentiation (81). Most striking, mice carrying a homozygous mutation in the c-*myb* gene die in utero and exhibit severe anemia and other defects in hematopoiesis that are detectable by days 14 to 15 of development (48).

Although c-Myb expression is downregulated in mature cells, transient c-Myb expression occurs in lymphocytes and other cells in response to mitogenic stimulation (40, 63, 67, 71, 74, 77). Reduced c-Myb expression appears to inhibit the growth of hematopoietic cells in vitro (2, 10, 24), and there is evidence that c-Myb plays a role in the traversal from the G_1 to the S phase of the cell cycle (23, 40, 56, 71). Thus, Myb appears to play an important role in cell proliferation that may be distinct from its role in development.

Recent studies have shown that Myb proteins bind specifically to the nucleotide sequence PyAACG/TG (6) and can transactivate gene expression through this site (19, 33, 36, 50, 51, 69, 83). However, only a limited number of cellular genes that are known targets of Myb have been identified.

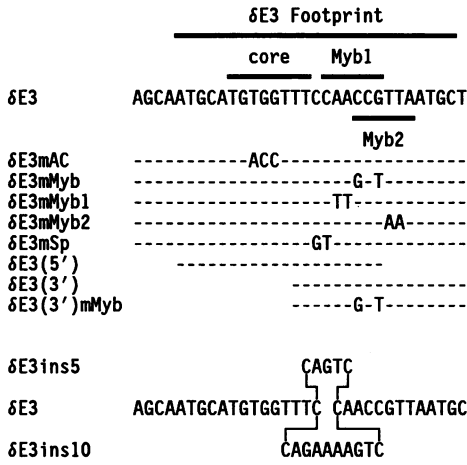

FIG. 1. Wild-type and mutant δE3 sites tested. The actual oligonucleotides used in this study include flanking *Bam*HI sites appended to facilitate cloning and radiolabeling. The extent of the δE3-binding site, as defined by DNase I footprinting (60), and the locations of the core and Myb sites are shown.

These include two genes specifically expressed in hematopoietic cells, *mim-1* (50) and CD4 (69).

In this work, we analyzed the role of potential Myb-binding sites within the 3′ end of the δE3 element of the human TCR δ enhancer. We show that c-Myb regulates TCR δ enhancer activity by binding specifically to δE3 and suggest that coordinate expression of c-Myb and CBFs may play an important role in T-cell development.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The δE3 and related binding sites used in this study are shown in Fig. 1. Complementary oligonucleotides representing each binding site included appended *Bam*HI-compatible overhangs (not shown). Complementary oligonucleotides containing one Myb-binding site (1×Myb) were 5′-GATCCTAGGAATAACGGAAT-3′ and 5′-GATCATTCCGTTATTCCTAG-3′ as described previously (8). Complementary oligonucleotides containing two Myb-binding sites (2×Myb) were 5′-TAGAATAACGGAAG CAATAACGGAA-3′ and 5′-TAGTTCCGTTATTGCTTCC GTTATT-3′, as described previously (41). The analogous oligonucleotides containing two mutant Myb-binding sites [m(2×Myb)] were 5′-TAGAATCCAGGAAGCAATCCAG GAA-3′ and 5′-TAGTTCCTGGATTGCTTCCTGGATT-3′. The δE6 binding site was formed by using the complementary oligonucleotides 5′-GATCTCTCAAGCAGGTTGAAA GCAGGTTCCAAGA-3′ and 5′-GATCTCTTGGAACCTGC TTTCAACCTGCTTGAGA-3′.

Plasmid V_δ1-CAT and versions of this plasmid carrying the monomeric 35-bp δE3 site, the monomeric 35-bp $\delta E3^{mAC}$ site, the intact 370-bp TCR δ enhancer, or the mutated 370-bp fragment 370^{mAC} have been described previously (61, 62). Oligonucleotides $\delta E3^{mMyb}$, $\delta E3^{mMyb1}$, $\delta E3^{mMyb2}$, δE3ins5, δE3ins10, and $\delta E3^{mSp}$ were treated with T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.), annealed, and ligated into *Bam*HI-digested and phosphatase-treated V_δ1-CAT plasmid. The polymerase chain reaction (PCR) was used to generate the Myb-binding-site mutation in the 370-bp fragment of the TCR δ enhancer (370^{mMyb}). The oligonucleotides 5′-GGGAAGCTTGGAAACCACATGCAT TGC-3′ (MybPCRup) and 5′-GGGAAGCTTTAATGCTAG AGTTATCACT-3′ (MybPCRdn) include a 2-bp change in the δE3 Myb site that also generates a *Hin*dIII site. Linearized pBluescript KS+ containing the 370-bp fragment cloned in the *Sma*I site was subjected to PCR, with either MybPCRup oligonucleotide and the −40 primer or MybPCRdn and the reverse primer, to obtain fragments representing the 5′ and 3′ portions of the 370-bp enhancer. The 5′ fragment was digested with *Bam*HI and *Hin*dIII, the 3′ fragment was digested with *Eco*RI and *Hin*dIII, and the two were ligated together into *Bam*HI-*Eco*RI-digested pBluescript KS+ to re-create the 370-bp enhancer, including the mutation. The insert was subjected to dideoxynucleotide sequence analysis to confirm its structure and was then excised with *Not*I and *Eco*RV and cloned into V_δ1-CAT plasmid previously treated with *Bam*HI, the Klenow fragment of DNA polymerase I (New England Biolabs), and *Not*I. The plasmid c-fos-CAT is a derivative of J21 (84). 3×δE3-c-fos-CAT, which carries a δE3 trimer excised from the plasmid 3×δE3-V_δ1-CAT (62) and subcloned into the *Eco*RV site of c-fos-CAT, was generated by J. M. Redondo. V_δ1-CAT-Eα, which carries a 0.7-kb *Bst*XI fragment of the human TCR α enhancer (30), was generated by P. Lauzurica. This fragment was excised from pUC13 as a *Bam*HI fragment, treated with Klenow fragment to generate blunt ends, and subcloned into *Kpn*I-digested and blunt-ended V_δ1-CAT. To generate the plasmid pCMV4-c-Myb, a 2.7-kb fragment encoding c-Myb was excised from the plasmid pRmb3SVneo (19) by digestion with *Spe*I, treatment with Klenow, and redigestion with *Hin*dIII and was then ligated into *Hin*dIII-*Sma*I-digested pCMV4 (1).

DNA-binding assays. Preparation of nuclear extracts and radiolabeling of binding-site probes with the Klenow fragment of DNA polymerase I and [α-^{32}P]dGTP (Dupont-New England Nuclear, Boston, Mass.) were done as described previously (62). Electrophoretic mobility shift assays were performed as described previously (68), with some modifications. A 2.2-μg sample of nuclear protein or 1 μl of 1/10-diluted bacterial extracts (kindly provided by John Cogswell, University of North Carolina at Chapel Hill) (22) was incubated with 1 μg of bovine serum albumin and 0.5 μg of poly(dI-dC) DNA carrier in 25 μl of reaction mix containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 2% glycerol for 15 min on ice in the presence or absence of competitors or monoclonal antibodies (MAbs). A murine MAb that recognizes the C-terminal region of murine c-Myb (UBI, Lake Placid, N.Y.) and a control immunoglobulin G1 MAb were used. Labeled binding-site probes (6 fmol) were then added for an additional 30 min of incubation at 4°C, and samples were electrophoresed at 416 V through a 4% polyacrylamide gel containing 22.5 mM Tris-borate and 0.5 mM EDTA at 4°C. The experiment analyzing v-Myb and CBF binding to the δE3, δE3ins5, and δE3ins10 probes (see Fig. 9B) was performed as described previously (61, 62), except that 0.6 fmol of binding-site probe was used and all incubations and electrophoresis experiments were carried out at 4°C. Purified CBF was kindly provided by Nancy Speck, Dartmouth University. Gels were incubated in fixative (10% acetic acid, 30% methanol) for 15 min, dried under vacuum, and exposed to X-ray film (KODAK XAR) at −70°C with an intensifying screen.

Immunoprecipitation assays. Cells (5×10^6) were washed in phosphate-buffered saline (PBS) and incubated for 1 h at 37°C in 2.5 ml of cysteine- and methionine-free minimal essential medium containing 10% dialyzed fetal bovine se-

rum. A total of 125 μCi [^{35}S]cysteine and [^{35}S]methionine (Expre^{35}S^{35}S; Dupont-New England Nuclear) was added for an additional 3.5 h of incubation at 37°C. Cells were washed in PBS and lysed in 2 ml of cold 150 mM NaCl–50 mM Tris-HCl (pH 8.0)–10 mM iodoacetamide–0.1 mM phenylmethylsulfonyl fluoride–1% Nonidet P-40, 0.5% sodium deoxycholate–0.1% sodium dodecyl sulfate (SDS) for 30 min on ice. Lysates were centrifugated for 15 min at 13,000 × *g* at 4°C to remove the insoluble material. Aliquots of lysates normalized for radioactivity were precleared by incubation with 5 μl of normal rabbit serum and Pansorbin (Calbiochem, La Jolla, Calif.) for 2 h at 4°C. Precleared lysates were then incubated with 0.5 μg of anti-c-Myb MAb or control immunoglobulin G1 MAb followed by 150 μl of culture supernatant of rat anti-murine kappa light-chain hybridoma 187.1 (ATCC HB 58) and 100 μl of protein A–Sepharose CL-4B (10% suspension) (Pharmacia, Piscataway, N.J.), with each incubation continuing for 1 h at 4°C. The immunoadsorbent was washed four times in NET (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.02% azide) supplemented to 0.65 M NaCl and then twice with NET. Immunoprecipitated proteins were eluted by boiling in reducing SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and then subjected to electrophoresis through a 10% Laemmli slab gel (37). Following fluorography, the gel was dried and exposed to X-ray film.

Transfections and CAT assays. The human T leukemia cell line Jurkat was grown in RPMI 1640 medium (GIBCO-BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (FBS; Intergen, Purchase, N.Y.) and penicillin-streptomycin (GIBCO-BRL). For chloramphenicol acetyltransferase (CAT) assays, cells were transfected with CsCl-purified plasmid DNA by using Lipofectin (GIBCO-BRL) as described previously (60) or by the DEAE-dextran method (15, 43). Cell extracts were normalized on the basis of protein concentration by the Bradford method (Bio-Rad, Richmond, Calif.). Acetylation of [^{14}C]chloramphenicol (Dupont-New England Nuclear) was assayed as described previously (60) and quantified by using a Betascope (Betagen, Waltham, Mass.).

Treatment of transfected cells with sense and antisense c-*myb* oligonucleotides. The sense and antisense c-*myb* oligomers (codons 2 to 7) were described previously (2, 10, 24). Phosphorothioate-modified oligodeoxynucleotides were *n*-butanol extracted, ethanol precipitated, and dissolved in RPMI 1640. Jurkat cells were preincubated in culture medium at 5 × 10^6 cells per ml for 3 to 4 h at 37°C in presence or absence of 50 μM oligonucleotide. Cells were washed three times and were divided into aliquots of 10^6 cells for transfection with 2 μg of plasmid in the presence of 50 μg of DEAE-dextran. After transfection, cells were resuspended in 0.5 ml of culture medium and incubated at 37°C for 1 h in a 24-well tissue culture plate. Oligonucleotides were then added to 50 μM for the 48-h culture period. Protein assays to normalize cell extracts were performed in microtiter plates by using an automated plate reader.

RESULTS

v-Myb binds specifically to the δE3 element. In previous experiments, an intact core sequence (TGTGGTTT) within the 5′ portion of δE3 was shown to be necessary but not sufficient for transcriptional activation by the δE3 minimal enhancer fragment (61). This suggested that one or more additional proteins might functionally interact with sequences within δE3. We noted an imperfect match to the consensus Myb site on the top strand of δE3 (CAACCG, denoted Myb1) and an overlapping perfect match to the consensus Myb site on the bottom strand of δE3 (TAACGG, denoted Myb2), located 3′ to the core site (Fig. 1). We wondered whether protein binding to these sites might be important for enhancer function. Because of the previously observed complexity of protein binding to a 35-bp δE3 site probe, we generated a double-stranded 18-bp oligonucleotide encompassing the 3′ end of δE3 to be used in electrophoretic mobility shift assays (EMSA).

We first investigated whether the v-Myb protein could specifically bind to δE3 by using a bacterial extract containing v-Myb. We compared the δE3-binding-site probe [δE3(3′)] with a control 16-bp oligonucleotide containing a characterized Myb-binding site (1×Myb) in EMSA (Fig. 2A). No complexes were detected when the radiolabeled binding-site probes were incubated with control bacterial extracts (−). However, complexes of identical mobility were formed when the 1×Myb and δE3(3′) oligonucleotide probes were incubated with bacterial extracts containing v-Myb (V). In each case one abundant complex and an array of minor complexes with higher electrophoretic mobility were observed. The minor complexes probably reflect the presence of v-Myb degradation products within the extracts. All of the complexes are specific, since competition was observed when using an excess of unlabeled oligonucleotide containing two consensus Myb binding sites (2×Myb) but was not observed when using equivalent amounts of the same oligonucleotide with mutated Myb-binding sites [m(2×Myb)]. We generated a mutant version of the δE3(3′) oligonucleotide with both potential Myb-binding sites eliminated [δE3(3′)^{mMyb} (Fig. 1)]. Whereas the wild-type δE3(3′) site served as a competitor in the binding reaction, the mutant site, δE3(3′)^{mMyb}, did not (Fig. 2A). In addition, no complexes were detected when a radiolabelled δE3(3′)^{mMyb}-binding site was used as a probe.

We then generated two additional δE3 mutants (δE3^{mMyb1} and δE3^{mMyb2} [Fig. 1]) predicted to selectively eliminate either the Myb1 site or the Myb2 site. Each of the mutant binding-site probes retained the capacity to bind v-Myb, indicating that the Myb1 and Myb2 sites within δE3 can both serve as v-Myb-binding sites (Fig. 2B). We assessed the relative affinities of v-Myb for the δE3^{mMyb1} and δE3^{mMyb2} sites in cross-competition experiments. The two binding sites displayed similar potencies as competitors in binding reactions, arguing that v-Myb binds with similar affinity to the two sites (Fig. 2B). Our results argue against simultaneous occupancy of the two sites on a wild-type δE3 element, because the complex formed between v-Myb and the 1×Myb probe (with one Myb site) and the complex formed between v-Myb and the δE3(3′) probe (with two Myb sites) displayed identical electrophoretic mobilities (Fig. 2A). We conclude that v-Myb can specifically bind to either of two overlapping Myb sites within δE3.

c-Myb binds specifically to the δE3 element. We next asked whether proteins in Jurkat T-cell nuclear extract bound with similar specificity to δE3 (Fig. 3). When the δE3(3′) probe was incubated with Jurkat nuclear extract, a series of protein-DNA complexes were detected. Three of these complexes were judged to be specific, on the basis of the ability of unlabeled δE3(3′) oligonucleotide but not the unrelated unlabeled δE6 oligonucleotide to compete for complex formation. Of these specific complexes, one (marked c-Myb) displayed a lower electrophoretic mobility than the complex formed by the same probe with bacterial v-Myb. This would be expected of a complex containing

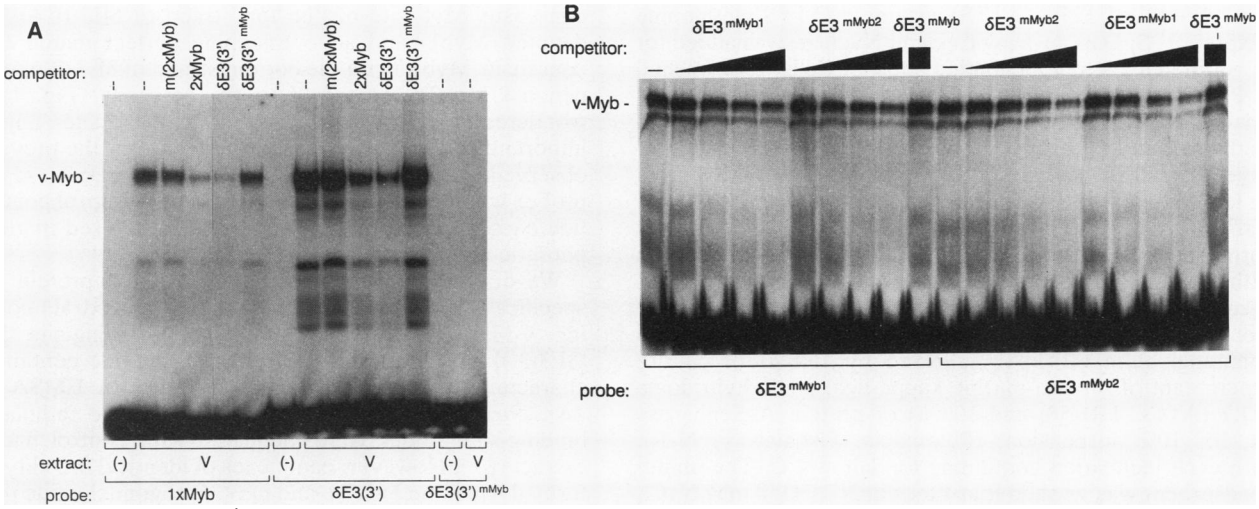

FIG. 2. Binding of v-Myb to δE3. (A) Radiolabeled δE3(3′), δE3(3′)^{mMyb}, and 1×Myb oligonucleotides were incubated with bacterial extract containing v-Myb (V) or control bacterial extract (−) in the absence or presence of unlabeled competitors. 2×Myb and m(2×Myb) were used at 500-fold molar excess, and δE3(3′) and δE3(3′)^{mMyb} were used at 1,000-fold molar excess. DNA-protein complexes were resolved by electrophoresis. The v-Myb-containing DNA-protein complex is marked. (B) Radiolabeled δE3^{mMyb1} and δE3^{mMyb2} oligonucleotides were analyzed for v-Myb binding as above. Unlabeled δE3^{mMyb1} and δE3^{mMyb2} competitors were used at 50-, 250-, 500-, and 1,000-fold molar excess. Unlabeled δE3^{mMyb} competitor was used at 1,000-fold molar excess.

c-Myb, since the v-Myb protein (45 kDa) is a truncated form of c-Myb (75 kDa). Importantly, the formation of this complex was efficiently inhibited by an excess of unlabeled δE3(3′) or 2×Myb oligonucleotides but not by the same oligonucleotides with mutant Myb-binding sites [δE3(3′)^{mMyb} and m(2×Myb), respectively] (Fig. 3). The two additional specific complexes detected by the δE3(3′) probe were of higher electrophoretic mobility but appeared to display the same binding specificity (arrowheads, Fig. 3). These complexes might result from proteolysis of components of the major specific complex or, alternatively, from the binding of distinct nuclear factors.

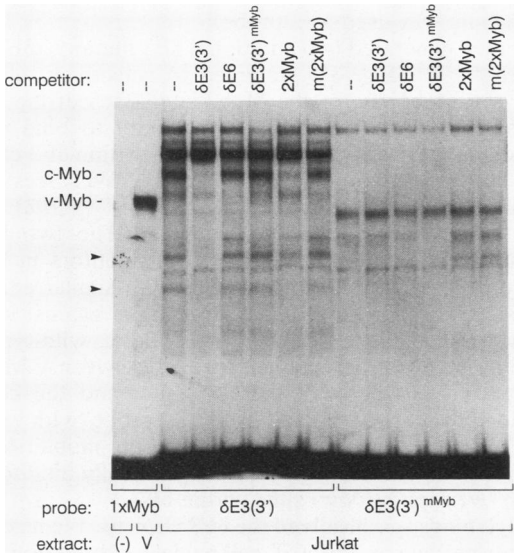

FIG. 3. Binding of Jurkat T-cell nuclear factors to the δE3(3′) site. Radiolabeled δE3(3′) and δE3(3′)^{mMyb} oligonucleotides were incubated with Jurkat nuclear extracts in the absence of competitor or in the presence of a 50-fold molar excess of the indicated competitors. For comparison, a radiolabeled 1×Myb probe was incubated with control bacterial extract (−) or bacterial extract containing v-Myb (V). DNA-protein complexes were resolved by electrophoresis. The v-Myb-containing DNA-protein complex and a putative c-Myb-containing DNA-protein complex are marked. Arrowheads denote additional specific complexes of higher electrophoretic mobility.

To confirm these data, we tested the ability of radiolabeled δE3(3′)^{mMyb} oligonucleotide to form specific complexes with proteins in Jurkat nuclear extract (Fig. 3). As expected, this probe did not detect the complexes defined in the above competition studies to reflect a specific interaction of nuclear proteins with the δE3 Myb site. However, this probe also failed to detect some complexes that were detected by the δE3(3′) probe but were judged to be nonspecific on the basis of competition experiments. These included a highly abundant complex of low electrophoretic mobility, as well as some minor complexes. We suspect that these complexes reflect the specific binding of highly abundant and perhaps low-affinity nuclear proteins that cannot be inhibited from the radiolabeled δE3 probe by standard amounts of unlabeled competitor. We conclude from these experiments that a number of proteins in Jurkat nuclear extract specifically interact with the δE3 Myb sites.

To prove that c-Myb is present in one or more of the specific complexes formed between the δE3(3′) probe and proteins in Jurkat T cell nuclear extracts, we used an anti-c-Myb MAb in EMSA supershift experiments (Fig. 4). Inclusion of this MAb in binding experiments resulted in a shift of the major specific complex (marked c-Myb) to one of lower electrophoretic mobility (arrow). This effect was not observed when using a control MAb of the same isotype. Further, we observed no effect of antibody on the migration of the δE3A and δE3C complexes formed with a radiolabeled δE3(5′) probe (Fig. 4). We conclude that a protein that is identical or very closely related to c-Myb binds specifically to δE3.

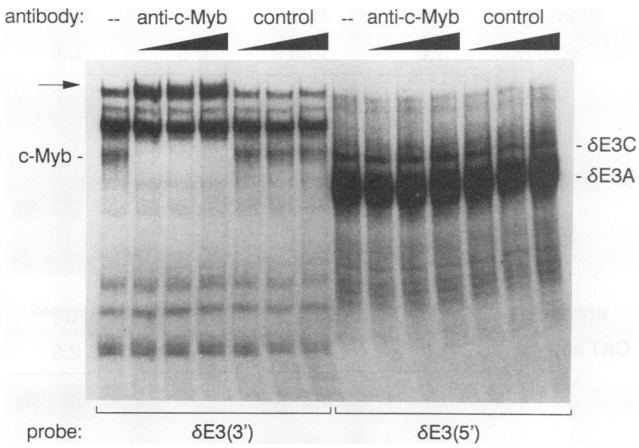

FIG. 4. c-Myb in Jurkat nuclear extract binds to the δE3(3′) site. Radiolabeled δE3(3′) and δE3(5′) were incubated with Jurkat nuclear extracts in the presence of increasing amounts of anti-c-Myb MAb or control MAb (0.1, 0.2, and 0.4 μg each). DNA-protein complexes were resolved by electrophoresis. The putative c-Myb complex with δE3(3′) and the δE3A and δE3C complexes with δE3(5′) are marked. The arrow denotes a new complex formed with the δE3(3′) probe in the presence of anti-c-Myb MAb.

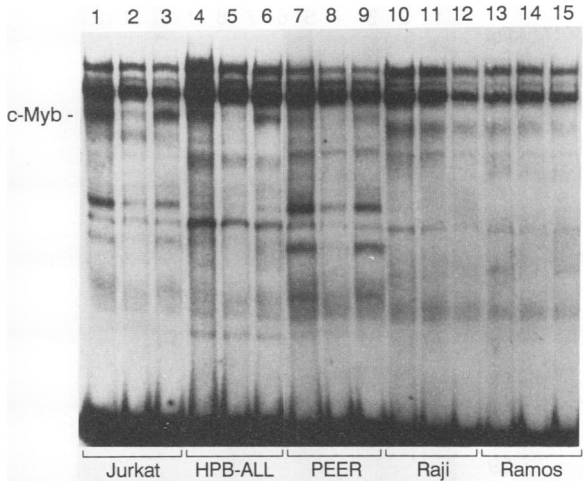

FIG. 5. Cell distribution of δE3(3′)-binding factors. Radiolabeled δE3(3′) oligonucleotide was incubated with nuclear extracts from a series of human T- and B-cell lines in the absence or presence of 50-fold molar excess of competitor. DNA-protein complexes were resolved by electrophoresis. Nuclear extracts of three T-cell lines (Jurkat, HPB-ALL, and PEER) and two B-cell lines (Raji and Ramos) were tested. Lanes: 1, 4, 7, 10, and 13, no competitor; 2, 5, 8, 11, and 14, competition with 2×Myb oligonucleotide; 3, 6, 9, 12, and 15, competition with m(2×Myb) oligonucleotide. The c-Myb-containing complex is marked.

We did not observe an effect of the anti-c-Myb MAb on the migration of any of the other complexes, including the specific complexes of relatively high electrophoretic mobility (Fig. 4). This could indicate that these complexes reflect the binding of distinct, c-Myb-related factors. However, this result would also be consistent with proteolysis of c-Myb, since the antibody detects an epitope in the C-terminal region that is distinct from the DNA-binding domain. Variable yield of these complexes in different experiments is perhaps consistent with the latter possibility, but the precise nature of these complexes is uncertain.

The EMSA supershift data suggest that c-Myb cannot bind to the δE3(5′) probe, even though the Myb1 site is included in this probe. This probably results from the position of the Myb1 site at the end of the probe and reflects a requirement for flanking sequences for efficient c-Myb binding. In support of this interpretation, we also failed to detect v-Myb binding to the δE3(5′) probe in direct-binding experiments, although a weak interaction could be detected when this binding site was used as an unlabeled competitor at high concentrations (data not shown).

Nuclear extracts from additional cell lines were examined for the ability to form the c-Myb-containing complex. On the basis of electrophoretic mobility and competition, three T-cell nuclear extracts tested (HPB-ALL, PEER, and MOLT-13) formed this complex, whereas two B-cell nuclear extracts (Raji and Ramos) did not (Fig. 5; data not shown). Metabolic labeling and immunoprecipitation with the anti-c-Myb MAb were then used to assay c-Myb protein expression in these cell lines. A protein of the expected size, 75 kDa, was specifically immunoprecipitated from lysates of the T-cell lines but not the B-cell lines (Fig. 6). Thus, formation of the specific complex containing c-Myb correlates with c-Myb expression in these cell lines. The detection of c-Myb expression in immature T leukemia cell lines is consistent with previous data (82).

The Myb binding sites within the δE3 element are essential for transcriptional activity of the TCR δ enhancer. To evaluate the functional importance of protein binding to the Myb sites within δE3, we cloned wild-type and mutant δE3 elements upstream of the $V_\delta 1$ promoter in the enhancer-dependent test construct $V_\delta 1$-CAT and transiently transfected the constructs into the Jurkat T-cell line to measure their activities (Fig. 7). Consistent with results of previous experiments, a single copy of the 35-bp δE3 element efficiently activated transcription from the $V_\delta 1$ promoter (10.1-fold induction), whereas a version of this element with a mutation in the core site, $\delta E3^{mAC}$, displayed no activity (0.8-fold induction). Strikingly, a single copy of the 35-bp δE3 element carrying the 2-bp mutation shown to destroy the Myb-binding sites also eliminated enhancer activity (0.5-fold induction).

We also examined the effect of the Myb-binding-site mutation in the context of the 370-bp TCR δ enhancer fragment shown to display maximal enhancer activity (60, 62) (Fig. 7). Consistent with results of previous studies, the 370-bp fragment displayed potent enhancer activity when cloned upstream of the $V_\delta 1$ promoter (43.7-fold induction), and this activity was almost completely eliminated by the core site mutation (370^{mAC}; 2.9-fold induction). We used PCR to introduce the mutation in the Myb-binding sites into the 370-bp fragment and found that this mutation completely abolished transcriptional activity (1.1-fold induction). We conclude that an intact binding site for Myb within the δE3 element is essential for the activity of the TCR δ enhancer in Jurkat cells.

c-Myb regulates the TCR δ enhancer through the δE3 Myb sites. Although the above experiments establish that c-Myb specifically binds to a site within δE3 that is essential for enhancer activity, they do not formally prove that c-Myb itself can regulate enhancer activity through this site. To address this issue, we first tested the ability of c-Myb to regulate δE3 enhancer activity in cotransfection experiments. We chose EL4 for these experiments because this cell line expresses low endogenous levels of c-Myb (13), high

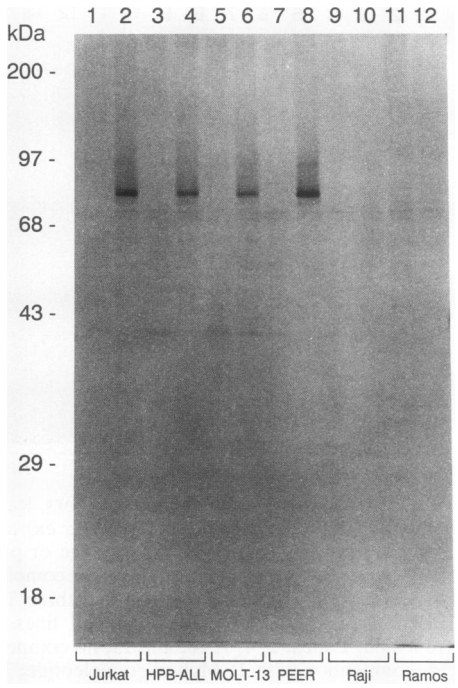

FIG. 6. Expression of c-Myb in human T- and B-cell lines. Cells from four T-cell lines (Jurkat, HPB-ALL, MOLT-13, and PEER) and two B-cell lines (Raji and Ramos) were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine, lysed, and subjected to immunoprecipitation with a control MAb (lanes 1, 3, 5, 7, 9, and 11), or an anti-c-Myb MAb (lanes 2, 4, 6, 8, 10, and 12). Immunoprecipitates were resolved by SDS-PAGE.

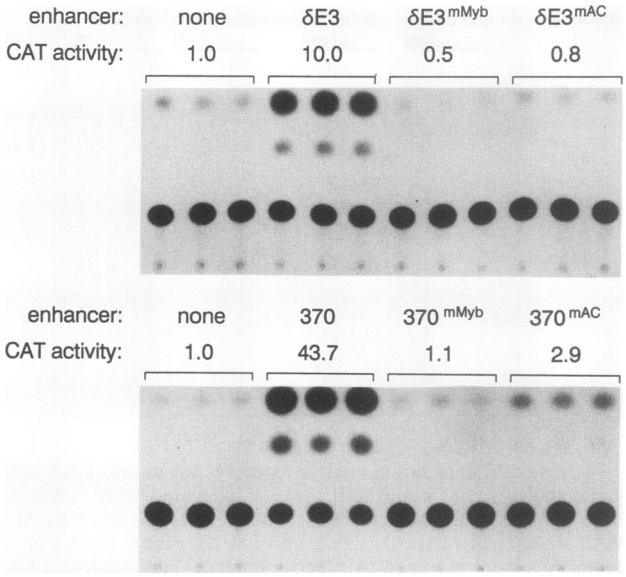

FIG. 7. Transcriptional activation by wild-type and mutant TCR δ enhancer fragments. The indicated sites were cloned as monomers upstream of V_δ1 promoter in the enhancer-dependent test construct V_δ1-CAT. Constructs were transfected in triplicate into the Jurkat T-cell line, and values for percent chloramphenicol acetylation were averaged and then normalized to the activity of the enhancerless V_δ1-CAT construct.

endogenous levels of the T-cell-specific CBF (54, 55), and minimal δE3 enhancer activity (data not shown). Three copies of the δE3 element were cloned into a plasmid carrying a minimal c-*fos* promoter driving CAT expression to generate the plasmid 3×δE3–c-fos-CAT. A c-Myb expression plasmid was then cotransfected with these plasmids at 0.5:1 or 1:1 ratios into EL4. We found that the 3×δE3-c-fos-CAT reporter was transactivated by 3.8- and 7.1-fold, respectively (Fig. 8A). In contrast, the c-fos-CAT reporter lacking δE3 was transactivated by 1.9- and 3.0-fold. Thus, δE3 imparts 2.0- to 2.5-fold greater inducibility to the construct, arguing that c-Myb can activate transcription by binding to δE3. The background inducibility of c-fos-CAT might result from an interaction of c-Myb with additional plasmid sequences.

We then examined the ability of antisense c-*myb* oligonucleotide to inhibit δE3 enhancer function in Jurkat cells. Jurkat cells were transiently transfected with the reporter constructs V_δ1-CAT, 3×δE3-V_δ1-CAT, and V_δ1-CAT-Eα (containing the TCR α enhancer) and were incubated in culture medium containing sense oligonucleotide, antisense oligonucleotide, or no oligonucleotide (Fig. 8B). Sense oligonucleotide at 50 μM did not inhibit the activities of any of the constructs tested. Antisense oligonucleotide at 50 μM had no effect on V_δ1-CAT or V_δ1-CAT-Eα but reproducibly inhibited the activity of 3×δE3-V_δ1-CAT by 50 to 60%. This corresponded to a partial (75%) inhibition of c-Myb expression after 48 h of antisense oligonucleotide treatment, as judged by Western blot analysis relative to vinculin and tubulin internal controls (data not shown). The lack of an effect on V_δ1-CAT-Eα argues that the observed inhibition of 3×δE3-V_δ1-CAT does not result from a general inhibition of cell proliferation or transcription but, rather, reflects a specific inhibition of δE3 function.

We observed no effects of 50 μM antisense oligonucleotide on the proliferation of transfected Jurkat cells, but we did observe significant inhibition at higher concentrations. This prevented us from using higher concentrations to demonstrate a more complete but nevertheless specific reduction of δE3 activity. Of note, other investigators have shown that far lower concentrations of the same antisense oligonucleotide have dramatic effects on T-cell proliferation and c-Myb expression (23). However, we have observed that whereas the proliferation of transfected Jurkat cells is relatively insensitive to antisense oligonucleotide treatment, the proliferation of untransfected Jurkat cells is very sensitive. We suspect that the cells become partially refractory to oligonucleotide uptake as a result of transfection, thus requiring the use of larger amounts of antisense oligonucleotide for transfected cells. Nevertheless, the specific inhibition observed in our experiments supports the idea that δE3 enhancer activity in Jurkat cells is dependent on the expression of c-Myb. On the basis of both the transactivation and antisense approaches, we conclude that c-Myb regulates TCR δ expression by binding to the Myb sites within δE3.

The organization of the core and Myb sites is critical for δE3 enhancer activity. Since δE3 contains two overlapping binding sites for Myb proteins, it was not clear whether enhancer function required occupancy of one or the other of the two sites. To address this, we cloned monomers of the δE3^{mMyb1} and δE3^{mMyb2} oligonucleotides upstream of the V_δ1 promoter in the V_δ1-CAT vector and assayed for enhancer activity following transient transfection into Jurkat cells. Elimination of the Myb1 site had a small effect on δE3 enhancer activity, whereas elimination of the Myb2 site resulted in a dramatic reduction in activity (Fig. 9A). Thus,

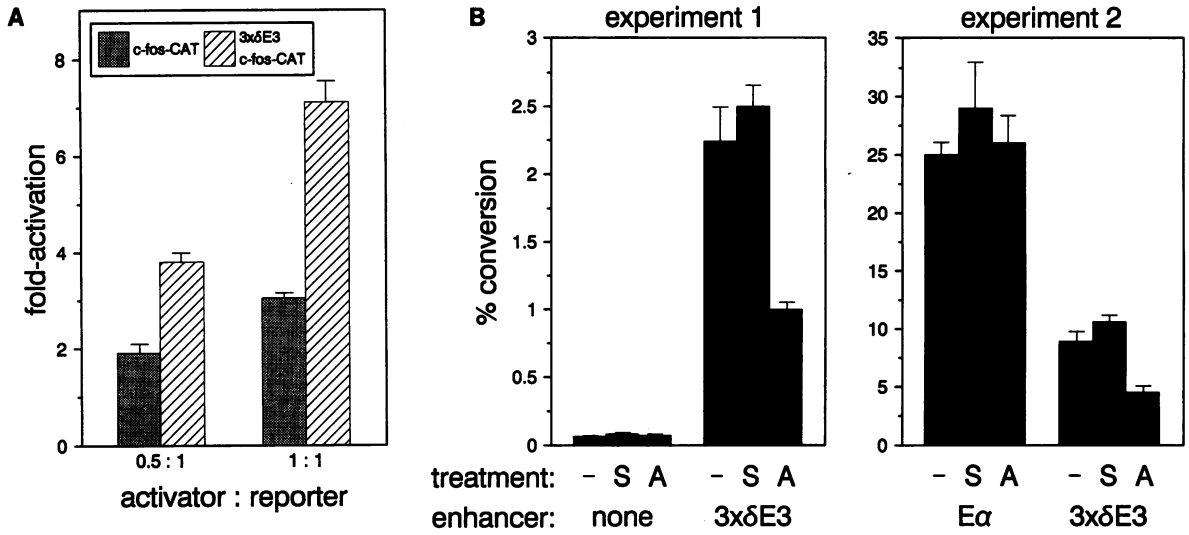

FIG. 8. c-Myb regulates TCR δ enhancer function through δE3. (A) EL-4 cells were transfected with the reporter plasmids c-*fos*–CAT or 3×δE3–c-*fos*–CAT, without or with the activator plasmid pCMV4-c-Myb at the indicated ratios. The plasmid pCMV4 was included as needed to maintain a constant amount of total plasmid DNA. For each reporter, the percent chloramphenicol acetylation in the presence of activator is normalized to the percent chloramphenicol acetylation without activator (fold-activation). Activity determinations were performed in triplicate in each experiment, and the results represent the mean and standard error for three experiments. (B) Jurkat cells were transfected with $V_\delta 1$-CAT reporter constructs containing no enhancer, a trimer of δE3 (3×δE3), or a TCR α enhancer fragment (Eα) and were incubated with sense and antisense oligonucleotides as indicated. The results of two experiments are presented as the mean percent chloramphenicol acetylation and standard error of triplicate samples.

enhancer activity is dependent primarily on c-Myb binding to the Myb2 site.

Because the affinities of the two sites for v-Myb (and presumably c-Myb) are comparable (Fig. 2B), the above results argue that the position of the Myb2 site may be important for enhancer function. We asked whether there was a required spatial relationship between the core and Myb sites by constructing two δE3 mutants with an altered distance between the core and Myb sites (5 bp or half helical turn [δE3ins5] and 10 bp or full helical turn [δE3ins10]) (Fig. 1). When the mutant δE3 elements were subcloned into $V_\delta 1$-CAT and tested by transient transfection of Jurkat cells, both were found to be inactive (Fig. 9A).

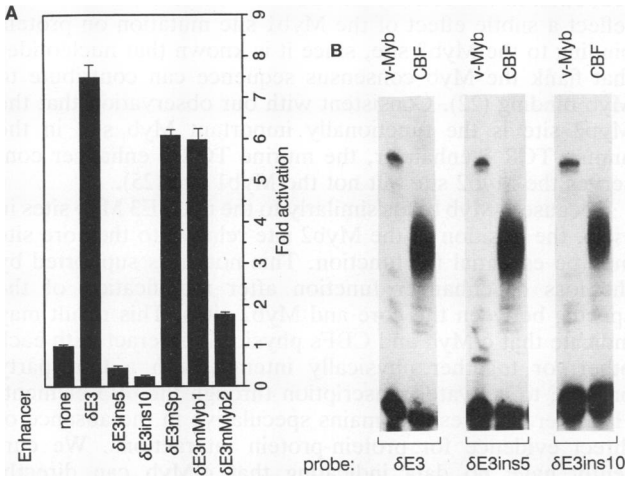

FIG. 9. Core and Myb site organization is critical for δE3 enhancer activity. (A) The indicated binding sites were tested for activation of the $V_\delta 1$ promoter in the test construct $V_\delta 1$-CAT. Constructs were transfected into the Jurkat T-cell line, and values for percent chloramphenicol acetylation of the test constructs were normalized to the activity of the enhancerless $V_\delta 1$-CAT construct. The results are presented as the mean and standard error for three to nine determinations. (B) Radiolabeled binding sites were incubated with bacterial extract containing v-Myb or with purified CBF, and DNA-protein complexes were resolved by electrophoresis.

Loss of enhancer function occurred despite the presence of intact Myb and core sites, as assayed by their ability to bind v-Myb and CBF (61, 78) in EMSA (Fig. 9B). Further, loss of enhancer function did not appear to result from any disruption of binding of functionally important factors to the spacer region between the Myb and core sites. Thus, a 2-bp substitution introduced at the site of the insertion mutations ($\delta E3^{mSp}$ [Fig. 1]) displayed enhancer activity that was only slightly reduced compared with that of the wild-type δE3 element (Fig. 9A). Because the $\delta E3^{mSp}$ and $\delta E3^{mMyb1}$ mutations both displayed strong enhancer activity, none of the 4 bp that lie between the defined core and Myb2 sites are involved in essential contacts with proteins required for enhancer activity. We conclude that the insertion mutations abolish enhancer function because they alter the precise spacing and alignment of the Myb and core sites within δE3, presumably by disrupting protein-protein interactions that are essential for transcriptional activation.

DISCUSSION

The 30-bp δE3 site was previously identified as an essential element for transcriptional activation by the human TCR δ enhancer (61, 62). The core sequence TGTGGTTT in the 5′ end of this fragment was shown to bind a T-cell-specific nuclear factor, and an intact core sequence was found to be necessary but not sufficient for transcriptional activation (61, 62). This suggested that an additional factor that bound to 3′ sequences within δE3 might also be necessary for enhancer

activity. In this work, we showed that c-Myb binds to and regulates δE3 enhancer function through a consensus Myb site located 3′ of the δE3 core site. Intact and appropriately positioned binding sites for c-Myb and the T-cell-specific CBF are both necessary and sufficient for enhancer activity. Thus, these two factors cooperate functionally to mediate T-cell-specific expression of the TCR δ gene.

Many studies have shown that Myb proteins are sequence-specific DNA-binding proteins that function as *trans*-activators of gene expression and play a crucial role in the differentiation and development of hematopoietic cells (42, 66). However, only two cellular genes that are specifically expressed in hematopoietic cells are known to be targets of c-Myb regulation (50, 69). One of these genes, *mim-1*, is promyelocyte specific; however, the function of this gene and its role in development are unknown (50). The other, CD4, is expressed primarily in T cells and encodes a cell surface molecule important in antigen recognition (69). Our data identifying the human TCR δ gene as a target of Myb regulation are consistent with an important role for Myb as a regulator of gene expression and differentiation of hematopoietic cells.

Our previous studies indicated a complicated pattern of protein binding to the δE3 element but did not identify a complex containing c-Myb (62). Those studies were performed with a 35-bp binding site including the entire δE3 element. In this study we used shorter binding-site probes corresponding to the 5′ 20 bp and 3′ 18 bp of δE3. As seen in Fig. 4, the electrophoretic mobility of the complex formed between the δE3(3′) probe and c-Myb is identical to that of the δE3C complex formed with the δE3(5′) probe. Coelectrophoresis of the complex containing c-Myb with the ubiquitous δE3C complex using the 35-bp site is the most likely explanation for our inability to detect c-Myb binding previously.

Treatment of transfected Jurkat cells with antisense c-*myb* oligonucleotide inhibited δE3 enhancer activity, arguing that endogenous c-Myb regulates TCR δ enhancer function in these cells. However, we could not achieve greater than 50 to 60% inhibition of δE3 activity without the complication of more pleiotropic effects on cell growth. Under these conditions, c-Myb protein expression was only partially inhibited. Because of this experimental limitation, we could not establish whether c-Myb is the only factor in Jurkat cells that transactivates through the δE3 Myb site. Thus it remains possible that other factors, either related to c-Myb or not, could regulate δE3 function by binding to the Myb site. Although the product of the B-*myb* gene was shown to bind but not transactivate gene expression through Myb sites (20, 80), no data are available for the product of the A-*myb* gene.

Our data argue that the c-Myb cooperates functionally with the T-cell-specific CBF to mediate transcriptional activation, because intact binding sites for both factors are required for δE3 activity. Previous work indicated cooperation of Myb with Ets transcription factors. The E26 virus encodes a Gag-Myb-Ets fusion protein that is required for leukemogenicity (46, 47), and the combination of c-Myb and Ets-2 was shown to activate the *mim-1* promoter (16). Most recently, Myb was shown to cooperate with C/EBP transcription factors to activate the *mim-1* promoter, and the combination of Myb with the myeloid cell-specific factor C/EBPβ was suggested to play an important role in myeloid cell-specific gene expression (9, 49). Hence, collaboration with additional transcription factors may be a general requirement for transcriptional activation by Myb. The T-cell-specific CBF described in this and previous studies plays an important role in transcriptional activation of the TCR δ enhancer (61, 62), the TCR β enhancer (57), the TCR γ enhancer (31), the long terminal repeats of murine leukemia viruses (7, 32, 75, 78), and perhaps additional T-cell-specific genes (12). The required cooperation with c-Myb for efficient TCR δ enhancer activity does not simply reflect a nonspecific requirement for multimerization of protein-binding sites, since a trimer of δE3(5′) (which lacks the functionally important Myb site) is much less active than a monomer of δE3 (which has both core and Myb sites) (61). Rather, our data argue for a specific functional relationship between proteins that bind to the two sites. Functional cooperation between c-Myb and CBF may be of general importance, since binding sites for these factors also appear to be juxtaposed in the murine TCR δ enhancer (25) and in the murine TCR γ enhancer (70). These observations indicate that this combination of factors could play an important role in orchestrating the coordinate activation of sets of genes during thymic differentiation of T lymphocytes.

We note that the myeloid-specific murine myeloperoxidase promoter carries a core site (that presumably binds the ubiquitous CBF AML1 [3]) and an adjacent Myb site, positioned almost exactly as found in δE3 (73). This suggests the possibility that cooperation between members of the Myb and core-binding families of transcription factors could play important roles in gene expression in non-T-lineage cells as well.

Although we identified two overlapping Myb sites within δE3, only one of these, termed Myb2, is important for enhancer activity. EMSA data suggest that v-Myb binds with similar affinity to both sites in vitro but cannot occupy both sites simultaneously. Assuming mutually exclusive binding to the two sites, fractional occupancy of the Myb2 site in vivo would predict that the activity of the wild-type δE3 would be intermediate between the activities of $\delta E3^{mMyb1}$ and $\delta E3^{mMyb2}$, which was not observed. Thus, the functional data are most easily interpreted as indicating that the Myb2 site is the primary site occupied in vivo. The small reduction in the activity of $\delta E3^{mMyb1}$ relative to δE3 could reflect a subtle effect of the Myb1 site mutation on protein binding to the Myb2 site, since it is known that nucleotides that flank the Myb consensus sequence can contribute to Myb binding (22). Consistent with our observation that the Myb2 site is the functionally important Myb site in the human TCR δ enhancer, the murine TCR δ enhancer conserves the Myb2 site but not the Myb1 site (25).

Because v-Myb binds similarly to the two δE3 Myb sites in vitro, the position of the Myb2 site relative to the core site may be essential for function. This notion is supported by the loss of enhancer function after modification of the spacing between the core and Myb2 sites. This result may indicate that c-Myb and CBFs physically interact with each other, or together physically interact with a third-party protein, to activate transcription through the δE3 element. However, this result remains speculative in the absence of direct evidence for protein-protein interactions. We currently have no data indicating that c-Myb can directly interact with CBF, and to our knowledge, there are no data indicating that c-Myb can interact directly with other transcription factors for which functional cooperation can be demonstrated.

c-Myb is expressed at constitutively high levels in immature thymocytes and becomes downregulated during terminal differentiation (65, 74, 82). In mature cells, including peripheral blood T lymphocytes, c-Myb expression is coupled to the cell cycle, occurring in G_1/S-phase proliferating

cells (23, 40, 56, 71). Our finding that the TCR δ enhancer is regulated by c-Myb is somewhat perplexing since we are not aware of evidence for cell cycle control of TCR δ gene expression. Specifically, it is unclear how expression is maintained in resting, G_0 T cells. It is possible that a Myb-related factor such as A-Myb, which is expressed in G_0 cells (26), plays a role in maintaining TCR δ gene expression in these cells. However, it is also possible that the regulation of TCR δ expression in immature and mature cells is distinct and that our studies with the T leukemic cells lines Jurkat and Molt-13 have mapped only the *cis*-acting elements of the TCR δ enhancer that are important for TCR δ expression in immature cells. These will be important issues to address in future studies.

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REFERENCES

1. **Andersson, S., D. L. Davis, H. Dahlback, H. Jornvall, and D. W. Russell.** 1989. Cloning, structure, and expression of the mitochondrial cytochrome P-450 stero 26-hydrolase, a bile acid biosynthetic enzyme. J. Biol. Chem. **264**:8222–8229.
2. **Anfossi, G., A. M. Gerwirtz, and B. Calabretta.** 1989. An oligomer complementary to c-myb-encoded mRNA inhibits proliferation of human myeloid leukemia cell lines. Proc. Natl. Acad. Sci. USA **86**:3379–3383.
3. **Bae, S. C., Y. Yamaguchi-Iwai, E. Ogawa, M. Maruyama, M. Inuzuka, H. Kagoshima, K. Shigesada, M. Satake, and Y. Ito.** 1993. Isolation of PEBP2αB cDNA representing the mouse homolog of human acute myeloid leukemia gene, AML1. Oncogene **8**:809–814.
4. **Beug, H., P. D. Blundell, and T. Graf.** 1987. Reversibility of differentiation and proliferative capacity in avian myelomonocytic cells transformed by ts-E26 leukemia virus E26. Genes Dev. **1**:227–286.
5. **Beug, H., A. Leutz, P. Kahn, and T. Graf.** 1984. Ts mutants of E26 leukemia virus allow transformed myeloblasts, but not erythroblasts or fibroblasts, to differenciate at the non-permissive temperature. Cell **39**:579–588.
6. **Biedenkapp, H., U. Borgmeyer, A. E. Sippel, and K.-H. Klempnauer.** 1988. Viral myb oncogene encodes a sequence-specific DNA-binding activity. Nature (London) **335**:835–837.
7. **Boral, A. L., S. A. Okenquist, and J. Lenz.** 1989. Identification of the SL3-3 virus enhancer core as a T-lymphoma cell-specific element. J. Virol. **63**:76–84.
8. **Bortner, D. M., and M. C. Ostrowski.** 1991. Analysis of the v-myb structural components important for transactivation of gene expression. Nucleic Acids Res. **19**:1533–1539.
9. **Burk, O., S. Mink, M. Ringwald, and K.-H. Klempnauer.** 1993. Synergistic activation of the chicken mim-1 gene by v-myb and C/EBP transcription factors. EMBO J. **12**:2027–2038.
10. **Citro, G., D. Perroti, C. Cucco, I. D'Agnano, A. Sacchi, G. Zupi, and B. Calabretta.** 1992. Inhibition of leukemia cell proliferation by receptor-mediated uptake of c-myb antisense oligodeoxynucleotides. Proc. Natl. Acad. Sci. USA **89**:7031–7035.
11. **Clarke, M. F., J. F. Kukowska-Latallo, E. Westin, M. Smith, and E. W. Prochownik.** 1988. Constitutive expression of a c-myb cDNA blocks Friend murine erythroleukemia cell differentiation. Mol. Cell. Biol. **8**:884–892.
12. **Clevers, H., M. Lonberg, S. Dunlap, E. Lacy, and C. Terhorst.** 1989. An enhancer located in a CpG-island 3′ to the TCR/CD3-ε gene confers T lymphocyte-specificity to its promoter. EMBO J. **8**:2527–2535.
13. **Cogswell, J. P., P. C. Cogswell, W. M. Kuehl, A. M. Cuddihy, T. M. Bender, U. Engelke, K. B. Marcu, and J. P.-Y. Ting.** 1993. Mechanism of c-*myc* regulation by c-Myb in different cell lineages. Mol. Cell. Biol. **13**:2858–2869.
14. **Davis, M. M., and P. J. Bjorkman.** 1988. T-cell antigen receptor genes and T-cell recognition. Nature (London) **334**:395–402.
15. **Dorsett, D. L., I. Keshet, and E. Winocour.** 1983. Quantitation of a simian virus 40 nonhomologous recombination pathway. J. Virol. **48**:218–228.
16. **Dudek, H., R. V. Tantravahi, V. N. Rao, E. S. P. Reddy, and E. P. Reddy.** 1992. Myb and Ets proteins cooperate in transcriptional activation of the mim-1 promoter. Proc. Natl. Acad. Sci. USA **89**:1291–1295.
17. **Duprey, S. A., and D. Boettiger.** 1985. Developmental regulation of c-myb in normal myeloid progenitor cells. Proc. Natl. Acad. Sci. USA **82**:6937–6941.
18. **Durban, E., and D. Boettiger.** 1981. Differential effects of transforming avian RNA tumor viruses on avian macrophages. Proc. Natl. Acad. Sci. USA **82**:6937–6941.
19. **Evans, J. L., T. L. Moore, W. M. Kuehl, T. Bender, and J. P.-Y. Ting.** 1990. Functional analysis of c-*myb* protein in T-lymphocytic cell lines shows that it *trans*-activates the c-*myc* promoter. Mol. Cell. Biol. **10**:5747–5752.
20. **Foos, G., S. Grimm, and K.-H. Klempnauer.** 1992. Functional antagonism between members of the myb family: B-myb inhibits v-myb-induced gene activation. EMBO J. **11**:4619–4629.
21. **Fowlkes, B. J., and D. M. Pardoll.** 1989. Molecular and cellular events of T cell development. Adv. Immunol. **44**:207–264.
22. **Garcia, A., K. LaMontagne, D. Reavis, U. Stober-Grasser, and J. S. Lipsick.** 1991. Determinants of sequence-specific DNA-binding by p48v-myb. Oncogene **6**:265–273.
23. **Gewirtz, A. M., G. Anfossi, D. Venturelli, S. Valpreda, R. Sims, and B. Calabretta.** 1989. G1/S transition in normal human T-lymphocytes requires the nuclear protein encoded by c-myb. Science **245**:180–183.
24. **Gewirtz, A. M., and B. Calabretta.** 1988. A c-myb antisense oligodeoxynucleotide inhibits normal human hematopoiesis in vitro. Science **242**:1303–1306.
25. **Gill, L. L., D. Zaninetta, and K. Karjalainen.** 1991. A transcriptional enhancer of the mouse T cell receptor δ gene locus. Eur. J. Immunol. **21**:807–810.
26. **Golay, J., A. Capucci, M. Arsura, M. Castellano, V. Rizzo, and M. Introna.** 1991. Expression of c-myb and B-myb, but not A-myb, correlates with proliferation in human hematopoietic cells. Blood **77**:149–158.
27. **Golay, J., M. Introna, and T. Graf.** 1988. A single point mutation in the v-ets oncogene affects both erythroid and myelomonocytic cell differentiation. Cell **55**:1147–1158.
28. **Gonda, T. J., and D. Metcalf.** 1984. Expression of myb, myc and fos proto-oncogenes during the differentiation of a murine myeloid leukemia. Nature (London) **310**:249–251.
29. **Gonda, T. J., D. K. Sheiness, and J. M. Bishop.** 1982. Transcripts from cellular homologs of retroviral oncogenes: distribution among chicken tissues. Mol. Cell. Biol. **6**:617–624.
30. **Ho, I.-C., L.-H. Yang, G. Morle, and J. M. Leiden.** 1989. A T-cell-specific transcriptional enhancer element 3′ of C_α in the human T-cell receptor α locus. Proc. Natl. Acad. Sci. USA **86**:6714–6718.
31. **Hsiang, Y. H., D. Spencer, S. Wang, N. A. Speck, and D. H. Raulet.** 1993. The role of viral enhancer "core" motif-related sequences in regulating T cell receptor-τ and -δ gene expression. J. Immunol. **150**:3905–3916.
32. **Kamachi, Y., E. Ogawa, M. Asano, S. Ishida, Y. Murakami, M. Satake, Y. Ito, and K. Shigesada.** 1990. Purification of a mouse nuclear factor that binds to both the A and B cores of the polyomavirus enhancer. J. Virol. **64**:4808–4819.
33. **Klempnauer, K.-H., H. Arnold, and H. Biedenkapp.** 1989. Activation of transcription by v-myb: evidence for two different

mechanisms. Genes Dev. 3:1582–1589.
34. **Klempnauer, K.-H., T. J. Gonda, and J. M. Bishop.** 1982. Nucleotide sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: the architecture of a transduced oncogene. Cell 31:453–463.
35. **Klempnauer, K.-H., G. Ramsay, J. M. Bishop, M. G. Moscovici, C. Moscovici, J. P. McGrath, and A. D. Levinson.** 1983. The product of the retroviral transforming gene v-myb is a truncated version of the protein encoded by the cellular oncogene c-myb. Cell 33:345–355.
36. **Ku, D.-H., S.-C. Wen, A. Engelhard, N. C. Nicolaides, K. E. Lipson, T. A. Marino, and B. Calabretta.** 1993. c-myb transactivates cdc-2 expression via myb binding sites in the 5′ flanking region of the human cdc2 gene. J. Biol. Chem. 268:2255–2259.
37. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
38. **Leiden, J. M.** 1992. Transcriptional regulation during T-cell development: the α TCR gene as a molecular model. Immunol. Today 13:22–30.
39. **Liebermann, D. A., and B. Hoffman-Liebermann.** 1989. Proto-oncogene expression and dissection of the myeloid growth to differentiation developmental cascade. Oncogene 4:583–592.
40. **Lipsick, J. S., and W. J. Boyle.** 1987. c-*myb* protein expression is a late event during T-lymphocyte activation. Mol. Cell. Biol. 7:3358–3360.
41. **Luscher, B., E. Christenson, D. W. Litchfield, E. G. Krebs, and R. N. Eisenman.** 1990. Myb DNA binding inhibited by phosphorilation at a site deleted during oncogenic activation. Nature (London) 344:517–522.
42. **Luscher, B., and R. N. Eisenman.** 1990. New light on Myc and Myb. II. Myb. Genes Dev. 4:2235–2241.
43. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. 11:1295–1308.
44. **McClinton, D., J. Stafford, L. Brents, T. P. Bender, and W. M. Knehl.** 1990. Differentiation of mouse erythroleukemia cells is blocked by late up-regulation of a c-*myb* transgene. Mol. Cell. Biol. 10:705–710.
45. **McMahon, J., K. M. Howe, and R. J. Watson.** 1988. The induction of Friend erythroleukemia differentiation is markedly affected by expression of a transfected c-myb cDNA. Oncogene 3:717–720.
46. **Metz, T., and T. Graf.** 1991. Fusion of the nuclear oncoproteins v-Myb and v-Ets is required for the leukemogenicity of E26 virus. Cell 66:95–105.
47. **Metz, T., and T. Graf.** 1991. v-myb and v-ets transform chicken erythroid cells and cooperate both in trans and in cis to induce distinct differentiation phenotypes. Genes Dev. 5:369–380.
48. **Mucenski, M. L., K. McLain, A. B. Kier, S. H. Swerdlow, C. M. Schreiner, T. A. Miller, D. W. Pietryga, W. J. Scott, and S. S. Potter.** 1991. A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. Cell 65:677–689.
49. **Ness, S. A., E. Kowenz-Leutz, T. Casini, T. Graf, and A. Leutz.** 1993. Myb and NF-M: combinatorial activators of myeloid genes in heterologous cell types. Genes Dev. 7:749–759.
50. **Ness, S. A., A. Marknell, and T. Graf.** 1989. The v-myb oncogene product binds to and activates the promyelocyte-specific mim-1 gene. Cell 59:1115–1125.
51. **Nishina, Y., H. Nakagoshi, F. Imamoto, T. J. Gonda, and S. Ishii.** 1989. Trans-activation by the c-myb proto-oncogene. Nucleic Acids Res. 17:107–117.
52. **Nomura, N., M. Takahashi, M. Matsui, S. Ishii, T. Date, S. Sasamoto, and R. Ishizaki.** 1988. Isolation of human cDNA clones of myb-related genes, A-myb and B-myb. Nucleic Acids Res. 16:11075–11089.
53. **Nunn, M. F., and T. Hunter.** 1989. The ets sequence is required for induction of erythroblastosis in chickens by avian retrovirus E26. J. Virol. 63:398–402.
54. **Ogawa, E., M. Inuzuka, M. Maruyama, M. Satake, M. Naito-Fujimoto, Y. Ito, and K. Shigesada.** 1993. Molecular cloning and characterization of PEBP2β, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2β. Virology 194:314–331.
55. **Ogawa, E., M. Maruyama, H. Kagoshima, I. Inuzuka, J. Lu, M. Satake, K. Shigesada, and Y. Ito.** 1993. PEBP2/PEA2 represents a new family of transcription factor homologous to the products of the Drosophila runt and the human AML1. Proc. Natl. Acad. Sci. USA 90:6859–6863.
56. **Pauza, C. D.** 1987. Regulation of human T-lymphocyte gene expression by interleukin-2: immediate-response genes include the proto-oncogene c-*myb*. Mol. Cell. Biol. 7:342–348.
57. **Prosser, H. M., D. Wotton, A. Gegonne, J. Ghysdael, S. Wang, N. A. Speck, and M. J. Owen.** 1992. A phorbol ester response element within the human T-cell receptor β-chain enhancer. Proc. Natl. Acad. Sci. USA 89:9934–9938.
58. **Radke, K., H. Beug, S. Kornfeld, and T. Graf.** 1982. Transformation of both erythroid and myeloid cells by E26, an avian leukemia virus that contains the myb gene. Cell 31:643–653.
59. **Raulet, D. H., D. M. Spencer, Y.-H. Hsiang, J. P. Goldman, M. Bix, N. S. Liao, M. Zijlstra, R. Jaenisch, and I. Correa.** 1991. Control of τδ T-cell development. Immunol. Rev. 120:185–204.
60. **Redondo, J. M., S. Hata, C. Brocklehurst, and M. S. Krangel.** 1990. A T cell specific transcriptional enhancer within the human T cell receptor δ locus. Science 247:1225–1229.
61. **Redondo, J. M., J. L. Pfohl, C. Hernandez-Munain, S. Wang, N. A. Speck, and M. S. Krangel.** 1992. Indistinguishable nuclear factor binding to functional core sites of the T-cell receptor δ and murine leukemia virus enhancers. Mol. Cell. Biol. 12:4817–4823.
62. **Redondo, J. M., J. L. Pfohl, and M. S. Krangel.** 1991. Identification of an essential site for transcriptional activation within the human T cell receptor δ enhancer. Mol. Cell. Biol. 11:5671–5680.
63. **Reed, J. C., J. D. Alpers, P. C. Nowell, and R. G. Hoover.** 1986. Sequential expression of protooncogenes during lectin-stimulated mitogenesis of normal human lymphocytes. Proc. Natl. Acad. Sci. USA 83:3982–3986.
64. **Rosson, D., and E. P. Reddi.** 1986. Nucleotide sequence of chicken c-myb complementary DNA and implications for myb oncogene activation. Nature (London) 319:604–606.
65. **Sheiness, D., and M. Gardinier.** 1984. Expression of a proto-oncogene (proto-*myb*) in hematopoietic tissues of mice. Mol. Cell. Biol. 4:1206–1212.
66. **Shen-Ong, G. L. C.** 1990. The myb oncogene. Biochim. Biophys. Acta 1032:39–52.
67. **Shipp, M. A., and E. L. Reinherz.** 1987. Differential expression of nuclear proto-oncogenes in T cells triggered with mitogenic and nonmitogenic T3 and T11 activation signals. J. Immunol. 139:2143–2148.
68. **Singh, H., R. Sen, D. Baltimore, and P. Sharp.** 1986. A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. Nature (London) 319:154–158.
69. **Siu, G., A. L. Wurster, J. S. Lipsick, and S. M. Hedrick.** 1992. Expression of the CD4 gene requires a Myb transcription factor. Mol. Cell. Biol. 12:1592–1604.
70. **Spencer, D. M., Y.-H. Hsiang, J. P. Goldman, and D. P. Raulet.** 1991. Identification of a T-cell-specific transcriptional enhancer located 3′ of $C_\tau 1$ in the murine T-cell receptor τ locus. Proc. Natl. Acad. Sci. USA 88:800–804.
71. **Stern, J. B., and K. A. Smith.** 1986. Interleukin-2 induction of T-cell G1 progression and c-myb expression. Nature (London) 233:203–206.
72. **Strominger, J. L.** 1989. Developmental biology of T cell receptors. Science 244:943–950.
73. **Suzow, J., and A. D. Friedman.** 1993. The murine myeloperoxidase promoter contains several functional elements, one of which binds a cell type-restricted transcription factor, myeloid nuclear factor 1 (MyNF1). Mol. Cell. Biol. 13:2141–2151.
74. **Thompson, C. B., P. B. Challoner, P. E. Neiman, and M. Groudine.** 1986. Expression of the c-myb proto-oncogene during cellular proliferation. Nature (London) 319:374–380.
75. **Thornell, A., B. Hallberg, and T. Grundstrom.** 1991. Binding of SL3-3 enhancer factor 1 transcriptional activators to viral and chromosomal enhancer sequences. J. Virol. 65:42–50.

76. **Tokodoro, K., R. J. Watson, H. Higo, H. Amanuma, S. Kuramochi, H. Yanagisawa, and Y. Ikawa.** 1988. Down-regulation of c-myb gene expression is a prerequisite for erythropoietin-induced erythroid differentiation. Proc. Natl. Acad. Sci. USA **85**:8900–8904.
77. **Torelli, G., L. Selleri, A. Donelli, S. Ferrari, G. Emilia, D. Venturelli, L. Moretti, and U. Torelli.** 1985. Activation of c-*myb* expression by phytohemagglutinin stimulation in normal human T lymphocytes. Mol. Cell. Biol. **5**:2874–2877.
78. **Wang, S., and N. A. Speck.** 1992. Purification of core-binding factor, a protein that binds the conserved core site in murine leukemia virus enhancers. Mol. Cell. Biol. **12**:89–102.
79. **Wang, S., Q. Wang, B. E. Crute, I. N. Melnikova, S. R. Keller, and N. A. Speck.** 1993. Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. Mol. Cell. Biol. **13**:3324–3339.
80. **Watson, R. J., C. Robinson, and E. W.-F. Lam.** 1993. Transcription regulation by murine B-myb is distinct from that by c-myb. Nucleic Acids Res. **21**:267–272.
81. **Weber, B. L., E. H. Westin, and M. F. Clarke.** 1990. Differentiation of mouse erythroleukemia cells enhanced by alternatively spliced c-myb mRNA. Science **249**:1291–1293.
82. **Westin, E. H., R. C. Gallo, S. K. Arya, A. Eva, L. M. Souza, M. A. Baluda, S. A. Aaronson, and F. Wong-Staal.** 1982. Differential expression of the amv gene in human hematopoietic cells. Proc. Natl. Acad. Sci. USA **79**:2194–2198.
83. **Weston, K., and J. M. Bishop.** 1989. Transcriptional activation by the v-myb oncogene and its cellular progenitor, c-myb. Cell **58**:85–93.
84. **Winoto, A., and D. Baltimore.** 1989. A novel, inducible and T cell-specific enhancer located at the 3′ end of the T cell receptor α locus. EMBO J. **8**:729–733.