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Identification of an Inducible Regulator of c-*myb* Expression during T-Cell Activation

SEE-CHUN PHAN, BRIAN FEELEY, DONALD WITHERS,† AND LINDA M. BOXER*

Center for Molecular Biology in Medicine, Palo Alto VAMC, Palo Alto, California 94304, and Department of Medicine, Stanford University School of Medicine, Stanford, California 94305

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Resting T cells express very low levels of c-Myb protein. During T-cell activation, c-*myb* expression is induced and much of the increase in expression occurs at the transcriptional level. We identified a region of the c-*myb* 5′ flanking sequence that increased c-*myb* expression during T-cell activation. In vivo footprinting by ligation-mediated PCR was performed to correlate in vivo protein binding with functional activity. A protein footprint was visible over this region of the c-*myb* 5′ flanking sequence in activated T cells but not in unactivated T cells. An electrophoretic mobility shift assay (EMSA) with nuclear extract from activated T cells and an oligonucleotide of this binding site demonstrated a new protein-DNA complex, referred to as CMAT for c-*myb* in activated T cells; this complex was not present in unactivated T cells. Because the binding site showed some sequence similarity with the nuclear factor of activated T cells (NFAT) binding site, we compared the kinetics of induction of the two binding complexes and the molecular masses of the two proteins. Studies of the kinetics of induction showed that the NFAT EMSA binding complex appeared earlier than the CMAT complex. The NFAT protein migrated more slowly in a sodium dodecyl sulfate-polyacrylamide gel than the CMAT protein did. In addition, an antibody against NFAT did not cross-react with the CMAT protein. The appearance of the CMAT binding complex was inhibited by both cyclosporin A and rapamycin. The CMAT protein appears to be a novel inducible protein involved in the regulation of c-*myb* expression during T-cell activation.

Interaction of antigen with the antigen receptor of T lymphocytes initiates a series of changes resulting in cell division and immunologic function. The antigen receptor rapidly induces the production of cytokines that control cell proliferation, cell fate decisions, and the differentiation of B cells, granulocytes, macrophages, and other cell types involved in the immune response (3, 13, 30). A number of genes are activated in T cells, including the proto-oncogenes c-*myc* and c-*myb* (17, 33). The products of these genes provide a link with surface receptor-mediated activation events and prepare the cell for subsequent DNA replication and proliferation.

The interleukin-2 gene is active only in T cells that have been stimulated through the antigen receptor, and it plays a major role in T-cell proliferation. The induction of the interleukin-2 gene is mediated by a transcription factor, the nuclear factor of activated T cells (NFAT) (9, 35, 40). The NFAT complex is found in nuclei of activated T cells; it binds to the promoter of the interleukin-2 gene and transmits signals initiated at the T-cell antigen receptor. NFAT is assembled from a preexisting, T-cell-restricted cytoplasmic factor and an inducible ubiquitous nuclear component consisting of AP1 factors within 30 min after activation of the antigen receptor (10, 29). The cytoplasmic component ($NFAT_c$) is translocated to the nucleus in response to calcium signaling, and this process is inhibited by the immunosuppressive drugs cyclosporin A and FK506.

c-Myb is a sequence-specific DNA-binding protein with the ability to transactivate promoters with the specific consensus sequence PyAACG/TG. The c-*myb* gene product is a highly conserved 75- to 83-kDa phosphoprotein which is predominantly expressed in hematopoietic tissues and localized to the nucleus (4, 5, 18, 19, 23). Reduction of c-*myb* expression results in a block to hematopoietic precursor cell proliferation, and homozygous c-*myb* mutant mice demonstrate greatly impaired fetal hepatic hematopoiesis. The c-*myb* gene product plays an important role in regulating lymphocyte proliferation. c-*myb* mRNA levels vary during the cell cycle of normal T lymphocytes and reach a peak in S phase (37, 38). Resting T cells express very low levels of c-Myb. During T-cell activation, levels of c-*myb* mRNA increase manyfold (8, 31, 36–38). Antisense experiments have demonstrated that the c-*myb* gene product is required for T cells to enter S phase of the cell cycle (12). Reduction of c-Myb levels does not interfere with T lymphocyte activation or early molecular events, but it does block the T cells in late G_1 of the cell cycle (12).

The transcriptional control of c-*myb* expression has been examined in several studies (14, 16, 22, 24, 26, 27), but it is not known how c-*myb* expression is controlled during T-cell activation. We have identified a region of the c-*myb* promoter that mediates an increase in c-*myb* expression during T-cell activation. An inducible protein, CMAT (c-*myb* in activated T cells), binds to this sequence. The CMAT binding site shows some sequence similarities with the NFAT binding site, but we demonstrate that the CMAT binding activity is distinct from the NFAT binding activity. The CMAT complex is present only in activated T cells.

MATERIALS AND METHODS

Activation of T cells. The Jurkat TagC cell line (29) was obtained from G. Crabtree (Stanford University). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U of penicillin G per ml, and 50 μg of streptomycin per ml. For activation, 20 ng of phorbol myristate acetate (PMA) per ml and 1 μM ionomycin were added. The protein synthesis inhibitor anisomycin was added at 100 μM 5 min prior to activation in the gel shown in Fig. 6, lane 4. Cyclosporin A or rapamycin at the concentrations indicated in Fig. 7A was added 5 to 15 min prior to activation where indicated in

* Corresponding author. Mailing address: Division of Hematology, S-161, Stanford University School of Medicine, Stanford, CA 94305-5112. Phone: (415) 493-5000, ext. 63126. Fax: (415) 858-3982. Electronic mail address: hf.lmb@forsythe.stanford.edu.

† Present address: The Biomembrane Institute, University of Washington, Seattle, WA 98119.

the figure. Nuclear extracts were prepared at the times indicated in Fig. 5A or at 8 h after treatment of the cells. Peripheral T cells were isolated from buffy coats by Ficoll-Hypaque density gradient centrifugation. Monocytes were removed by adherence to plastic flasks, and B lymphocytes were depleted by nylon wool columns. The cells were then further purified by discontinuous Percoll density gradient centrifugation. Purity was greater than 95% as assessed by flow cytometry. Activation was performed as described above.

Construction of reporter plasmids. The *Hin*dIII-*Bam*HI fragment of the luciferase reporter vector was derived from pSV232AL-AΔ5′ (obtained from D. Helinski, University of California, San Diego). The fragment was cloned into pUC18 and subsequently recut as a *Hin*dIII-*Kpn*I fragment and ligated into the multiple cloning site of Bluescript II KS+. The 2.3-kb *Eco*RI-*Eco*RI 5′ flanking region of human c-*myb* (a kind gift from R. Dalla-Favera, Columbia University) was ligated into the *Eco*RI site 5′ of the luciferase gene in Bluescript. To remove a confounding ATG site, the *Hin*dIII-*Nco*I fragment was removed, *Hin*dIII linkers were added, and the plasmid was religated. The deletion constructs of the c-*myb* promoter were made by PCR from this original construct. All numbers are relative to the translation start site.

Mutagenesis of the CMAT binding site was achieved by a technique previously described by Higuchi (15). Mutants were screened by restriction enzyme analysis and subsequently sequenced with a Fmol sequencing kit (Promega) or a Sequenase kit (U.S. Biochemicals). Compressions were resolved with dITP or deaza-dGTP. The oligonucleotide sequence used for the PCR primer (with mutated bases shown in boldface type) was GGTGGCGGCCGAAGAAGAA**TT**AAAA AAAA.

Transfections and luciferase assays. Transfections were performed on cells in log phase. Cells were washed and resuspended in unsupplemented RPMI 1640 medium to a final concentration of 2×10^7 cells per ml and incubated for 10 min at room temperature after the addition of 2 μg of DNA. Electroporations were carried out with a Bio-Rad Gene Pulser at 250 mV and 960 μF. The cells were then incubated again for 10 min at room temperature. Transfected cells were cultured in 24 ml of supplemented RPMI 1640 medium for 48 h. Cells were either unactivated or activated with PMA and ionomycin for 10 h. Cell lysis and luciferase assays were conducted according to the protocol and with reagents supplied with Promega's luciferase assay system. Luciferase measurements were performed on a model LKB 1251 luminometer. A plasmid expressing β-galactosidase was cotransfected as an internal control of the transfection efficiency. Each transfection was repeated at least three times in duplicate with at least three different DNA preparations. The average values with the standard deviations were plotted.

EMSA. The following oligonucleotides were used for the electrophoretic mobility shift assays (EMSA):

CMAT	AAGAAGAAGGAAAAAAAACCCTAGC
	TTCTTCTTCCTTTTTTTTGGGATCG
NFAT	TCTAAGGAGGAAAAACTGTTTCATG
	AGATTCCTCCTTTTTGACAAAGTAT
Mutated CMAT	AAGAAGAA**TT**AAAAAAAACCCTAGC
	TTCTTCTT**AA**TTTTTTTTGGGATCG

The oligonucleotides were synthesized with 5′ overhangs and end labeled with [α-^{32}P]dCTP and Klenow fragment. The binding reaction mixture contained 10 mM Tris-HCl (pH 7.5), 30 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1 μg of poly(dI-dC), 6 μg of bovine serum albumin (BSA), 0.5 ng (10^4 cpm) of end-labeled DNA oligonucleotide probe, and 5 to 10 μg of protein from crude nuclear extract. The binding reaction was conducted at room temperature for 60 min, and the samples were loaded onto a 0.5× Tris-borate-EDTA–5% polyacrylamide gel. The samples were electrophoresed at 30 mA at 4°C. For the competition studies in Fig. 2A and C, the indicated molar excesses of unlabeled competitor oligonucleotide were added to the binding reaction mixture. For the supershifts, the binding reaction was performed as described above with incubation for 60 min at room temperature. Antibody was added, and the incubation was continued for 1 h at 4°C. The cytosolic and preexisting NFAT ($NFAT_c$ and $NFAT_p$, respectively) antibodies (28) were generously provided by G. Crabtree (Stanford University). Antibodies against Fos, Jun, and Ets were obtained from Santa Cruz Biotechnology.

UV cross-linking and SDS-polyacrylamide gel electrophoresis. An EMSA was performed as described above. The wet gel was exposed to film to locate the complexes. UV cross-linking was performed essentially as previously described (6) on a short-wave UV light box at 4°C for 90 min. The regions of the gel containing the complexes were cut out, and the complexes were eluted at room temperature overnight in 50 mM Tris-HCl (pH 7.9)–0.1% sodium dodecyl sulfate (SDS)–0.1 mM EDTA–5 mM dithiothreitol–150 mM NaCl–0.1 mg of BSA per ml. The eluted protein was precipitated with 4 volumes of acetone, washed with ethanol, and air dried. After resuspension in Laemmli loading buffer, SDS-polyacrylamide gel electrophoresis was performed and the labeled proteins were visualized by autoradiography.

Preparation and analysis of RNA. Total cellular RNA was isolated by the guanidinium thiocyanate method (7). Ten micrograms of RNA per sample was separated on 1.2% agarose–formaldehyde gels. The RNA was blotted onto Hybond-N filters (Amersham) and processed according to the manufacturer's instructions. The c-*myb* probe was an *Eco*RI fragment of human c-*myb* cDNA (20) labeled by random priming. Human glyceraldehyde-3-phosphate dehydrogenase cDNA cloned into the *Pst*I site of pBR322 (39) was used as the probe for a housekeeping mRNA.

In vitro methylation interference. 5′ end-labeled oligonucleotide (labeled with T4 kinase) was methylated with 0.5% dimethyl sulfate for 2 min at room temperature. This probe was used in an EMSA as described above. The wet gel was exposed to locate the complexes, and both the bound and free probes were excised and transferred to DEAE membranes. The DNA was eluted and cleaved with piperidine, and equal counts of bound and free samples were resolved in a 15% acrylamide sequencing gel.

In vivo dimethyl sulfate treatment and DNA isolation. DNA isolation after dimethyl sulfate treatment was performed as previously described (1, 2). Cleavage with piperidine was performed according to the Maxam-Gilbert procedure (21).

Ligation-mediated PCR. Chemically modified and cleaved DNA was subjected to amplification by ligation-mediated PCR essentially as described by Mueller and Wold (25), Pfeifer et al. (32), and Garrity and Wold (11). Sequenase was used for first-strand synthesis, and *Taq* DNA polymerase was used for PCR. Conditions used for amplification were 95°C for 2 min, 61°C for 2 min, and 76°C for 3 min. After 20 to 22 cycles of PCR, samples were hybridized with end-labeled primers (primer 3 of each primer set) and amplified by one more cycle of PCR. The reaction mixtures were resolved in a 6% polyacrylamide denaturing gel. The common linkers used were GCGGTGACCCGGGAGATCTGAATTC and GAATTCAGATC. The primers for the coding strand were: TGGAGACG GGGAAATTAGGAGTTGG, GTTGGAGGAGTAGGGGATGTGAGC, and GTTGGAGGAGTAGGGGATGTGAGCATATG.

RESULTS

Identification of a site that mediates activation of the c-*myb* promoter during Jurkat T-cell activation. To examine the contribution of transcriptional initiation to the increased expression of c-*myb* during Jurkat T-cell activation, promoter-reporter constructs were made with the c-*myb* 5′ flanking sequence and the luciferase gene. We identified a region of the c-*myb* promoter that mediated an increase in promoter activity during Jurkat T-cell activation. The location of this region in the c-*myb* promoter is illustrated in Fig. 1A. Inclusion of a region between positions −784 and −758 resulted in a 9.2-fold increase in the promoter activity in activated Jurkat cells and only a 1.4-fold increase in the promoter activity in unactivated Jurkat cells (Fig. 1B). Further experiments were performed to characterize this regulatory element.

A specific complex is formed with the −784-to-−758 region in EMSA with nuclear extract from activated T cells. Nuclear extracts were prepared from unactivated and activated Jurkat or primary T cells and used in EMSA with the −784-to-−758 region of the c-*myb* promoter. Similar results were obtained with both Jurkat and primary T cells. A single complex of retarded mobility was observed with nuclear extract from unactivated T cells (Fig. 2A, lane 1). The same complex was observed with nuclear extract from activated T cells, but in addition, a more slowly migrating complex was also present (Fig. 2A, lane 2). EMSA with nuclear extracts prepared from several other hematopoietic cell lines did not reveal any complex with the migration pattern of the more slowly migrating activated-T-cell complex (data not shown). We refer to this complex as CMAT.

The guanine residues at positions −779 and −778 are required for binding, as demonstrated in Fig. 2A, lanes 3 and 4. The mutated oligonucleotide did not compete against the CMAT complex (Fig. 2A, lane 3), and the CMAT complex was not formed when the mutated oligonucleotide was used as the probe for EMSA (Fig. 2A, lane 4). In vitro methylation interference confirmed that the guanines at −779 and −778 were required for protein binding (Fig. 2B). In addition, methylation of the guanine residue at −782 interfered with protein binding (Fig. 2B). Formation of the constitutive complex was not affected by methylation of any of these guanine residues. We did observe a consistent decrease in intensity of the guanine at

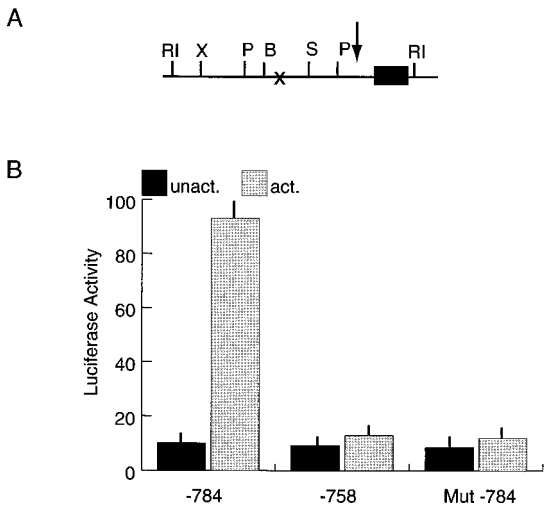

FIG. 1. Results of transient transfection experiments with the c-*myb* 5′ flanking sequence during Jurkat T-cell activation. (A) Diagram of c-*myb* 5′ flanking sequence and first exon. The arrow marks the transcription initiation site, and the X across the line marks the location of the CMAT site. The transcription initiation site is 200 bp upstream from the ATG codon. Restriction sites: RI, *Eco*RI; X, *Xba*I; P, *Pst*I; B, *Bam*HI; S, *Sac*I. (B) Transient transfection analysis of the c-*myb* promoter-luciferase constructs spanning the CMAT site. The results are shown relative to the luciferase activity of a promoterless construct. The CMAT site is at −782. Mut-784 is the −784 construct with the guanines at positions −779 and −778 changed to thymidines. All numbers are relative to the translation start site. unact., unactivated; act., activated.

−694 at the 3′ end of the noncoding strand. None of the guanines on the noncoding strand showed any decrease in intensity. Although we have not defined the binding site for the constitutive complex, we have shown that sequences at the 3′ end of the CMAT oligonucleotide are able to compete for binding of the constitutive complex in EMSA (data not shown).

To determine whether CMAT binding by EMSA correlated with the function of the CMAT site, the two guanine residues at −779 and −778 were mutated in the c-*myb* promoter-reporter construct. It can be seen in Fig. 1B that this mutation abolished the increase in the c-*myb* promoter activity level during Jurkat T-cell activation.

Comparison of the CMAT and NFAT complexes. Because the CMAT sequence showed some similarity to that of the NFAT site and the NFAT complex is also present only in activated T cells, we decided to investigate whether the same proteins were present in both complexes. As shown in Fig. 2C, the NFAT complex migrated more slowly than the CMAT complex in EMSA (compare lane 2 with lane 6). The CMAT complex competed weakly for binding with the NFAT complex at a 75-fold molar excess (Fig. 2C, lane 3), and the NFAT complex also demonstrated weak competition with the CMAT complex (Fig. 2C, lane 7). At a 20-fold molar excess of self competitor, the complex disappeared completely (Fig. 2C, lanes 4 and 8).

Supershift EMSA were performed with antibodies against NFAT to determine whether the CMAT complex contained the NFAT protein. Antibodies against both $NFAT_p$ and $NFAT_c$ produced supershifted bands with the NFAT probe and nuclear extract from activated T cells (Fig. 3, lanes 3 and 4). No supershifted band was visible with the antibody against either $NFAT_p$ or $NFAT_c$ with the CMAT complex (Fig. 3, lanes 7 and 8). No supershifted complex was visible with antibodies against Fos, Jun, or Ets with the CMAT complex (data not shown).

To determine the molecular mass of the CMAT protein, UV cross-linking of the EMSA complex followed by denaturing SDS-polyacrylamide gel electrophoresis was performed (Fig. 4). After correction for the bound oligonucleotide, a molecular

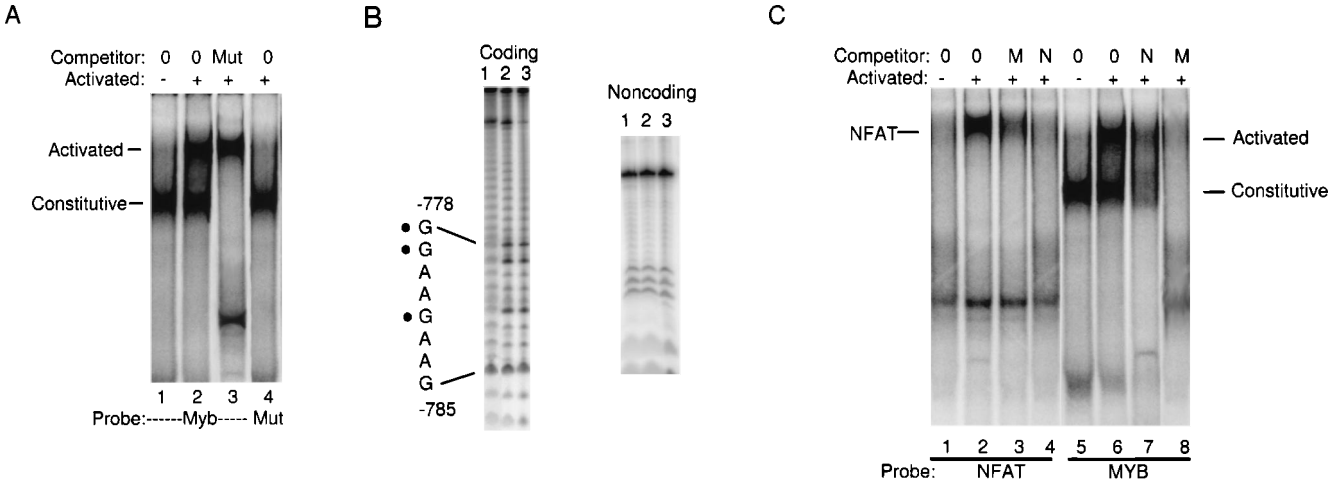

FIG. 2. Definition of the CMAT binding site by EMSA and methylation interference. (A) EMSA analysis with nuclear extracts from unactivated and activated Jurkat cells and the CMAT sequence (Myb, lanes 1 to 3). Labeled CMAT oligonucleotide was incubated with nuclear extract from unactivated (−) and activated (+) T cells in lanes 1 and 2, respectively, without competitor oligonucleotide (0); lane 3's content is the same as that of lane 2 except for the addition of a 75-fold molar excess of unlabeled oligonucleotide with the guanines at −778 and −779 changed to thymidines (Mut). The mutated CMAT oligonucleotide was labeled in lane 4. The activated and constitutive complexes are labeled. (B) Methylation interference analysis of the CMAT and constitutive complexes formed with activated Jurkat cell nuclear extract. The coding and noncoding strands are shown. The protected guanine residues in the coding strand are marked with filled circles. There are no protected guanine residues in the noncoding strand. Lanes 1 contain the CMAT complex, lanes 2 contain free oligonucleotide, and lanes 3 contain the constitutive complex. The nucleotide sequence and position in the coding strand of the c-*myb* promoter where methylation interference occurred are indicated. (C) EMSA of the NFAT (lanes 1 to 4) and c-*myb* CMAT (lanes 5 to 8) site probes with nuclear extracts from unactivated (−) and activated (+) Jurkat cells. Cross-competition studies were performed with a 75-fold molar excess of the c-*myb* CMAT site (M) (lane 3) or the NFAT site (N) (lane 7). A 20-fold molar excess of the c-*myb* CMAT site (lane 8) or the NFAT site (lane 4) was used for self-competition. The activated and constitutive complexes formed with the *myb* CMAT probe are labeled. 0, no oligonucleotide competitor added.

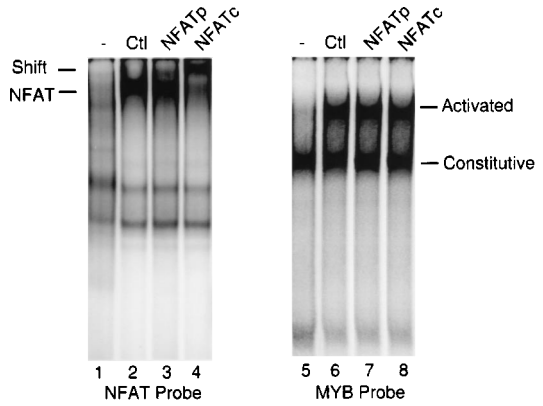

FIG. 3. Effects of antibodies on the EMSA complexes formed with the NFAT and *myb* CMAT probes and Jurkat nuclear extract. Nuclear extract from unactivated Jurkat cells (−) was used in lanes 1 and 5, and nuclear extract from activated Jurkat cells was used in lanes 2 to 4 and 6 to 8. The following antibodies were added: preimmune serum (Ctl) in lanes 2 and 6, antibody against $NFAT_p$ in lanes 3 and 7, and antibody against $NFAT_c$ in lanes 4 and 8. The NFAT complex, the supershifted NFAT complex, and the activated and constitutive *myb* CMAT complexes are labeled.

mass of 110 kDa was found for the NFAT protein. This is in agreement with previous reports (29). The CMAT protein migrated more rapidly; its estimated molecular mass was 90 kDa. We also analyzed the constitutive complex and found a molecular mass of 60 kDa for this complex. We conclude that the CMAT complex does not contain the same proteins as the NFAT complex does.

Appearance of CMAT binding activity over time. To determine when CMAT binding activity first appears after PMA and ionomycin treatment of T cells, nuclear extracts were prepared at various times after T-cell activation. An EMSA was performed with these nuclear extracts and the CMAT probe. As shown in Fig. 5A, the CMAT complex is visible at 1.5 h after treatment of the Jurkat T cells. The CMAT binding activity peaks at 8 h posttreatment, but the complex is still visible at 16 to 24 h. The same nuclear extracts were used in an EMSA with the NFAT probe. Consistent with a previous report (35), the NFAT complex appeared 15 to 30 min after the activation of Jurkat T cells. The CMAT complex appeared approximately 75 min after the NFAT complex did. This is consistent with the fact that the interleukin-2 gene is induced earlier than the c-*myb* gene during T-cell activation.

Figure 5B shows the time course of induction of c-*myb* mRNA in primary T cells during activation. An increase in the level of c-*myb* mRNA is seen at 5 h, and it peaks between 15 to 24 h. The time course of induction of c-*myb* mRNA in activated Jurkat cells is similar to that of primary T cells; however, the level of c-*myb* mRNA is higher in unactivated Jurkat cells than in resting primary T cells.

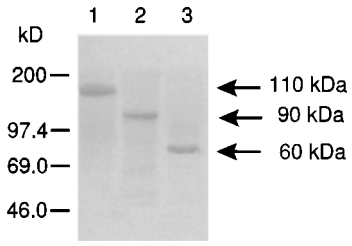

FIG. 4. Denaturing SDS-polyacrylamide gel analysis of the UV cross-linked EMSA complexes formed with Jurkat nuclear extracts and the NFAT and *myb* CMAT probes. Protein from the NFAT complex is shown in lane 1, protein from the activated *myb* CMAT complex is shown in lane 2, and protein from the constitutive *myb* complex is shown in lane 3. The positions of the molecular mass markers are shown. After correction for the bound oligonucleotide, the molecular mass of the CMAT protein is 90 kDa.

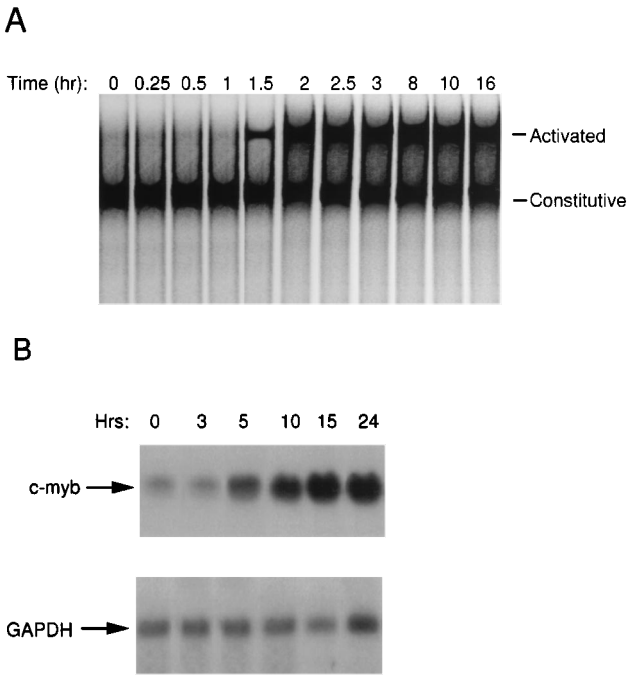

FIG. 5. Appearances of the CMAT EMSA complex and endogenous c-*myb* mRNA over time. (A) Appearance of the CMAT complex over time by EMSA. Nuclear extracts were prepared from Jurkat cells treated with PMA and ionomycin for the indicated times. The activated and constitutive complexes are labeled. (B) Time course of the induction of c-*myb* mRNA in primary T cells treated with PMA and ionomycin for the indicated times. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

New protein synthesis is required for the appearance of CMAT binding activity. We investigated the requirements for CMAT binding activity in activated Jurkat cells using anisomycin. Anisomycin inhibits ribosomal subunit association and blocks 98% of protein synthesis in Jurkat cells at 100 μM in 5 min (35). Cells were treated with anisomycin 5 min prior to the addition of PMA and ionomycin, and nuclear extracts were prepared after 8 h. An EMSA with this nuclear extract demonstrated that CMAT binding activity was inhibited by approximately 98%, but there was little effect on the constitutive complex (Fig. 6, lane 4).

The effect of the immunosuppressive drugs cyclosporin A and rapamycin on CMAT binding activity. To determine whether immunosuppressive drugs have any effect on CMAT binding activity, Jurkat cells were incubated with several different concentrations of cyclosporin A and rapamycin. At a concentration of 10 ng of cyclosporin A per ml, the CMAT complex was not visible (Fig. 7A). There was no effect on the constitutive complex. Rapamycin at concentrations above 1 ng/ml prevented the formation of the CMAT complex.

The induction of c-*myb* promoter activity with activation was also abolished by cyclosporin A at 10 ng/ml and by rapamycin at 5 ng/ml (Fig. 7B). Western blot (immunoblot) analysis of T-cell nuclear extracts confirmed that no induction of c-*myb* expression occurred with treatment with anisomycin, cyclosporin A, or rapamycin at these concentrations (data not shown).

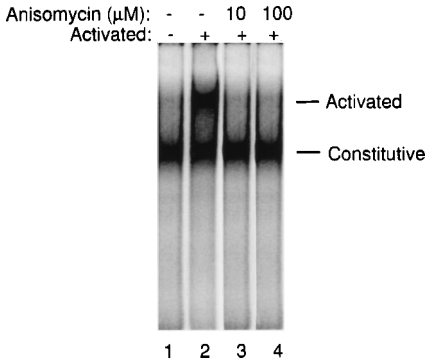

FIG. 6. Effects of inhibition of protein synthesis on CMAT complex binding activity. Jurkat cells were incubated with 10 or 100 μM anisomycin for 5 min prior to the addition of PMA and ionomycin (lanes 3 and 4). Nuclear extracts were prepared after 8 h and used in an EMSA with the c-*myb* CMAT probe.

An in vivo footprint is located over the CMAT site in activated T cells. We wished to determine whether the CMAT site was occupied in vivo in activated T cells. In vivo footprinting by ligation-mediated PCR was used to examine the region around the CMAT site in both unactivated and activated T cells. As shown in Fig. 8, there was no protection of guanine residues in the CMAT site in resting T cells. The guanine residues in the Myb I and II sites were protected. We have previously shown that these sites function as negative regulatory elements in T cells (14). In activated T cells, the Myb I and II sites were not protected, but the CMAT site was protected. The three guanine residues required for binding in vitro were protected in vivo. These findings are consistent with the results from EMSA that demonstrated that the CMAT binding complex was present only in activated T cells. We did not find any evidence for in vivo binding of the constitutively expressed 60-kDa protein in resting T cells, although from our in vitro binding studies, we know that methylation protection is not the best technique to assess binding of the constitutive complex. Treatment of the cells with cyclosporin A prior to activation resulted in no footprint over the CMAT site (Fig. 8, lane C); the footprint results were very similar to those obtained with resting T cells. These results support the contention that the in vivo footprint is due to the CMAT protein and not the constitutive protein. In vivo footprinting has been performed with both Jurkat cells and primary T cells with similar results. The footprints from primary T cells are shown in Fig. 8.

DISCUSSION

The c-*myb* gene product is required by activated T cells for entry into S phase of the cell cycle. We wished to determine the mechanism of regulation of the c-*myb* gene during T-cell activation. In transient transfection experiments into Jurkat cells, we identified a region located between positions −784 and −758 upstream of the ATG codon that mediated a 9.2-fold increase in the c-*myb* promoter activity level. We were able to identify two potential CMAT sites with conserved contact bases in the murine c-*myb* promoter. One is located 640 bp upstream of the ATG codon near two potential Myb binding sites, and the other is 570 bp upstream of the ATG codon. In the chicken c-*myb* promoter, there is a potential CMAT site 1,200 bp upstream from the ATG codon. Although c-Myb is induced in murine and chicken T cells, we have not investigated whether these potential CMAT sites play any role in the induction.

EMSA analysis with nuclear extract from activated T cells demonstrated that a new protein complex bound to the CMAT site. A more rapidly migrating constitutive complex was formed with nuclear extract from both unactivated and activated T cells. Although there is a potential Ets site in this region, we have no evidence from antibody studies that either of these proteins is an Ets family member.

Further confirmation of the function of the CMAT binding complex in activated T cells was obtained by in vivo footprint analysis. The CMAT site was occupied in activated T cells but not in resting T cells. Resting T cells express low levels of c-Myb, and we did observe that the Myb I and II sites were occupied in resting T cells but not in activated T cells. Our previous studies have shown that these sites function as negative regulatory regions in T cells (14). Although a constitutively expressed binding activity is observed in EMSA, we could find no evidence for binding to this site in resting T cells in vivo by methylation protection. Although it is possible that the in vivo footprint observed in activated T cells represents binding of the constitutively expressed protein, we believe that it is the CMAT protein because its appearance correlates with the appearance of the CMAT EMSA complex and the same three guanine residues are required for binding in vitro. In addition, no footprint is visible over the CMAT site if the T cells are

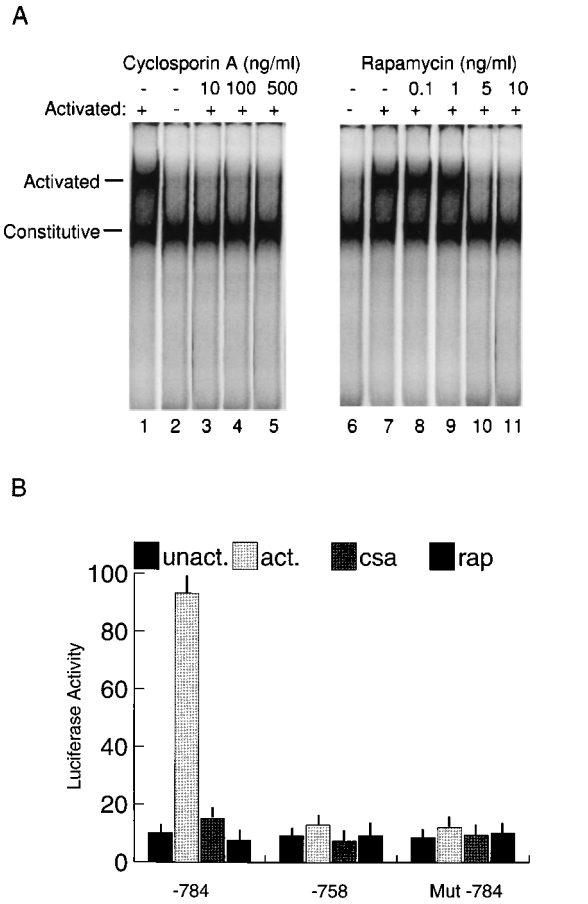

FIG. 7. Effects of the immunosuppressants cyclosporin A and rapamycin on the CMAT binding complex. (A) Jurkat cells were incubated with 10, 100, or 500 ng of cyclosporin A per ml (lanes 3 to 5) or with 0.1, 1, 5, and 10 ng of rapamycin per ml (lanes 8 to 11) for 5 min prior to the addition of PMA and ionomycin. Nuclear extracts were prepared after 8 h and used in an EMSA with the c-*myb* CMAT probe. (B) Transient transfection analysis of the c-*myb* promoter activity in the presence of cyclosporin A or rapamycin. Cyclosporin A (csa) at 100 ng/ml or rapamycin (rap) at 5 ng/ml was added 15 min prior to the addition of PMA and ionomycin. unact., unactivated; act., activated.

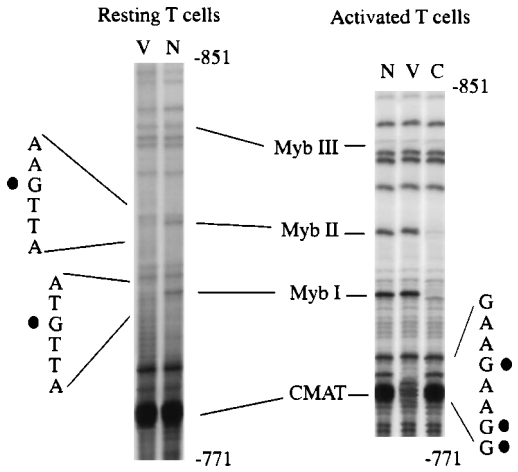

FIG. 8. In vivo footprint analysis by ligation-mediated PCR of the c-*myb* CMAT and Myb sites in resting and activated primary T cells. The coding strand is illustrated; there are no guanine residues in the noncoding strand in this region. The nucleotide numbers are relative to the translation start site. N denotes in vitro-methylated DNA, V denotes in vivo-methylated DNA, and C denotes treatment of the T cells with 100 ng of cyclosporin A per ml for 15 min prior to the addition of PMA and ionomycin. The protected guanines are marked by filled circles. Protection of guanine is 64% at position −798 (the Myb I site in resting T cells), 58% at position −812 (the Myb II site in resting T cells), 68% at positions −779 and −778, 82% at position −782 (the CMAT site in activated T cells), 69% at position −798 (the Myb I site in cyclosporin-treated cells), and 61% at position −812 (the Myb II site in cyclosporin-treated cells).

treated with cyclosporin A prior to activation. The protected guanines at −779 and −778 are required for binding of the CMAT complex and for transcriptional activity.

Although the binding sites for NFAT and CMAT show some sequence homology, we have demonstrated that the CMAT protein is distinct from the NFAT protein. There is a difference in the protected guanines (underlined) in each of the binding sites: CMAT, GAAGGAAAA; and NFAT, AGAGGAAAA. Antibodies against either $NFAT_p$ or $NFAT_c$ did not react with the CMAT complex. UV cross-link analysis demonstrated that the CMAT protein migrated more rapidly than the NFAT protein. In addition, during T-cell activation the CMAT binding complex was induced slightly later than the NFAT complex was.

New protein synthesis was required for the appearance of CMAT binding activity as demonstrated by the fact that no CMAT complex was observed in cells treated with anisomycin prior to activation. Treatment of the T cells with cyclosporin A prior to activation prevented the appearance of the CMAT complex in EMSA and the in vivo footprint over this site. Cyclosporin treatment also abolished the induction of c-*myb* promoter activity. Treatment with rapamycin had a similar effect. Thus, inhibition of either of two different pathways involved in T-cell activation can interfere with c-*myb* expression. Similar results have been reported for another nuclear oncoprotein, c-*jun* (34).

On the basis of the molecular mass and the binding site of the CMAT protein, we believe that it represents a novel inducible transcription factor. Because activated T cells require c-Myb to enter S phase of the cell cycle, identification of the factors that induce c-Myb expression is essential for an understanding of the events involved in T-cell activation. The CMAT protein appears to be another component of the T-cell activation cascade. Further characterization of this novel inducible transcription factor and a determination of the role it plays in T-cell activation are under way.

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