Identification of the Major Positive Regulators of c-myb Expression in Hematopoietic Cells of Different Lineages*

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The c-myb gene is primarily expressed in hematopoietic cells, and it is overexpressed in many leukemias. The regulation of its expression is of critical importance in hematopoietic cells. We identified the major positive regulatory sites in the 5′-flanking sequence of the human c-myb gene, and we found that the positive regulators differed in cells of different lineages. In the Molt-4 T-cell line, two Ets-like binding sites were required for the expression of c-myb. The 5′ site played a minor role in the regulation of c-myb expression, and we demonstrated that a protein of 67 kDa bound to this site. Antibodies against Ets proteins showed no cross-reactivity with this protein. We showed that Ets-1 bound to the 3′-regulatory site in the c-myb promoter by electrophoretic mobility shift assay and antibody studies. Both of these Ets-like binding sites were nonfunctional in the DHL-9 B-cell line and the K562 myeloid cell line. We identified a novel transcription factor of 50.5 kDa that was required for expression of c-myb in these cell lines.

The c-myb protooncogene is the cellular homologue of the avian myeloblastosis virus and avian leukemia virus (E26) transforming genes (1, 2). The c-myb gene product is a 75–83-kDa phosphoprotein that is predominantly expressed in hematopoietic cells and in leukemic cells. c-Myb is a sequence-specific DNA-binding protein with the ability to transactivate promoters with the specific consensus sequence PyAAC(G/T)G (3, 4). It is involved in the regulation of a number of cellular genes associated with proliferation and cell growth as well as genes that are expressed in a lineage-specific manner (4–8).

Reduction of c-myb expression results in a block to hematopoietic precursor cell proliferation (9), and homozygous c-myb mutant mice demonstrate greatly impaired fetal hepatic hematopoiesis (10). The importance of the c-myb gene product in leukemic cell proliferation is demonstrated by the inhibition of cellular proliferation by c-myb antisense oligonucleotides (11). Leukemic cells were shown to be more sensitive to this inhibitory effect than normal hematopoietic cells (12).

c-Myb plays a central role in the regulation of hematopoietic cell development, and the control of its expression is critically important. The regulation of c-myb expression is not well understood; it appears to be complex and occurs at several levels. An important mechanism for regulation of murine c-myb expression is a block to transcription elongation within the first intron of the c-myb locus, recognized as a pause site (13–15). A correlation between protein binding to the intron 1 pause site and c-myb mRNA levels has been demonstrated using DNA mobility shift assays (16).

In cotransfection studies c-Myb is involved in positive auto-regulation of the c-myb gene in hamster fibroblasts (17). We have shown that two c-Myb binding sites function as negative regulators of c-myb expression in T-cell lines (18). The Wilms’ tumor gene product, WT1, is also a negative regulator of c-myb expression in both T- and B-cell lines (19). Further studies of the regulation of expression of c-myb have shown that c-Jun and JunD are positive regulators of the c-myb promoter in hamster fibroblasts. A second promoter in the 3′ end of intron 1 has been identified (20), and there are regulatory elements in the first intron as well (21, 22).

The Ets family of transcription factors is comprised of proteins that bind to the core sequence GGA (for reviews, see Janknecht and Nordheim (23) and Wasylyk et al. (24)). The Ets-1 protein, which is predominantly expressed in B and T lymphocytes, has been found to act as a transcriptional activator of several T-cell-specific genes, including the T-cell receptors α and β and CD4 (25–29). Mutation of the two guanines in the core binding area results in a loss of 95% of the promoter activity of the T-cell receptor α gene (25). Studies of lymphocytes that lack Ets-1 have shown a significant increase in apoptosis and a subsequent decrease in the number of T-cells (30, 31).

In this report we have characterized the major positive regulatory sites in the human c-myb 5′-flanking sequence. In the T-cell line, Molt-4, we find that two Ets-like binding sites are required for c-myb expression. These Ets binding sites are nonfunctional in both a B- and myeloid cell line, and we find that a novel transcription factor is required for c-myb expression in these cell lines.

MATERIALS AND METHODS

Cell Lines—Molt-4, DHL-9, Jurkat, Nalm-6, and K562 cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 units/ml penicillin and 50 μg/ml streptomycin. For activation of Jurkat cells, 20 ng of phorbol myristate acetate/ml and 1 μM ionomycin were added for 8 h prior to nuclear extract preparation.

Construction of Reporter Plasmids—The construction of the human c-myb promoter luciferase construct has been described previously (18, 19). A deletion of the 5′-flanking region was made using a unique BamHI-restriction site located at −910 from the ATG codon. Further deletions were made using Bal-31 endonuclease. Deletions were made at 30-nt intervals from BamHI-linearized plasmid. The deleted end was then treated with Klenow polymerase, cut with HindIII, and separated by gel electrophoresis. A vector was prepared by removing the HindIII/Ncol (Ncol site treated with Klenow polymerase) fragment of the lucif-
The oligonucleotide sequences used were (mutated base pairs are in **boldface** type and position in the **c-myb** promoter in parentheses. 

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**TABLE I**

**Oligonucleotides**

| Oligonucleotide       | Sequence\(^a\)          |
|-----------------------|-------------------------|
| 5′ T-cell site        | (−785) GAAGAGGAAAAAACC  |
| 3′ T-cell site        | (−476) TTTCTAGAAGGGGGCC  |
| Mut 5′ T-cell site     | (−785) GAAGAAATTTTTTTTTGG |
| Mut 3′ T-cell site     | (−785) TTTCTAGAAGGGGGCC  |
| CMAT site             | (−787) AAGAGGAAAAAATTTTAC   |
| Consensus Ets-1 site  | TGTTCAAGAGTTTCCC          |
| Irrelevant oligo      | TCTTCTTGTAGAACC           |
| B-myeloid cell site   | (−290) AAGGGGCCGTTTCGATCT  |
| Mut B-myeloid site     | (−290) AAGGGCGGCTACACCTGCA |

\(^a\) Mutated bases in boldface type and position in the **c-myb** promoter in parentheses.

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The oligonucleotide sequences used were (mutated base pairs are in boldface type): 5′ **T-cell site**, GAAGAGGAAAAAACC; 3′ **T-cell site**, TTTCTAGAAGGGGGCC; **Mut 5′ T-cell site**, GAAGAAATTTTTTTTTGG; **Mut 3′ T-cell site**, TTTCTAGAAGGGGGCC; **CMAT site**, AAGAGGAAAAAATTTTAC; **Consensus Ets-1 site**, TGTTCAAGAGTTTCCC; **Irrelevant oligo**, TCTTCTTGTAGAACC; **B-myeloid cell site**, AAGGGGCCGTTTCGATCT; **Mut B-myeloid site**, AAGGGCGGCTACACCTGCA. Mutants were screened by restriction enzyme analysis and subsequently sequenced using the Fmol sequencing kit (Promega Biotech Inc.) or the Sequenase kit (U.S. Biological Corp.). Compressions were resolved with dITP or Fmol sequencing kit (Promega Biotech Inc.).

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**RESULTS**

**Location of the Positive Regulatory Regions of the c-myb Promoter in T-, B-, and Myeloid Cell Lines**—Transient transfections with a number of deletion constructs of the **c-myb** promoter were performed in Molt-4 cells. As shown in Fig. 1A, there are two major regions of positive regulatory activity. These are located between −784 and −758 (8.5-fold change in activity) and −474 and −455 (36-fold change in activity). Comparison of the sequence in each of these regions revealed potential Ets binding sites with the core sequence GGA.

Since **c-myb** is expressed in all hematopoietic cells, we wished to determine whether the same promoter regions were involved in positive regulation in **B-** and myeloid cell lines. We found that the two active regions in T-cells showed no activity in either DHL-9 B-cells (Fig. 1B) or K562 myeloid cells (Fig. 1C). The major positive regulatory region in each of these cell lines was located between −292 and −271. This region mediated a 12-fold change in activity in DHL-9 cells and a 5-fold change in K562 cells. Comparison of the sequence in this region with both of the positive regulatory regions identified in T-cells revealed no similarities, and no known transcription factor binding sites were identified in this region.

**Characterization of the Protein That Binds to the 5′ Site in Molt-4 Cells**—**c-myb** was expressed in an oligonucleotide that encompassed the 5′ region of positive regulatory activity in Molt-4 cells. As shown in Fig. 2A, lane 1, two specific complexes were formed. Competition with a 100-fold molar excess of unlabeled cold oligonucleotide prevented formation of the two complexes (Fig. 2A, lane 2) while competition with an irrelevant oligonucleotide (Fig. 2A, lane 5) had no effect. To determine whether the two guanine residues at −779 and −778 were required for binding, we used an oligonucleotide with the two guanine residues replaced by thymines. This oligonucleotide did not form the same two complexes as the wild-type oligonucleotide did (Fig. 2A, lane 6), and it did not compete against the two complexes formed by the wild-type
A are constructs with the 5' mutated (guanines at two complexes. Jurkat cells, and a 60-kDa protein was constitutively expressed. We wished to determine whether the EMSA complexes formed in Molt-4 cells were similar to the ones we had observed in Jurkat cells. As shown in Fig. 2B, the mobility of the EMSA complexes formed with Molt-4 nuclear extract differed from that of the complexes formed with either unactivated or activated Jurkat nuclear extracts (Fig. 2B, lanes 1–3 and 4–6). This was true with both the 5' site probe or the CMAT site probe. (The CMAT site probe is 7 base pairs longer than the 5' site probe.)

To determine the molecular mass of the protein that bound to the 5' site, EMSA followed by UV cross-linking was performed. Both the upper and lower EMSA complexes yielded a single protein that migrated at 79 kDa (Fig. 3, lanes 1 and 2). When corrected for the bound oligonucleotide, the molecular mass was 67 kDa. The corrected molecular mass of the CMAT protein was 90 kDa (Fig. 3, lane 3) and that of the constitutively expressed protein was 60 kDa (Fig. 3, lane 4). Because the binding site contains a potential Ets sequence, we performed a Western blot on the UV cross-linked protein with an antibody that cross-reacts with many Ets proteins. The cross-linked proteins were not reactive with this antibody. In addition, no supershifted complex was seen when this antibody was used in EMSA with either the 5' or CMAT site.

Methylation interference was performed to determine which guanine residues contacted the protein that bound to the 5' site. On the coding strand, methylation of the two guanines in the GGA sequence (−779 and −778) interfered with binding (Fig. 4). Methylation of the other two guanine residues had no effect on binding of the protein. There are no guanines in this region of the noncoding strand.

To confirm the importance of this site, we mutated the guanines at −779 and −778 in the c-myb promoter-luciferase construct. The activity of this construct was 9.5-fold lower than the construct with the wild-type site (Fig. 1A, Mut 5').

Characterization of the Protein That Binds to the 3' Site in Molt-4 Cells—EMSA was performed with an oligonucleotide of

2 B. Feeley and L. M. Boxer, unpublished data.
Fig. 3. Denaturing SDS-polyacrylamide gel analysis of the UV cross-linked EMSA complexes formed with Molt-4 and Jurkat nuclear extracts and the 5’ site. The positions of the molecular mass markers are shown. After correction for the bound oligonucleotide, the molecular mass of the protein in Molt-4 nuclear extract is 67 kDa.

Fig. 4. Methylation interference analysis of the two EMSA complexes formed with Molt-4 nuclear extract and the 5’ site. 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. The lane labeled L is the lower EMSA complex (more rapidly migrating), the lane labeled U is the upper EMSA complex, and the lane labeled F is unbound oligonucleotide.

the positive regulatory sequence located more 3’ in the c-myb promoter. With nuclear extracts from Molt-4 cells, two complexes were visible (Fig. 5A, lane 4). Further studies demonstrated that only the slower migrating complex was a specific one (marked with an arrow in Fig. 5). Competition with a 100-fold molar excess of unlabeled oligonucleotide prevented formation of only the slower migrating complex (Fig. 5A, lane 5). Competition with an irrelevant oligonucleotide or the 5’ c-myb site did not prevent formation of either complex (Fig. 5A, lanes 9 and 7, respectively). EMSA with an oligonucleotide in which the two guanines at -469 and -468 were changed to thymines revealed only the nonspecific complex. 2 The mutated oligonucleotide did not compete against the slower migrating complex (Fig. 5A, lane 8). We used an Ets-1 consensus sequence as a competitor and found that it prevented formation of the slower migrating complex (Fig. 5A, lane 6). When the Ets-1 sequence was labeled and used in EMSA with Molt-4 nuclear extract, two complexes were visible. The faster migrating complex co-migrated with the specific complex formed with the c-myb 3’ site (Fig. 5A, lanes 1 and 4). In addition, the c-myb 3’ site competed against the labeled Ets-1 consensus site in EMSA (Fig. 5A, lane 3).

An antibody that is specific for Ets-1 was used in EMSA experiments with the 3’ c-myb site. As shown in Fig. 5B, lane 2, a supershifted complex was formed and the original complex disappeared when this antibody was added. This antibody also supershifted the complexes formed with the consensus Ets-1 oligonucleotide (Fig. 5, lane 5), and a complex formed with a truncated Ets-1 protein (Fig. 5B, lane 8).

UV cross-linking followed by SDS denaturing gel analysis of the specific complex formed with the 3’ site and the two complexes formed with the Ets-1 consensus site revealed a single protein of the same molecular mass in each case. After correction for the bound oligonucleotide, the molecular mass was 55 kDa, which is the size reported for Ets-1. 2 Methylation interference demonstrated that the two guanines at -470 and -469 in the coding strand were required for binding of this protein (Fig. 6). The guanines at -474 and -472 in the noncoding strand were also required for protein binding (Fig. 6).

We changed the two guanines at -470 and -469 to thymines in the c-myb-promoter luciferase construct. The activity of the promoter was decreased by 18-fold compared to the wild-type -523 promoter (Fig. 1A, Mut 3’ -523) and by 25-fold compared to the wild-type -474 promoter.

Characterization of the Protein That Interacts with the c-myb-positive Regulatory Site in DHL-9 and K562 Cells—EMSA analysis with an oligonucleotide that encompassed the positive regulatory sequence from the c-myb promoter in DHL-9 cells revealed a single specific complex (Fig. 7A, lanes 1 and 4). A 100-fold molar excess of unlabeled oligonucleotide prevented formation of this complex (Fig. 7A, lane 2). We changed the guanines at -280 and -278 to adenines and found that this sequence no longer competed against the wild-type sequence in
EMSA (Fig. 7A, lane 3). The specific complex was not seen in EMSA with the labeled mutant sequence (Fig. 7A, lane 5).

EMSA with K562 nuclear extract revealed a single specific complex with the same mobility as the one formed with DHL-9 nuclear extract (Fig. 7B, lanes 1 and 4, and C, lanes 1 and 2). The mutant sequence did not compete against this complex (Fig. 7B, lane 3) or form a similar complex when labeled (Fig. 7B, lane 5). EMSA with nuclear extracts from two T-cell lines and another B-cell line revealed that a complex of identical mobility was formed with the wild-type oligonucleotide (Fig. 7C).

EMSA followed by UV cross-linking and denaturing SDS gel electrophoresis was performed to determine the molecular mass of this protein. A single protein of 63.5 kDa was observed (see Fig. 8 for results with DHL-9 cells). When corrected for the bound oligonucleotide, the molecular mass of this protein was 50.5 kDa.

Methylation interference was performed to determine the guanine residues that contact the protein (Fig. 9). On the coding strand, methylation of guanines −282, −280, and −278 interfered with binding, and on the noncoding strand, methylation of guanines −279 and −277 interfered with binding.

We changed the guanines at −280 and −278 to adenines in the c-myb promoter-luciferase construct and found that the promoter activity decreased by 14-fold in DHL-9 cells (Fig. 1B, Mut) and in K562 cells by 5.7-fold compared to the wild-type −455 construct (Fig. 1C, Mut −455) and by 6-fold compared to the wild-type −292 construct (Fig. 1C, Mut −292).

**DISCUSSION**

We have identified the major positive regulatory regions of the c-myb promoter in T-, B-, and myeloid cell lines. Although c-myb is expressed in all hematopoietic lineages, the regulation in T-cells is different from that in B- and myeloid cells. We have previously shown that c-Myb negatively regulates the c-myb promoter only in T-cells (18). We have also shown that WT1 is a negative regulator of c-myb expression in both T- and B-cell lines, but that a different WT1 site is functional in each cell lineage (19).

There are two major positive regulators of c-myb expression in T-cells. One is a 67-kDa protein that appears to be a novel transcription factor. Although the recognition sequence contains a potential Ets binding site, we have not been able to demonstrate that this protein is a member of the Ets family. Our studies have shown that the 67-kDa protein does not comigrate with either CMAT or the 60-kDa protein found in Jurkat cells on UV cross-linking SDS gel analysis. Both the 67-kDa protein and CMAT contact the two guanines at −779 and −778 and the CMAT protein also contacts guanine −782, while the constitutively expressed protein does not contact any of these guanine residues (35). In Jurkat cells, the 5′ region appears to be important only for the increase in expression seen with activation and there is very little constitutive activity associated with this region (35). The 3′ region is the major positive regulatory region in Jurkat cells.3

The two c-Myb binding sites between −815 and −793 are responsible for the negative regulatory activity seen in Fig. 1A between constructs −910 and −784 (18). The WT1 site between −630 and −621 is responsible for the negative regulatory activity seen in Fig. 1A between the −758 and −594 constructs (19). There appears to be another region of negative regulatory activity between −594 and −523; we have not characterized this region further. It is possible that another transcription factor binds near the Ets site in the region from −523 to −474. We noted a small decrease in activity when this region was

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3 S.-C. Phan and L. M. Boxer, unpublished data.

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**Fig. 6.** Methylation interference analysis of the EMSA complex formed with the 3′ site and Molt-4 nuclear extract. The coding and noncoding strands are shown. The protected guanine residues are marked with filled circles. Lane F is unbound oligonucleotide, and lane B is bound oligonucleotide.

**Fig. 7.** EMSA of the oligonucleotide of c-myb promoter sequence from −290 to −271 with DHL-9 and K562 nuclear extracts. A. EMSA with DHL-9 nuclear extract. Lane 1 is the labeled −290 to −271 oligonucleotide, lanes 2–4 are the same as lane 1 except for the addition of a 100-fold molar excess of unlabeled −290 to −271 oligonucleotide (Self), an oligonucleotide with the guanines at −280 and −278 changed to adenines (Mut), and an irrelevant oligonucleotide (Irr). Lane 5 is the labeled mutant oligonucleotide. The specific complex is labeled with an arrow, and the nonspecific complexes are labeled NS. B. EMSA with K562 nuclear extract. The lanes are the same as in A. C. EMSA with the −290 to −271 oligonucleotide and different hematopoietic cell line nuclear extracts. Lane 1 is nuclear extract from DHL-9 cells, lane 2 is K562 cells, lane 3 is Jurkat T-cells, lane 4 is Molt-4 cells, and lane 5 is Nalm-6 B-cells.
deleted, and mutation of the Ets site did not decrease the promoter activity to the base-line level when this region was present (Fig. 1A, Mut 3′–523).

Although we demonstrated that Ets-1 bound to the 3′-regulatory site in vitro, it is possible that a different Ets family member is a positive regulator of the c-myb promoter through this site. From the results of chimeric mice with Ets-1-deficient lymphocytes, it has been proposed that Ets-1 serves to keep T-cells as a resting, G0 cell cycle stage (30, 31). It would thus seem less likely that Ets-1 would positively regulate the expression of a gene such as c-myb since the c-Myb protein is a positive regulator of cell proliferation. However, Ets-1-deficient T-cells were also very susceptible to cell death (30, 31). While the c-Myb protein is involved in the positive regulation of cell proliferation, it also plays a role in the induction of programmed cell death if conditions are not right for proliferation (36). It is interesting to note that Ets-1 was thought to regulate the T-cell receptor gene α by binding to a specific site in the enhancer (25). In Ets-1-deficient T-cells, the T-cell receptor α gene is expressed (30, 31), suggesting that Ets-1 is not required for this expression or that another Ets protein can replace Ets-1. It is possible that there are several Ets proteins in T-cells that play overlapping roles in the regulation of a set of genes expressed in T-cells.

The 5′- and 3′-positive regulatory sites identified in Molt-4 T-cells are not functional in either the DHL-9 B-cell line or the K562 myeloid cell line. Instead a transcription factor binding to a sequence from –282 to –277. Based on the molecular mass and the DNA recognition sequence, we believe that this is a novel transcription factor. It is expressed in different hematopoietic lineages, but this site in the c-myb promoter is nonfunctional in T-cell lines. In the DHL-9 B-cell line, the WT1 site between 455 and 447 serves as a negative regulatory element (19). Neither of the WT1 sites is functional in the myeloid cell line, K562.

We did not find any evidence for a functional role for either the E2F site at –259 or the AP-1 site at –370 in the human c-myb promoter. The activity of the E2F site was described in a glioblastoma cell line (37), and the activity of the AP-1-like site was described in fibroblasts (38). It is interesting to note that the two binding sites for the zinc finger protein MZF1 were not functional in Molt-4, DHL-9, or K562 cells. These sites appear to be negative regulators of c-myb expression and are required during hematopoietic cell development from embryonic stem cells (39). It is possible that these sites are no longer functional in more differentiated hematopoietic cells.

In summary, we have identified the major positive regulatory sites in the human c-myb 5′-flanking sequence. In Molt-4 T-cells, two Ets-like sites are required, although we have no evidence that an Ets protein binds to the 5′ site. In both DHL-9 B-cells and K562 myeloid cells, the Ets-like sites are nonfunctional. A novel transcription factor is involved in the regulation of c-myb expression in these cell lines.

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