Repression of the c-myb Gene by WT1 Protein in T and B Cell Lines

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The c-myb gene is primarily expressed in immature hematopoietic cells, and it is overexpressed in many leukemias. We have investigated the role of negative regulatory sites in the c-myb promoter in the Molt-4 T cell line and in the DHL-9 B cell line. A potential binding site for either the EGR-1 or WT1 protein was identified by in vivo footprinting in the 5′-flanking region of c-myb in a region of negative regulatory activity in T cells. We showed by electrophoretic mobility shift assay and electrophoretic mobility shift assay Western that WT1, EGR-1, and Sp1 bound to this site. A mutation of this site which prevented protein binding increased the activity of the c-myb promoter by 2.5-fold. In the DHL-9 B cell line, this site was nonfunctional; however, we found a potential EGR-1-WT1 site located more 3′ in a region of negative regulatory activity. We showed that WT1, EGR-1, and Sp1 bound to this site, and that mutation of this site increased the activity of the c-myb promoter by 3.2-fold. Cotransfection of a WT1 expression vector repressed the activity of the c-myb promoter in both cell lines, and this repression was relieved when the EGR-1/WT1 sites were removed. Cotransfection of either an EGR-1 or Sp1 expression vector had no significant effect on the activity of the c-myb promoter. We conclude that WT1 is a negative regulator of c-myb expression in both T and B cell lines.

The c-myb protooncogene is the cellular homologue of the avian myeloblastosis virus and avian leukemia virus (E26 transforming genes (1, 2). 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). Reduction of c-myb expression results in a block to hematopoietic precursor cell proliferation (5), and homozygous c-myb mutant mice demonstrate greatly impaired fetal hepatic hematopoiesis (6). 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 (7). Leukemic cells were shown to be more sensitive to this inhibitory effect than normal hematopoietic cells (8).

The central role that c-Myb plays in the regulation of hematopoietic cell development has fueled research into the regulation of its expression. The regulation of c-myb expression appears to be complex and occurs at several levels. An important mechanism for regulation of mouse c-myb expression is a block to transcription elongation within the first intron of the c-myb locus, recognized as a pause site (9–11). A correlation between protein binding to the intron 1 pause site and c-myb mRNA levels has been demonstrated using DNA mobility shift assays (12).

It has been shown that in vitro translated c-Myb can bind to Myb binding sequences found in the c-myb 5′-flanking region and that in cotransfection studies c-Myb is involved in positive autoregulation of the c-myb gene in hamster fibroblasts (13). Recent studies conducted in mouse T cell lines suggest that murine c-myb expression is dependent on a GC-rich sequence of the 5′-flanking region and that the c-myb promoter is functional in diverse T cell lines (14). We have shown that two Myb binding sites function as negative regulators of c-myb expression in T cell lines (15). 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 recently (16).

The putative Wilms' tumor suppressor gene (wt1) encodes a zinc finger DNA-binding protein that functions as a transcriptional repressor (17, 18). The WT1 protein binds to the target sequence GCCGGGGCG which is also recognized by the zinc finger transcription factors EGR-1, EGR-2, and EGR-3. The wt1 gene is mainly expressed in the developing kidney, testis, ovary, and spleen (19).

In this report we have characterized a site identified by in vivo footprinting in T cells. We show that WT1 binds to this site in vitro and, by cotransfection experiments, that WT1 negatively regulates c-myb expression. In the DHL-9 B cell line, a similar sequence located farther downstream was protected in vivo. We found that WT1 bound to this site in vitro, and, in cotransfection experiments, it negatively regulated c-myb expression in B cells through this site.

MATERIALS AND METHODS

Cell Lines—Molt-4 and DHL-9 cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum, 2 mm l-glutamine, and 0.5 μg/ml penicillin/streptomycin.

Construction of Reporter Plasmids—The HindIII-BamHI fragment of the luciferase reporter vector was derived from pSV232AL-A5† (obtained from D. Helinski, UCSD). The fragment was cloned into pUC18 and subsequently recut as a HindIII-KpnI fragment and ligated into the multiple cloning site of Bluescript II KS †. The 2.3-kilobase EcoRI-EcoRI 5′-flanking region of human c-myb (a kind gift from R. Dalla-Favera, Columbia) was ligated into the EcoRI site 5′ of the luciferase gene in Bluescript. To remove a confounding ATG site, the HindIII-NcoI fragment was removed, HindIII linkers were added, and the plasmid was religated. 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 Bal31 endonuclease. Deletions were made at 30-s intervals from BamHI-linearized plasmid. The deleted end was then treated with Klenow polymerase, cut with HindIII, and sep-
arated by gel electrophoresis. A vector was prepared by removing the HindIII/NorI (NorI site treated with Klenow polymerase) fragment of the luciferase Bluescript construct, and the fragment and luciferase vector were ligated. Other deletions at specific locations were made by the polymerase chain reaction (PCR)1 and confirmed by sequence analysis.

Mutagenesis of the WT1 binding sites was achieved using a technique previously described by Higuchi (20). Mutants were screened by restriction enzyme analysis and subsequently sequenced using the fmol Sequencing Kit (Promega) or the Sequenase Kit (U. S. Biochemical Corp.). Compressions were resolved with dTP or daesa-dGTP. The oligonucleotide sequences used for PCR primers are (mutated bases are in boldface).

5′ WT site: CCGCTTTGCCACCCCTTAGACGTG
3′ WT site: GCCGGCGCCACCTTGGCCACCC

The WT1, EGR-1, and Sp1 expression vectors consisted of the full-length coding region of each gene under the control of the cytomegalovirus immediate-early promoter (21).

In Vivo Dimethylsulfate Treatment and DNA Isolation—DNA isolation after dimethylsulfate treatment was performed as described previously (22-24). Cleavage with piperidine was performed according to the Maxam-Gilbert procedure (25).

Ligation-mediated PCR—Chemically modified and cleaved DNA was then subjected to amplification by ligation-mediated PCR essentially as described by Mueller and Wold (26), Pfeifer et al. (27), and Garrity and Wold (28). 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. Footprinting on each strand was repeated at least four times with genomic DNA samples prepared from at least three separate batches of dimethylsulfate-treated cells. The primers used for PCR were synthesized in an Applied Biosystems 380B DNA synthesizer and purified on Applied Biosystems oligonucleotide purification cartridges. The common linkers used were GCGGTGACCCGGGAGATCTGAATTC and CAGGCGGGCGGGCATT.

Quantitation of footprints was performed as described previously (22) with ImageQuant software version 4.15 (Molecular Dynamics). Percent quantitation of footprints was performed as described previously (22).

RESULTS

Identification of in Vivo Footprints over EGR-1/WT1 Sites—In vivo footprinting by ligation-mediated PCR was performed on the c-myb 5′-flanking region in Molt-4 T cells and in DHL-9 cells. A protected region extended from −630 to −621 in Molt-4 cells (all numbers are relative to the translation start site) (Fig. 1). This sequence is identical with the EGR-1/WT1 consensus binding site. In the B cell line, DHL-9, this region did not show any protection. A similar sequence extending from −455 to −446 was protected (Fig. 2). This site differs by one base from the EGR-1/WT1 consensus binding sequence. We also found that the −455 to −446 sequence was not protected in Molt-4 cells.

The Region of the c-myb Promoter Containing the EGR-1/WT1 Site Functions as a Negative Regulator in Molt-4 T Cells—To determine whether the regions identified by in vivo footprinting correlated with functional activity of the c-myb promoter, a number of deletion constructs of the 5′-flanking region of c-myb were made and linked to the luciferase gene. The activities of several of these constructs in Molt-4 cells are shown in Fig. 3A. A region of negative regulatory activity was observed between −632 and −594. The EGR-1/WT1 binding site is located in this region. To determine whether this site was responsible for the negative activity of this region, we made a construct with a mutated EGR-1/WT1 site. As shown in Fig. 3B, the activity of the promoter construct with the mutated EGR-1/WT1 site increased by approximately 2.5-fold. The increase in activity with mutation of the EGR-1/WT1 site accounted for essentially all of the negative regulatory activity in this region.

1 The abbreviations used are: PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay.
WT1 Represses c-myb Expression

The 3' EGR-1/WT1 Site in the c-myb Promoter Functions as a Negative Regulator in DHL-9 B Cells—The region between −632 and −594 had no functional activity in DHL-9 cells. A negative regulatory region located between −455 and −444 was identified (see Fig. 4A). The site which was protected in DHL-9 cells in vivo is located in this region. To determine whether this was a functional site, the sequence was mutated. As shown in Fig. 4B, the activity of the promoter construct with the mutated site increased by approximately 3.2-fold. Mutation of the EGR-1/WT1 site accounted for all of the negative regulatory activity in the region from −455 to −444 in DHL-9 cells.

Both Sequences Bind the WT1 Protein—Because WT1 has been shown to act as a transcriptional repressor, we investi-

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expression vector at a ratio of 1:1. As shown in Fig. 7A, cotransfection of the WT1 expression vector in Molt-4 cells repressed the c-myc promoter until the WT1 site was removed in the −594 construct. Similarly, in DHL-9 cells, cotransfection of the WT1 expression vector repressed the c-myc promoter until the functional WT1 site was removed in the −444 construct. These results demonstrate that increased levels of WT1 protein repress the activity of the c-myc promoter. As shown in Fig. 7C, cotransfection of either an EGR-1 or Sp1 expression vector had no significant effect on the c-myc promoter activity in Molt-4 cells. Similar results were obtained in the DHL-9 cell line (Fig. 7D).

DISCUSSION

The c-myc gene is expressed primarily in immature hematopoietic cells. We have shown previously that two Myb binding sites in the 5'-flanking region are negative regulators of c-myc expression in T cells (15). We have now characterized another negative regulatory region of the c-myc promoter and have shown by cotransfection experiments that WT1 is a negative regulator of c-myc expression in T cells. Mutation of this site increased c-myc expression by 2.5-fold. Mutation of the two Myb binding sites increased expression by 1.85-fold (15); together, the deletion of the Myb and WT1 sites brings the c-myc promoter activity to its maximal value. These results suggest that these sites are major negative regulatory sites for the c-myc promoter in T cells.

We have shown by EMSA and Western analysis that the WT1 protein binds to this site in the c-myc promoter. Three bands of altered mobility are observed with both WT1 site oligonucleotides. We have demonstrated that WT1 protein is found in the fastest migrating band. The EGR-1 protein is located in the intermediate complex, and Sp1 is found in the slowest migrating EMSA complex.

The 5' EGR-1/WT1 site was not functional in the DHL-9 B cell line. A region of negative regulatory activity was mapped further 3' in the promoter. A potential binding site for EGR-1/
WT1, which differed from the consensus sequence by one base, was located in this region. We demonstrated that WT1, EGR-1, and Sp1 bound to this site by EMSA and by Western analysis. This site is identical with the B2 site in the insulin-like growth factor II gene which binds purified WT1 protein (33). Increased expression of WT1 in both DHL-9 and Molt-4 cells led to repression of the c-myc promoter while cotransfection of EGR-1 or Sp1 expression vectors had little effect.

In vivo footprinting by ligation-mediated PCR demonstrated that the 5′ EGR-1-WT1 site was protected in Molt-4 cells and that the 3′ EGR-1-WT1 site was protected in DHL-9 cells. We had initially thought that the EGR-1 protein might be responsible for the in vivo footprints because it is a transcriptional activator and the c-myc gene is expressed at high levels in both cell lines. Our transient transfection experiments demonstrated that both the 5′ and 3′ EGR-1-WT1 sites were negative regulatory elements in the Molt-4 and DHL-9 cell lines, respectively. Although we cannot be certain that the in vivo footprints represent binding of WT1 and not EGR-1 or Sp1 to these sites, the fact that the sites are negative regulatory elements makes it more likely that WT1 is responsible for this activity. In addition, we demonstrated that cotransfection of a WT1 expression vector with the c-myc promoter-luciferase constructs caused repression of luciferase activity while EGR-1 and Sp1 expression vectors had no significant effect.

It has been shown previously that WT1 represses a platelet-derived growth factor gene (34), the colony-stimulating factor 1 gene (35), the transforming growth factor β1 gene (36), the insulin-like growth factor I receptor (37), and the insulin-like growth factor II gene (33, 38) at the transcriptional level. It is interesting to note that WT1 has been shown to regulate negatively genes that encode positive regulators of cell growth. Both WT1 and c-Myc are expressed in immature hematopoietic cells. We have now demonstrated by cotransfection experiments that the c-myc gene, which also encodes a protein involved in cell proliferation, is negatively regulated by the WT1 protein in T and B cell lines.

In summary, we have characterized several negative regulatory factors involved in the control of c-myc expression in both B and T cell lines. In T cells, both Myb and WT1 are negative regulators, while in B cells WT1 is a negative regulator of c-myc expression. We have preliminary evidence that the positive regulators of c-myc expression differ in T versus B cell lines as well.

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