Differential Regulation of c-Myb-induced Transcription Activation by a Phosphorylation Site in the Negative Regulatory Domain*

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Mark R. Miglarese†‡§, Alison F. Richardson‡, Natasha Aziz¶, and Timothy P. Bender†**
From the †Departments of Microbiology and ¶Pharmacology, University of Virginia, Charlottesville, Virginia 22908

The c-myb proto-oncogene encodes a highly conserved 75–89-kDa transcription factor that contains three functional domains, an amino-terminal DNA binding domain (DBD), a central acidic transactivation domain, and a carboxyl-terminal negative regulatory domain (NRD). Two acute transforming retroviruses, avian myeloblastosis virus and the E26 leukemia virus, transduced portions of c-myb and encode Myb proteins that are truncated in both the DBD and the NRD. Several conserved potential sites for phosphorylation by proline-directed serine/threonine protein kinases reside in or near the NRD, suggesting that phosphorylation might play a role in regulating c-Myb. We have previously demonstrated that serine 528, located in the NRD, is a target for phosphorylation by proline-directed serine/threonine protein kinases and that serine 528 substitution, suggesting that phosphorylation might mediate its effect on the transcription transactivation of c-Myb to transactivate a series of cellular target promoters. We have tested the ability of S528A Myb to transactivate a synthetic promoter containing five copies of the mim-1A Myb-responsive element and a minimal herpes tk promoter. We have tested the ability of S528A Myb to transactivate a series of cellular target promoters and report that the serine to alanine substitution increased the ability of Myb to activate transcription from the CD34 promoter but not the c-myb or mim-1A promoters. This suggests that phosphorylation of serine 528 may differentially regulate c-Myb activity at different promoters. The DNA binding and multimerization activities of c-Myb appear to be unaffected by the S528A substitution, suggesting that phosphorylation of serine 528 may mediate its effect on the transcription transactivating activity of c-Myb by regulating interactions with other proteins.

The c-myb proto-oncogene encodes a nuclear DNA binding protein that functions as both a transcriptional activator and repressor (1, 2). Expression of c-myb is detected primarily in hematopoietic tissue although c-myb mRNA has been reported in primary chicken embryo fibroblasts (3), smooth muscle cells (4), and several non-hematopoietic human tumors including neuroblastoma (5), colon carcinoma (6), small cell lung carcinoma (7), and breast carcinoma (8). The down-regulation of c-myb expression is associated with hematopoietic maturation, and in each hematopoietic lineage examined the expression of c-myb mRNA and protein is highest in immature normal tissue and tumor cell lines (1). This pattern of expression led to the hypothesis that the c-myb gene product would play a role in regulating hematopoiesis, and this has been supported experimentally in several systems. First, c-myb antisense oligodeoxynucleotides inhibit both erythroid and myeloid colony formation in vitro (9). Second, murine erythroleukemia cells and leukemic myeloid cells stably transfected with either constitutively expressed or inducible c-myb expression vectors were blocked in their ability to terminally differentiate in response to chemical inducing agents in vitro (10, 11). Third, transgenic mice lacking a functional c-myb gene developed normally to day 14 and after that they died with severely disrupted patterns of erythroid and myeloid development (12). Finally, Badiani et al. (13) demonstrated that transgenic mice carrying a dominant interfering c-myb allele under the control of a CD2 promoter had disrupted patterns of T-lymphogenesis.

The murine c-Myb protein is a 636-amino acid peptide of approximately 75 kDa (1). Chicken c-myb was identified as the cellular homologue of two viral myb genes carried by the avian myeloblastosis virus (AMV)1 and the E26 virus (14, 15). These viruses independently transduced portions of the c-myb gene, deleting both amino- and carboxyl-terminal coding sequences. Three major functional domains have been defined on the c-Myb protein: 1) a DNA binding domain, 2) an acidic transactivation domain, and 3) a negative regulatory domain. The DNA binding domain (DBD) is located near the amino-terminal end of the protein and is a highly conserved region consisting of three imperfect repeats (R1–3) of 50–52 amino acids each. The DNA binding domain defines a family of Myb-related proteins that have been identified in humans, mice, chickens, Drosophila, yeast, slime molds, and plants (1). Pienckenap et al. (16) demonstrated that Myb bound DNA specifically to the consensus sequence PyAAC(G/T)G. This led to the finding that Myb could transactivate transcription when this element, referred to as the Myb-responsive element (MRE), was ligated to various test promoters (17, 18). Although the DBD provides the only sequences required for DNA binding, it does not appear to activate transcription by itself. Deletion analysis and linker scanning of both c- and AMV v-Myb led to the identification of an approximately 50-amino acid acidic region, carboxyl-terminal to the DBD, that is required for transactivation by Myb (17–19). The third major functional region is the broadly de

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‡ Supported in part by Public Health Service Training Grant GM-07055 from the National Institutes of Health. Present address: DNAX Research Institute, 901 California Ave., Palo Alto, CA 94304.

§ Supported in part by Public Health Service Training Grant CA-09109 from the National Cancer Institute. Present address: Pfizer Central Research, Eastern Point Road, Groton, CT 06340.

¶ Supported in part by Public Health Service Training Grant GM-07055 from the National Institutes of Health. Present address: DNAX Research Institute, 901 California Ave., Palo Alto, CA 94304.

** To whom correspondence should be addressed: Dept. of Microbiology, University of Virginia, Box 444, 1300 Jefferson Park Ave., Charlottesville, VA 22908. Tel.: 804-924-1246; Fax: 804-982-1071; E-mail: tpb3e@virginia.edu.

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1 The abbreviations used are: AMV, avian myeloblastosis virus; DBD, DNA binding domain; MRE, Myb-responsive element; NRD, negative regulatory domain; NF-M, nuclear factor-myeloid; EMSA, electrophoretic mobility shift assay; bp, base pair; GST, glutathione S-transferase; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
fined negative regulatory domain (NRD). Deletion of this region results in up to a 10-fold increase in Myb-induced transcription activation when assayed by transient co-transfection with several test promoters (18–20). This region contains a leucine zipper-like motif (16), and point mutations of key residues in this motif increase both the transcription activating and transforming activity of murine Myb (21). Thus, it is of particular interest that potential protein-protein interactions have been identified in blotting studies using a peptide containing this motif to probe HeLa cell extracts (21). Several other features of the NRD are also consistent with it playing a role in regulating c-Myb activity. First, it is a site of proviral integration as well as modification by alternative splicing (15). Second, disruption of the carboxy-terminal portion of AMV v-Myb (which retains a portion of the NRD) by linker insertion results in an increase in the transactivating activity of AMV v-Myb (22). Third, both amino- and carboxy-terminal deletions have been reported to independently activate the transforming potential of c-Myb. Finally, cells transformed by differentially truncated forms of c-Myb differ phenotypically (23, 24). This finding suggests that changes in the structure of c-Myb may be important in determining cell lineage and stage of development in hematopoietic cells presumably by altering the array of genes regulated by c-Myb.

The sequence-specific DNA binding by Myb proteins led to the demonstration that both c- and v- myb encoded transcription factors (17, 18), and a number of candidate target promoters have been identified, including mim-1 (25), c-myec (26), CD3 (27), CD4 (28), T cell receptor-β (29), and a murine thymic locus control region (30). Each of these promoters requires the DBD for transcription by Myb and contains at least one Myb binding site. The sequence of these Myb binding sites is surprisingly variable, and Myb proteins bind these sites with varying affinity (31). In the case of the mim-1 promoter there are three MRE’s that bind Myb with different affinities (25, 31). Mutation of the highest affinity site (mim-1A) abolates activation by Myb proteins while mutation of the lower affinity sites has less of an effect. In some cases, c-Myb has been demonstrated to activate transcription in cooperation with Ets-2, core binding factor and NF-M (31). We now demonstrate that serines 11 and 12 are phosphorylated in vivo and are targets for phosphorylation in vitro by casein kinase II. Mutation of these sites results in decreased cooperativity with NF-M (32, 33). In contrast, the NBD is not required for the activation of transcription from the human hsp70 promoter or the avian MD1 promoter, and it has been suggested that c-Myb may bind and inactivate a negative acting transcription factor (34, 35). Thus, c-Myb may regulate gene expression via at least two distinct mechanisms. However, little is known about how c-Myb activates transcription or the mechanisms that regulate c-Myb activity.

Both v- and c-Myb are phosphorylated on serine and threonine at multiple sites in vitro (36–39). Lüsch et al. (36) demonstrated that serines 11 and 12 are phosphorylated in vivo and are targets for phosphorylation in vitro by casein kinase II. Phosphorylation of serines 11 and 12 results in decreased sequence-specific DNA binding in vitro, and substitution of these sites by alanine results in decreased cooperativity with NF-M (40). In addition, c-Myb becomes hyperphosphorylated at several unidentified sites during mitosis, and mitotic c-Myb binds DNA less efficiently than interphase c-Myb (41). We have previously demonstrated that murine c-Myb is phosphorylated on serine 528 (which lies within the NRD) in vivo and that it is a target for phosphorylation by p42MAP kinases in vitro. Substitution of serine 528 by alanine results in a 3–7-fold increase in the ability of c-Myb to activate transcription from an artificial promoter/reporter construct consisting of five copies of the mim-1A MRE and a minimal herpesvirus tk promoter (38, 42). We now demonstrate that substitution of serine 528 by alanine modulates the transcription activating properties of c-Myb on some target promoters, but not others, suggesting the phosphorylation of serine 528 provides a mechanism to differentially regulate c-Myb activity. Interestingly, this substitution does not affect the ability of c-Myb to bind DNA or to form multimerized complexes. We suggest that phosphorylation of serine 528 may serve to regulate the interaction between c-Myb and other proteins that modulate c-Myb activity.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—The African green monkey kidney cell lines, CV-1 and CMT3 COS, were obtained from Dr. David Rekosh (University of Virginia) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone) and 2 mM L-glutamine (Life Technologies, Inc.) in a humidified incubator at 37 °C in 10% CO₂. The CMT3 COS cell line is a derivative of CV-1 and is transformed by SV40 large T antigen driven by the mouse metallothionein I promoter (43).

Plasmids—The murine c-Myb expression vector pRMb3SV.wt (44) contains the entire murine c-Myb coding sequence driven by the Rous sarcoma virus long terminal repeat and sequences for polyadenylation from SV40. The empty control vector pRSV which lacks c-Myb coding sequences was generated by Neo1 digestion, which removes the c-Myb coding sequences, and religation. Oligodeoxynucleotide mutagenesis was used to create alanine (pRMb3SV.AS528) and aspartate (pRMb3SV.AS528) substitution mutations at serine 528 of murine c-Myb. Briefly, the 2-kilobase pair Neo1 fragment, containing the murine c-Myb coding sequences, was isolated from pRMb3SV.wt (44, 45) and subcloned into pALTER (Promega). Oligodeoxynucleotide-directed mutagenesis was carried out using the Altered Sites II System (Promega) per the manufacturer’s instructions. Mutant cDNAs were then subcloned into pRSV SV via their Neo1 sites, creating pRMb3SV.SS528A and pRMb3SV.AS528. Insert orientation was confirmed by restriction endonuclease digestion, and the presence of appropriate mutations was confirmed by double-stranded dideoxynucleotide chain termination DNA sequencing using a Sequenase version 2.0 kit per the manufacturer’s instructions (U.S. Biochemical Corp.).

The SV40 origin of replication was introduced into pRSV, pRMb3SV.wt, pRMb3SV.SS528A, and pRMb3SV.SS528 for autonomous replication in the SV40 large T antigen-transformed CMT3 COS cell line. Briefly, pSV4Ori, which carries a 376-bp EcoRI/HindIII fragment containing the SV40 origin of replication ligated into pUC18, was obtained from Dr. David Rekosh (University of Virginia). PSV4Ori was digested with EcoRI and HindIII and made blunt-ended by filling in with Klenow fragment, and the 376-bp fragment was isolated by agarose gel electrophoresis. pRSV, pRMb3SV.wt, pRMb3SV.SS528A, and pRMb3SV.SS528 were linearized with BamHI, blunt-end-filled by using Klenow fragment, treated with calf intestinal phosphatase, and ligated with the 376-bp fragment to yield pRSVori, pRMb3SVori.wt, pRMb3SVori.SS528A, and pRMb3SVori.9. In pRMb3SV-based plasmids, the unique BamHI site lies immediately 3’ to the polyadenylation sequences.

To generate pORigGL.Lwt and pORigGL.LS528A, pRSVori was linearized by digestion with HindIII and BglII and treated with calf intestinal phosphatase. Polymerase chain reaction was used to amplify sequences encoding GST-fusion proteins from pG2T.wt, pG2T.SS528A, pGEX-2T, and pGEX2TK.E1A12S. The pG2T.wt and pG2T.SS528A plasmids encode GST fused amino-terminally to full-length wild type or SS528A murine c-Myb, respectively (42). pGEX2TK.E1A12S was obtained from Dr. Daniel Engle (University of Virginia) and encodes GST fused amino-terminally to the full-length adenovirus Type 2 E1A 128 coding sequence. Forward (forex2: 5’-GACAAGCTTGGCCATG-3’) and reverse (revgex: 5’-CA-AGAGATTCAGCAGCATGCAAGATGCAATCC-3′) oligodeoxynucleotide primers for polymerase chain reaction amplification were commercially synthesized (Oligos Etc.). Forex2 includes sequences beginning at the translation initiation codon of GST (nucleotide position 270 in pGEX2T, underlined above) and ending within the GST coding region at nucleotide position 294. Forex2 also contains a HindIII site near the 5’ end of the fragment and a minimal Kozak sequence (4B) (corresponding to nucleotides −3 through −1 in murine c-Myb) immediately 5’ to the GST translation initiation codon. Revgex contains sequences corresponding to nucleotides 961 through 939 in the polylinker of pGEX2T plus a BglIII site at the 5’-end. Each 100-μl reaction included 500 ng each of forex2 and revgex primers, 20 ng of DNA template, 200 μM dNTPs, 10 μl of 10 × reaction buffer (100 mM KCl, 100 mM (NH₄)₂SO₄, 200 μM
Regulation of c-Myb Activity

Triton-X (pH 8.8 at 25°C), 20 mM MgSO₄, 0.1% Trition X-100 and 0.5 μl (1X) Promega Dual-labeled DNA polymerase (New England Biolabs) and 0.01% sodium bisulfite. Daltonic MgSO₄ was added to bring the final concentration to 6 mM. Amplification was carried out in a Perkin-Elmer DNA thermocycler under the following conditions: 30 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 4 min, followed by one extension cycle at 72°C for 15 min and a 4°C soak cycle. Polymerase chain reaction products were extracted with phenol/chloroform, precipitated with ethanol, and digested with HindIII and BglII. The digested DNA products were purified by agarose gel electrophoresis and ligated into the pRS3Vorl vector. The presence of appropriate mutations was confirmed by DNA sequencing as described above. The pOri.GSTFLwt and pOri.GSTFLS528A plasmids consist of pRS3Vorl with the GST wild type c-Myb or the GST-S528A c-Myb fusion insert, respectively. pOri.GST contains only the empty pRS3V expression vector. Transfections for luciferase assays were performed in triplicate.

Cell lysates were prepared and luciferase assays performed using the luciferase assay kit (Promega) per the manufacturer's instructions. Transfected cells were washed once with PBS and incubated in 400 μl of 1× Reporter Lysis Buffer (Promega) for 15 min at 27°C. Cells were harvested with a rubber policeman into the centrifuge tubes, and frozen in a dry ice/ethanol bath. The lysates were thawed and clarified by centrifugation in an Eppendorf microcentrifuge tubes, and frozen in a dry ice/ethanol bath. The lysates were visualized using enhanced chemiluminescence per the manufacturer's instructions (Amersham Corp.). To detect GST and GST fusion proteins, nitrocellulose membranes were exposed to XAR-5 film (Kodak) at 27°C for 4–16 h.

Detection of Myb Multimers in Transfected Cell Lysates—Detection of Myb-mRNA interactions was performed essentially as described (49) with several modifications. CMT3COS cells were seeded at 5 × 10⁵ cells per 100-mm dish 20 h prior to transfection by the CaPO₄ method (48). For each plasmid, 3 μg of pCD34[-3.7k/2.2] or the pRMb3SVori.wt or pRMb3SVori.S528A. At 48 h post-transfection, the supernatants were fractionated by 7.5% SDS-PAGE, electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell), and immunoblotted with anti-Myb or anti-GST antibodies, provided by Dr. Daniel Engle (University of Virginia), who was used as a nonspecific competitor (upper strand, 5'-GTCCCCCGTACGTCAC-CCCGGAG-3'). For supershifts, cell extract was preincubated with 20 μg of anti-Myb Type I monoclonal antibody (UBI) for 30 min at 4°C. Reactions were loaded onto a 4% polyacrylamide gel and run at 40 V/cm in prechilled (4°C) 0.25× TBE buffer (12.5 mM Tris-HCl, pH 8.5, 12.5 mM NaCl, 0.1 mM EDTA) at 3°C. The gel was stained with 0.05% Coomassie blue and destained with 30% acetic acid until the bands were visible.
Regulation of c-Myb Activity

RESULTS

Transcription Transactivating Activities of Wild Type and S528A c-Myb—Considerable evidence indicates that the NRD functions to influence c-Myb activity, yet little is known about how sequences in this region function. We have previously demonstrated that sequences in the negative regulatory domain serve as substrate for phosphorylation by p42MAPK in vivo and that at least one of these sequences, serine 528, is phosphorylated in vivo (see Ref. 42 and Fig. 2). Substitution of serine 528 with alanine results in a 3–7-fold increase in the ability of Myb to activate transcription from an artificial promoter containing one or five copies of the mim-1A Myb response element (1 × MRE or 5 × MRE) inserted into a minimal herpesvirus thymidine kinase promoter (38). This suggests that phosphorylation at serine 528 serves to negatively regulate c-Myb transcription transactivating activity. To determine whether serine 528 may be involved in regulating the ability of c-Myb to activate transcription from cellular promoters, we have used a transient transfection assay to compare the ability of wild type and S528A Myb to transactivate several Myb-responsive cellular promoters. The promoters tested in this study (see Fig. 1) include the human CD34 hematopoietic stem cell antigen promoter (pCD34[-3.7k/+302]d.luc), the chicken mim-1 promoter (pRL.Luc), and the murine c-myc promoter (pMycBg.luc). As a negative control, the empty expression vector lacking c-myc coding sequences (pRSV) was included in each assay.

To determine whether wild type, S528A, and S528D c-Myb proteins were expressed at similar levels in transfected CV-1 cells, anti-Myb immunoblots of transfected CV-1 cell lysates were performed. CV-1 cells were transiently transfected with pRMb3SV.wt, pRMb3SV.S528A, or pRMb3SV.S9 and harvested 48 h post-transfection. Lysates from 2 × 10^6 cells per transfection were fractionated by 8% SDS-PAGE and immunoblotted to detect c-Myb proteins. As demonstrated in Fig. 3A, c-Myb expression is not detected in lysates from CV-1 cells transfected with the empty control plasmid pRSV (Fig. 3A, lane 4). In contrast, wild type c-Myb and S528A c-Myb are readily detectable in CV-1 cells transfected with pRMb3SV.wt and pRMb3SV.S528A (Fig. 3A, lanes 1 and 2, respectively). Furthermore, the amounts of wild type and S528A c-Myb expressed in transfected CV-1 cells are equivalent. The S528D c-Myb protein encoded by pRMb3SV.S9 was not detected in transfected CV-1 cells (Fig. 3A, lane 3) and was not used in transfections designed to assay transcription transactivating activities. Results from this experiment demonstrated that wild type and S528A c-Myb were expressed at similar levels in transfected CV-1 cells and allowed for a direct comparison of their relative transcription transactivating activities in the cotransfection assays described below.

We first examined the relative abilities of wild type and S528A Myb to activate transcription from the human CD34 promoter. CD34 is a cell surface glycoprotein whose pattern of expression in early hematopoietic precursors in bone marrow is similar to that of c-Myb (50) and transcription from the CD34 promoter is activated by c-Myb (27). As demonstrated in Fig. 3B, both wild type and S528A c-Myb activated transcription from the CD34 promoter at each concentration of transfected expression plasmid tested. However, S528A c-Myb activated transcription from the CD34 promoter approximately 3–4-fold more effectively than did wild type c-Myb. These results are representative of three independent experiments and are similar to our previous results using a synthetic 5 × MRE containing promoter/reporter construct (38).

To extend our analysis to another known Myb-responsive cellular promoter, the relative abilities of wild type and S528A c-Myb to transactivate the chicken mim-1 promoter were examined. Although the mim-1 promoter contains three potential MREs referred to as mim-1A, mim-1B, and mim-1C (Fig. 2), the mim-1A MRE is predominantly responsible for activation by c-Myb (51). The mim-1 promoter also contains two binding sites for the NF-M myeloid-specific transcription factor (see Fig. 2), and c-Myb and NF-M synergistically activate transcription of mim-1 in myeloid cells (32, 51). In contrast to the results obtained using the 5 × MRE and CD34 promoters, wild type and S528A c-Myb equivalently activated transcription from the mim-1 promoter at each amount of expression plasmid tested (Fig. 3C). Thus, in transfected CV-1 cells, there is no significant difference between the abilities of wild type and S528A c-Myb to activate transcription from the intact mim-1 promoter. The relative abilities of wild type and S528A c-Myb to transactivate the murine c-myc promoter were also examined. The murine c-myc promoter contains 16 Myb binding sites located in two
Regulation of c-Myb Activity

Fig. 2. Schematic representation of murine c-Myb. The c-Myb protein is depicted schematically as a box with regions transduced and expressed by the E26 and AMV avian acute transforming retroviruses represented by lines above c-Myb. The three tandem repeats comprising the DNA binding domain (DNAB) are represented as black triangles. The transactivation (TA) and negative regulatory (NR) domains are also shown. The two nuclear localization (NL) motifs are shown as stippled rectangles and the leucine zipper (LZ) is depicted as a striped rectangle. Five previously reported potential sites for phosphorylation by proline-directed serine/threonine protein kinases (42) are labeled with the single-letter amino acid code followed by their position in murine c-Myb (e.g., S528 represents serine at position 528). T486 lies within a TP motif (small stippled box), S459 and S528 lie within SP motifs (small black circles) and T462 and T486 lie within PXTP motifs (small stippled triangles). Amino acid positions delineating the amino terminus, DNAB domain, TA domain, NR domain and carboxyl terminus are indicated on the scale below c-Myb (19, 45, 72–75).

clusters. The cluster distal to the transcription start site contains 10 and the cluster proximal to the site of transcription contains 6 Myb binding sites (Fig. 2) (52). The results of transient transfection assays obtained using the murine c-myc promoter were similar to those obtained using the chicken mim-1 promoter (Fig. 3D). The relative abilities of wild type and S528A c-Myb to transactivate the murine c-myc promoter were equivalent throughout the range of expression plasmid amounts tested. Both proteins effectively activated transcription of the c-myc promoter. These results indicate that phosphorylation of serine 528 may provide a mechanism to differentially regulate c-Myb transcription transactivating activity at different promoters.

Substitution of Serine 528 Does Not Affect DNA Binding—To determine whether the different transactivation activities observed for wild type and S528A c-Myb was reflected by a differential ability to bind DNA, EMSAs were performed. For these experiments, wild type c-Myb and the Myb mutants were transiently expressed in CMT3COS cells. Ser-528 is phosphorylated in CMT3COS cells, and the differential transcription activation properties of wild type and S528A c-Myb are similar in CV-1 and CMT3COS cells (38). As demonstrated in Fig. 4C, all three proteins, including S528D, were expressed in CMT3COS cells. The S528D c-Myb substitution mutant was also tested in these experiments since it was stably expressed in CMT3COS cells. A single major complex with retarded mobility was detected in extracts from CMT3COS transfected with each expression vector (Fig. 4, A and B). This complex was not detected in cells transfected with the empty pRSV control vector. The presence of c-Myb in the major protein-DNA complex was demonstrated by a supershift of the protein-DNA complex when the lysate was preincubated with a c-Myb monoclonal antibody. The specificity of this interaction was demonstrated by cold competition assay, and the binding of DNA to each Myb protein was effectively competed by preincubation with unlabelled MRE oligodeoxynucleotide at a molar excess of 100–500-fold (Fig. 4B). In contrast, unlabeled cyclic AMP response element oligodeoxynucleotide, at the same molar excess, did not compete for DNA binding (Fig. 4B). Scatchard analysis, using a more extensive range of competitor concentrations, revealed no differences between the affinities of these three proteins for several MREs. In addition, no difference in the on or off rates of these proteins was detected. Thus, the difference in the abilities of wild type and S528A c-Myb to transactivate the CD34 and synthetic 5 × MRE containing promoters does not appear to be due to differences in DNA binding due to substitution of serine 528 with alanine, suggesting that phosphorylation of serine 528 does not regulate c-Myb sequence-specific DNA binding activity.

Substitution of Serine 528 Does Not Affect the Ability of c-Myb to Form Multimerized Complexes—It has been reported that c-Myb can homodimerize in vitro via a leucine zipper-like motif (amino acids 375–403) in the NRD (53). Those authors reported that c-Myb homodimers cannot bind DNA in a sequence-specific manner and are therefore prevented from activating transcription from Myb-responsive promoters. Since the leucine zipper of c-Myb resides in close proximity to serine 528, we tested whether substitution of serine 528 by alanine affected the ability of c-Myb to multimerize in transfected CMT3COS cell lysates. A similar approach to that reported by Nomura et al. (53) was used, with two modifications. First, CMT3COS cells were used instead of NIH-3T3 cells for high level expression of transfected gene products since it is not known whether serine 528 of murine c-Myb would be a target for phosphorylation in NIH-3T3 cells. Second, our experiments utilized full-length rather than truncated Myb proteins to address the issue of Myb multimerization.

CMT3COS cells were transiently cotransfected with plasmids encoding wild type or S528A c-Myb and individual plasmids encoding either GST wild type c-Myb, GST S528A c-Myb, GST E1A, or GST. Whole cell lysates were prepared and incubated with glutathione-agarose to harvest the GST-containing proteins. After a series of washes, proteins bound to the beads were released by boiling in sample buffer, fractionated by 8% SDS-PAGE, and transferred to nitrocellulose for detection by either anti-c-Myb or anti-GST monoclonal antibodies. GST c-Myb fusion proteins can be distinguished from native c-Myb proteins by their slower mobility during SDS-PAGE (GST c-Myb migrates at approximately 100 kDa and c-Myb migrates at approximately 75 kDa). Whereas GST E1A and GST did not form complexes with wild type or S528A c-Myb (Fig. 5A, lanes 4–7), both GST wild type c-Myb and GST S528A c-Myb associated with c-Myb (Fig. 5A, lanes 1 and 2). Thus, the S528A substitution did not have an apparent effect on the ability of c-Myb to multimerize with GST wild type c-Myb. To determine whether S528A substitutions on both partners would affect multimerization, we also tested the ability of GST S528A c-Myb to form a complex with S528A c-Myb. As shown in lane 3 of Fig. 5A, GST S528A c-Myb also associated with S528A c-Myb to a similar extent as observed using one or two wild type partners (compare Fig. 5A, lanes 1, 2, and 3). The 75-kD proteins detected in lanes 1–3 of Fig. 5A are full-length Myb proteins and not degradation products of the GST c-Myb proteins because they were not detected in lysates from CMT3COS cells.

A. F. Richardson and T. P. Bender, unpublished observations.
transfected with plasmids encoding GST c-Mybs alone. Neither wild type nor S528A c-Myb bound to glutathione agarose in the absence of any GST-fusion protein.

To ensure that the negative control GST fusion proteins were appropriately expressed in this assay, the nitrocellulose filter was stripped and reprobed with the 9D9 anti-GST mAb that detects each GST fusion protein used in this assay. Fig. 5B demonstrates that all of the GST fusion proteins were appropriately expressed in this experiment. In fact, GST E1A and GST were more effectively harvested by incubation with glutathione-agarose than were GST wild type c-Myb and GST S528A c-Myb (Fig. 5B, lanes 1–7). Together, these data indicate that full-length c-Myb multimerized with GST c-Myb and that the S528A substitution does not affect multimerization. Thus, the difference in the abilities of wild type and S528A c-Myb to transactivate the 5 × MRE and CD34 promoters was not reflected by an apparent difference in their ability to participate in Myb-Myb interactions.

**DISCUSSION**

We have examined the potential role of serine 528 in regulating c-Myb transcription transactivating activity on a series of cellular promoters that are known to be activated by c-Myb. Substitution of serine 528 for alanine (S528A c-Myb) resulted in a substantial increase in c-Myb-activated transcription from the human CD34 promoter (see Fig. 3B) as well as from the previously reported synthetic Myb-responsive promoters containing one or five copies of a mim-1A-based MRE (38). These

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3 M. R. Miglarese and T. P. Bender, unpublished observations.
results suggest that phosphorylation of serine 528 may act to suppress c-Myb transcription transactivating activity at these promoters. In contrast to the results obtained using the CD34 promoter, S528A c-Myb and wild type c-Myb were equally effective at stimulating transcription from the chicken mim-1 and murine c-myc promoters (see Fig. 3, C and D) indicating that phosphorylation of serine 528 does not play a role in regulating c-Myb-activated transcription from either the mim-1 or c-myc promoters. These results strongly support the notion that phosphorylation of serine 528 provides a mechanism to differentially regulate c-Myb activity by modifying the structure of the NRD.

The observation that wild type and S528A c-Myb equivalently transactivated the murine c-myc promoter is consistent with previously published data demonstrating that truncation of the NRD does not potentiate c-Myb-activated c-myc trans-
post-translational modification under some circumstances. In mature hematopoietic and some non-hematopoietic cell lines, the activity of c-Myb appears to be regulated primarily by changes in c-Myb expression during the cell cycle (55, 56). However, c-Myb expression does not appear to be regulated during the cell cycle in immature hematopoietic cells (3, 57). If c-Myb plays a similar role in regulating gene expression during the cell cycle in both immature and mature hematopoietic cells, then a mechanism must be available to regulate c-Myb activity during the cell cycle in immature hematopoietic cells. Phosphorylation provides a rapid and efficient mechanism by which c-Myb activity may be regulated. Indeed, Lüscher and colleagues (37) have recently reported that hyperphosphorylation of c-Myb during mitosis, at unidentified sites, in a pre-B cell lymphoma cell line correlates with decreased DNA binding activity. These observations, along with the data presented in this article, suggest that phosphorylation may serve to negatively regulate c-Myb activity. However, it should be noted that these observations have been made using a relatively restricted set of Myb target promoters and that phosphorylation at these sites, or other unidentified sites, may serve to increase the ability of c-Myb to activate transcription at other promoters in a lineage or differentiation stage-specific fashion.

How phosphorylation of serine 528 may regulate c-Myb transcription transactivating activity is not understood. Serine 528 resides within a region (residues 495–554) that was previously demonstrated to have a negative effect on c-Myb transcription transactivating activity (58). Phosphorylation of c-Myb serine 528 may mediate the direct physical interaction between the NRD and the transactivation domain, thus masking the transactivation domain and suppressing the transcription transactivating activity of c-Myb. Release of phosphate from serine 528 would then allow c-Myb to assume a more active conformation. However, we do not favor this model because it fails to account for the differential activity of wild type and S528A c-Myb at different promoters in the same cell type.

Phosphorylation has been shown to negatively regulate the DNA binding activities of several transcription factors, including c-Myb, c-Jun, Oct-1, and Ets-1 (59–62). However, our results indicate that serine 528 does not play a significant role in regulating the DNA binding activity of c-Myb. This is consistent with the findings of Dubendorff et al. (58) who demonstrated that amino acids 495–554 of avian c-Myb (serine 528 of murine c-Myb corresponds to serine 533 of avian c-Myb) suppress c-Myb transactivating activity in-cis and in-trans without affecting DNA binding activity. However, since the MRE consensus sequence is degenerate, phosphorylation of serine 528 may affect c-Myb DNA binding activity at only a subset of MREs that have not yet been examined. In addition, it should be noted that in the EMSA experiments presented in this study, c-Myb was expressed at very high levels in transfected CMT3COS cells. Thus, it is possible that high level expression of c-Myb effectively titrated out the protein kinase(s) responsible for phosphorylating serine 528. However, this is unlikely as c-Myb is phosphorylated on Ser-528 in CMT3COS cells, and the differences between the transcription activating properties of wild type and S528A Myb are similar in CV-1 and CMT3COS cells (58).

Mutations in the c-Myb leucine zipper that abolish the ability to homodimerize in vitro also increase the transcription transactivating, transforming, and DNA binding activities of c-Myb (53). Since serine 528 lies in close proximity to the leucine zipper within the NRD (residues 374–403), phosphorylation of serine 528 might regulate the ability of the leucine zipper to mediate homodimerization. However, data presented in this article do not support a role for phosphorylation of serine 528 in regulating c-Myb homodimerization. Although the ability of c-Myb to homodimerize per se was not tested in vitro using purified proteins, the S528A substitution in c-Myb had no measurable effect on the detection of steady state complexes containing two or more c-Myb proteins in transfected CMT3COS cells.

We favor a model in which phosphorylation of serine 528 regulates the interaction between c-Myb and other transcription activators or repressors. Phosphorylation has been demonstrated to regulate the interaction of several transcription factors, including NF-κB, E2F, and PU.1, with heterologous binding partners (63–65). For example, the PU.1 transcription factor binds to its cognate sequence on the immunoglobulin 3′ κ enhancer where it can be phosphorylated on serine 148. This functions to recruit NFEM-5 to its cognate DNA element directly 3′ to the PU.1 site and stimulate transcription (64). Studies performed in our laboratory and by others (66) indicate that the c-Myb NRD can associate with a number of as yet uncharacterized cellular proteins. While our data suggest that phosphorylation at serine 528 does not affect the interaction between c-Myb and a general inhibitor of transcription, it may limit the ability of c-Myb to interact with specific transcription repressors, thus differentially regulating c-Myb activity.

The c-Myb NRD is commonly truncated in oncogenic Myb proteins generated by either retroviral transduction or integration, thus deleting serine 528 (see Fig. 2). This suggests that the inability of these proteins to be phosphorylated on serine 528 might contribute to their oncogenic activation. However, substitution of chicken c-Myb serine 533 with alanine (chicken c-Myb serine 533 corresponds to murine c-Myb serine 528) is not sufficient to transform chicken yolk sac cells in vivo. 4 A potential explanation for this observation is that phosphorylation of serine 528 may not regulate c-Myb-activated transcription of genes directly involved in transformation. Alternatively, deletion or substitution of serine 528 may contribute to Myb oncogenicity only in the context of other lesions in the carboxy-terminal region or amino-terminal truncation. Indeed, carboxy-terminal truncated c-Myb is weakly transforming compared with amino-terminal truncated c-Myb, whereas truncation of both termini synergize to oncogenically activate Myb, indicating that intramolecular interactions may play a key role in the regulation of c-Myb function.

While the data presented in this article support a role for phosphorylation of serine 528 in regulating c-Myb transcription transactivating activity, other phosphorylation sites will also likely be important for the regulation of c-Myb activity. Indeed, phosphorylation of serines 11 and 12 by CK II in vitro inhibits the sequence-specific DNA binding activity of c-Myb, and mutation of these sites to alanine decreases the ability of c-Myb to activate transcription (36). These sites are also phosphorylated in vivo in at least two cell lines, and their substitution by alanine results in increased DNA binding and transcription transactivating activity (36, 40). In addition, common and lineage-specific phosphopeptides have been identified by our laboratory and others (36–38). This raises the possibility that lineage-specific phosphorylation may regulate c-Myb function in a cell type-specific manner. It will be of considerable interest to identify these sites of phosphorylation and assess their roles in the regulation of c-Myb activity in different cell types.

p42mapk is generally viewed as a cytoplasmic protein kinase that is activated during entry into the cell cycle (67). However, it has been reported to translocate to the nucleus upon activa-

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4 J. Lipeck, M. R. Miglarese and T. P. Bender, unpublished observations.

5 N. Aziz, M. R. Miglarese and T. P. Bender, unpublished observation.
Regulation of c-Myb Activity

6 N. Aziz and T. P. Bender, unpublished observation.

22705

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