The c-myb protooncogene encodes a highly conserved
75–89-kDa transcription factor that contains three func-
tional 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 myeloblas-
tosis virus and the E26 leukemia virus, transduced por-
tions of c-myb and encode Myb proteins that are trun-
cated 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 in vitro. Serine 528 is phosphorylated in vivo
in several cell lines, and substitution of serine 528 to ala-
nine (S252A) resulted in an increased ability of Myb to
transactivate a synthetic promoter containing five cop-
ies of the mim-1A Myb-responsive element and a mini-
mal herpes th promoter. We have tested the ability of S528A Myb to transactivate a series of cellular target
promoters and report that the serine to alanine substi-
tution increased the ability of Myb to activate transcrip-
tion from the CD34 promoter but not the c-myec or mim-1
promoters. This suggests that phosphorylation of serine
528 may differentially regulate c-Myb activity at differ-
ent 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 transac-
tivating 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 carci-
noma (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 exper-
imentally in several systems. First, c-myb antisense oligode-
oxynucleotides inhibit both erythroid and myeloid colony for-
mation in vitro (9). Second, murine erythroleukemia cells and
leukemic myeloid cells stably transfected with either constitu-
tively 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
erthroid 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) 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 transacti-
vation 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, Drosoph-
ila, yeast, slime molds, and plants (1). Biedenkapp et al. (16)
demonstrated that Myb bound DNA specifically to the consen-
sus 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 vari-
ous 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-termi-
nal 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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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-trans-
ferase; mAb, monoclonal antibody; PAGE, polyacrylamide gel electro-
phoresis; PBS, phosphate-buffered saline.
fined negative regulatory domain (NRD). Deletion of this region results in up to a 10-fold increase in c-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 c-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 carboxyl-terminal portion of AMV c-Myb (which retains a portion of the NRD) by linker insertion results in an increase in the transcription activating activity of AMV c-Myb (22). Third, both amino- and carboxyl-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-myc (26), CD34 (27), CD4 (28), T cell receptor-ß (29), and a murine thymic locus control region (30). Each of these promoters requires the DBD for transactivation 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 (31). Mutation of the highest affinity site (mim-1A) abrogates 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 (25, 29, 32, 33). In contrast, the DBD is not required for the activation of transcription from the human hsp 70 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 vivo (36–39). Lüserher 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 vitro and that it is a target for phosphorylation by p42 MAPK in vitro. Substitution of serine 528 by alanine results in 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 CMT3COS, 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 glutamine (Life Technologies, Inc.) in a humidified incubator at 37 °C in 10% CO2. The CMT3COS 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 that lacks c-myb coding sequences was generated by Neol digestion, which removes the c-myb coding sequences, and religation. Oligodeoxynucleotide mutagenesis was used to create alanine substituted (pRMb3SV.S528A) (pRMb3SV.9) substitution mutations at serine 528 of murine c-Myb. Briefly, the 2-kilobase pair Neol 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 via their Neol sites, creating pRMb3SV.S528A and pRMb3SV.9. 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.S528A, and pRMb3SV.9 for autonomous replication in the SV40 large T antigen-transformed CMT3COS 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 by using Klenow fragment, and the 376-bp fragment was isolated by agarose gel electrophoresis. pRSV, pRMb3SV.wt, pRMb3SV.S528A, and pRMb3SV.9 were linearized with BamHI, made blunt-ended by filling using Klenow fragment, treated with calf intestinal phosphatase, and ligated with the 376-bp fragment to yield pRSVori, pRMb3SVori.wt, pRMb3SVori.9, and pRMb3SVori.9. In pRMb3SV-based plasmids, the unique BamHI site lies immediately 3′ to the polyadenylation sequences.

To generate pORIGFL.wt and pORIGFLSLS298A, 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.SLS298A, pGEX-2T, and pGE2TK.E1A125S. The pG2T.wt and pG2TSLS298A plasmids encode GST fused amino-terminally to full-length wild type or S528A murine c-Myb, respectively (42). pGE2TK.E1A125S was obtained from Dr. Daniel Engle (University of Virginia) and encodes GST fused amino-terminally to the full-length adenovirus Type 2 E1A 125S coding sequence. Forward (for2x: 5′-GACAAGCTTGCCATG-3′) and reverse (rev2x: 5′-GGAGATCTTACGATCAGATGAAATTC-3′) oligodeoxynucleotide primers for polymerase chain reaction amplification were commercially synthesized (Oligos Etc.). For2x includes sequences beginning at the translation initiation codon of GST (nucleotide position 270 in pGEX-2T, underlined above) and ending within the GST coding region at nucleotide position 294, For2x also contains a HindIII site near the 5′-end (underlined above) and a minimal Kozak sequence (48) (corresponding to nucleotides −3 through −1 in murine c-myc) immediately 5′ to the GST translation initiation codon. Rev2x contains sequences corresponding to nucleotides 961 through 939 in the polynucleotide of pGEX-2T plus a BglII site at the 5′-end. Each 100-μl reaction included 500 ng each of for2x and rev2x primers, 20 ng of DNA template, 200 μM dNTPs, 10 μl of 10 × reaction buffer (100 mM KCl, 100 mM (NH4)2SO4, 200 mM...
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Tri-tin (PH 8.8 at 25 °C), 20 mM MgSO4, 0.1% Triton X-100 and 0.5 μM (1 μl) of DNAse (New England Biolabs) was added to the reactions using NenSorb columns per the manufacturer’s instructions (DuPont NEN). The mim-1A MRE oligodeoxynucleotide was commercially synthesized with four base overhangs (Oligo’s Etc). EMSA reactions contained 1 μl of transfected CMT3COS cell lysate, 2 μl of 10 × buffer (100 mM Tris-HCl, pH 7.9, 500 mM NaCl, 10 mM EDTA, 0.5% nonfat dry milk, 50% glycerol, 0.1% saturated bromphenol blue), 2 μl of 100 μM dithiothreitol (Boehringer Mannheim), 1 μl of ligation buffer (4°C) (Sigma), 13 μl of H2O, 1 μl (approximately 0.1 ng or 10,000 cpm) of 32P-labeled probe and were incubated at 27 °C for 45 min. For competition assays, cell extracts were preincubated with unlabeled competitor oligodeoxynucleotide for 30 min at 27 °C at which time labeled probe was added to the reaction for an additional 15 min. A double-stranded oligodeoxynucleotide containing the Myb responsive region was provided by Dr. Daniel Engel (University of Virginia), was used as a nonspecific competitor (upper strand, 5'-GTCCCCCGTACGCTACCCGGGAG-3'). For supershifts, cell extract was preincubated with 20 μg of anti-Myb Type 1 monoclonal antibody (UBI) for 30 min at 4 °C. Reactions were loaded onto a 4% polyacrylamide gel and run at 40 Vcm in prechilled (4 °C) 0.25 × TBE buffer (12.5 mM Tris-HCl, pH 8.5, 12.5 mM Na2HPO4, 500 μM NaCl, 0.1% SDS) and transferred to a Hybond-C Sorbet membrane (Amersham Corp.). To detect GST and GST fusion proteins, filters were stripped for 30 min at 70°C in strip buffer (2% Triton X-100, 500 mM NaCl, 0.15 mM benzamidine, 2.8 mM sodium orthovanadate, 1% Triton X-100, 1 mM EDTA, 1% Triton X-100) and incubated in blocking buffer for 1 h, rinsed three times with four base overhangs (Oligo’s Etc). EMSA reactions contained 1 μl of each of transfected plasmids. Precleared lysates were incubated with 50 μl of GST-Sepharose 4B beads or the GST/S2A c-Myb fusion insert, respectively. pGstGST was cotransfected with 1 μg of either pRMSb3Vori.wt or pRMSb3Swi528A. At 48 h post-transfection, the cells were harvested by gentle scraping in PBS and transferred to a 50-ml conical tube (Sarstedt). Cells were pelleted by centrifugation at 500 × g for 10 min at 4 °C, resuspended in 1 ml of ice-cold PBS, and transferred to a microcentrifuge tube. Cells were then pelleted by brief microcentrifugation, resuspended in 300 μl of HNNE* (10 mM HEPES, pH 7.9, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.01 unit/ml α2-macroglobulin, 50 mM β-glycerophosphate (Calbiochem), 100 mM nicotinamide (Calbiochem)), and mixed by inversion for 2 h at 4 °C. Lysates were clarified by centrifugation in a refrigerated Eppendorf microcentrifuge at the maximum setting for 10 min at 4 °C and the supernatants then transferred to fresh microcentrifuge tubes and kept on ice. Lysates were precleared with 100 μl of a 1:1 slurry of protein A-Sepharose 4B beads (Pharmacia Biotech Inc.) in 150 mM NaCl, 10 mM Tris-HCl, pH 8, for 1 h at 4 °C on a rotating wheel. Protein A-Sepharose 4B beads were removed by brief microcentrifugation, and the supernatants were transferred to a microcentrifuge tube. Thirty-microliter aliquots of each supernatant were removed from precleared lysates as controls for protein expression from transfected plasmids. Precleared lysates were incubated with 50 μl of a 1:1 slurry of preequilibrated glutathione-Sepharose 4B (Pharmacia) for 2 h at 4 °C. Glutathione-Sepharose 4B beads were preequilibrated in TNE (20 mM Tris-HCl, pH 8, 1% Nonidet P-40, 1 mM EDTA), blocked in TNE plus 4% nonfat dry milk for 1 h, washed once with TNE, and resuspended as a 1:1 slurry in TNE. The beads were harvested by brief centrifugation and washed three times with 1 ml of TNE per wash. After the final wash, the beads were resuspended in 100 μl of 2 × 5B (20% glycerol, 124 mM Tris-HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 0.1% saturated bromphenol blue), boiled for 5 min, and briefly microcentrifuged. The supernatant was fractionated by 7.5% SDS-PAGE, electrophoretically transferred to nitrocellulose filters (Schleicher & Schuell), and immunoblotted with anti-Myb or anti-GST monoclonal antibodies as described below.

Protein Immunoblotting—Nitrocellulose filters were blocked in TBS (50 mM Tris-HCl, pH 8, 150 mM NaCl) plus 5% nonfat dry milk for 1 h at room temperature or overnight at 4 °C. After several rinses with TBS, filters were incubated with an anti-Myb mAb at 1 μg/ml (Type I anti-Myb mAb, UBI) for 1 h at room temperature. Filters were washed at room temperature for 30 min with several changes of TBS and incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Corp., diluted 1:1333) for 1 h at room temperature. Filters were washed with TBS as described above, and Myb proteins were visualized using enhanced chemiluminescence reagents per the manufacturer’s instructions (Amersham Corp.). To detect GST and GST fusion proteins, filters were stripped for 30 min at 70 °C in strip buffer (2% SDS, 0.7% β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8), rinsed several times in TBS, incubated in blocking buffer for 1 h, rinsed three times in TBS, and incubated for 2 h at 27 °C with 9D9 mouse anti-GST hybridoma supernatant, provided by Dr. J. Thomas Parsons (Univer-
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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 protein kinase C (38). As a consequence of these facts, sequence from the c-Myb promoter approximates a 3–4 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 (pCD34d.luc), and the murine c-myc promoter (pMycBg.luc). As a negative control, the empty expression vector lacking c-myc coding sequences (pRS3V) 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.9 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 pRS3V (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.9 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 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
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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 fusion proteins. GST c-Myb fusion proteins can be distinguished from native c-Myb proteins by their slower mobility during 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-kDa 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.
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

\[ \text{Equation} \]

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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 transcription (54). Thus, it appears that the NRD does not act as a negative regulator in the context of Myb-activated c-myc transcription, further supporting our hypothesis that the c-Myb NRD is differentially active at different promoters. The finding that S528A c-Myb and wild type c-Myb were equally effective at stimulating transcription from the chicken mim-1 promoter was unexpected since the c-Myb NRD has previously been demonstrated to negatively regulate the synergistic activation of mim-1 transcription by c-Myb and CEBP/β (also referred to as NF-M) (32). Although the S528A substitution in c-Myb had no measurable effect on transactivation of the mim-1 promoter in the current study, CV-1 cells do not express CEBP/β.

It should be noted that promoter context contributed to the differential activity of wild type and S528A c-Myb, since the dominant mim-1A MRE from the mim-1 promoter was differentially responsive to these proteins within the context of a heterologous minimal promoter containing one or five copies of the mim-1A MRE in the absence of CEBP/β. It is not surprising that c-Myb activity might be regulated by...
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 transactivation activating properties of wild type and S528A Myb are similar in CV-1 and CMT3COS cells (38).

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-kB, 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 function 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 vitro. 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 carboxyl-terminal region or amino-terminal truncation. Indeed, carboxyl-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. Lipieck, M. R. Miglarese and T. P. Bender, unpublished observations.

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

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