Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 6429–6433, July 1995
Biochemistry

Modulation of c-Myb-induced transcription activation by a phosphorylation site near the negative regulatory domain

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Communicated by Oscar L. Miller, Jr., University of Virginia, Charlottesville, VA, March 30, 1995

ABSTRACT **The c-*myb* protooncogene encodes a highly conserved transcription factor that functions as both an activator and a repressor of transcription. The v-*myb* oncogenes of E26 leukemia virus and avian myeloblastosis virus encode proteins that are truncated at both the amino and the carboxyl terminus, deleting portions of the c-Myb DNA-binding and negative regulatory domains. This has led to speculation that the deleted regions contain important regulatory sequences. We previously reported that the 42-kDa mitogen-activated protein kinase ($p42^{mapk}$) phosphorylates chicken and murine c-Myb at multiple sites in the negative regulatory domain *in vitro*, suggesting that phosphorylation might provide a mechanism to regulate c-Myb function. We now report that three tryptic phosphopeptides derived from *in vitro* phosphorylated c-Myb comigrate with three tryptic phosphopeptides derived from metabolically labeled c-Myb immunoprecipitated from murine erythroleukemia cells. At least two of these peptides are phosphorylated on serine-528. Replacement of serine-528 with alanine results in a 2- to 7-fold increase in the ability of c-Myb to transactivate a Myb-responsive promoter/reporter gene construct. These findings suggest that phosphorylation serves to regulate c-Myb activity and that loss of this phosphorylation site from the v-Myb proteins may contribute to their transforming potential.**

The c-*myb* protooncogene encodes a highly conserved 75- to 89-kDa transcription factor which is expressed primarily in immature cells and cell lines of each hematopoietic lineage, where it plays an important role in proliferation and differentiation (reviewed in ref. 1). The c-Myb protein contains three functional domains: an amino-terminal DNA-binding domain, a centrally located acidic transcription activation domain, and a carboxyl-terminal negative regulatory domain (1). Two acute transforming retroviruses, avian myeloblastosis virus (AMV) and the E26 avian leukemia virus, have independently transduced portions of the c-*myb* gene (2, 3). Both v-Myb proteins are truncated at their amino and carboxyl termini, and the loss of these sequences is thought to be responsible for their transforming activity (4, 5). Sequential deletion from the carboxyl terminus of c-Myb results in increased DNA-binding, transcription-activating, and transforming activities (6–9). These findings suggest that important regulatory sequences are located at both the amino and the carboxyl terminus of c-Myb. However, it is not understood how carboxyl-terminal sequences regulate c-Myb activity.

Both v- and c-Myb are phosphorylated on serine and threonine but not tyrosine (10, 11). Lüscher *et al.* (10) have reported that c-Myb is a substrate for casein kinase II *in vitro* at serine-11 and -12 and that these serines are targets for kinase activity in cultured cells. Phosphorylation of these residues inhibits c-Myb sequence-specific DNA binding. These authors also have reported that c-Myb is hyperphosphorylated during mitosis in an avian lymphoma cell line at sites other than serine-11 and -12, suggesting that changes in the state of c-Myb phosphorylation may alter c-Myb transcription activating activity during the cell cycle (12). However, a relationship between the state of c-Myb phosphorylation and its transcription activating activity was not demonstrated. We have identified serine-528, which is located near the murine c-Myb negative regulatory domain, as a phosphorylation site in cultured cells. Replacement of serine-528 with alanine results in a 2- to 7-fold increase in the ability of c-Myb to transactivate a synthetic promoter containing five copies of the *mim*-1A Myb-responsive element (MRE). Thus, a phosphorylation site near the negative regulatory domain modulates c-Myb transcription-activating properties, suggesting that changes in the state of phosphorylation may serve to regulate c-Myb function.

MATERIALS AND METHODS

Cell Culture, Metabolic Labeling, and Preparation of Whole Cell Extracts. The C19.396 cell line is a stable transfectant of the C19 murine erythroleukemia (MEL) cell line carrying a murine c-*myb* transgene driven by the inducible mouse metallothionein I promoter (13). C19.396 MEL cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 μM 2-mercaptoethanol, and G418 at 200 μg/ml, in 5% CO_2 atmosphere. To induce expression of the c-*myb* transgene, $ZnCl_2$ was added (50 μM) 6–8 hr prior to metabolic labeling. CV-1 and CMT3COS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM glutamine in 10% CO_2.

For metabolic labeling, C19.396 cells were incubated for 2 hr in phosphate-free RPMI 1640 supplemented with 2 mM glutamine, 50 μM $ZnCl_2$, 1.5% bovine serum albumin, and G418 at 200 μg/ml. Cells were then resuspended in fresh phosphate-free RPMI 1640 (supplemented as above) with 8 mCi of [^{32}P]orthophosphate (DuPont/NEN; 8500–9120 Ci/mmol; 1 Ci = 37 GBq) per 10^8 cells and labeled for 4 hr. CMT3COS cells were cultured in phosphate-free DMEM supplemented with 2 mM glutamine and 1.5% bovine serum albumin for 2 hr and labeled with 12.5 mCi of [^{32}P]orthophosphate per 10^7 cells at 32–44 hr posttransfection. After labeling, both C19.396 and CMT3COS cells were resuspended at 1–5 × 10^7 cells per ml in YRIPA [12 mM Na_2HPO_4/4 mM NaH_2PO_4/1% (vol/vol) Nonidet P-40/1% (wt/vol) sodium deoxycholate/0.1% (wt/vol) SDS/2 mM EDTA, pH 8/1.5 mM

Abbreviations: GST, glutathione *S*-transferase; MEL, murine erythroleukemia; MRE, Myb-responsive element; $p42^{mapk}$, 42-kDa mitogen-activated protein kinase.

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EGTA/150 mM NaCl/20 mM NaF/50 mM β-glycerophosphate/100 mM microcystin/1 mM Na_3VO_4/1 mM phenylmethylsulfonyl fluoride with leupeptin at 10 μg/ml, aprotinin at 0.1 unit/ml, and α_2-macroglobulin at 0.01 unit/ml], sonicated, clarified by centrifugation, and precleared with protein A-Sepharose (Pharmacia).

Purification of Glutathione *S*-Transferase (GST) Fusion Proteins and Production of Antibodies. Plasmid pGSTNR, which contains cDNA encoding murine c-Myb aa 326–636 (including the negative regulatory domain) cloned into pGEX3X, and plasmid pGSTDB, which encodes the murine c-Myb DNA-binding domain, have been described (14). A point mutant of pGSTNR encoding a serine-to-alanine substitution at murine c-Myb serine-528 (S528A) was created with the Altered Sites II *in vitro* mutagenesis system (Promega). Expression and purification of GST fusion proteins have been described (14, 15). Antiserum against GSTDB prepared in New Zealand White rabbits and partially purified on protein G-Sepharose is referred to as WK serum.

Immunoprecipitation and SDS/PAGE. ^{32}P-labeled c-Myb was immunoprecipitated from whole cell extracts with WK serum for 1 hr at 4°C. Protein A-Sepharose was added for 15 min at 4°C and the lysates were briefly microcentrifuged at 4°C. Immune complexes were then washed three times: first with YRIPA, then with HSB [2 M NaCl/10 mM Tris, pH 7.4/1% (vol/vol) Nonidet P-40/0.5% (wt/vol) sodium deoxycholate], and then with YRIPA again. The beads were resuspended in 2× sample buffer [20% (vol/vol) glycerol/124 mM Tris, pH 6.8/4% (wt/vol) SDS/10% (vol/vol) 2-mercaptoethanol/0.1% (wt/vol) bromophenol blue], boiled for 3–5 min, and fractionated in an SDS/7.5% (wt/vol) polyacrylamide gel.

***In Vitro* Phosphorylation of GST Fusion Proteins and Tryptic Peptide Mapping.** GSTNR and the S528A mutant were phosphorylated *in vitro* by 42-kDa mitogen-activated protein kinase ($p42^{mapk}$) purified from EL-4 cells and were fractionated by SDS/PAGE as described (14). For tryptic phosphopeptide mapping, immunoprecipitated c-Myb or labeled GST-Myb fusion proteins were excised from the gels and tryptic phosphopeptide mapping was carried out as described (14) except that isobutyric acid was used in the thin-layer chromatography buffer (16).

Peptide Sequencing. Approximately 24 μg of GSTNR was phosphorylated *in vitro* by $p42^{mapk}$, fractionated by SDS/PAGE, and transferred to nitrocellulose. ^{32}P-labeled GSTNR protein was excised and active sites on the nitrocellulose were blocked by treatment with 500 μl of 0.5% (wt/vol) polyvinylpyrrolidone/0.6% (vol/vol) acetic acid for 30 min at 37°C. The nitrocellulose was washed with water, suspended in 50 mM NH_4HCO_3 buffer, and digested with 1.2 μg of sequencing-grade trypsin (Promega) for 12 hr at 37°C. The supernatant was collected, concentrated, and fractionated by HPLC on an Applied Biosystems OD-300 C_{18} column (particle size, 7 μm; 2.1 mm × 30 mm). Peptides were eluted with a 40-min, 0–60% gradient of solvent B (solvent A, 0.1% trifluoroacetic acid; solvent B, 0.085% trifluoroacetic acid/60% acetonitrile) at a flow rate of 200 μl/min. Fractions were collected at 1-min intervals, and those containing labeled phosphopeptides were identified by Cerenkov counting. Aliquots (1/20th) of fractions containing the highest radioactivity (cpm) were individually loaded onto a 75-μm-i.d., 190-μm-o.d. microcapillary column packed with a 13-cm bed of PRP-1 (Alltech Associates). Peptides were eluted with a 12-min linear gradient of 0–80% solvent B (solvent A, 0.1 M acetic acid; solvent B, acetonitrile; flow rate, 0.5 μl/min) into a TSQ 700 triple-quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with an electrospray ion source.

Transient Transfection and Luciferase Assays. Expression vectors encoding wild-type or S528A c-Myb were cotransfected with a promoter/reporter plasmid containing a minimal herpes simplex virus thymidine kinase promoter and five copies of the *mim*-1A MRE upstream of the firefly luciferase gene (p5XMRE-A-luc, gift of B. Lüscher, Medizinische Hochshule, Hannover, Germany) into CV-1 cells. CV-1 cells were transfected in triplicate by the calcium phosphate method (17) and harvested 48 hr after transfection. Cell extracts were normalized for protein content by a Coomassie blue G-250-based protein assay (Bio-Rad). Luciferase assays were performed with a commercial kit (Promega) and quantified in a RackBeta scintillation counter (LKB).

RESULTS

Phosphorylation of c-Myb in MEL Cells. Although the negative regulatory domain represses the DNA-binding, transcription activating, and transforming properties of c-Myb, the mechanism by which these effects are mediated is not understood (1). We have reported (14) that $p42^{mapk}$ phosphorylates c-Myb *in vitro* on several tryptic peptides in the negative regulatory domain which are absent from the v-Myb proteins (Fig. 1). To determine whether the phosphorylation sites identified in a cell-free system were phosphorylated in cultured cells ("*in vivo*"), c-Myb immunoprecipitated from logarithmically growing ^{32}P-labeled C19.396 MEL cells was fractionated by SDS/PAGE and subjected to tryptic phosphopeptide mapping alone and in combination with the carboxyl-terminal portion of c-Myb (GSTNR) which had been phosphorylated *in vitro* by $p42^{mapk}$ (Fig. 2*a*). Five major tryptic phosphopeptides can be detected when GSTNR is phosphorylated *in vitro* with $p42^{mapk}$ (ref. 14 and Fig. 2*a*, *in vitro* panel). The same pattern of tryptic phosphopeptides is observed with a GST fusion protein comprising full-length c-Myb (14). ^{32}P-labeled c-Myb immunoprecipitated from C19.396 cells contained at least five tryptic phosphopeptides (Fig. 2*a, in vivo* panel). When a mixture of *in vitro* phosphorylated GSTNR and metabolically labeled c-Myb from C19.396 cells was analyzed by two-dimensional tryptic phosphopeptide mapping, three tryptic phosphopeptides (labeled A–C in Fig. 2*a*) derived from *in vitro* phosphorylated GSTNR comigrated with tryptic phosphopeptides derived from *in vivo* labeled c-Myb (Fig. 2*a*, mix panel). Phosphopeptides A–C were also detected by tryptic phosphopeptide mapping with ^{32}P-labeled c-Myb immunoprecipitated from both the parental C19 cell line and the murine pre-B-cell lymphoma cell line 70Z/3.12 (data not shown). Tryptic phosphopeptides E and F were not detected on tryptic phosphopeptide maps from *in vivo* labeled c-Myb, indicating that they are not targets of phosphorylation in the cell lines examined. These findings demonstrate that c-Myb is phosphorylated in at least two hematopoietic cell lines at one or more sites which serve as targets for phosphorylation by $p42^{mapk}$ *in vitro*.

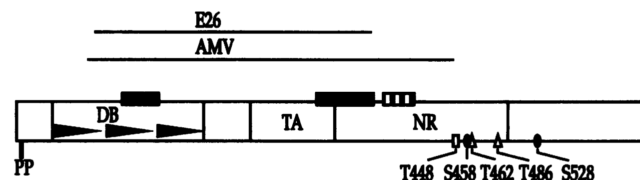

FIG. 1. Diagram of the murine c-Myb protein. Murine c-Myb (rectangular box) and sequences of the c-Myb protein present in the E26 $p135^{gag-myb-ets}$ and avian myeloblastosis virus (AMV) $p48^{v-myb}$ proteins (labeled lines above) are shown. The DNA-binding domain (DB) with three tandem repeats (solid triangles), transactivation domain (TA), negative regulatory domain (NR), leucine zipper (striped box), nuclear localization domains (solid rectangles), and amino-terminal sites of phosphorylation (PP) (10) of c-Myb are also indicated. Potential sites of $p42^{mapk}$ phosphorylation are indicated with the one-letter amino acid code and number of the target murine residue; small open rectangle, TP motif; open triangles, PXTP motifs; solid ovals, SP motifs (5, 6, 10, 14, 18–20).

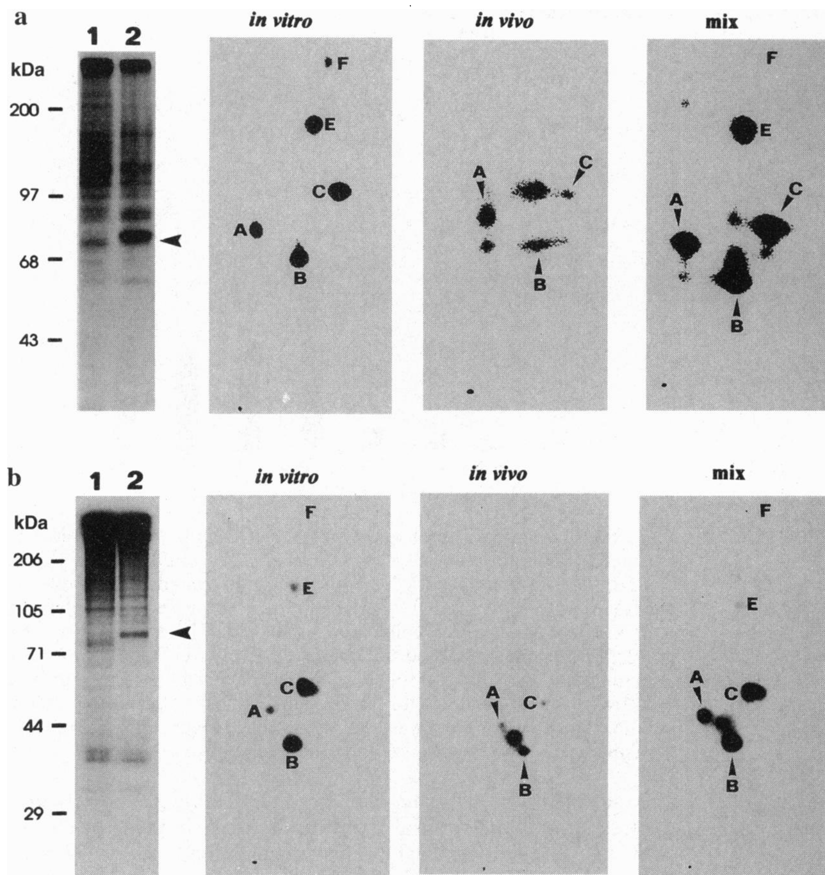

FIG. 2. Phosphorylation of c-Myb *in vivo*. Tryptic phosphopeptide maps of ^{32}P-labeled c-Myb immunoprecipitated from C19.396 MEL cells (*a*) or c-*myb*-transfected CMT3COS cells (*b*) are compared with maps of GSTNR phosphorylated *in vitro* with p42^{mapk}. ^{32}P-labeled cells were lysed and immunoprecipitation was carried out with normal rabbit serum (*a* and *b*, lane 1) or WK serum (*a* and *b*, lane 2) and the precipitates were analyzed by SDS/PAGE. Regions of the gel containing c-Myb (arrowheads, lane 2 in *a* and *b*) were excised and subjected to tryptic peptide mapping. Tryptic phosphopeptide maps of GSTNR phosphorylated *in vitro* with p42^{mapk} (*in vitro*), metabolically labeled c-Myb (*in vivo*), and a mixture of equal cpm of both *in vitro* and *in vivo* phosphorylated Myb proteins (mix) are presented. The major phosphopeptides A, B, C, E, and F are indicated. The origin is marked by a small dot at the bottom of each map. Phosphopeptide maps were visualized with a Molecular Dynamics PhosphorImager and IMAGEQUANT software.

Serine-528 of Murine c-Myb Is Phosphorylated *in Vivo*. We have reported (14) that phosphopeptides A–C are lost by deletion of aa 499–558 in chicken c-Myb and that these phosphopeptides are most likely the result of incomplete digestion by trypsin. This region contains a single conserved SP motif which is a potential site of phosphorylation by p42^{mapk} and other proline-directed serine/threonine kinases (chicken serine-533, corresponding to murine serine-528). The tryptic phosphopeptide map generated from GSTNR-S528A lacked phosphopeptides A–C (Fig. 3), which are normally present both on *in vitro* maps from wild-type GSTNR and on maps generated from *in vivo* phosphorylated c-Myb (Fig. 2). Using both Edman degradation analysis (data not shown) and microcapillary HPLC–electrospray ionization tandem mass spectrometry, we identified serine-528 as the phosphorylated residue in peptides B and C (Fig. 4). Since phosphopeptide B is QEVES^{528}PTEK (Fig. 4 *Upper*) and phosphopeptide C is IKQEVES^{528}PTEK (Fig. 4 *Lower*), we conclude that phosphopeptide C arises from incomplete digestion by trypsin.

Modulation of c-Myb Transcription-Transactivating Activity by Serine-528. As serine-528 is a target for phosphorylation *in vivo* and is located near the negative regulatory domain, we determined whether c-Myb transactivating activity is affected by this site. We compared the ability of wild-type and S528A c-Myb to transactivate a reporter construct containing five copies of the *mim*-1A MRE (5×MRE) ligated to a minimal herpes simplex virus thymidine kinase promoter. For expression in eukaryotic cells, cDNAs encoding wild-type c-Myb or the S528A mutant were cloned downstream of the Rous sarcoma virus long terminal repeat in pRSV (13). Each transfection mixture contained 1.0 μg of reporter plasmid, 0.5–10 μg of the indicated expression vector, and sufficient empty vector to bring the total DNA to 11 μg. CV-1 cells were used for these experiments because they do not contain detectable endogenous c-Myb (data not shown). Both wild-type and S528A c-Myb transactivated the promoter/reporter construct (Fig. 5). However, S528A c-Myb transactivated this reporter construct 2- to 7-fold more efficiently than wild-type c-Myb. This difference in transactivation efficiency was not due to differences in expression between wild-type and S528A c-Myb, as determined by immunoblotting (data not shown).

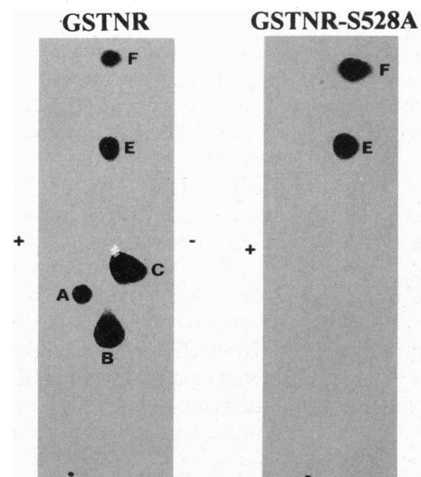

FIG. 3. Tryptic phosphopeptide maps of the wild-type c-Myb negative regulatory domain and a serine-528 point mutant. The carboxyl-terminal portion of murine c-Myb (aa 326–636) expressed as a GST fusion protein [GSTNR, previously described as GST-muNR (14)] and a mutant protein with a serine-to-alanine substitution at position 528 (GSTNR-S528A, right panel) were phosphorylated by p42^{mapk} *in vitro*, fractionated by SDS/PAGE, eluted from the gel, and subjected to tryptic phosphopeptide mapping. The origin is indicated by a small dot at the bottom of each peptide map. Anode (+) and cathode (−) are indicated. The five major tryptic phosphopeptides identified are labeled A, B, C, E, and F.

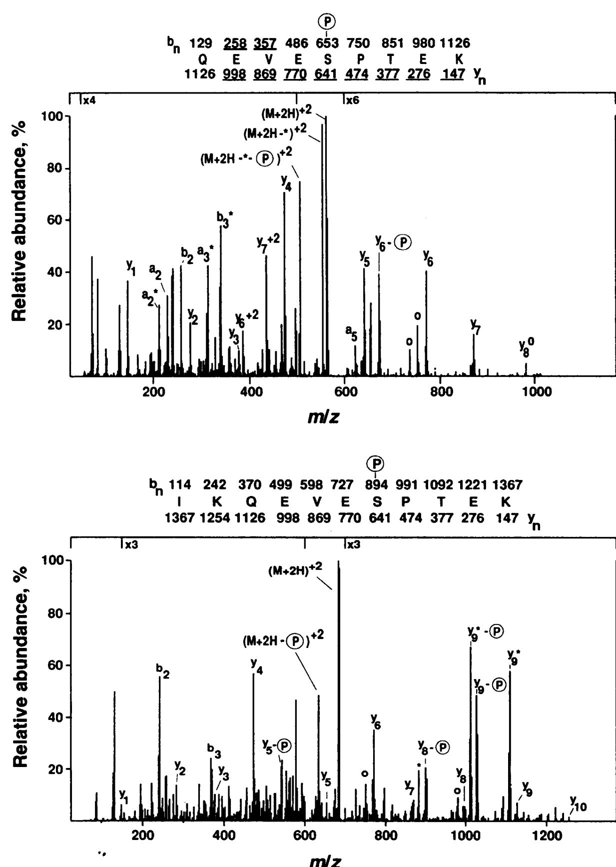

FIG. 4. Collision-activated dissociation mass spectrum from murine c-Myb tryptic phosphopeptides B (*Upper*) and C (*Lower*). Fragment ions of type b contain the amino terminus plus one or more additional residues. Fragment ions of type y contain the carboxyl terminus plus one or more additional residues. The deduced amino acid sequence (one-letter amino acid code) is shown above each spectrum; circled P denotes a phosphate group. Neutral losses of water and ammonia are denoted by o and *, respectively. Phosphoserine gives a characteristic neutral loss of H_3PO_4.

To determine whether serine-528 can be phosphorylated in African green monkey kidney cells, pRSVMyb was modified to contain a simian virus 40 origin of replication and transiently transfected into CMT3COS cells. The CMT3COS cell line, a derivative of the CV-1 cell line, was used for high-level expression of transfected c-Myb (Fig. 2*b*; ref. 21). Tryptic phosphopeptides A–C detected in tryptic digests of *in vitro* phosphorylated c-Myb comigrated with three phosphopeptides derived from ^{32}P-labeled c-Myb immunoprecipitated from CMT3COS cells (Fig. 2*b*). Thus, serine-528 is a target for phosphorylation *in vivo* and negatively regulates the ability of c-Myb to transactivate the 5×MRE reporter construct in the CV-1 cell line. This suggests that phosphorylation of serine-528 negatively regulates c-Myb activity.

DISCUSSION

Several lines of evidence indicate that protein sequences at both the amino and the carboxyl terminus of c-Myb exert a negative influence on c-Myb activity. First, Myb proteins encoded by avian myeloblastosis virus and the E26 avian leukemia virus are truncated at both the amino and the carboxyl terminus with respect to c-Myb (1). Second, retrovirus-induced tumors in both the chicken and the murine system commonly harbor insertions that result in expression of Myb proteins truncated at the amino or carboxyl terminus (22–28). Third, truncation of either the amino or the carboxyl terminus

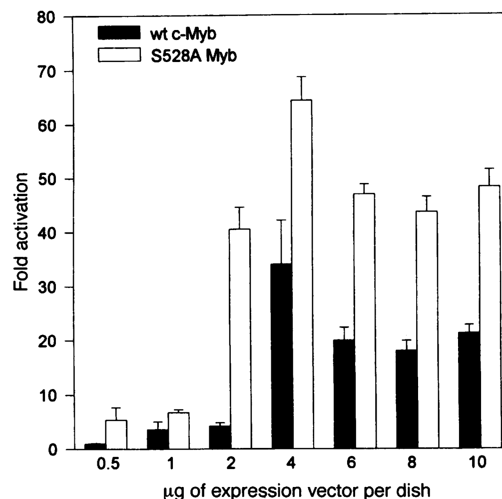

FIG. 5. Comparison of wild-type (wt) and S528A c-Myb transcriptional activation potentials. CV-1 African green monkey kidney cells were cotransfected with various amounts of pRMb3SV.wt or pRMb3SV.S528A and 1 μg of the promoter/reporter plasmid p5XMRE-A-luc. CV-1 cells were transfected in triplicate by the calcium phosphate method and harvested 48 hr after transfection. Cell extracts were normalized for protein content. Each bar represents the average fold induction (for three samples) over the empty vector controls with error bars representing standard error of the mean. The results are representative of three independent experiments.

is sufficient to activate c-Myb transforming potential and, interestingly, differential truncation results in transformation of different cell types (9). This suggests that modification of these regions might serve to regulate c-Myb activity. How these sequences exert their influence on c-Myb activity and how their function is regulated are not understood. We have demonstrated that murine c-Myb is phosphorylated in cultured cells at serine-528 and that substitution of alanine for serine-528 resulted in a 2- to 7-fold increase in transcription-transactivating activity. Thus, phosphorylation at serine-528 may provide a mechanism to negatively regulate c-Myb function. The effects of phosphorylation at serine-528 on c-Myb activity may prove to be promoter-dependent and phosphorylation may provide a mechanism to differentially regulate c-Myb activity.

Metabolically labeled c-Myb immunoprecipitated from C19.396 MEL cells and CMT3COS cells yielded distinct but overlapping sets of tryptic phosphopeptides, suggesting that c-Myb may contain common and lineage-specific phosphorylation sites. Differential phosphorylation may provide a mechanism to alter c-Myb activity in a lineage-specific fashion, in response to growth and differentiation factors. Indeed, Lüscher and Eisenman (29) identified changes in the pattern of c-Myb phosphorylation detected on interphase and mitotic cells, suggesting that phosphorylation may alter c-Myb activity during the cell cycle.

The carboxyl-terminal region of c-Myb appears to contain at least two elements that are involved in regulating c-Myb activity. Sakura *et al.* (6) used a series of sequential carboxyl-terminal deletions to broadly define a region spanning murine c-Myb aa 326–500 that is referred to as the negative regulatory domain (see Fig. 1). Kalkbrenner *et al.* (8) subsequently identified a region, aa 496–640, of the human c-Myb protein that also negatively influenced the ability of c-Myb to transactivate. These reports suggested that two essentially nonoverlapping regions in the carboxyl-terminal portion of c-Myb might play a role in regulating c-Myb function. Dubendorff *et al.* (30) created a series of small deletions in the chicken c-Myb carboxyl-terminal region and identified two regions that suppressed c-Myb transactivation activity. One region, aa 425–

464, is included within the previously defined negative regulatory domain. The second region, aa 499–558, is immediately carboxyl-terminal to the negative regulatory domain in the region defined by Kalkbrenner *et al.* (8) and includes serine-533 (the chicken homologue of murine c-Myb serine-528). These results suggest that the carboxyl-terminal region may contain at least two distinct negative regulatory elements. Alternatively, the two regions may interact to mediate a negative influence on c-Myb activity.

How phosphorylation in the carboxyl-terminal region regulates c-Myb transcription activating properties is not clear. Several groups have demonstrated that progressive carboxyl-terminal truncations result in Myb proteins with increased sequence-specific DNA-binding activity, and phosphorylation at serine-528 might have a similar effect. However, we have been unable to demonstrate a difference in DNA-binding activity between wild-type c-Myb and the S528A mutant in extracts from transfected CMT3COS cells (A. Richardson, M.R.M., and T.P.B., unpublished data). In addition, Dubendorff *et al.* (30) have reported that a 60-aa deletion of chicken c-Myb which includes serine-533 does not affect DNA-binding activity *in vivo*. Thus, it is unlikely that the major effect of phosphorylation at serine-528 is to regulate DNA-binding activity. Phosphorylation might serve to regulate interactions between c-Myb and other proteins or to suppress the activity of the transactivation domain. Two previous reports have provided evidence that c-Myb may interact with two proteins of 150 and 67 kDa via its leucine zipper (31, 32). Preliminary data indicate that the c-Myb carboxyl terminus interacts with several additional cellular proteins (A. Richardson and T.P.B., unpublished data). It will be of interest to determine whether phosphorylation of serine-528 regulates interactions between c-Myb and these proteins. Regarding potential suppression of the transactivation domain by phosphorylation at serine-528, Dubendorff *et al.* (30) reported the ability of chicken c-Myb carboxyl-terminal sequences to suppress transactivation by Myb in trans. The carboxyl-terminal sequence of primary importance (aa 499–558) includes serine-533, whereas the target of suppression includes the acidic sequences of the transactivation domain. This suppression could be mediated by direct interaction between the region containing serine-533 and the transactivation domain or by another protein that interacts with the transactivation domain and is stabilized by the phosphorylated serine-533 region (30).

Serine-528 lies in a region of the c-Myb protein that is highly conserved among chicken, human, and murine c-Myb homologues, human A-myb, and BAS1 (30). However, while the SP motif in this region is conserved in the chicken, human, and murine c-Myb homologues, this motif is not found in other Myb family proteins. Thus, phosphorylation of serine-528 may serve a unique role in regulating c-Myb. It is now important to understand how and when phosphorylation at serine-528 is regulated. While we have demonstrated that c-Myb is a substrate for $p42^{mapk}$ *in vitro*, it should be stressed that it is not clear whether $p42^{mapk}$ is responsible for phosphorylation of serine-528 *in vivo*. Other serine/threonine kinases might phosphorylate serine-528 *in vivo*, including proline-directed serine/threonine kinases of the mitogen-activated and cyclin-dependent protein kinase families. Interestingly, the carboxyl terminus contains consensus sequences for a number of serine/threonine kinases, including protein kinase A, glycogen synthase III, casein kinase II, and protein kinase C. Thus, the carboxyl terminus of c-Myb may serve as an integrator of multiple signal transduction pathways where different phosphorylation or dephosphorylation events collaborate to regulate c-Myb activity.

We thank Gwen Harley for expert technical assistance. This work was supported by U.S. Public Health Service Grants GM37537 and AL33993 to D.F.H. and CA40042 to T.P.B. T.P.B. was also supported by American Cancer Society Grants FRA-425 and DB-92. M.R.M. was supported by Department of Health and Human Services Training Grant CA09109. N.A. and M.R.M. made equal contributions to this work and are listed alphabetically.

1. Graf, T. (1992) *Curr. Opin. Genet. Dev.* **2,** 249–255.
2. Roussel, M., Saule, S., Lagrou, C., Rommens, C., Beug, H., Graf, T. & Stehelin, D. (1979) *Nature (London)* **281,** 452–455.
3. Klempnauer, K. H. & Bishop, J. M. (1983) *J. Virol.* **48,** 565–572.
4. Gonda, T. J. & Bishop, J. M. (1983) *J. Virol.* **46,** 212–220.
5. Klempnauer, K. H., Gonda, T. J. & Bishop, J. M. (1982) *Cell* **31,** 453–463.
6. Sakura, H., Kanei Ishii, C., Nagase, T., Nakagoshi, H., Gonda, T. J. & Ishii, S. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 5758–5762.
7. Hu, Y. L., Ramsay, R. G., Kanei Ishii, C., Ishii, S. & Gonda, T. J. (1991) *Oncogene* **6,** 1549–1553.
8. Kalkbrenner, F., Guehmann, S. & Moelling, K. (1990) *Oncogene* **5,** 657–661.
9. Grasser, F. A., Graf, T. & Lipsick, J. S. (1991) *Mol. Cell. Biol.* **11,** 3987–3996.
10. Lüscher, B., Christenson, E., Litchfield, D. W., Krebs, E. G. & Eisenman, R. N. (1990) *Nature (London)* **344,** 517–522.
11. Bading, H., Beutler, C. & Moelling, K. (1989) *Oncogene* **4,** 33–38.
12. Litchfield, D. W., Lüscher, B., Lozeman, F. J., Eisenman, R. N. & Krebs, E. G. (1992) *J. Biol. Chem.* **267,** 13943–13951.
13. Cuddihy, A. E., Brents, L. E., Aziz, N., Bender, T. P. & Kuehl, W. M. (1993) *Mol. Cell. Biol.* **13,** 3505–3513.
14. Aziz, N., Wu, J., Dubendorff, J. W., Lipsick, J. S., Sturgill, T. W. & Bender, T. P. (1993) *Oncogene* **8,** 2259–2265.
15. Smith, D. B. & Johson, K. S. (1988) *Gene* **67,** 31–40.
16. Boyle, W. J., van der Geer, P. & Hunter, T. (1991) *Methods Enzymol.* **201,** 110–149.
17. Van den Elsen, P., Houweling, A. & Van deer Eb, A. (1983) *Virology* **128,** 377–390.
18. Ibanez, C. E. & Lipsick, J. S. (1988) *J. Virol.* **62,** 1981–1988.
19. Nunn, M. F., Seeburg, P. H., Moscovici, C. & Duesberg, P. H. (1983) *Nature (London)* **306,** 391–395.
20. Nunn, M., Weiher, H., Bullock, P. & Duesberg, P. (1984) *Virology* **139,** 330–339.
21. Gerard, R. D. & Gluzman, Y. (1985) *Mol. Cell. Biol.* **5,** 3231–3240.
22. Kanter, M. R., Smith, R. E. & Hayward, W. S. (1988) *J. Virol.* **62,** 1423–1432.
23. Pizer, E. & Humphries, E. H. (1989) *J. Virol.* **63,** 1630–1640.
24. Wolff, L. & Koller, R. (1990) *J. Virol.* **64,** 155–160.
25. Mukhopadhyaya, R. & Wolff, L. (1992) *J. Virol.* **66,** 6035–6044.
26. Shen Ong, G. L., Morse, H. C., III, Potter, M. & Mushinski, J. F. (1986) *Mol. Cell. Biol.* **6,** 380–392.
27. Weinstein, Y., Ihle, J. N., Lavu, S. & Reddy, E. P. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5010–5014.
28. Weinstein, Y., Cleveland, J. L., Askew, D. S., Rapp, U. R. & Ihle, J. N. (1987) *J. Virol.* **61,** 2339–2343.
29. Lüscher, B. & Eisenman, R. N. (1992) *J. Cell Biol.* **118,** 775–784.
30. Dubendorff, J. T., Whitakker, L. J., Eltman, J. T. & Lipsick, J. S. (1993) *Genes Dev.* **6,** 2524–2535.
31. Kanei-Ishii, C., MacMillan, E. A., Nomura, T., Sarai, A., Ramsay, R. G., Aimoto, S., Ishii, S. & Gonda, T. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3088–3092.
32. Favier, D. & Gonda, T. J. (1994) *Oncogene* **9,** 305–311.