Phosphorylation of B-Myb Regulates Its Transactivation Potential and DNA Binding*

(Received for publication, September 8, 1999, and in revised form, September 22, 1999)

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The transcription factor B-Myb is a cell cycle-regulated phosphoprotein and a potent regulator of cell cycle progression. Previous studies demonstrated that B-Myb was phosphorylated at the onset of S phase, suggesting that it could be due to cyclin-dependent kinases. We identified 10 B-Myb phosphorylation sites by automated peptide radiosequencing of tryptic phosphopeptides derived from in vivo 32P-labeled B-Myb. Each B-Myb phosphorylation site contained a phosphoserine or phosphothreonine followed by a proline, suggesting that this phosphorylation is due to a proline-directed kinase. Cyclin A-Cdk2 and cyclin E-Cdk2 complexes each phosphorylated B-Myb in a cell-free system on the same sites as in intact cells. Furthermore, the ability of B-Myb to activate a reporter plasmid was enhanced by the cotransfection of cyclin A, whereas mutagenesis of the 10 identified phosphorylation sites from B-Myb blocked the effect of cyclin A coexpression. Additional analysis revealed that the effect of phosphorylation on B-Myb transactivation potential was enhanced by phosphorylation sites in its carboxyl-terminal half. One phosphorylation site (Ser281) appeared to negatively regulate DNA binding, as mutation of this site enhanced the ability of B-Myb to bind a Myb-binding sequence. These data suggest that B-Myb is a target for phosphorylation by cyclin-Cdk2 and that phosphorylation of B-Myb regulates its transcriptional activity.

* This work was supported in part by a pilot project from the University of Nebraska Medical Center/Eppley Cancer Center, Grant BE-260 from the American Cancer Society, and NCI Grant P30 CA36727 from the National Institutes of Health (to the Eppley Cancer Institute). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by NCI Health Training Grant CA09476 from the National Institutes of Health.

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1 The abbreviations used are: MBS, Myb-binding site(s); CR, conserved region; HPLC, high pressure liquid chromatography; ECF, enhanced chemiluminescence.
medium supplemented with 10% fetal bovine serum and incubated at 37 °C in 5% CO2. Sf9 cells were maintained in Grace's medium supplemented with 10% fetal bovine serum and incubated at 27 °C.

Generation of B-Myb Antiserum—A rabbit was immunized with a gluthathione S-transferase fusion protein containing amino acids 553–611 of B-Myb together with Freund's adjuvant. After 16 weeks, the immune serum was harvested and tested for its ability to immunoprecipitate B-Myb.

Constitution of B-Myb Expression Plasmids and DNA Mutagenesis—A FLAG epitope tag was added to the amino terminus of mouse B-Myb cDNA (a gift of R. J. Watson (9)) using a polymerase chain reaction strategy. The forward primer (5′-CCAGGATCTCTGCCCAGGGACGACGATGACAAGTCTCGGCGGACGCGCTGCG-3′) contains an EcoRI site and encodes the FLAG epitope; the reverse primer was 5′-GGTTGACATTTGGGCGATCT. The reverse primer was 5′-GGTTGACATTTGGGCGATCT. The forward primer (5′-GGTTGACATTTGGGCGATCT) encodes the FLAG epitope; the reverse primer was 5′-GGTTGACATTTGGGCGATCT. The polymerase chain reaction product was cut with EcoRI and XhoI to remove FLAG-B-Myb from pCMV5. This fragment was ligated into the site of pcDNA3 to produce pcDNA3-FLAG-B-Myb. DNA mutagenesis was performed with the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's directions. Primers were designed to encode changes from serine to alanine or threonine to valine and to make a silent mutation to create or delete a restriction endonuclease site and to facilitate the identification of mutagenized clones. Template DNA was either pcDNA3-FLAG-B-Myb or one of the previously mutated B-Myb cDNAs. All mutations were confirmed by DNA sequencing. The forward primers for mutagenesis were 5′-GGGGAGAAGTGCGCGTTCCGGAGCCCCCAGAATCTCTCAAGCCG-3′ (T267V, creates a Bsp EI site), 5′-CCAGGATCTCTGCCCAGGGACGACGATGACAAGTCTCGGCGGACGCGCTGCG-3′ (S396A, creates a SacII site), 5′-GCTGATCCCATCGCTCGGCGATCT (3TV, creates a Bsp EI site), and 5′-GGTTGACATTTGGGCGATCT. The reverse primers were the complements of the forward primers. All polymerase chain reaction primers and DNA sequencing were done by the University of Nebraska Medical Center Molecular Biology Core Laboratory.

Transfection, Expression, and Immunoprecipitation of B-Myb Proteins—B-Myb proteins were transfected by the calcium phosphate method (21). Cells were assayed 48 h after transfection. The cells were solubilized in lysis buffer and immunoprecipitated overnight at 4 °C with anti-FLAG M2 antibodies (Kodak Scientific Imaging Systems) conjugated to agarose and washed as described previously (22) before addition of sample buffer and electrophoresis.

Metabolic Labeling of B-Myb Proteins in Intact Cells—Metabolic labeling of B-Myb with [35S]methionine was performed as described previously (23). 293T cells transiently expressing B-Myb or mutated B-Myb proteins were incubated in phosphate-free Dulbecco's modified Eagle's medium containing [32P]orthophosphate (carrier-free; ICN) at a final concentration of 2.5 mCi/ml for 4 h at 37 °C. Cells were lysed and immunoprecipitated as described previously (22). Phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography or with storage phosphor technology (Molecular Dynamics, Inc.).

Phosphorilation of B-Myb in a Cell-free System—293T cells transiently expressing B-Myb were lysed and immunoprecipitated as described above. Sf9 cells were co-infected with recombinant baculovirus containing cDNAs for either cyclin A and Cdk2 or cyclin E and Cdk2 (a gift of S. H. Lee). After 48 h, the infected Sf9 cells were lysed. Sf9 cell lysates were incubated with immunoprecipitated B-Myb in a kinase reaction. Alternatively, Sf9 cell lysates were immunoprecipitated with anti-cyclin E or anti-cyclin A antibodies together with anti-FLAG antibodies on protein G-agarose beads. Sf9 cell lysates were also immunoprecipitated with anti-cyclin A or anti-cyclin E antibodies and incubated with 3 μg of histone H1 in a kinase reaction. Immunoprecipitates were washed with phosphate-buffered saline. The immunoprecipitates were then washed with 10 μl of kinase reaction buffer (20 mM Tris, pH 7.5, 10 mM MgCl2, 5 μM ATP, and 1 μM of [γ-32P]ATP (10 μCi/μl, 6000 Ci/mmol; Amersham Pharmacia Biotech)). The reaction was allowed to proceed for 20 min at 37 °C. Electrophoresis sample buffer was added to stop the reaction, and the samples were heated at 110 °C for 2 min by SDS-polyacrylamide gel electrophoresis.

HPLC Phosphopeptide Mapping and Phosphoamino Acid Analysis—[32P]-Labeled B-Myb proteins were recovered from polyacrylamide gels by the method of Hunter and co-workers (24) and then digested with trypsin and separated on a peptide C18 column as described previously (22). One-dimensional phosphoamino acid analysis was performed on the tryptic phosphopeptides that were desalted (22). Preparation and Sequencing of B-Myb Phosphopeptides for Radiosequencing—Five 10-cm dishes of 293T cells transiently expressing pcDNA3-FLAG-B-Myb were prepared for each experiment. Each dish was labeled with 5 μCi of [32P]orthophosphate for 4 h at 37 °C. 32P-Labeled B-Myb proteins were immunoprecipitated and separated by SDS-polyacrylamide gel electrophoresis as described above. Tryptic phosphopeptides generated from radiolabeled B-Myb were separated on a Vydac C8 column and subjected to amino-terminal Edman degradation as described previously (22). A portion of each sequencing fraction was collected and subjected to scintillation counting for radioactivity from phosphoserine and phosphothreonine.

Promoter/Reporter Assays—U2-OS cells (in 60-mm dishes) were co-transfected with 10 μg of 3x-ACT-Luc (a gift of S. A. Nussenzweig) (25), 6 μg of pcDNA3-FLAG-B-Myb or pcDNA3-B-Myb 10Min, 10 μg of pRc/cyclin A, 4 μg of Rous sarcoma virus-β-galactosidase, and, where necessary, a balancing amount of empty pcDNA3 vector. After 48 h, cells were assayed using the luciferase assay system (Promega) according to the manufacturer's directions. Luciferase activity was assayed in a Monolight 2010 luminometer (Analytical Luminescence Laboratory). β-Galactosidase activity was assayed (26) to normalize for transfection efficiency.

Western Blot Analysis—Proteins were transferred to polyvinylidene difluoride membranes and blotted using anti-FLAG M2 antibodies (1:3000) and alkaline phosphatase-conjugated goat anti-mouse secondary antibodies (1:2000). Blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

DNA Affinity Purification—DNA affinity purification using biotinylated oligonucleotides was performed as described previously (27) with the following modifications. Four biotinylated DNA probes were synthesized, MBS-1 (AGAATGTGTGTCAGTTAGGGTGTAGAG) and its complement (26) and mutant MBS-1 containing a scrambled MBS sequence (AGAATGTGTGTCAGTTAGGGTGTAGAC) were used. After 24 h of incubation, the double-stranded oligonucleotides were incubated with immobilized streptavidin (Roche Molecular Biochemicals) for 1 h at 4 °C and washed as described by the manufacturer. 293T cells were transfected with wild-type or mutated B-Myb as described above and then lysed in KCl extraction buffer (27). Insoluble matter was pelleted by centrifugation; the KCl concentration was diluted to 200 mM, and lysates were precleared with immobilized streptavidin for 1 h at 4 °C. B-Myb from 30 μl of each cleared lysate was detected by ECF (Amersham Pharmacia Biotech) Western analysis and quantified with a Storm PhosphoImager (Molecular Dynamics, Inc.). Equal amounts of B-Myb protein from each lysate were added to the immobilized DNA with 0.5 μg of salmon sperm DNA and 1 μg/μl bovine serum albumin for 1 h at 4 °C and then washed four times with DNA affinity wash buffer (10 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, and 0.1% Triton X-100). B-Myb bound to the DNA was analyzed by ECF Western blotting and then quantified.

RESULTS

Identification of B-Myb Phosphorylation Sites—Previous studies have shown that B-Myb is phosphorylated in a cell cycle-dependent manner at the onset of S phase (19) and that phosphorylation may control the biological activity of B-Myb (8, 11, 28). The goal of this study was to identify the specific amino acids phosphorylated on B-Myb in intact cells. B-Myb was transiently expressed in cells metabolically labeled with [35S]methionine or [32P]orthophosphate. The radiolabeled proteins were immunoprecipitated with anti-B-Myb or preimmune serum. Immunoprecipitation of [35S]methionine-labeled B-Myb with either immuno or preimmune serum from cells transiently expressing B-Myb or transfected with the vector only demonstrated that the anti-B-Myb antibody but not the preimmune immunoprecipitated B-Myb (Fig. 1A, left panel). Immunoprecipitation of B-Myb from [32P]-labeled cells under the same conditions demonstrated that B-Myb was phosphorylated in intact cells (Fig. 1A, right panel). Phosphoamino acid analysis of the [32P]-labeled B-Myb protein revealed the presence of phosphoserine and phosphothreonine (Fig. 1B).
To identify specific B-Myb phosphorylation sites, B-Myb was expressed in the human embryonic kidney cell line 293T. 32P-Labeled B-Myb was immunoprecipitated and separated by SDS-polyacrylamide gel electrophoresis, and the proteins were visualized with a ZnCl2 stain. The gel piece was digested with trypsin, and the radiolabeled phosphopeptides were extracted and subjected to HPLC analysis. The major radiolabeled peaks were sequenced and identified by automated Edman degradation (Fig. 2). The presence of the radiolabeled phosphate group on the amino acid residue prevents detection of the amino acid in that cycle, although subsequent amino acids can be identified. A fraction of each sequencing cycle was also counted for radioactivity released by β-elimination to identify the cycle containing the specific amino acid phosphorylated within each peptide. The 32P released in each cycle was compared with the peptide sequence determined for each phosphopeptide to identify each phosphorylation site (Fig. 3). Phosphoamino acid analysis done on the HPLC fractions confirmed the prediction of phosphoserine or phosphothreonine in the phosphopeptide sequence determined for each phosphopeptide to identify each phosphorylation site (Fig. 3). Phosphoamino acid sequence on B-Myb is consistent with the possibility that B-Myb is phosphorylated by a proline-directed kinase. The fact that B-Myb is phosphorylated in a cell cycle-dependent manner suggests that it may be phosphorylated by cyclin-dependent kinases (1, 8, 19, 28), members of the family of proline-directed kinases. Previous work indicated that B-Myb is phosphorylated at the onset of the S phase of the cell cycle (19), coincident with increased cyclin A-Cdk2 kinase activity. Phosphorylation of serine or threonine by Cdk2 requires phosphorylation of serine or threonine followed directly by a proline residue, suggesting that B-Myb is phosphorylated by a proline-directed kinase. The location of the predicted Cdk2 phosphorylation site was confirmed by comparing the HPLC profiles of mutated B-Myb proteins to that of the wild-type protein (Fig. 4). Single phosphopeptide peaks are absent from the HPLC profile. Amino acids listed in parentheses were inferred from the known sequence of B-Myb.

**Fig. 1.** Metabolic labeling of B-Myb in intact cells. A, immunoprecipitation by anti-B-Myb or preimmune serum of [35S]methionine or [32P]-labeled B-Myb proteins from COS-1 cells transfected with pCMV5-B-Myb or pCMV5 only; B, phosphoamino acid analysis of [32P]-labeled B-Myb from A. The locations of phosphoamino acid standards are indicated.

**Fig. 2.** HPLC separation of B-Myb phosphopeptides. B-Myb tryptic phosphopeptides from 32P-labeled 293T cells were analyzed by HPLC. Tryptic peptides were detected by absorbance at 210 nm and collected by a peak-actuated fraction collector. The labeled peaks were sequenced by automated Edman degradation. The sequence of each identified peptide and the location of the predicted phosphorylation sites was confirmed by comparing the HPLC profiles of mutated B-Myb proteins to that of the wild-type protein (Fig. 4). Single phosphopeptide peaks are absent from the HPLC profile. Amino acids listed in parentheses were inferred from the known sequence of B-Myb.

Serine or threonine followed directly by a proline residue, suggesting that B-Myb is phosphorylated by a proline-directed kinase. The fact that B-Myb is phosphorylated in a cell cycle-dependent manner suggests that it may be phosphorylated by cyclin-dependent kinases (1, 8, 19, 28), members of the family of proline-directed kinases. Previous work indicated that B-Myb is phosphorylated at the onset of the S phase of the cell cycle (19), coincident with increased cyclin A-Cdk2 kinase activity. Phosphorylation of serine or threonine by Cdk2 requires phosphorylation of serine or threonine followed directly by a proline residue, suggesting that B-Myb is phosphorylated by a proline-directed kinase. The location of the predicted Cdk2 phosphorylation site was confirmed by comparing the HPLC profiles of mutated B-Myb proteins to that of the wild-type protein (Fig. 4). Single phosphopeptide peaks are absent from the HPLC profile. Amino acids listed in parentheses were inferred from the known sequence of B-Myb.

Mutagenesis of B-Myb Phosphorylation Sites—Biochemical identification of each phosphorylation site was confirmed by site-directed mutagenesis. Isosteric substitution of alanine or valine for serine or threonine, respectively. Each site was mutated independently, except Thr519, Thr522, and Thr524, which were all altered in a single mutagenesis event. The combined mutation of Thr519, Thr522, and Thr524 was made due to their proximity to one another and because the sequencing data (Fig. 3) suggested that these amino acids may be alternative phosphorylation sites for each other. A B-Myb construct that removed all 10 of the phosphorylation sites identified was also created. The mutated B-Myb proteins were transiently expressed in 32P-labeled 293T cells. The radiolabeled B-Myb proteins were isolated and digested with trypsin, and the tryptic phosphopeptides were analyzed by reverse-phase HPLC. The loss of specific phosphorylation sites was confirmed by comparing the HPLC profiles of the mutated B-Myb proteins to that of the wild-type protein (Fig. 4). Single phosphopeptide peaks are absent from the HPLC phosphopeptide profiles of B-Myb S283A (Fig. 4A), B-Myb T267V (Fig. 4A), and B-Myb S581A (Fig. 4D). Ser396 and Thr408 reside in the same tryptic peptide.

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Elimination of either site resulted in a partial decrease and slight shift in the position of the corresponding phosphopeptide peak (Fig. 4B). The B-Myb S455A protein did not have a phosphopeptide map discernibly different from that of intact 32P-labeled B-Myb (Fig. 4C). The phosphopeptide containing Ser455 eluted at the end of the HPLC gradient, far later than would be predicted from the sequence composition. This observation suggests that, when it was sequenced, the Ser455 phosphopeptide was digested incompletely by trypsin. It is also possible that the Ser455 phosphopeptide interacted with other peptide fragments of B-Myb to delay its elution from the column. Either explanation suggests that elution of the phosphopeptide containing Ser455 from the HPLC gradient may vary and could prevent resolution of the site by mapping of the B-Myb S455A protein.

The 3TV mutation removed three peaks that were present in intact B-Myb (Fig. 4D). The small phosphopeptide remaining in the second peak is Thr497, which coeluted with the Thr524 phosphopeptide of the 3TV mutant (Fig. 2, peak B). Valine substitution at position 497 resulted in a partial reduction of the appropriate phosphopeptide peak of B-Myb T497V in Fig. 4C. Mutation of all 10 identified sites eliminated the corresponding peaks and led to a relative increase in phosphorylation of minor sites (Fig. 4E).

Phosphorylation of B-Myb by Cdk2 in a Cell-free System—B-Myb has been suggested as a potential substrate for the cyclin-dependent kinase Cdk2 (1, 8, 19, 28). Two different approaches were used to assess the ability of Cdk2 to phosphorylate B-Myb in a cell-free system. In the first approach, B-Myb immunoprecipitated from 293T cells was mixed with lysate from SF9 cells co-infected with baculovirus containing cDNAs for Cdk2 and cyclin A or cyclin E. Kinase assays indicated that B-Myb was phosphorylated preferentially in this approach by the cyclin A-Cdk2 complex (Fig. 5A, lanes 1 and 2). Histone H1 was also phosphorylated by each kinase complex to demonstrate that Cdk2 was active (Fig. 5B, lanes 1 and 2). In the second approach, the B-Myb substrate and the cyclin-Cdk2 kinase complex were immunoprecipitated together by combining antibodies to the FLAG epitope on B-Myb and antibodies to cyclin A or cyclin E with protein G-agarose. The phosphorylation reaction was performed with the immobilized substrate and kinase. With this protocol, cyclin A-Cdk2 and cyclin E-Cdk2 were both capable of phosphorylating B-Myb (Fig. 5A, lanes 3 and 5). The ability of the cyclin E-Cdk2 complex to phosphorylate B-Myb to a greater extent than the cyclin A-Cdk2 complex corresponds to the increased activity of the cyclin E-Cdk2 on the histone H1 substrate (Fig. 5B, lanes 3 and 4). Our results indicate that B-Myb can be phosphorylated in a cell-free system by both cyclin A-Cdk2 and cyclin E-Cdk2 complexes.

HPLC phosphopeptide maps were generated to determine the extent to which B-Myb phosphorylated in a cell-free system by Cdk2 mimics the phosphorylation of B-Myb in intact cells. B-Myb phosphorylated in the immune complex by cyclin A-Cdk2 or cyclin E-Cdk2 was isolated, digested with trypsin, and...
were transfected with wild-type (WT) 293T cells. Phosphorylation mutants were labeled with $^{32}$P and lysed, and B-Myb proteins were immunoprecipitated with an antibody to the FLAG epitope. Phosphorylated proteins were resolved by electrophoresis and detected by autoradiography. Tryptic phosphopeptides from $^{32}$P-labeled B-Myb bands were analyzed by HPLC. Fractions were collected in 1-min increments. Peaks eliminated by the point mutants are indicated by arrows, and samples are offset by 1000 cpm for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity. HPLC phosphopeptide profiles of $^{32}$P-labeled wild-type B-Myb are shown for clarity.

The HPLC profiles were compared with the profile of B-Myb phosphorylated in intact cells. B-Myb phosphorylated in the immune complex by cyclin A-Cdk2 or cyclin E-Cdk2 contained most of the peaks found in B-Myb phosphorylated in intact cells. However, the relative stoichiometry of phosphorylation differed between B-Myb phosphorylated in the immune complex and B-Myb phosphorylated in intact cells (Fig. 5C). The decrease in stoichiometry of B-Myb phosphorylation in the immune complex may be due, in part, to the presence of endogenous phosphate retained on the isolated B-Myb proteins. It is also possible that Cdk2 phosphorylates only a subset of the phosphorylation sites on B-Myb in intact cells.

Transcriptional Activities of Intact B-Myb and B-Myb Lacking Phosphorylation Sites—B-Myb can transactivate reporter plasmids containing multiple MBS (4, 6). The promoter/reporter construct 3xA-TK-luc contains three MBS from the mim-1 promoter in tandem followed by the thymidine kinase promoter of herpes simplex virus controlling the luciferase gene (25). Transfection of B-Myb with 3xA-TK-luc enhanced the transcriptional activity of the promoter/reporter construct by 6-fold (Fig. 6). B-Myb was unable to activate a promoter/reporter construct lacking the MBS (data not shown). Expression of cyclin A with B-Myb resulted in a 15-fold increase in transcription from 3xA-TK-luc, consistent with previous observations (8). Transfection of cyclin A alone had no effect on the activity of the promoter/reporter construct (Fig. 6). In combination with the observation that cyclin A-Cdk2 phosphorylates isolated B-Myb on sites also phosphorylated in the intact cell (Fig. 5), these data suggest that the ability of B-Myb to transactivate the mim-1 promoter is enhanced by B-Myb phosphorylation.

We tested this possibility by measuring the ability of cyclin A to augment the transactivation potential of a mutated B-Myb protein that lacks the 10 identified phosphorylation sites (10Mut) (Fig. 6). Transfection of the B-Myb 10Mut construct with the promoter/reporter plasmid resulted in a 3-fold increase in luciferase activity. In contrast to experiments with intact B-Myb, the coexpression of cyclin A enhanced the ability of the B-Myb 10Mut protein to activate transcription by ~2-fold. The contribution of phosphorylation sites within each region of B-Myb to the overall transcriptional activity was examined. Deletion of sites within the acidic region (positions 267 and 283) did not have any effect on the transactivation potential of B-Myb (Fig. 7A). The remaining phosphorylation sites in the carboxyl-terminal half of B-Myb all appeared to contribute partially to its ability to transactivate the 3xA-TK-luc reporter gene. The combined mutation of three phosphorylation sites in the region between the acidic and conserved domains (positions 396, 408, and 455; see Fig. 9) reduced the transactivation potential of B-Myb by 25% (Fig. 7A). Similarly, loss of sites within the conserved region (positions 497 and 3TV) or of a single site in the carboxyl-terminal tail (position 581) reduced activity by an additional 21% (Fig. 7A). The combined mutation of B-Myb phosphorylation sites in these three regions (8Mut) can account for the full loss of activity (53%) that was seen with B-Myb 10Mut (Fig. 7B). These data suggest that phosphorylation of multiple sites regulates B-Myb transactivation potential. Furthermore, these data support the conclusion that cyclin-Cdk2-induced phosphorylation of B-Myb enhances the transactivation potential of B-Myb.

DNA Binding of B-Myb and B-Myb Lacking Phosphorylation Sites—The ability of B-Myb to transactivate the MBS reporter plasmid is dependent upon its ability to interact with the MBS. To determine if the decreased transactivation potential observed with B-Myb 10Mut was due to a decreased binding to the MBS, we analyzed the ability of each B-Myb mutant to interact with the MBS by DNA affinity purification. Wild-type B-Myb or the phosphorylation mutants were precipitated by an immobilized double-stranded DNA probe containing the MBS.
but not by a probe containing a scrambled MBS (data not shown). To compare the DNA-binding ability of wild-type B-Myb with that of the phosphorylation mutants, the amount of B-Myb present in each lysate was measured by ECF Western blotting. Equal amounts of B-Myb protein were added to each precipitation reaction. B-Myb proteins bound to the double-stranded MBS oligonucleotide were detected by ECF Western blotting and quantified (Fig. 8). Both B-Myb 10Mut and 8Mut increased the ability of B-Myb to interact with the MBS by 2-fold. This increased binding of B-Myb to the MBS appeared to be due to Ser581, as mutation of this site alone increased DNA binding by −3-fold (Fig. 8). Mutation of the phosphorylation sites that affect B-Myb transactivation potential (Fig. 7A) had no effect on the ability of B-Myb to bind DNA. This suggests that the decreased transactivation potential observed with B-Myb 10Mut is not due to an inability to interact with the MBS. Furthermore, mutation of a specific phosphorylation site (Ser581) increases its ability to bind the MBS (Fig. 8), but does not lead to increased transactivation potential by itself.

**DISCUSSION**

We have identified 10 sites phosphorylated on B-Myb in the human embryonic kidney cell line 293T. All 10 phosphorylation sites identified in mouse B-Myb contain the consensus sequence Ser(P)/Thr(P)-Pro and are conserved in human B-Myb. Phosphorylation of these sites in vivo is mimicked by the phosphorylation of B-Myb in an immune complex by the proline-directed kinase Cdk2 bound to cyclin E or cyclin A. Moreover, removal of the 10 identified phosphorylation sites from B-Myb blocks the ability of cyclin A to potentiate B-Myb-mediated transactivation of a Myb-responsive promoter (Fig. 6). This effect is due specifically to phosphorylation sites in the carboxy-terminal half of B-Myb, not to sites within the putative transactivation domain (Fig. 7). Furthermore, loss of the phosphorylation site at Ser581 increases B-Myb binding to the MBS by 3-fold (Fig. 8). These results suggest that phosphorylation of B-Myb by cyclin-Cdk2 may be important in regulating its biological activity.

Several previous studies have proposed that B-Myb is a substrate of Cdk2 in intact cells (8, 28, 30, 31). Experiments coexpressing cyclin A and Cdk2 with B-Myb demonstrate a mobility shift in B-Myb indicative of phosphorylation, and an increased ability of B-Myb to transactivate a Myb-responsive promoter/reporter is coincident with this shift (8, 28). Three-dimensional phosphopeptide mapping and mutagenesis (30, 31) indirectly identified phosphorylation sites at Thr443, Thr447, Thr490, Thr497, Thr524, and Ser581. HPLC mapping and

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direct amino acid sequencing of in vivo labeled phosphopeptides suggested that three of these amino acids (Thr443, Thr447, and Thr490) are less prominent phosphorylation sites on B-Myb (Fig. 4E). Phosphorylation of Thr 497 and Ser581 enhances the ability of B-Myb to transactivate the MBS reporter construct (Fig. 7A) (30). We have identified additional sites that affect B-Myb transactivation. Mutation of the three phosphorylation sites identified between the acidic region and the CR (Ser396, Thr408, and Thr455) inhibits B-Myb transactivation as much as mutation of phosphorylation sites in the CR (Thr 497, Thr 519, Thr522, and Thr 524) or Ser581 (Fig. 7A). Indirect analysis suggested that mutation of Thr524 decreased transactivation of a Gal4/B-Myb fusion construct (31), but this effect was detected only when Thr524 was mutated in combination with Thr519 and Thr522 in the CR. HPLC analysis and direct sequencing indicated that Thr519 is the primary site for phosphorylation on this peptide (Fig. 2).

Phosphorylation of B-Myb may have opposing functions. Mutation of the phosphorylation site at Ser581 decreases B-Myb transactivation potential (Fig. 4E) (30), but enhances binding to the MBS by 3-fold (Fig. 8). Data demonstrating that phosphorylation of Ser581 inhibits B-Myb binding to the B-Myb DNA-binding site (Fig. 8) suggest that B-Myb fusion constructs may not be entirely appropriate models for analyzing the effect of phosphorylation on B-Myb biology.

Our data suggest that multiple B-Myb phosphorylation sites may be required for the synergistic action of cyclin A on B-Myb transcriptional activity. Previous studies implicated the acidic region and the carboxy-terminal portion of B-Myb as potential regulators of B-Myb transcriptional activity (4, 6–8, 28). All 10 of the phosphorylation sites that we identified reside within these two regions of B-Myb. No phosphorylation sites were identified in the DNA-binding domain (Fig. 9). Deletion mutagenesis of the acidic region in B-Myb impairs its ability to transactivate Myb-responsive promoters (4). However, our results suggest that phosphorylation of Thr497 and Ser528 in the acidic region of B-Myb is not responsible for the enhanced transactivation potential of B-Myb when coexpressed with cyclin A (Fig. 7A). Phosphorylation of these sites could, however, affect the function of B-Myb in a manner not detected by the promoter/reporter construct.

Eight phosphorylation sites were found in the carboxyl-terminal half of B-Myb, including three sites in the region be-
Strated that mutation of Ser581 increases the DNA-binding subcellular distribution of a constitutively expressed B-
putative nuclear localization signals (NLS), the putative nuclear localization signals (NLS). AA, amino acid.

tween the transactivation domain and the CR (Ser\textsuperscript{396}, Ser\textsuperscript{408}, and Ser\textsuperscript{455}), four phosphorylation sites in the CR (Thr\textsuperscript{497}, Thr\textsuperscript{519}, Thr\textsuperscript{522}, and Thr\textsuperscript{524}), and an additional site in the carboxyl-terminal tail (Ser\textsuperscript{581}) (Fig. 9). The loss of phosphorylation sites in any one of these three regions caused an ~20% decrease in the transactivation capacity of B-Myb (Fig. 7A). However, the full contribution of phosphorylation to B-Myb-induced transactivation was observed only when mutation of the phosphorylation sites in each of these regions was combined. This loss of transactivation potential of B-Myb 10Mut was not due to an impaired ability to interact with the MBS (Fig. 8).

Our data suggest that phosphorylation of the carboxyl-terminal half of B-Myb may block the negative regulation by the CR (see below), as loss of phosphorylation sites led to a decreased transactivation potential of B-Myb (Fig. 7). Within the carboxyl terminus of mouse B-Myb, the CR (amino acids 471–549) is 46% conserved with the mouse c-Myb CR (amino acids 414–494), which is known to inhibit the transcriptional activity of c-Myb (32, 33). The sequence containing these phosphorylation sites, (T/S)P(R/H)TP(T/S)PFK (amino acids 459–467 in c-Myb and 519–527 in B-Myb), is the stretch of greatest amino acid homology between the CRs of B-Myb and c-Myb. The conservation of phosphorylation sites in the CR supports the idea that these phosphorylation sites may be critical to the normal function of Myb proteins.

Phosphorylation of the B-Myb CR may regulate B-Myb function through several different mechanisms. First, phosphorylation of sites in the CR could affect intramolecular interactions within B-Myb. Ness (34) proposed a model for regulation of Myb proteins in which the carboxyl-terminal half of each protein folds back over the DNA-binding and transactivation domains, thus inhibiting its ability to bind and transactivate promoters. Release of this steric inhibition could be mediated either directly through phosphorylation or indirectly through other proteins. Application of this model to B-Myb is supported by studies demonstrating that deletion of the carboxyl terminus of B-Myb increases its ability to transactivate artificial promoters in transfected cells (6–8).

A second consequence of B-Myb phosphorylation may be an alteration in B-Myb subcellular distribution. Two nuclear localization sequences in the carboxyl-terminal half of B-Myb (Fig. 9, NLS\textsubscript{1} and NLS\textsubscript{2}) restrict it to the nucleus of the cell (4). One of the phosphorylation sites identified, Ser\textsuperscript{581} (Fig. 9), resides within nuclear localization sequence-2 (amino acids 569–585). It is possible that phosphorylation of Ser\textsuperscript{581} in B-Myb is necessary to maintain its nuclear localization and transcriptional activity. One prediction from this hypothesis is that the subcellular distribution of a constitutively expressed B-Myb transgene might change during the cell cycle. We have demonstrated that mutation of Ser\textsuperscript{581} increases the DNA-binding ability of B-Myb; however, this is unlikely to be due to a change in subcellular distribution since we assayed B-Myb binding in cellular lysates.

A third possibility is that phosphorylation of B-Myb may affect its activity by altering its ability to interact with other proteins. It has been suggested that accessory proteins are necessary for the cellular activity of B-Myb and that these proteins are expressed at different levels in different cell types (7). Phosphorylation of B-Myb may enhance its interactions with unidentified proteins, leading to an increased transactivation potential of B-Myb. Alternatively, phosphorylation of B-Myb may block its interaction with inhibitory proteins that suppress its transcriptional activity. For example, p107 binds to B-Myb \textit{in vitro} and can suppress the ability of B-Myb to transactivate an artificial promoter in transfected cells (10, 35). The sequences in B-Myb that interact with p107 have not been identified. However, it is possible that the phosphorylation of B-Myb might inhibit its association with p107 as a mechanism for enhancing B-Myb transactivation potential.

B-Myb may play a critical role in cell proliferation and may be an essential component of the molecular mechanism linking the cell cycle engine with coordinated gene expression during S phase (1). Consistent with a role for phosphorylation by cyclin-dependent kinases to enhance the biological activity of B-Myb, coexpression of B-Myb with cyclin A enhances transactivation by B-Myb (Fig. 6) as well as S phase progression (8, 11). Phosphorylation of B-Myb at the G\textsubscript{1}/S interface coincides with the activation of Cdk2 (19). Thus, activation of B-Myb by phosphorylation may be one mechanism used by Cdk2 in complex with cyclin A or cyclin E to promote S phase. Conversely, the observation that a B-Myb transgene is capable of overcoming p53-induced growth arrest in a glioblastoma cell line (15) may argue against an obligate role of Cdk2-mediated phosphorylation in enhancing B-Myb function. Induction of p53 induces expression of the cyclin-dependent kinase inhibitor p21\textsuperscript{Cip1} with a concomitant inhibition of cyclin-dependent kinase activity (15). Surprisingly though, this suppression of cyclin-dependent kinase activity does not inhibit the ability of ectopic B-Myb to overcome p53-induced growth arrest. The identification of specific phosphorylation sites on B-Myb provides a valuable tool for resolving this issue and dissecting the biochemical mechanisms underlying B-Myb function.

Acknowledgments—We thank Laurey Steinke and Han Huang (Protein Structure Core Facility, Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center) for analysis of protein sequences. We also thank Roger Watson for the B-Myb cDNA; Scott Ness for the 3xA-TK-luc reporter plasmid; S. H. Lee for recombinant baculovirus encoding cyclin A, cyclin E, and Cdk2; and Rockefeller University for the human embryonic kidney 293T cells.

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