Mitosis-specific Phosphorylation of the Nuclear Oncoproteins Myc and Myb

Bernard Lüscher and Robert N. Eisenman

Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104

Abstract. The c-*myc* and c-*myb* proto-oncogenes encode phosphorylated nuclear DNA binding proteins that are likely to be involved in transcriptional regulation. Here we demonstrate that both Myc and Myb proteins are hyperphosphorylated during mitosis. In the case of Myb, hyperphosphorylation is accompanied by the appearance of three M phase-specific tryptic phosphopeptides. At least one of these phosphopeptides corresponds to a phosphopeptide generated after phosphorylation of Myb in vitro by $p34^{cdc2}$ kinase. By contrast, the mitotic hyperphosphorylation of Myc does not correlate with the appearance of unique phosphopeptides, suggesting that M phase and interphase sites may be clustered within the same peptides. In addition Myc does not appear to be a target for $p34^{cdc2}$ phosphorylation.

The hyperphosphorylated forms of Myc and Myb from mitotic cells are functionally distinct from the corresponding interphase proteins in that the former have reduced ability to bind nonspecifically to double-stranded DNA cellulose. Furthermore, mitotic Myb binds poorly to oligodeoxynucleotides containing an Myb response element. We surmise that the decreased DNA binding capacity of hyperphosphorylated Myb and Myc during M phase may function to release these proteins from chromatin during chromosome condensation.

THE notion that the complex series of nuclear and cytoplasmic events that constitute mitosis (M phase) in eukaryotes may be triggered by a single signal, or a small number of signals, dates back to cell fusion experiments in the slime mold Physarum as well as mammalian cells (Johnson and Rao, 1970, Rausch et al., 1966). Fusion experiments between cells in G2/M phase and cells in other cell cycle phases revealed that the former possess a dominant factor(s) which can act upon interphase nuclei to induce premature chromosome condensation and nuclear envelope breakdown (Johnson and Rao, 1970). This idea has been borne out by recent work leading to the identification of a protein complex whose action appears to control entry into M phase and possibly other cell cycle phases as well. A key constituent of this complex is a highly conserved protein kinase ($p34^{cdc2}$). This supports the notion that the mitotic "trigger" may function through a phosphorylation cascade (for recent reviews see Nurse, 1990, Moreno and Nurse, 1990, Pines and Hunter, 1990).

It has long been thought that protein phosphorylation is involved in mitosis. Several studies had demonstrated a general increase in phosphorylation during entry of mammalian tissue culture cells into M phase (Davis et al., 1983). Furthermore, a phosphorylated epitope present on multiple proteins during mitosis appeared to be specifically recognized by mAbs prepared against an M phase HeLa cell extract (Davis et al., 1983). One of these antibodies, MPM-2, was found to produce diffuse immunofluorescent staining during interphase, while during M phase it reacted strongly with kinetochores, centrosomes, midbodies and other nuclear proteins (Vandre et al., 1984). Specific target proteins for M phase phosphorylation have also been identified including microtubules (Piras and Piras, 1975), histone H1 (for review see Wu et al., 1986), and the nuclear lamins (Gerace and Blobel, 1980). In the case of the lamins, M phase-specific hyperphosphorylation appears to be responsible for lamin disassembly, and subsequent dephosphorylation is required for lamin reassembly at the end of mitosis (for review see Burke and Gerace, 1986; Ottaviano and Gerace, 1985; Suprynowicz and Gerace, 1986; Fisher, 1987).

The concept that cycles of phosphorylation and dephosphorylation initiate and regulate key cell cycle events has been greatly bolstered by the finding that the $p34^{cdc2}$ kinase is a critical component of the mitotic activating complex. An important goal has become to identify primary substrates of $p34^{cdc2}$ and to relate their phosphorylation to specific cell cycle changes. Recent work (for review see Moreno and Nurse, 1990) has demonstrated a number of interesting substrates for the mitotic kinase including the nuclear lamins (Heald and McKeon, 1990; Lüscher et al., 1991; Peter et al., 1990), histone H1 (Langån et al., 1989), RNA polymerase II (Cisek and Corden, 1989), HMGI (Reeves et al., 1991), caldesmon (Yamashiro et al., 1991; Mak et al., 1991) GTP-binding proteins (Bailly et al., 1991), and SWI5 (Moll et al., 1991). The studies on the nuclear lamins indicate that phosphorylation by $p34^{cdc2}$ is crucial for nuclear lamina disas-

Bernard Lüscher's present address is Institute for Molecular Biology, Medizinische Hochschule Hannover OE5250, Konstanty-Gutschow Str 8, Postfach 61 01 80, D-3000 Hannover 61 Germany.

© The Rockefeller University Press, 0021-9525/92/08/775/10 $2.00

sembly (Heald and McKeon, 1990; Peter et al., 1990). For SWI5, phosphorylation by cdc28 seems to regulate the subcellular localization, the phosphorylated form being cytoplasmic and the dephospho form nuclear (Moll et al., 1991). For the other substrates it is not yet completely clear what role, if any, $p34^{cdc2}$ phosphorylation plays in the function of the other substrates.

Given that oncogenes appear to be involved in the regulation of cell proliferation, it is of interest to determine whether their protein products are also differentially phosphorylated during mitosis. Recent studies have demonstrated M phase hyperphosphorylation for $pp60^{src}$ and several other *src* family protein tyrosine kinases, mos, $p150^{c\text{-}Abl}$ (for extensive review see Shalloway and Shenoy, 1991), and the tumor suppressor proteins Rb (Chen et al., 1989; Lin et al., 1991) and p53 (Thilner et al., 1990; Brischoff et al., 1990; Stirzbecher et al., 1990). Phosphopeptide mapping experiments indicate that these proteins may be directly phosphorylated by $p34^{cdc2}$. In the case of $pp60^{src}$ the M phase phosphorylation results in a significant increase in kinase activity possibly mediated through a secondary event involving loss of phosphate from tyrosine 527 (Bagrodia et al., 1991).

The oncogene-encoded proteins examined for M phase hyperphosphorylation thus far have been primarily cytoplasmic kinases (for review see Shalloway and Shenoy, 1991). Here we report studies on the phosphorylation state of the proteins encoded by the c-*myc* and c-*myb* protooncogenes (referred to as Myc and Myb proteins, respectively). Both of these proteins are sequence-specific DNA binding proteins that have been implicated in the control of cell differentiation and proliferation. They are thought to function as transcription factors, although roles in DNA replication have also been considered (for recent reviews see Lüscher and Eisenman, 1990*b,c*). Previous work has demonstrated that Myc and Myb are phosphorylated in exponentially growing cells by casein kinase II (CKII) (Lüscher et al., 1990, 1989). Phosphorylation by CKII inhibits the sequence-specific binding of Myb and it has been suggested that the frequent loss of the CKII phosphorylation site may contribute to oncogenic activation (Lüscher et al., 1990). For Myc the functional significance of CKII phosphorylation has not been resolved (Street et al., 1990). Here we show that the onset of mitosis correlates with additional phosphorylation events on Myc and Myb as well as with a reduced ability of both these proteins to bind DNA.

Materials and Methods

Cell Culture and Labeling

HeLa cells were grown in DME, supplemented with 10% FBS. The human Burkitt's lymphoma cell line Manca was maintained in DME supplemented with 10% bovine serum. The chicken bursal lymphoma cell line BK3A has been described (Hihara et al., 1974; Lüscher et al., 1991). For mitotic arrest experiments, exponentially growing HeLa cells were arrested at the G1/S border of the cell cycle by treatment with 1 mM thymidine for 16 h. Then the cells were washed and incubated for 8 h in regular medium to allow transition through S phase before the addition of 100 ng/ml of nocodazole (Sigma Chemical Co., St. Louis, MO). After 5 h in nocodazole, the mitotic cells were removed from the monolayer by shake-off. Exponentially growing BK3A cells were treated for 10–11 h with 100 ng/ml nocodazole. The mitotic index of the nocodazole-treated cells was determined by DAPI staining of methanol/acetone fixed cells. More than 95% of the HeLa cells and between 90–85% of the BK3A cells displayed regularly condensed chromosomes. Cell labeling with [^{35}S] methionine and with ^{32}P-orthophosphate was performed as described previously (Lüscher et al., 1991; Lüscher and Eisenman, 1988).

Antibodies

The antipeptide antibodies used were affinity-purified rabbit antibodies prepared against synthetic peptides corresponding to the 12 COOH-terminal amino acids of human or chicken Myc, respectively (Hann et al., 1983; Hann and Eisenman, 1984). The Myb-specific polyclonal rabbit antiserum raised against a bacterial fusion protein has been described (Lüscher and Eisenman, 1988). The Myb-specific mAb 2.27, raised against a bacterial fusion protein, was obtained from G. Ramsay (University of California, San Francisco) (Evan at al., 1984). The polyclonal rabbit antiserum recognizing enolase was obtained from J. Cooper (Fred Hutchinson Cancer Research Center) (Cooper et al. 1983). The anti-$p34^{cdc2}$ antibodies used were affinity-purified rabbit antibodies prepared against a synthetic peptide corresponding to the 8 COOH-terminal amino acids of human p34 and was obtained from J. Wang (University of California, San Diego) (Lin et al., 1991).

Immunoprecipitation and Immunoblotting

All the procedures were as described previously (Lüscher et al., 1991; Lüscher et al., 1990, 1988; Lüscher and Eisenman, 1990*a*).

Kinase Assays

$p34^{cdc2}$ kinase assays using purified enzyme (Brizuela et al., 1989) were performed on immunoprecipitated Myc or Myb as described for lamin B2 (Lüscher et al., 1991). Alternatively, Myc or Myb were first immunoprecipitated onto protein A–Sepharose 4B beads and the $p34^{cdc2}$ was immunoprecipitated from nocodazole-treated Manca cells onto the same Sepharose beads. The kinase reactions were performed as described (Lüscher et al., 1991). Casein kinase II assays were done as described before (Lüscher et al., 1989). For Glycogen synthase kinase 3 (GSK3) kinase assays, Myc was immunoprecipitated onto protein A–Sepharose 4B beads and washed. Before the kinase reaction, the immunocomplexes were washed twice in 20 mM imidazole-HCl, pH 7.4, 5 mM $MgCl_2$, 0.02 mM EDTA, and then taken up in 20 μl of the same buffer and 2 μl of purified GSK3 (from rabbit muscle, 250 U/ml against Mg/ATP–dependent protein phosphatase, obtained from T. Haystead, University of Washington). The reaction was performed at 30°C for 15 min and stopped by the addition of SDS-gel sample buffer.

Phosphopeptide Mapping and Phosphoamino Acid Analysis

Two-dimensional phosphopeptide mapping and phosphoamino acid analysis were performed as described before (Firzlaff et al., 1989; Lüscher et al., 1991). One-dimensional phosphopeptide mapping was done as described previously (Lüscher et al., 1989), except that proteinase K (10 ng/sample) was used instead of *S. aureus* V8 protease.

DNA Binding

For DNA-cellulose chromatography assays, 20–30 × 10^6 exponentially growing or nocodazole-arrested BK3A cells were labeled with $^{32}P_i$ for 2 h. The labeled cells were then resuspended in 0.5 ml of buffer C (30 mM Tris-HCl, pH 7.4, 5 mM NaF, 10 mM b-glycerophosphate, 15 aprotinine) containing 400 mM NaCl and broken by sonication. The cell lysates were cleared by centrifugation at 16,000 *g* for 15 min. The supernatants were diluted with buffer C to 50 mM NaCl (loading buffer) and loaded onto a double-stranded DNA cellulose column (bed volume 1 ml, obtained from Sigma Chemical Co.). The lysate was passed three times over the column. After washing with loading buffer the bound proteins were eluted stepwise with increasing salt concentrations. Individual fractions were adjusted to 0.5% of NP-40, DOC, and SDS. Myc and Myb were successively immunoprecipitated from individual fractions using specific antibodies. The gel retardation assays are performed as described previously (Lüscher et al., 1990). In brief, Myb was immunoprecipitated from detergent lysates of BK3A cells. The immunoprecipitated protein was released from the antibody by treatment with 6M guanidine-HCl, 0.1% β-mercaptoethanol. The denatured proteins were renatured by dialysis against gel mobility shift buffer.

Results

Myc in Interphase and Mitotic HeLa Cells

In our initial studies we examined the synthesis of Myc in mitotic and interphase HeLa cells. Mitotic cells were prepared as described in detail in Materials and Methods. To increase the number of cells in mitosis we first treated the exponentially growing HeLa cell monolayer cultures with excess thymidine for 16 h in order to block most cells at the G1/S border. After wash-out of the thymidine the partially synchronized population was allowed to continue growth through S phase for an additional 8 h before treatment with nocodazole. After 5 h in nocodazole the mitotic cells were harvested by shake-off, labeled with ^{32}P-orthophosphate ($^{32}P_i$) or [^{35}S]methionine, lysed in detergents, and immunoprecipitated with specific anti-Myc antibody. Fig. 1 shows an SDS-PAGE analysis of anti-Myc immunoprecipitates [^{35}S]methionine labeled from mitotic (lane *4*) and interphase HeLa cells (lane *3*). In this experiment we estimated that 99% of the harvested cells contained condensed chromatin and were in mitosis as judged by DAPI staining (data not shown). The two major c-*myc* primary translation products p64$^{c\text{-}Myc}$ and p67$^{c\text{-}Myc}$ are clearly detected in the interphase cells along with a series of faint background bands unrelated to Myc. By contrast, the mitotic cells appear to lack any labeled protein bands co-migrating with p64/p67$^{c\text{-}Myc}$, although many of the faint background bands are still visible. The absence of [^{35}S]methionine labeled Myc in mitotic HeLa cells is not simply due to shut-off of protein synthesis in mitosis since the immunoprecipitation was performed on mitotic and interphase extracts whose volumes were adjusted for equal incorporation of the radioactive label. Furthermore, when equal quantities of total radioactive extract were electrophoresed we clearly detected synthesis of the major interphase proteins, with some exceptions, during mitosis, although overall translation was depressed several fold in this experiment (Fig. 1, lanes *1* and *2*).

Although newly synthesized Myc could not be detected in these cells blocked in mitosis, we reasoned that a potentially stable pool of Myc, such as that observed by anti-Myc immunofluoresence analysis of mitotic cells (Eisenman et al., 1985; Winqvist et al., 1984), might be detectable through phosphate exchange after ^{32}Pi labeling. SDS-PAGE comparison of equal aliquots of ^{32}Pi labeled mitotic and interphase HeLa cells showed an apparent increase in phosphorylation in the mitotic cells, including several strongly labeled bands (Fig. 1, lanes *5* and *6*). Immunoprecipitation with anti-Myc revealed the expected ^{32}Pi-labeled p64/p67$^{c\text{-}Myc}$ bands in interphase cells (Fig. 1, lane *9*). Treatment of the immunocomplex with alkaline phosphatase before denaturation and electrophoresis resulted in the loss of most but not all of the ^{32}Pi-label, with no change in electrophoretic mobility of the residual labeled material (Fig. 1, lane *10*). Anti-Myc immunoprecipitates from mitotic cells however showed a specific ^{32}Pi-labeled doublet with the major labeled species migrating ~1 kD more slowly than p64 (Fig. 1, lane *7*). Again, alkaline phosphatase treatment removed most of the

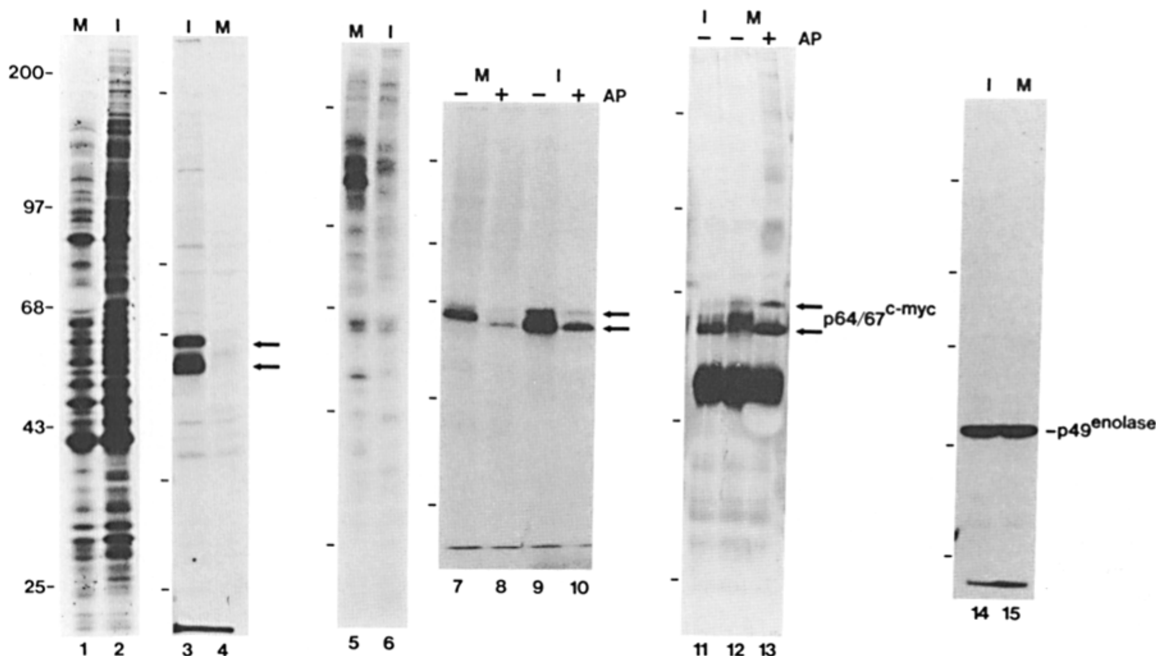

Figure 1. Myc in mitotic HeLa cells. Logarithmically growing HeLa cells (*I*) or cells arrested in metaphase by nocodazole treatment (*M*) were labeled with [^{35}S]methionine (lanes *1–4*), with $^{32}P_i$ (lanes *5–10*), or were unlabeled (lanes *11–15*). Total labeled cell lysates were run in lanes 1, 2, 5, and 6, and unlabeled cell lysate was run in lanes 14 and 15. Myc was immunoprecipitated using affinity-purified antibodies raised against the 12 COOH-terminal amino acids of human Myc (lanes *3*, *4*, *7–13*). After separation on an SDS-polyacrylamide gel, the samples in lanes 11–15 were transferred to nitrocellulose and probed with anti-Myc antibodies (lanes *11–13*) or with antienolase antiserum (lanes *14* and *15*). The immunoprecipitates in lanes 8, 10, and 13 were treated with alkaline phosphatase (*AP*) before separation on SDS-polyacrylamide gels. Samples were analyzed on 10% SDS-PAGE. The following prestained molecular mass markers were used for this and all other analyses: myosin (200 kD), phosphorylase B (97.4 kD), BSA (68 kD), ovalbumin (43 kD), and α-chymotrypsinogen (25.7 kD).

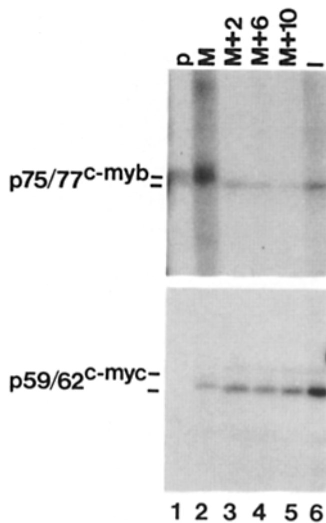

Figure 2. Phosphorylation of Myb and Myc in the chicken bursal lymphoma cell line BK3A. Myb and Myc were immunoprecipitated from logarithmically growing BK3A cells and phosphorylated in an in vitro kinase reaction by $p34^{cdc2}$ (lane *1*). Myb and Myc were immunoprecipitated from $^{32}P_i$-labeled BK3A cells arrested in mitosis by nocodazole treatment (lane *2*), released from the nocodazole block for 2 (lane *3*), 6 (lane *4*), and 10 h (lane *5*), or from logarithmically growing cells (lane *6*). After the nocodazole block, cells were labeled during the last 2 h of the incubation time. The lysates were sequentially immunoprecipitated with anti-Myc antibodies and then with anti-Myb antiserum. Samples were analyzed by 10% SDS-PAGE.

label but in this case the mobility of the residually labeled proteins was shifted to that found in the interphase cells (Fig. 1, lanes *8–10*).

To determine whether the mobility shift of phosphate-labeled Myc in mitotic relative to interphase cells was an artifact due to labeling of a small population of Myc in mitosis, we analyzed total Myc in mitotic and interphase cells using immunoblotting. Since the very low levels of Myc preclude direct immunoblotting of cell extracts, we first prepared anti-Myc immunoprecipitates from unlabeled mitotic and interphase HeLa cells which were fractionated on SDS-PAGE, transferred to nitrocellulose, and probed with anti-Myc antibodies followed by anti–rabbit Ig-alkaline phosphatase conjugate. The major band at 53 kD corresponds to the Ig heavy chains from the first immunoprecipitate. The $p64/p67^{c\text{-}Myc}$ proteins were visible in interphase cells (Fig. 1, lane *11*), while mitotic cells contain major anti-Myc detectable proteins shifted to ~1 kD higher molecular mass (Fig. 1, lane *12*). Alkaline phosphatase treatment of the initial immunocomplex again resulted in a shift to the interphase mobility (Fig. 1, lane *13*).

These results suggest that while there is little or no de novo synthesis of Myc in HeLa cells blocked in mitosis there is nonetheless a pool of potentially stable Myc. This pool of Myc differs from that found in interphase cells in that it has a slower electrophoretic mobility due to phosphorylation, a result reminiscent of that for hyperphosphorylated $pp60^{c\text{-}Src}$ and other proteins in mitotic cells (Shalloway and Shenoy, 1991). That the altered mobility of Myc is not due to some nonspecific effect of our procedure for inducing mitosis or is not simply a general modification of all cellular proteins was demonstrated by examining the cytoplasmic phosphorylated enzyme enolase which did not possess an altered electrophoretic mobility when mitotic and interphase cells were compared (Fig. 1, lanes *14* and *15*).

Nuclear Oncoprotein Phosphorylation in Avian Lymphoid Cells

Our initial studies using HeLa cells suggested that Myc was differentially phosphorylated in interphase and mitosis. For further experiments, we utilized the rapidly growing chicken B cell lymphoma cell line BK3A. The use of BK3A allowed us both to extend our initial observations to another cell type and to examine the Myb nuclear oncoprotein since c-*myb* expression is mainly restricted to hematopoietic cells (Lüscher and Eisenman, 1990*c*). After treatment of BK3A cells with nocodazole for 10–11 h, 90–95% of the harvested cells had condensed chromatin as determined by DAPI staining (data not shown). These cells were either labeled with $^{32}P_i$ for 2 h in the presence of nocodazole or released from the block for the times indicated and labeled during the final 2 h. Immunoprecipitates from these labeled cells were prepared first with an anti-Myc antiserum then with anti-Myb, and analyzed on SDS-PAGE. Both Myc and Myb were shifted to a slightly lower electrophoretic mobility in mitotic compared to interphase cells (Fig. 2). This shift in the apparent molecular weights of Myc and Myb was rapidly reversed after removal of nocodazol (Fig. 2, lanes *2–5*). In addition to the altered mobility of M phase Myc, a 60-kD species was observed which in I phase cells was only visible after prolonged exposure of the gel (see Figs. 5 *A*). The labeled protein species indicated as p46, p41, and p21 in Fig. 5 *A* are NH_2-terminal truncated forms of c-Myc as determined by their reactivity with a COOH-terminal antibody and by comparative phosphopeptide mapping (B. Lüscher unpublished observations). These forms are potential degradation products of Myc. In mitotic cells the p21 form was absent (see Fig. 5 *A*).

Since we need to treat cells with nocodazole in order to obtain sufficient quantities of mitotic cells, it is formally possible that the effects observed on Myc and Myb (also see below) might be due simply to nocodazole itself. However, in experiments where exponentially growing cells are treated with nocodazole for 1 h we find no evidence for Myb hyperphosphorylation. After 3 h of treatment we detected a small amount of hyperphosphorylated protein, probably due to accumulation of cells in mitosis. Thus the effects observed in nocodazole-blocked cells are likely to be due to the fact that the cells have entered mitosis rather than as a direct effect of nocodazole.

We next prepared two-dimensional tryptic phosphopeptide maps of the mitotic and interphase Myb and Myc proteins isolated from BK3A cells (Figs. 3 *A* and 5 *A*) to determine whether the increased incorporation of phosphate in the case of Myb, and the shift in apparent molecular weight of both proteins, during mitosis correlated with additional sites of phosphorylation. Fig. 3 *B* shows tryptic phosphopeptide maps of Myb protein immunoprecipitated from BK3A cells in interphase (Fig. 3 *I*) and nocodazole blocked in mitosis (Fig. 3 *M*). Interphase Myb displayed five phosphopeptides labeled 1–5. Spots 1 and 2 correspond to the previously characterized NH_2-terminal casein kinase II phosphorylation site (Lüscher et al., 1990). During mitosis this pattern was retained with the exception of spot 5 which decreased in intensity. However, mitotic Myb had clearly gained additional phosphopeptides indicated by spots 6–8. By 2 h after removal of nocodazole the additional mitotic-specific phosphopeptides had disappeared and the pattern resembled that found in interphase cells (data not shown). We also determined whether the additional phosphorylations of Myb in mitosis resulted in a change in the ratio of phosphoserine/phosphothreonine. The phosphoamino acid analysis, shown in Fig. 4, demonstrates that in I phase BK3A cells serine was predominantly phosphorylated with no detectable

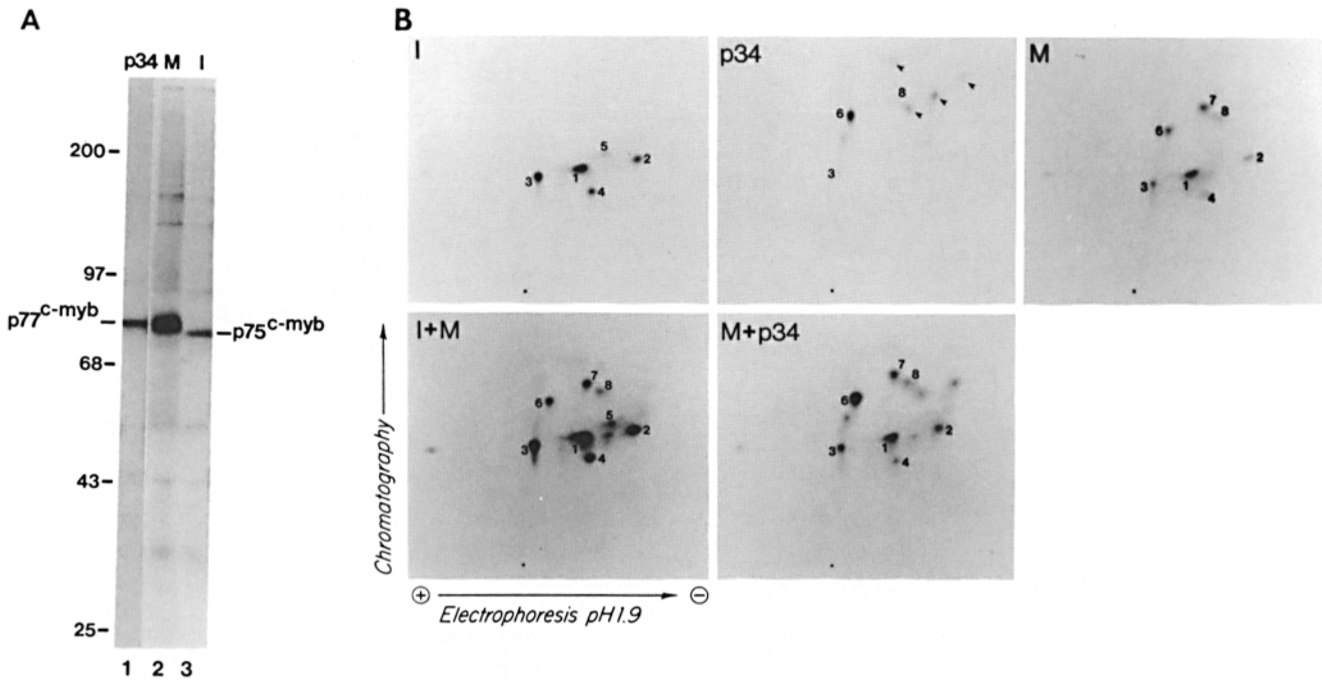

Figure 3. Hyperphosphorylation of mitotic Myb: involvement of $p34^{cdc2}$. (*A*) Myb was immunoprecipitated from logarithmically growing unlabeled BK3A cells and phosphorylated by the $p34^{cdc2}$ kinase (lane *1*), from M phase nocodazole-arrested or logarithmically growing $^{32}P_i$-labeled cells (lanes *2* and *3*, respectively). Samples were analyzed on a 10% SDS-PAGE. (*B*) The labeled Myb displayed in *A* was extracted from the gel, digested with trypsin, and the resulting peptides were analyzed on cellulose thin-layer plates in the first dimension by electrophoresis (*horizontal*, anode on the *left*) and in the second dimension by ascending chromatography (*vertical*).

phosphorylation of tyrosine or threonine (Fig. 4 *I*), while in mitotic cells there is a distinct increase in threonine phosphorylation (Fig. 4 *M*) which was reversed 2 h after removal of nocodazole (Fig. 4, *M + 2*). Thus the onset of mitosis is likely to bring about new phosphorylations on Myb involving at least threonine residues. Phosphoamino acid analysis of Myc isolated from either I or M phase cells revealed no differences between the two forms of the protein as in both instances Ser and Thr phosphorylation at equal ratio was detected (data not shown).

We also prepared one-dimensional partial phosphopeptide maps using proteinase K of mitotic and interphase Myc isolated from BK3A cells (Fig. 5 *B*, only the $p59^{c\text{-}Myc}$ species was used for peptide mapping). These were compared to Myc phosphorylated in vitro by CKII or GSK3. CKII phosphorylates two regions in Myc, one in the central acidic domain, and one near the COOH-terminus (Lüscher et al., 1989). GSK3 phosphorylates sites near the NH_2 terminus which are also modified in vivo (B. Lüscher, unpublished observations). Three major peptides were observed in these one-dimensional maps, labeled 1–3 (Fig. 5 *B*). Peptides 1 and 2 being derived from regions of the protein modified by CKII and peptide 3 from near the NH_2 terminus modified by GSKIII, myosin basic protein kinase (B. Lüscher, unpublished observations), or mitogen-activated protein kinase (Alvarez et al., 1991). The comparison showed that peptides 1 and 2 comigrated, whereas peptide 3 from M phase Myc had a reduced mobility when compared to I phase Myc. This suggested that the phosphorylation event responsible for the altered mobility on SDS-PAGE is occurring in the NH_2 terminal portion of the protein.

Next, we prepared two-dimensional phosphopeptide maps using proteinase K (Fig. 5 *C*). Surprisingly we could detect only minor differences in the pattern of peptides produced during these two cell cycle phases despite the evident shift in electrophoretic mobility which is sensitive to alkaline phosphatase treatment (Fig. 1) and the altered mobility of peptide 3 on one dimensional maps (Fig. 5 *B*). As proteinase K has a rather wide specificity, it should be mentioned that none of the phosphopeptides comigrated with phospho-Ser

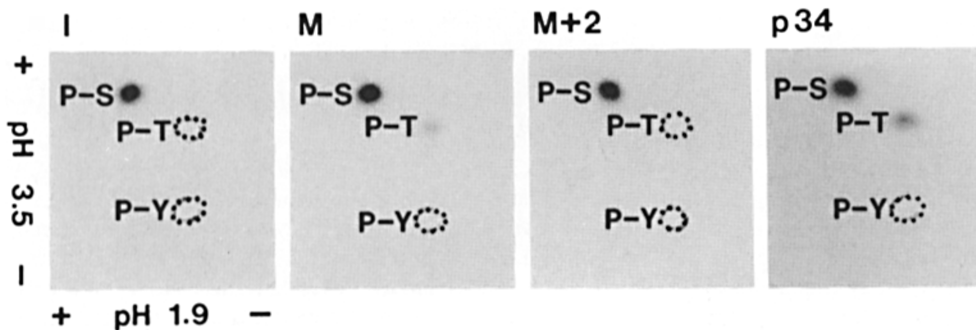

Figure 4. Phosphoamino acid analysis of Myb. $^{32}P_i$-labeled Myb was hydrolyzed in 6 M HCl for 3 h at 110°C and the resulting amino acids were separated by electrophoresis on cellulose thin-layer plates at pH 1.9 in the first dimension and at pH 3.5 in the second. The positions of unlabeled phosphoamino acids used as markers are indicated.

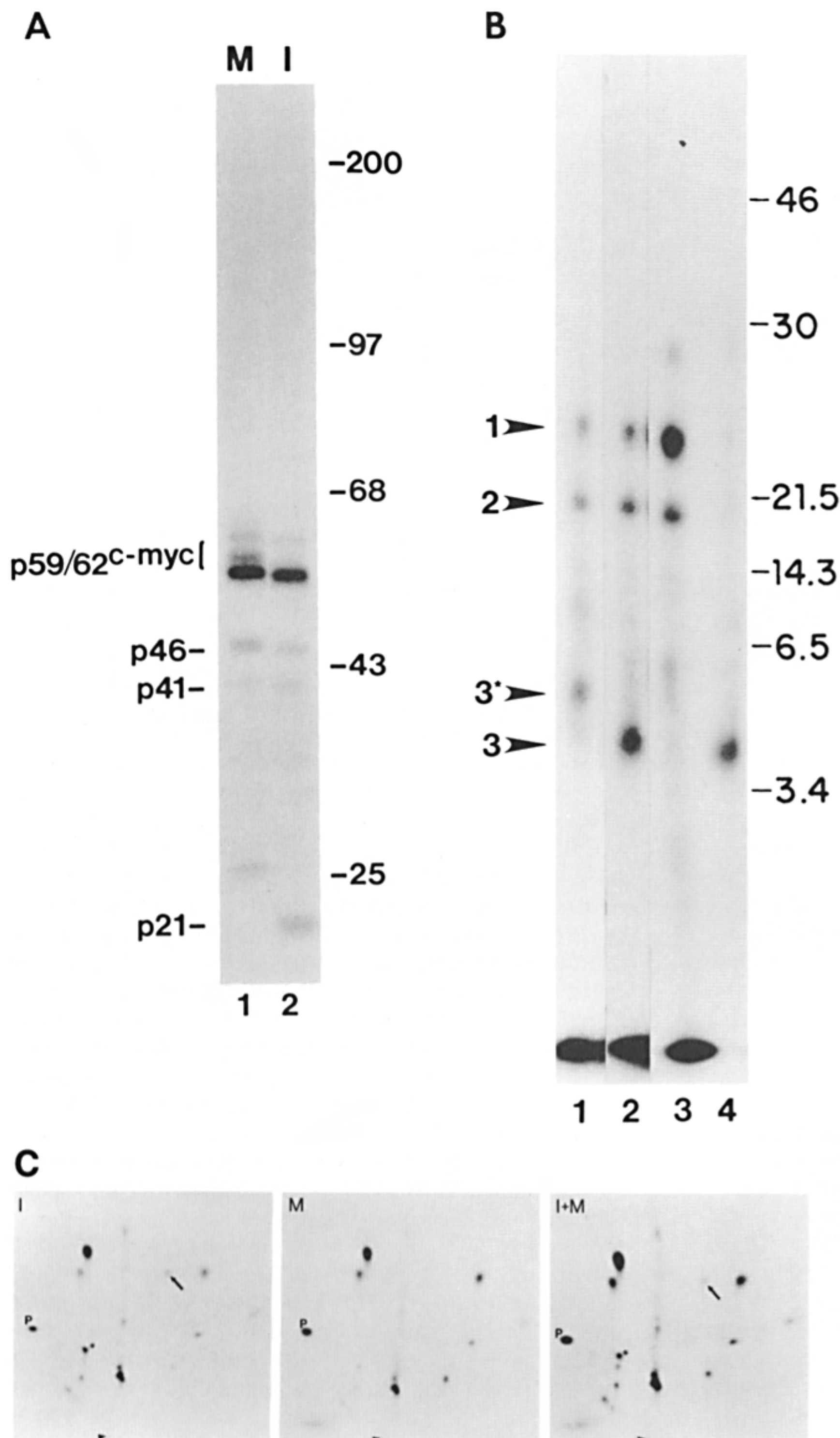

Figure 5. Analysis of mitotic Myc. (*A*) Myc was immunoprecipitated from $^{32}P_i$-labeled mitotically arrested BK3A cells or from logarithmically growing cells (lanes *1* and *2*, respectively). Samples were analyzed on a 10% SDS-PAGE. (*B*) The $p59^{c\text{-}Myc}$ species of Myc isolated from $^{32}P_i$-labeled I-phase (lane *2*) and M-phase (lane *1*) cells were cut out from an SDS-PAGE, digested with proteinase K, and reelectrophoresed on a 20% SDS-PAGE. As comparison $p59^{c\text{-}Myc}$ was immunoprecipitated and then labeled in an in vitro kinase reaction with CKII (lane *3*) or GSK3 (lane *4*). (*C*) The $p59^{c\text{-}Myc}$ species of Myc were eluted from the gel, digested with proteinase K, and the resulting peptides analyzed in two dimensions (see Fig. 3).

or -Thr. We also obtained identical interphase and mitotic phosphopeptide maps when the Myc proteins from BK3A cells were digested with trypsin, chymotrypsin, thermolysin, or endoproteinase Pro or when Myc proteins were analyzed from M phase or I phase HeLa cells after trypsin digestion (data not shown). At this point, the basis for the difference in the phosphorylation pattern between the M phase and I phase forms of Myc is unclear (see Discussion).

A Role for the $p34^{cdc2}$ Kinase in Mitotic Phosphorylation of Myb

The $p34^{cdc2}$ kinase has been demonstrated to specifically phosphorylate several proteins which are believed to be critically involved in regulating cell cycle events (for recent reviews see Enoch and Nurse, 1991; Moreno and Nurse, 1990; Pines and Hunter, 1990). In addition, a molecular complex containing $p34^{cdc2}$ appears to be responsible for

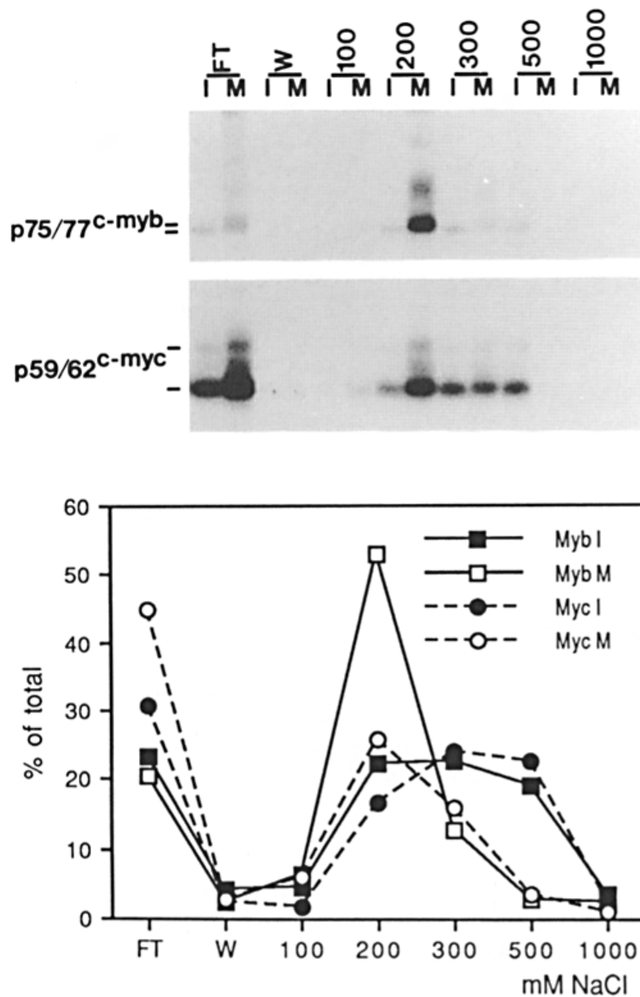

Figure 6. Nonspecific DNA binding of Myb and Myc. Mitotically arrested BK3A cells or logarithmically growing cells were labeled for 2 h with $^{32}P_i$. The cells were then disrupted by sonication and soluble proteins were chromatographed on double-stranded calf thymus DNA coupled to Sepharose 4b beads. Myc and Myb were immunoprecipitated from the individual fractions and analyzed on 10% SDS-PAGE. The NaCl concentrations used to elute are indicated. *FT*, flow through; *W*, wash. The bands corresponding to Myc and Myb from all the lanes were cut out and counted. The sum of all the counts for I phase Myb, M phase Myb, I phase Myc, or M phase Myc (Myb I, Myb M, Myc I, or Myc M, respectively) was set at 100%. The percentage of label in each fraction relative to the total counts of the combined fractions is displayed. A graphical representation of these data is shown in the lower panel. The 100% value represents 40.1% of the total I phase Myc radioactivity (9,310 cpm); 42.6% of the total M phase Myc (13,310); 22.3% of the total I-phase Myb (4,070 cpm), and 24.0% of the total M phase Myb (6,540 cpm). The input cpm were determined by taking 10% of the lysate applied to the column, immunoprecipitating the Myc and Myb proteins, and determining the amount of radioactivity in the relevant SDS-PAGE gel slice by means of Cerenkov counting.

driving mitosis (Pines and Hunter, 1990). Our observations on the differential phosphorylation of Myc and Myb during mitosis impelled us to test the possibility that these proteins might be targets for the $p34^{cdc2}$ kinase. We therefore prepared specific immunocomplexes containing Myc and Myb from BK3A cells and either added purified $p34^{cdc2}$ (Brizuela et al., 1989), or coimmunoprecipitated $p34^{cdc2}$ onto the protein A–Sepharose beads containing the Myb or Myc immunocomplexes, and incubated the samples with [$\gamma^{32}P$]-ATP. Fig. 2 shows that under the former conditions labeled phosphate was incorporated into Myb but not into Myc (lanes *1*, note that both conditions gave identical results). Using the same procedure lamin B2 was also shown to be a substrate for $p34^{cdc2}$ (Lüscher et al., 1991). This result is shown more clearly for Myb in Fig. 3 *A* where in vivo $^{32}P_i$-labeled interphase and mitotic Myb is compared with Myb labeled in vitro with $p34^{cdc2}$. The increased incorporation of phosphate label and the shift to lower electrophoretic mobility in vivo is apparent. Interestingly, the mobility shift is closely mimicked by phosphorylation in vitro with $p34^{cdc2}$ (compare Fig. 3 *A*, lanes *1–3*). However, whereas the $p34^{cdc2}$ phosphorylated Myb appeared as a single species, M phase Myb was composed of two closely spaced protein forms which are not well resolved in $^{32}P_i$ labeled samples but clearly separated in the immunoblot shown in Fig. 7. This mobility shift is not induced by all kinases which phosphorylate Myb since we have previously shown that the specific CKII phosphorylation which occurs both in vitro and in vivo does not result in a substantial mobility shift (Lüscher et al., 1990).

Phosphorylation of Myb in vitro by $p34^{cdc2}$ might simply be nonspecific and not correspond to actual in vivo phosphorylation sites. We therefore compared the tryptic phosphopeptide maps of $p34^{cdc2}$-labeled Myb with Myb protein from I and M phase cells. The phosphopeptide patterns, shown in Fig. 3, indicated that $p34^{cdc2}$ generated one major and six minor phosphopeptides (Fig. 3, *p34*). Three of these peptides, numbered 3, 6, and 8 co-migrated with three of the in vivo labeled peptides found in mitotic Myb (Fig. 3, *M* and *M+p34*). Four of the peptides weakly phosphorylated in vitro by $p34^{cdc2}$ did not appear to correspond to any in vivo phosphopeptides (Fig. 3, *arrowheads* in *p34*). Peptide 3 is also found in interphase Myb but 6 and 8 are specifically detected in mitosis (Fig. 3, *M*, and *I+M*). Of the remaining in vivo mitotic phosphopeptides that do not correspond to $p34^{cdc2}$ phosphopeptides, spots 1, 4, and 2 are also present in interphase. Only spot 7 is both specific to mitotic Myb and does not correspond to a $p34^{cdc2}$ phosphorylation in vitro. We had also demonstrated above (Fig. 4) that mitotic phosphorylation of Myb involved introduction of phosphate into threonine residues. When we carried out a phosphoamino acid analysis of Myb after in vitro phosphorylation by $p34^{cdc2}$ we also detected phosphorylation on both serine and threonine residues (Fig. 4, *p34*). In contrast, phosphorylation by CKII resulted only in phosphorylating serines (Lüscher et al., 1990).

Taken together these data indicate that $p34^{cdc2}$ can phosphorylate peptides which correspond to actual in vivo phosphopeptides present in interphase and mitotic Myb proteins. However, Myc does not appear to be a substrate for the mitotic kinase in vitro and, as shown in Fig. 5, we have been unable to detect major phosphopeptide differences between interphase and mitotic Myc.

Altered DNA Binding Properties of Mitotic Myb and Myc

Both Myb and Myc have been previously shown to bind to double stranded DNA. To test if mitotic phosphorylation alters the capacity of Myb or Myc to interact with DNA, I-phase and nocodazole-arrested M phase BK3A cells were

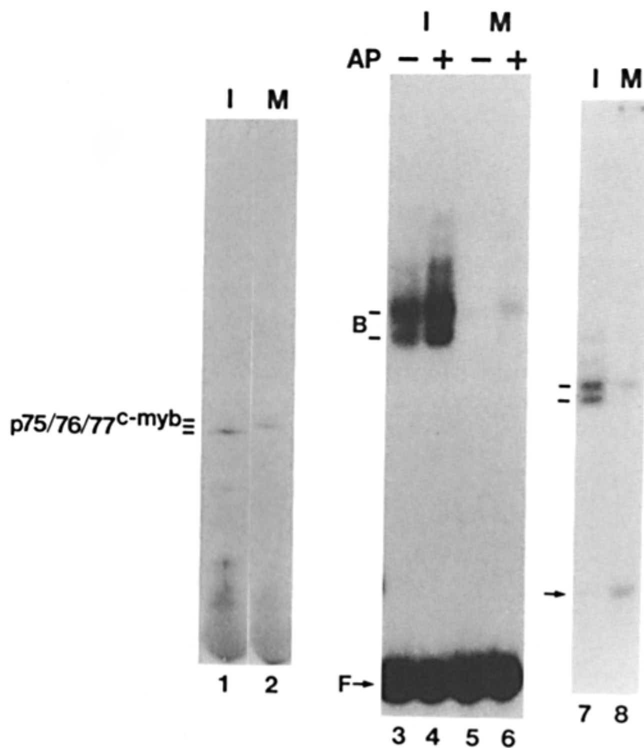

Figure 7. Specific DNA binding of M phase Myb. Myb proteins were prepared from logarithmically growing (lane *1*) or M phase-arrested (lane *2*) BK3A cells as described in Materials and Methods, and aliquots of these preparations were separated by 10% SDS-PAGE, transferred to nitrocellulose, and probed with an anti-Myb mAb. The same protein preparations (I-phase, lane *3*, *4*, and *7*; M phase, lanes *5*, *6*, and *8*) were used in gel mobility shift assays using MRE-1 (lanes *3*–*6*) or MRE-A (lanes *7* and *8*). Before the addition of probe, the protein samples in lanes 4 and 6 were pretreated with alkaline phosphatase (*AP*). *B*, bound probe; *F*, free probe.

labeled with $^{32}P_i$ and the proteins extracted in 400 mM NaCl after disruption of the cells by sonication. The soluble proteins were chromatographed on double-stranded calf thymus DNA cellulose columns. Myb and Myc were immunoprecipitated from individual fractions using specific antisera. The majority of both I phase Myb and Myc were eluted from the column between 200–500 mM NaCl (Fig. 6, lanes *I*). In contrast, the mitotic forms of the two proteins bound less tightly to double-stranded DNA cellulose. More than 50% of M phase Myb (two thirds of the bound protein) was eluted at a salt concentration of 200 mM. The amount of protein eluted at 300 and 500 mM salt was reduced by two- and sevenfold, respectively, when compared to I phase Myb. For Myc we also detected an increase in the amount of M phase protein eluting at 200 mM salt and a decrease at 300 and 500 mM salt compared to I phase Myc. In addition, we observed an increase in unbound M phase Myc. These data indicate that the specific modifications occurring during mitosis are sufficient to reduce the binding of Myc and Myb to DNA.

To further characterize the effect of M phase specific modifications, we compared the specific DNA binding properties of Myb immunopurified from either I or M phase cells to a Myb response element (MRE)[1] (Biedenkapp et al., 1988). Immunoprecipitated proteins were prepared for DNA binding by first treating with GuHCl to remove bound antibody, and then renaturing by dialysis (Lüscher et al., 1990). The immunopurified proteins were analyzed on immunoblots using a Myb-specific mAb. As shown in Fig. 7 mitotic Myb resolved into two distinct protein species with apparent molecular masses of 76 and 77 kD and displayed a reduced mobility compared to I phase Myb (75 kD). Treatment with alkaline phosphatase of M phase Myb resulted in an increased mobility on SDS-PAGE indistinguishable from I phase Myb (data not shown). These two protein preparations were then tested for their ability to interact with two short oligonucleotides containing different MREs (MRE-1 and MRE-A, respectively; Lüscher et al., 1990). For both MRE-1 and MRE-A Myb protein isolated from M phase cells showed a reduced specific DNA binding capacity (compare Fig. 7, lanes *3* and *5*, and *7* and *8*, respectively). It should be noted that we cannot at present distinguish between a direct effect of phosphorylation on DNA binding of the M phase form of Myb from an indirect effect (e.g., inappropriate refolding of the protein after the GuHCl treatment, see Materials and Methods). Nonetheless these results suggest that phosphorylation may have profound effects on Myb's structure.

In an effort to reactivate the DNA binding ability of the mitotic Myb, this protein preparation was pretreated with alkaline phosphatase or protein phosphatase type 2a before the addition of radiolabeled MRE-1 (Fig. 7, lanes *4* and *6*, and data not shown). I phase Myb showed increased binding which is thought to be the effect of removal of phosphate from the NH_2-terminal casein kinase II phosphorylation site. However, only a modest increase in specific DNA binding of the M phase Myb was observed. This is in contrast to the casein kinase II specific phosphorylation which can inhibit DNA binding but is almost completely reversed by the action of phosphatases (Lüscher et al., 1990). At this point, it is unclear if the inability to activate the DNA binding of mitotic Myb is a reflection of the relative insensitivity of the mitotic specific phosphorylation sites to phosphatases under the gel mobility shift conditions used or if other, as yet unidentified, M phase–specific modifications interfere with DNA binding.

Discussion

We have presented evidence that the two nuclear oncoproteins Myb and Myc are hyperphosphorylated during mitosis. In addition, their ability to bind to DNA is decreased during mitosis. During nocodazole-induced mitotic arrest both proteins display a phosphatase-reversible decrease in electrophoretic mobility in SDS-PAGE. In the case of Myb, the M phase phosphorylation corresponds to a clear change in its phosphopeptide map. However, for c-Myc no major changes in the phosphopeptide pattern were observed (see below). The mitotic forms of both of these proteins exhibited decreased binding to double-stranded DNA. In addition, M phase Myb had a reduced capacity to bind specific oligonucleotides containing the Myb response element. Thus entry into mitosis may have important consequences relating to the posttranslational modification and possibly the function of these two putative transcription factors.

A surprising result of this study is that the mitotic hyperphosphorylation of c-Myc does not result in an altered two-dimensional phosphopeptide map relative to I phase c-Myc.

1. *Abbreviation used in this paper*: MRE, Myb response element.

This is in contrast to c-Myb whose phosphopeptide map showed clear differences between I phase and M phase. For c-Myc none of the number of different proteolytic enzymes and electrophoresis conditions used detected a difference between the M phase and I phase forms. Nonetheless, the ability of phosphatase to reverse the shift in M phase Myc's electrophoretic mobility argues in favor of a difference in phosphorylation. Furthermore, a distinct mobility shift was detected in peptide 3 (Fig. 5 *B*) when I phase and M phase Myc were compared on a proteinase K one-dimensional map. One possible explanation for these data is that phosphorylation sites specific for I phase Myc and for M phase Myc are distinct yet located within the same peptide. The properties of the small peptide region might be expected to be the same when analyzed by two-dimensional fingerprinting. However, the characteristics in SDS-PAGE of the whole protein or the larger fragment produced by partial proteolysis might be affected. Alternatively, the increased apparent molecular weight may be the result of a combinatorial effect of several phosphorylation sites. In this model, individual c-Myc molecules within the interphase population are phosphorylated at different sites. During mitosis the same sites are phosphorylated, but each molecule would now have all sites occupied. This would produce identical I phase and M phase phosphopeptide maps but may well change the electrophoretic mobility of the protein population. At present we cannot distinguish between these possibilities. We have also tested c-Myc and c-Myb for reactivity with the MPM-2 and MPM-12 mAbs known to recognize a large subclass of M phase–specific phosphorylated epitopes (Davis et al., 1983). Neither mAb reacted with either I phase or M phase c-Myc and c-Myb proteins (B. Lüscher and P. N. Rao, unpublished observations).

Our inability to detect unique M phase phosphorylation sites in c-Myc prevents us from definitively determining the nature of the kinase involved in the hyperphosphorylation of c-Myc. Both c-Myc and c-Myb have been previously shown to be phosphorylated by casein kinase II (CKII) (Lüscher et al., 1989), glycogen synthase kinase 3 (GSK3) (B. Lüscher, unpublished results), and MAP kinase (Alvarez et al., 1991), all of which are thought to vary in activity in response to cell cycle or extracellular signals (Boyle et al., 1991; Carroll and Marshak, 1989; Sommercorn et al., 1987; Pulverer et al., 1991) and could therefore be responsible for M phase phosphorylation of Myc.

Another candidate for M phase–specific phosphorylation is the $p34^{cdc2}$ kinase. This kinase is a key regulator of mitosis which has been shown to phosphorylate an increasing number of substrate proteins (for review see Shalloway and Shenoy, 1991). Both Myb and Myc contain sequences that resemble the $p34^{cdc2}$ recognition motif (S/T P X K/R) as defined in histone H1, lamins, and $p60^{c\text{-}src}$ (Shalloway and Shenoy, 1991). It was therefore of interest to test if Myb or Myc could serve as substrates for this kinase. We have used both purified $p34^{cdc2}$-cyclin B complex from HeLa cells and $p34^{cdc2}$ kinase activity immunoprecipitated from nocodazole-arrested Manca cells to phosphorylate immunoprecipitates of either Myb or Myc. Whereas under these conditions Myb as well as lamin B_2 (Lüscher et al., 1991) were readily phosphorylated, Myc was not modified. As in the case of chicken lamin B_2 (Lüscher et al., 1991), $p34^{cdc2}$ phosphorylated only a subset of the M phase–specific phosphopeptides on Myb, leaving open the possibility that another kinase(s) is involved in the M phase–specific phosphorylation. It will be of interest to identify such enzymes and to test if they are regulated by $p34^{cdc2}$ or by some alternative pathway.

What biological role would mitotic hyperphosphorylation of proteins such as Myc and Myb play? One of the major events during mitosis is the condensation of the interphase chromatin to mitotic chromosomes, a process likely to facilitate efficient segregation of the chromosomes to the two daughter cells. During condensation, proteins that are involved in gene transcription, replication, and DNA repair may be released to allow efficient condensation. This process may be regulated at least in part by phosphorylation. It has been suggested that the mitotic hyperphosphorylation of histone H1 is important for chromosome condensation (Bradbury et al., 1974*a*,*b*; Inglis et al., 1976), however, no direct evidence has been obtained in support of this suggestion. Recently it has been shown that $p34^{cdc2}$ can phosphorylate the nonhistone high mobility group I protein in a manner that reduces its ability to bind DNA (Reeves et al. 1991). Our finding that Myb and Myc, which are involved in gene transcription, are hyperphosphorylated during mitosis adds these proteins to the growing list of M phase–specific substrates. The release from DNA of Myc and Myb, and possibly of many other DNA binding proteins, may be important in achieving chromosome compaction and possibly in reducing transcription during the transition from interphase to mitosis. Recently a similar observation has been made for Oct-1 which was shown to have a reduced DNA-binding capacity in M phase compared to interphase (Segil et al., 1991). Reduced DNA binding is also compatible with the observation that c-Myc is redistributed throughout the cell body during mitosis and little or no signal is detected associated with the condensed chromosomes (Eisenman et al., 1985; Winqvist et al., 1984).

In a recent series of experiments utilizing cell extracts to phosphorylate target proteins in vitro we have been able to demonstrate that mitotic, but not interphase extracts, can mediate phosphorylation of Myc, Myb, Jun, Fos, MyoD, lamin A and C, and the adenovirus E1a and 72-kD DNA binding proteins (our unpublished observations). In contrast, none of 10 cytoplasmically localized proteins, with the exception of $p60^{c\text{-}src}$, were hyperphosphorylated. These preliminary data indicate that many nuclear proteins may be potential substrates for M phase–specific kinases and provide support for the hypothesis that hyperphosphorylation may be a general mechanism to regulate transcription factor function during mitosis.

We would like to thank J. Cooper for the antienolase antiserum as well as for advice and encouragement throughout this study. We are also grateful to D. Beach for purified $p34^{cdc2}$ kinase, to P. Rao for the kind gift of MPM antibodies, to J. Y. Wang for anti-cdc2, T. Haystead for phosphatase, and to G. Ramsay for the monoclonal anti-Myb. S. Fisher provided expert technical assistance. We are also grateful to Alfred Nordheim for a critical reading of the manuscript.

This work was supported by grant CD59296 from the American Cancer Society and National Cancer Institute grant RO1CA20525 to R. N. Eisenman. Work from B. Lüscher's laboratory was supported by a grant from the Deutsche Forschungsgemeinschaft.

Received for publication 26 December 1992 and in revised form 13 April 1992.

References

Alvarez, E., I. C. Northwood, F. A. Gonzalez, D. A. Latour, A. Seth, C.

Abate, T. Curran, and R. J. Davis. 1991. Pro-Leu-Ser/Thr-Pro is a consensus primary sequence for substrate protein phosphorylation. *J. Biol. Chem.* 266:15277–15285.

Bagrodia, S., I. Chackalaparampil, T. E. Kmiecik, and D. Shalloway. 1991. Altered tyrosine 527 phosphorylation and mitotic activation of p60c-src. *Nature (Lond.).* 349:172–175.

Bailly, E., M. McCaffrey, N. Touchot, A. Zahraoui, B. Goud, and M. Bornens. 1991. Phosphorylation of two small GTP-binding proteins of the Rab family by p34cdc2. *Nature (Lond.).* 350:715–718.

Biedenkapp, H., U. Borgmeyer, A. E. Sippel, and K.-H. Klempnauer. 1988. Viral myb oncogene encodes a sequence-specific DNA binding activity. *Nature (Lond.).* 335:835–837.

Bischoff, J. R., P. N. Frieddman, D. R. Marshak, C. Prives, and D. Beach. 1990. Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. *Proc. Natl. Acad. Sci. USA.* 87:4766–4770.

Boyle, W. J., T. Smeal,, L. H. K. Defize, P. Angel, J. R. Woodgett, M. Karin, and T. Hunter. 1991. Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA binding activity. *Cell.* 64:573–584.

Bradbury, E. M., R. J. Inglis, and H. R. Matthews. 1974*a*. Control of cell division by very lysine rich histone (F1) phosphorylation. *Nature (Lond.).* 247:257–261.

Bradbury, E. M., R. J. Inglis, H. R. Matthews and T. A. Langan. 1974*b*. Molecular basis of control of mitotic cell division in eukaryotes. *Nature (Lond.).* 249:553–556.

Brizuela, L., G. Draetta, and D. Beach. 1989. Activation of human CDC2 protein as a histone H1 kinase is associated with complex formation with a p62 subunit. *Proc. Natl. Acad. Sci. USA.* 86:4362–4366.

Burke, B., and L. Gerace. 1986. A cell-free system to study reassembly of the nuclear envelope at the end of mitosis. *Cell.* 44:639–652.

Carroll, D., and D. R. Marshak. 1989. Serum stimulated cell growth causes oscillations in casein kinase II activity. *J. Biol. Chem.* 264:7345–7348.

Chen, P.-L., P. Scully, J.-Y. Shew, J. Y. J. Wang, and W.-H. Lee. 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell.* 58:1193–1198.

Cisek, L., and J. L. Corden. 1989. Phosphorylation of RNA polymerase by the murine homologue of the cell cycle control protein cdc2. *Nature (Lond.).* 339:679–684.

Cooper, J. A., N. A. Reiss, R. J. Schwartz, and T. Hunter. 1983. Three glycolytic enzymes are phosphorylated at tyrosine in cells transformed by Rouse sarcoma virus. *Nature (Lond.).* 302:218–223.

Davis, F. M., T. Y. Tsao, S. K. Fowler, and P. N. Rao. 1983. Monoclonal antibodies to mitotic cells. *Proc. Natl. Acad. Sci. USA.* 80:2926–2930.

Eisenman, R. N., C. Y. Tachibana, H. D. Abrams, and S. R. Hann. 1985. v-myc and c-myc-encoded proteins are associated with the nuclear matrix. *Mol. Cell. Biol.* 5:114–126.

Enoch, T., and P. Nurse. 1991. Coupling M phase and S phase: controls maintaining the dependence of mitosis in chromosome replication. *Cell.* 65:921–923.

Evan, G. I., G. K. Lewis, and J. M. Bishop. 1984. Isolation of monoclonal antibodies specific for products of the avian oncogene myb. *Mol. Cell. Biol.* 4:2843–2850.

Firzlaff, J. M., D. A. Galloway, R. N. Eisenman, and B. Luscher. 1989. The E7 protein of human papillomavirus type 16 is phosphorylated by casein kinase II. *New Biol.* 1:44–53.

Fisher, P. A. 1987. Disassembly and reassembly of nuclei in cell-free systems. *Cell.* 48:175–176.

Gerace, L., and G. Blobel. 1980. The nuclear envelope lamina is reversibly depolymerized during mitosis. *Cell.* 19:277–287.

Hann, S. R., and R. N. Eisenman. 1984. Proteins encoded by the human c-myc oncogene: differential expression in neoplastic cells. *Mol. Cell. Biol.* 4:2486–2497.

Hann, S. R., H. D. Abrams, L. R. Rohrschneider, and R. N. Eisenman. 1983. Proteins encoded by v-myc and c-myc oncogenes: identification and localization in acute leukemia virus transformants and bursal lymphoma cell lines. *Cell.* 34:789–798.

Heald, R., and F. McKeon. 1990. Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. *Cell.* 61:579–589.

Hihara, H., T. Shimizu, and H. Yamamoto. 1974. Establishment of tumor cells cultured from chickens with avian lymphoid leukosis. *Nat. Inst. Anim. Health. Quart.* 14:163–173.

Inglis, R. J., T. A. Langan, H. R. Matthews, D. G. Hardie, and E. M. Bradbury. 1976. Advance of mitosis by histone phosphokinase. *Exp. Cell Res.* 97:418–425.

Johnson, R. T., and P. N. Rao. 1970. Mammalian cell fusion: induction of premature chromosome condensation in interphase nuclei. *Nature (Lond.).* 226:717–722.

Langan, T. A., J. Gautier, M. Lohka, R. Hollingsworth, S. Moreno, P. Nurse, J. Maller, and R. A. Sclafani. 1989. Mammalian growth-associated H1 histone kinase: a homolog of cdc2+/CDC28 protein kinases controlling mitotic entry in yeast and frog cells. *Mol. Cell. Biol.* 9:3860–3868.

Lin, B. T.-Y., S. Gruenwald, A. Morla, W.-H. Lee, and J. Y. J. Wang. 1991. Retinoblastoma cancer suppressor gene product is a substrate of the cell cycle regulator cdc2 kinase. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:857–864.

Lüscher, B., and R. N. Eisenman. 1988. c-myc and c-myb protein degradation: effect of metabolic inhibitors and heat shock. *Mol. Cell. Biol.* 8:2504–2512.

Lüscher, B., and R. N. Eisenman. 1990*a*. Casein kinase II is involved in the regulation of several nuclear oncoproteins. *Contrib. Oncol.* 39:23–34.

Lüscher, B., and R. N. Eisenman. 1990*b*. New Light on Myc and Myb. Part I. Myc. *Genes Dev.* 4:2025–2035.

Lüscher, B., and R. N. Eisenman. 1990*c*. New Light on Myc and Myb. Part II. Myb. *Genes Dev.* 4:2235–2241.

Lüscher, B., E. A. Kuenzel, E. G. Krebs, and R. N. Eisenman. 1989. Myc oncoproteins are phosphorylated by casein kinase II. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1111–1119.

Lüscher, B., E. Christenson, D. W. Litchfield, E. G. Krebs, and R. N. Eisenman. 1990. Myb DNA binding inhibited by phosphorylation at a site deleted during oncogenic activation. *Nature (Lond.).* 344:517–522.

Lüscher, B., L. Brizuela, D. Beach, and R. N. Eisenman. 1991. A role for the p34cdc2 kinase and phosphatases in the regulation of phosphorylation and disassembly of lamin B2 during the cell cycle. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:865–875.

Mak, A. S., M. H. Watson, C. M. E. Litwin, and J. H. Wang. 1991. Phosphorylation of caldesmon by cdc2 kinase. *J. Biol. Chem.* 266:6678–6681.

Milner, J., A. Cook, and J. Mason. 1991. p53 is associated with p34cdc2 in transformed cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:2885–2889.

Moll, T., G. Tebb, U. Surana, H. Robitisch, and K. Nasmyth. 1991. The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the S. cerevisiae transcription factor SWI5. *Cell.* 66:743–758.

Moreno, S., and P. Nurse. 1990. Substrates for p34cdc2: in vivo veritas. *Cell.* 61:549–551.

Nurse, P. 1990. Universal control mechanism regulating onset of M-phase. *Nature (Lond.).* 344:503–508.

Ottaviano, Y., and L. Gerace. 1985. Phosphorylation of the nuclear lamins during interphase and mitosis. *J. Biol. Chem.* 260:624–632.

Peter, M., J. Nakagawa, M. Doree, J. C. Labbe, and E. A. Nigg. 1990. In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of the lamins by cdc2 kinase. *Cell.* 61:591–602.

Pines, J., and T. Hunter. 1990. p34cdc2: the S and M kinase? *New Biol.* 2:389–401.

Piras, R., and M. M. Piras. 1975. Changes in microtubule phosphorylation during cell cycle of HeLa cells. *Proc. Natl. Acad. Sci. USA.* 72:1161–1175.

Pulverer, B. J., J. M. Kyiakis, E. Nikolakaki, and J. R. Woodgett. 1991. Phosphorylation of *c-jun* mediated by MAP kinase. *Nature (Lond.).* 353:670–674.

Rausch, H. P., W. Sachsenmaier, K. Behrens, and V. Cruter. 1966. Synchronization of mitosis by the fusion of the plasmodia of *Physarum polycephalum*. *J. Cell Biol.* 31:204–209.

Reeves, R., T. A. Langan, and M. S. Nissen. 1991. Phosphorylation of the DNA-binding domain of nonhistone high-mobility group I protein by cdc2 kinase: reduction of binding affinity. *Proc. Natl. Acad. Sci. USA.* 88:1671–1675.

Segil, N., S. B. Roberts, and N. Heintz. 1991. Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. *Science (Wash. DC).* 254:1814–1816.

Shalloway, D., and S. Shenoy. 1991. Oncoprotein kinases in mitosis. *Adv. Cancer Res.* 57:185–225.

Sommercorn, J., J. A. Mulligan, F. J. Lozeman, and E. G. Krebs. 1987. Activation of casein kinase II in response to insulin and to epidermal growth factor. *Proc. Natl. Acad. Sci. USA.* 84:8834–8838.

Street, A. J., E. Blackwood, B. Lüscher, and R. N. Eisenman. 1990. Mutational analysis of the carboxy-terminal casein kinase II phosphorylation site in human c-myc. *Curr. Top. Microbiol. Immunol.* 166:251–258.

Stürzbecher, H. W., T. Maimets, P. Chumakov, R. Brain, C. Addison, V. Simanis, K. Rudge, R. Philp, M. Grimaldi, W. Court, and J. R. Jenkins. 1990. p53 interacts with p34cdc2 in mammalian cells: implications for cell cycle control and oncogenesis. *Oncogene.* 5:795–801.

Suprynowicz, F. A., and L. Gerace. 1986. A fractionated cell-free system for analysis of prophase nuclear disassembly. *J. Cell. Biol.* 103:2073–2081.

Vandre, D., F. M. Davis, P. N. Rao, and G. G. Borisy. 1984. Phosphoproteins are components of mitotic microtubule organizing centers. *Proc. Natl. Acad. Sci. USA.* 81:4439–4443.

Winqvist, R., K. Saksela, and K. Alitalo. 1984. The *myc* proteins are not associated with chromatin in mitotic cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2947–2950.

Wu, R. S., H. E. Panusz, C. L. Hatch, and W. H. Bonner. 1986. Histones and their modifications. *CRC Crit. Rev. Biochem.* 20:201–263.

Yamashiro, S., Y. Yamakita, H. Hosoya, and F. Matsumura. 1991. Phosphorylation of non-muscle caldesmon by p34cdc2 kinase during mitosis. *Nature (Lond.).* 349:169–172.