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^cd2 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^cd2 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.
assembly (Heald and McKeon, 1990; Peter et al., 1990). For SW15, phosphorylation by cdk28 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\textsuperscript{CDK2} 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\textsuperscript{c-src} and several other src family proteins tyrosine kinases, mos, p50\textsuperscript{map} (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\textsuperscript{CDK2}. In the case of pp60\textsuperscript{c-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 \textit{c-myc} and \textit{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, 1990b,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 Burkitt lymphoma cell line BK3A has been described (Hiirahara 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. 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% N-mercaptoethanol. The denatured proteins were renatured by dialysis against gel mobility shift buffer.

DNA Binding

For DNA-cellulose chromatography assays, 20–30 × 10⁶ exponentially growing or nocodazole-arrested BK3A cells were labeled with \textsuperscript{32}P 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 aprotinin) 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 washed three times through 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 were 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% N-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 32P-orthophosphate (32Pi) or [35S]methionine, lysed in detergents, and immunoprecipitated with specific anti-Myc antibody. Fig. 1 shows an SDS-PAGE analysis of anti-Myc immunoprecipitates [35S]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-Myc and p67/c-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-Myc, although many of the faint background bands are still visible. The absence of [35S]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 immunofluorescence analysis of mitotic cells (Eisenman et al., 1985; Winqvist et al., 1984), might be detectable through phosphate exchange after 32Pi labeling. SDS-PAGE comparison of equal aliquots of 32Pi 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 32Pi-labeled p64/p67/c-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 32Pi-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 32Pi-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 [35S]methionine (lanes 1-4), with 32Pi (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 1, 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 11 and 14). 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).
The major band at 53 kD corresponds to the Ig heavy chains from the first immunoprecipitate. The p64/p67c-uyo proteins were visible in interphase cells (Fig. 1, lane 1), while mitotic cells contain major anti-Myc detectable proteins shifted to ~1 kD higher molecular mass (Fig. 1, 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 antisera. Samples were analyzed on 10% SDS-PAGE.

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/p67c-Myc proteins were visible in interphase cells (Fig. 1, lane 1), while mitotic cells contain major anti-Myc detectable proteins shifted to ~1 kD higher molecular mass (Fig. 1, lane 2). Alkaline phosphatase treatment of the initial immunocomplex again resulted in a shift to the interphase mobility (Fig. 1, lane 3).

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

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, 1990c). 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 32P, 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 antiseraum 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 nocodazole (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 NH2-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 Myc 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 NH2-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 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<sup>cdc2</sup>. (A) Myb was immunoprecipitated from logarithmically growing unlabeled BK3A cells and phosphorylated by the p34<sup>cdc2</sup> kinase (lane 1), from M phase nocodazole-arrested or logarithmically growing 32P<sup>-</sup>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<sup>-</sup>Mr 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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. 32P-labeled Myb was hydrolyzied 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-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-Myc species of Myc isolated from $^{32}$P-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-Ras was immunoprecipitated and then labeled in an in vitro kinase reaction with CKII (lane 3) or GSK3 (lane 4). (C) The p59-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 p34cdc2 Kinase in Mitotic Phosphorylation of Myb

The p34cdc2 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 p34cdc2 appears to be responsible for
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<sup>cdc2</sup> kinase. We therefore prepared specific immunocomplexes containing Myc and Myb from BK3A cells and either added purified p34<sup>cdc2</sup> (Brizuela et al., 1989), or coimmunoprecipitated p34<sup>cdc2</sup> onto the protein A-Sepharose beads containing the Myb or Myc immunocomplexes, and incubated the samples with [γ<sup>32</sup>P]-ATP. Fig. 2 shows that under the former conditions labeled phosphate was incorporated into Myb but not into Myc (lanes I, note that both conditions gave identical results). Using the same procedure lamin B2 was also shown to be a substrate for p34<sup>cdc2</sup> (Lüscher et al., 1991). This result is shown more clearly for Myb in Fig. 3 A where in vivo <sup>32</sup>P labeled interphase and mitotic Myb is compared with Myb labeled in vitro with p34<sup>cdc2</sup>. 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<sup>cdc2</sup> (compare Fig. 3 A, lanes 1–3). However, whereas the p34<sup>cdc2</sup> phosphorylated Myb appeared as a single species, M phase Myb was composed of two closely spaced protein forms which are not well resolved in <sup>32</sup>P 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<sup>cdc2</sup> might simply be nonspecific and not correspond to actual in vivo phosphorylation sites. We therefore compared the tryptic phosphopeptide maps of p34<sup>cdc2</sup>-labeled Myb with Myb protein from I and M phase cells. The phosphopeptide patterns, shown in Fig. 3, indicated that p34<sup>cdc2</sup> 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 I+M). Four of the peptides weakly phosphorylated in vitro by p34<sup>cdc2</sup> 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<sup>cdc2</sup> 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<sup>cdc2</sup> 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<sup>cdc2</sup> 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<sup>cdc2</sup> 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
The soluble proteins were chromatographed on double-stranded calf thymus DNA cellulose. Myb and Myc were immunoprecipitated from individual fractions using specific antisera. The majority of both I phase Myb and Myc were eluted from the columns between 200–500 mM NaCl (Fig. 6, lanes 1). In contrast, the mitotic forms of both of these 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 threefold, 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) (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 (Lüsch 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-I and MRE-A, respectively; Lüsch et al., 1990). For both MRE-I 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 NH2-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 (Lüsch 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.
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<sup>cdc2</sup> 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<sup>cdc2</sup> recognition motif (S/T P X K/R) as defined in histone H1, laminas, and p60<sup>BH</sup> (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<sup>cdc2</sup>-cyclin B complex from HeLa cells and p34<sup>cdc2</sup> 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<sub>I</sub> (Lüscher et al., 1991) were readily phosphorylated, Myc was not modified. As in the case of chicken lamin B<sub>I</sub> (Lüscher et al., 1991), p34<sup>cdc2</sup> 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<sup>cdc2</sup> 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., 1974a,b; Inglis et al., 1976), however, no direct evidence has been obtained in support of this suggestion. Recently it has been shown that p34<sup>cdc2</sup> 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 of DNA from 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-l which was shown to have a reduced DNA-binding capacity in M phase compared to interphase (Segel 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<sup>BH</sup>, 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<sup>cdc2</sup> 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

Abate, T. Curran, and R. J. Davis. 1991. Pro-Leu-Ser/Thr-Pro is a consen-
sus primary sequence for substrate protein phosphorylation. J. Biol.
Chem. 266:15277–15285.
Bailly, E., M. McCaffrey, N. Tsuchot, A. Zahraraoui, B. Goud, and M. Bor-
ton. 1991. Phosphorylation of two small GTP-binding proteins of the Ral
Viral myb oncogene encodes a sequence-specific DNA binding activity.
Bischoff, J. R., P. N. Frieddman, D. R. Marshak, C. Prives, and D. Beach.
Nat. 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 phosphoryla-
tion 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. 1974a. Control of cell divi-
sion by very lysine rich histone (Fl) phosphorylation. Nature (Lond.).
Mole-
cular basis of control of mitotic cell division in eukaryotes. Nature (Lond.).
249:553–555.
Brizuela, L., G. Draetta, and D. Beach. 1989. Activation of human CDC2 pro-
tein as a histone H1 kinase is associated with complex formation with a p62
Burke, B., and L. Gerace. 1986. A cell-free system to study reassembly of the
phorylation of the retinoblastoma gene product is modulated during the cell
oncoproteins are phosphorylated by casin kinase II. EMBO (Eur. Mol.
Lüscher, T., E. Christensen, D. W. Litchfield, E. G. Krebs, and R. N. Eisen-
man. 1990. Myb DNA binding inhibited by phosphorylation at a site deleted
Lüscher, T., L. Brizuela, D. Beach, and R. N. Eisenman. 1991. A role for the
p34cdc2 kinase and phosphatases in the regulation of phosphorylation and
Milner, A., A. Cook, and J. Mason. 1991. p33 is associated with p34cdc2
phosphorylation and the CDC28 kinase protein in cell-cycle-regulated nu-
clear import of the S. cerevisiae transcription factor SWI5. Cell. 66:743–
758.
Ostuniavv, Y., and L. Gerace. 1985. Phosphorylation of the nuclear lamins
dissassembly of the nuclear lamina and M-phase-specific phosphoryla-
Pizar, R., and M. M. Pizar. 1975. Changes in microtubule phosphorylation
Pulverer, B. J., J. M. Kyriakis, E. Nikolskaki, and J. R. Woodgett. 1991. Phos-
phorylation of e-cjun mediated by MAP kinase. Nature (Lond.). 353:670–
674.
Rausch, H. P., W. Sachsemeier, K. Behrens, and V. Cruter. 1966. Synchroni-
zation of mitosis by the fusion of the plasmodia of Physarum polycephalum.
Reoves, R., T. A. Langan, and M. S. Nissen. 1991. Phosphorylation of the
DNA-binding domain of nonhistone high-mobility group I protein by cdc2
88:1671–1675.
Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. Science
cer Res. 57:185–222.
Sommercorn, J., J. A. Mulligan, F. J. Lozenza, and E. G. Krebs. 1987. Ac-
tivation of casein kinase II in response to insulin and to epidermal growth
Sturtebeker, H. W., T. Malnits, P. Chukamov, R. Brain, C. Addison, V.
1990. p33 interacts with p34cdc2 in mammalian cells: implications for cell
cycle control and oncogenesis. Oncogene. 5:795–801.
are components of mitotic microtubule organizing centers. Proc. Natl.
Acad. Sci. USA. 81:4439–4443.
Winquist, R., K. Saksela, and K. Alitalo. 1984. The c-myc proteins are not as-
sociated with chromatin in mitotic cells. EMBO (Eur. Mol. Biol. Organ.)
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. Phos-
phorylation of non-muscle caldesmon by p34cdc2 kinase during mitosis.