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Monoclonal antibodies to mitotic cells

(phosphoproteins/mitotic factors/immunofluorescence/immunoblots)

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ABSTRACT **Certain proteins or activities are present in mitotic cells but not in interphase cells. These proteins may be synthesized or activated, or both, just prior to mitosis and are responsible for the breakdown of the nuclear envelope and the condensation of chromosomes. To learn more about the nature of these proteins, we raised monoclonal antibodies to mitotic cells. Spleen cells from mice immunized with a 0.15 M NaCl extract of synchronized mitotic HeLa cells were fused with SP2/0-Ag14 mouse myeloma cells, and hybrids were selected in medium containing hypoxanthine, methotrexate, thymidine, and glycine. Two different hybridoma clones secreting antibodies reactive with mitotic and meiotic cells from every species tested were isolated. Chromosomes as well as cytoplasm in mitotic cells reacted with the antibodies, as detected by indirect immunofluorescence. The proteins from mitotic cells were separated by electrophoresis in $NaDodSO_4$/polyacrylamide slab gels, transferred to nitrocellulose sheets, and stained immunochemically. The two antibodies, designated MPM-1 and MPM-2, recognize a family of polypeptides with apparent molecular masses of 0.40 to >200 kilodaltons (kDa). Both antibodies reacted strongly with three polypeptide bands of 182 kDa, 118 kDa, and 70 kDa. Only mitotic cells exhibited the protein bands that were recognized by the antibodies. All these bands were found to be phosphoproteins as shown by ^{32}P labeling and autoradiography and their removal by alkaline phosphatase treatment.**

The nucleus of a mammalian cell undergoes profound reorganization when the cell enters mitosis. The nuclear envelope breaks down, and the chromatin condenses into discrete chromosomes. Although the initiation of mitosis is dependent on both RNA and protein synthesis until 2 hr and 1 hr, respectively, before mitosis, the addition of inhibitors of RNA and protein synthesis to cells after these times does not affect either initiation or completion of mitosis (1, 2).

The fusion of a mitotic cell with an interphase cell can induce nuclear envelope breakdown and premature chromosome condensation of the interphase nucleus (3). During this process, [^{3}H]tryptophan-labeled proteins from mitotic cells labeled at G_2 phase become associated with the prematurely condensed chromosomes, whereas the grain density on the metaphase chromosomes is not changed (4). Chromosome condensation and germinal vesicle (nucleus) breakdown can be induced in frog oocytes by the injection of extracts from mitotic but not interphase cells (5). The activity is labile to heat, sensitive to protease, insensitive to nuclease, and stabilized by phosphatase inhibitors and is found both in the cytoplasm and on the chromosomes of mitotic cells (6). These results suggest that certain nonhistone proteins, possibly phosphoproteins, of mitotic cells are not present in interphase cells. Al-Bader *et al.* (7) have reported the presence of such mitotic-specific proteins by using two-dimensional gel electrophoresis.

The objective of this study was to raise monoclonal antibodies to proteins present in mitotic cells but not in interphase cells. Two hybridoma clones that secreted antibodies reactive with mitotic and meiotic cells from every species tested were isolated. These two antibodies recognized a family of phosphorylated polypeptides found both in the cytoplasm and on the chromosomes of mitotic cells.

MATERIALS AND METHODS

Cells and Cell Synchrony. HeLa cells were maintained in monolayer culture and synchronized in S phase by using 2.5 mM thymidine (8) or in mitosis by using N_2O blockade (9) as described. The S phase and mitotic indices routinely ranged from 94% to 99%. Chinese hamster ovary (CHO) cells and a transformed mouse cell line (AKR-MCA) were synchronized in mitosis by using 0.1 μg of Colcemid per ml.

The mouse myeloma cell line, SP2/0-Ag 14, was maintained in McCoy's 5A medium with 2% rabbit serum (Kappa Scientific, Escondido, CA), 50 μM 2-mercaptoethanol, and 15 mM Hepes.

Preparation of Extracts from Mitotic Cells. Mitotic cells were washed in phosphate-buffered saline (P_i/NaCl; 10 mM Na_2HPO_4/0.15 M NaCl, pH 7.3) and resuspended at 2×10^7 cells per ml in P_i/NaCl and 1 mM phenylmethylsulfonyl fluoride. Cells were sonicated for 15 sec at 80 W with a Heat Systems–Ultrasonics cell disrupter to break >98% of the cells. After centrifugation of the mixture at 205,000 × *g* for 1 hr at 4°C in a Beckman L5-50 ultracentrifuge, the supernatant was retained. Protein concentrations of soluble mitotic extracts were determined to be ≈4 mg/ml by using the protein assay kit from Bio-Rad.

Immunization. Female BALB/c mice, 3 months old, were given an intraperitoneal injection of 0.25 ml of soluble mitotic extract emulsified with an equal volume of complete Freund's adjuvant (Miles). The mice were given two booster injections of 0.1 ml in the tail vein 3 weeks and 5 weeks later.

Cell Fusion and Cloning. Monoclonal antibodies were prepared by a method first described by Kohler and Milstein (10). Four days after the final booster injection, animals were killed by cervical dislocation, and spleens were removed aseptically and dissociated by mincing. Cells collected by centrifugation were resuspended in 0.17 M NH_4Cl at 4°C for 10 min to lyse erythrocytes (11). After being washed in medium without serum, spleen cells were mixed with SP2/0 cells at a ratio of 7:1 and pelleted by centrifugation. The pellet was gently resuspended at 5×10^8 cells per ml in 40% (wt/wt) polyethylene glycol 1000 in medium without serum (pH 7.6). After incubation at 30°C for 4 min, the cells were centrifuged at 22°C for 4 min at 300 × *g*, and the polyethylene glycol solution was removed. Cells

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Abbreviations: kDa, kilodaltons; P_i/NaCl, phosphate-buffered saline (10 mM Na_2HPO_4/0.15 M NaCl, pH 7.3); HATG medium, 100 μM hypoxanthine/0.4 μM methotrexate/5 μM thymidine/3 μM glycine.

were resuspended at 4×10^6 cells per ml in medium with 7.5% rabbit serum, 50 μM 2-mercaptoethanol, and 15 mM Hepes (complete medium), and 50-μl aliquots were distributed in 96-well microtiter plates. The following day, 50 μl of complete medium containing HATG medium (100 μM hypoxanthine/0.4 μM methotrexate/5 μM thymidine/3 μM glycine) at double strength was added to each well. An additional 0.1 ml of HATG medium was added 7–10 days after fusion. Colonies of 200 or more cells were visible by 12–14 days after fusion. Supernatants from these colonies were screened for antibodies to mitotic cells by indirect immunofluorescence. Positive colonies were subcloned twice by limiting dilution in HATG medium.

Ascites fluid was prepared by injecting 10^7 cells of selected hybridoma clones in 0.5 ml of saline into the peritoneal cavity of each mouse and harvesting the resultant ascites tumor 20–25 days later.

The antibody class and light chain type of the monoclonal antibodies was determined by immunoprecipitation in 1% agar as described (12).

Immunofluorescence and Immunoprecipitation. Indirect immunofluorescence was performed as described (12). Cells deposited on slides by using a cytocentrifuge were air dried, fixed in methanol for 10 min at 22°C, and air dried. The cells were overlayed with 75 μl of culture supernatant or an appropriate dilution of ascites fluid as primary antibody. Fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG, heavy and light chains (Miles), was used as the indicator antibody. Cell surface and intracellular antigens are stained by this method.

Measurement of the fluorescence intensity at 550–600 nm of individual cells was performed with a Leitz Orthoplan microscope equipped with epifluorescence, a "I2" filtercube, and an MPV microscope photometer. Blank readings were obtained from areas on the slide adjacent to the cells measured. Cells on slides stained with antisera obtained prior to immunization (preimmune) yielded readings equivalent to that of the blanks.

Immunoprecipitation of antigens from cell extracts was performed by the addition of 0.5% Nonidet P-40 detergent to the cell extract and the addition of ascites fluid containing an amount of protein equal to that of the cell extract. The mixture was incubated for 30 min at 30°C and then for 2 hr at 4°C. After the addition of 20 equivalents of anti-mouse IgG (Miles) and incubation at 4°C for 16 hr, the precipitate was recovered by centrifuging the mixture in a Beckman Microfuge at 4°C and washing it three times with P_i/NaCl containing 1 mM phenylmethylsulfonyl fluoride and 0.5% Nonidet P-40.

Polyacrylamide Gel Electrophoresis and Identification of Antibody-Reactive Polypeptides. Whole cells, after being washed three times in medium without serum, cell extracts, and immunoprecipitates, were solubilized in sample buffer containing 3% $NaDodSO_4$, and polypeptides were separated by electrophoresis in 6–20% gradient polyacrylamide gels as described (13). The following proteins (Pharmacia) were used as molecular size markers: thyroglobulin subunit [170 kilodaltons (kDa)], phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa). The polypeptides in the gel were then transferred electrophoretically to nitrocellulose paper as described (14, 15) for 4 hr at 300 mA. Then the nitrocellulose paper was cut into strips, which either were stained for protein in 0.1% napthol blue-black 10B (Sigma) in 45% CH_3COOH or were soaked for at least 24 hr in 3% bovine serum albumin (Sigma) in 10 mM Tris·HCl, pH 7.4/0.02% NaN_3 to block additional protein-binding sites on the nitrocellulose.

After the nitrocellulose was rinsed in 0.15 M NaCl/1 mM Tris·HCl, pH 7.4 (Tris/saline buffer), the strips were incubated for 16 hr at 25°C in 3% bovine serum albumin and 2% rabbit serum containing the monoclonal antibodies either as culture supernatants or as ascites fluid. We then washed the strips eight times over a 2-hr period by shaking in Tris/saline buffer. Individual strips were incubated for 2 hr at 25°C in peroxidase-conjugated rabbit anti-mouse IgG, heavy and light chains (Miles), while being shaken. After being washed as above, the strips were pooled in peroxidase substrate solution containing 0.07 mM *O*-dianisidine (Sigma), 9.2 mM H_2O_2, and 5 mM Tris·HCl (pH 7.4) and incubated while being shaken for 2 hr at 25°C.

^{32}P-Labeling, Autoradiography, and Phosphatase Treatment. Monolayers of HeLa cells synchronized in S phase by a double thymidine blockade were labeled with [^{32}P]orthophosphate (5 μCi/ml, New England Nuclear; 1 Ci = 3.7×10^{10} Bq) for 9–12 hr after reversal of the second blockade and collected in mitosis by exposure to 0.05 μg of Colcemid (Sigma) per ml. Autoradiography was performed by exposure of Kodak Safety Film L to nitrocellulose immunoblots of separated polypeptides. Nitrocellulose immunoblots or cell extracts were treated with bacterial alkaline phosphatase (Sigma) at 10 units/ml in 10 mM Tris·HCl (pH 8.0) at 25°C for 2 hr. Nitrocellulose immunoblots were treated with phosphatase and then washed in Tris/saline buffer before addition of primary antibodies.

RESULTS

Antibodies to Mitotic Cells. Two hybridoma clones secreting monoclonal antibodies that reacted specifically with mitotic cells by indirect immunofluorescence were isolated. Monoclonal antibody MPM-1 is an IgM antibody with κ light chains and antibody MPM-2 is an IgG_1 antibody with κ light chains. Analysis of the immunoglobulins produced by each of the clones by $NaDodSO_4$/polyacrylamide gel electrophoresis of culture supernatants and immunohistochemical staining with peroxidase-conjugated antimouse immunoglobulins revealed the presence of single light and heavy chain products from each clone, confirming the monoclonality of each clone. The antigens recognized by MPM-1 were present throughout the mitotic cells (Fig. 1). Chromosomes, as well as cytoplasm, were fluorescent. The nuclei of some interphase cells were also reactive. Similar results were obtained with MPM-2.

Measurement of the fluorescence intensity from individual cells in synchronized populations (Fig. 2) revealed that immunoreactivity was tightly coupled to mitosis. Metaphase cells were the most intensely fluorescent, having at least 10 times the intensity of S and G_1 cells. The average intensity of anaphase and telophase cells was about 2/3 of that in metaphase cells. G_2 cells had an average intensity 23% of that in metaphase cells. Thus, the antigenic reactivity detected by indirect immunofluorescence was first detected in the nuclei of G_2 cells and rapidly increased at mitosis. After reversal of the N_2O mitotic blockade, antigenic reactivity decreased with a half-time of approximately 1 hr. The measurement of the fluorescence intensity from cells stained with antibodies to nucleoli failed to reveal similar cell-cycle specificity, even though the nucleolar antigens are dispersed throughout the mitotic cells, as is the case for the antigens recognized by MPM-1 and MPM-2.

Absence of Species Specificity. The monoclonal antibodies to mitotic HeLa cells were tested for reactivity with mitotic cells from a variety of animal species, including human (normal and tumor cells), mouse, hamster, chicken, frog, nematode, and mosquito. The antibodies reacted specifically with mitotic cells. The antibodies also reacted with meiotic cells from rat testis. Indirect immunofluorescence of mosquito cells in mitosis is shown in Fig. 1 *B* and *D*.

Antibodies Recognize a Family of Polypeptides. The poly-

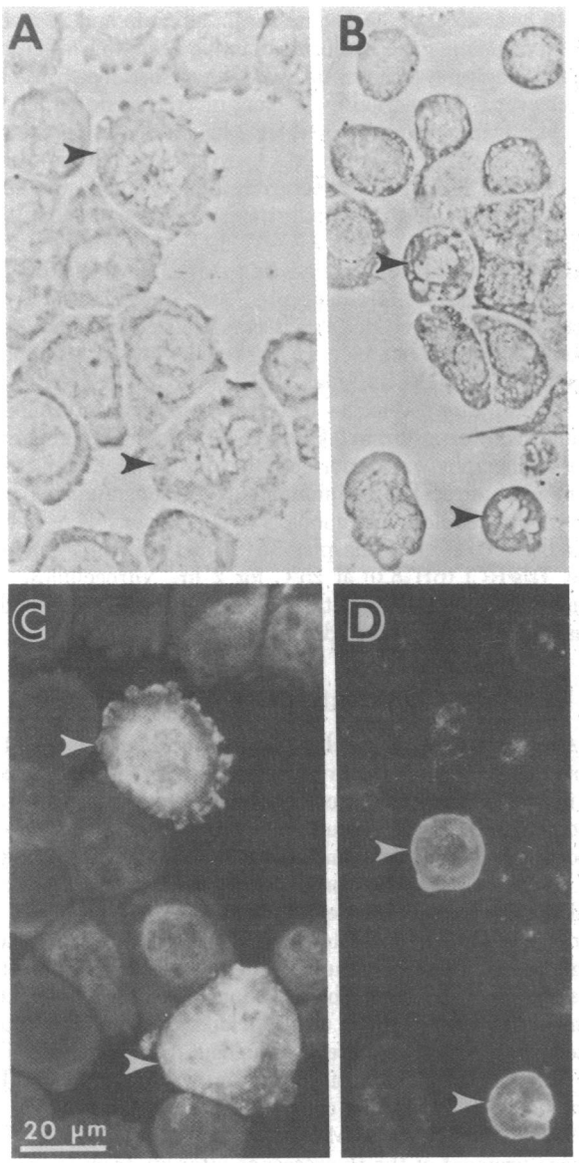

FIG. 1. Specificity of the antibodies to mitotic cells. Random populations of HeLa (*A* and *C*) and mosquito (*B* and *D*) cells were stained by indirect immunofluorescence with monoclonal antibody MPM-1. (*A* and *B*) Phase-contrast photomicrographs. (*C* and *D*) Fluorescent photomicrographs. Fluorescence is present both on the chromosomes and in the cytoplasm of the mitotic cells, which are indicated by arrows.

peptides from metaphase HeLa cells were electrophoretically separated on gradient $NaDodSO_4$/polyacrylamide gels, electrophoretically transferred to nitrocellulose paper, and incubated with antibodies to mitotic cells. Fig. 3 shows a family of at least 16 polypeptide bands with an apparent molecular mass range of 40 to >200 kDa that were recognized by the monoclonal antibodies. Three major reactive bands of 182 kDa, 118 kDa, and 70 kDa were recognized by both antibodies. There were some differences, however, in the reactivity of minor bands. Several experiments were performed to investigate the relationship between the bands because it is an uncommon occurrence for a monoclonal antibody to recognize more than one protein. Titration of antibodies failed to reveal differences in antibody affinity for the three major antigenic polypeptides. No evidence for the proteolysis of one major antigen into several smaller bands containing the same determinants was found by (*i*) resuspending viable mitotic cells in $NaDodSO_4$ sample buffer and boiling immediately for 3 min or (*ii*) by incubating an extract from mitotic cells at 30°C for 2 hr before preparing the sample for the gel. No apparent precursor–product relationship could be demonstrated from experiments with cells maintained in mitosis for various periods of time. Cell fractionation studies suggested that the 70-kDa antigen was enriched in chromo-

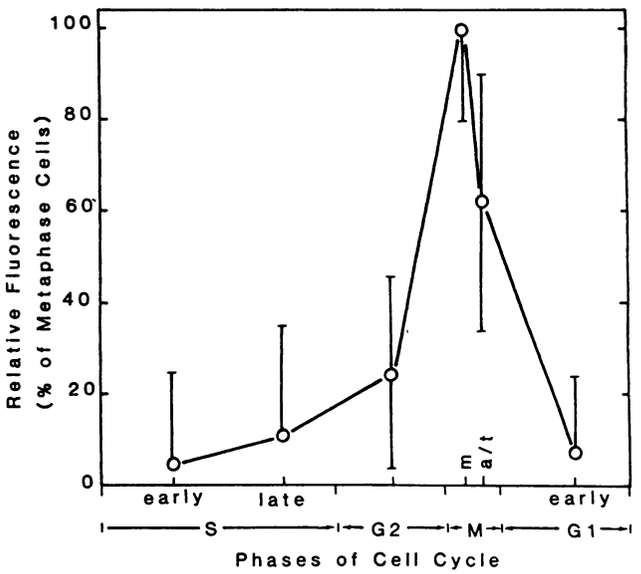

FIG. 2. Relative antigenic reactivity of synchronized HeLa cells. Cytocentrifuge preparations of synchronized HeLa cells were stained by indirect immunofluorescence with antibody MPM-1. The fluorescence intensity from 25 cells from each population was measured with a Leitz MPV microscope photometer. The fluorescence from blank areas on the slide adjacent to the cells was subtracted from each measurement. The fluorescence intensity from the nonmetaphase populations was normalized to that from 25 metaphase cells included on the same slide. The mean fluorescence intensities were plotted, and the bars represent the standard deviation. Metaphase cells (m) fluoresced more intensely than anaphase–telophase cells (a/t), and mitotic cells fluoresced more intensely than interphase cells.

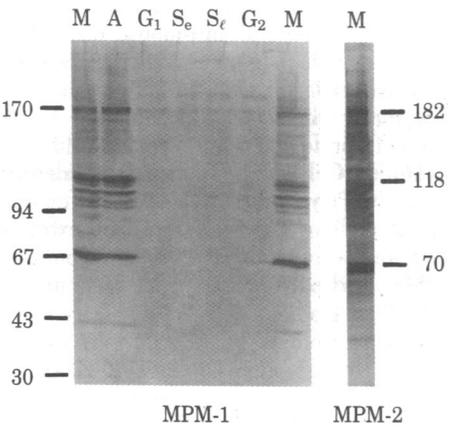

FIG. 3. Cell-cycle specificity of polypeptides recognized by the antibodies. Polypeptides from whole HeLa cells synchronized in various cell-cycle phases were separated in $NaDodSO_4$-gradient polyacrylamide gels, electrophoretically transferred to nitrocellulose sheets, and stained with monoclonal antibodies MPM-1 and MPM-2 by an indirect immunoperoxidase procedure. Approximately 25 μg of protein per slot was loaded. M, metaphase cells; A, anaphase cells 1 hr after reversal of N_2O blockade; G_1, cells in G_1 phase 3.5 hr after reversal of N_2O blockade; S_e, cells in early S phase 2 hr after reversal of second thymidine blockade; S_ℓ, cells in late S phase 6.5 hr after reversal of second thymidine blockade; G_2, cells in G_2 phase. Sizes shown in kDa are of marker proteins (on the left) and of the major antigens (on the right).

somes, but this polypeptide was also present in a soluble extract from mitotic cells.

The cell-cycle specificity of the antigenic polypeptides is also shown in Fig. 3. The 70-kDa and 118-kDa antigens were virtually absent during interphase, and only reduced amounts of the 182-kDa antigen were present. The small but detectable amount of the 70-kDa polypeptide in the G_2 lane was consistent with the 3–5% mitotic cell contamination of the G_2 cell population; mixing 5% mitotic cell protein with 95% S-phase cell protein also yielded faintly detectable bands. Cells irreversibly arrested in G_2 by incubation with *cis*-4-[{[(2-chloroethyl)nitrosoamino]carbonyl}amino]-cyclohexane carboxylic acid (7) for 48 hr did not have detectable levels of these antigens.

The three major antigenically-reactive polypeptides of 182 kDa, 118 kDa, and 70 kDa were found to be present in Colcemid-arrested metaphase cells of human, hamster, and mouse origin (Fig. 4). However, the apparent molecular weights and staining intensities of the minor bands differed between the species. Meiotic frog oocytes also contained the 70-kDa and 118-kDa antigens, whereas prophase cells from slime mold contained a single major antigenic polypeptide of 125 kDa.

Antibodies Recognize Phosphorylated Proteins. Two types of experiments were done to investigate whether the mitosis-specific antigens are phosphorylated because the activity from mitotic HeLa cells that induces chromosome condensation and nuclear envelope breakdown in frog oocytes is stabilized by phosphatase inhibitors (6). Phosphorylation of the antigens was studied by using synchronized HeLa cells labeled with ^{32}P during the G_2 phase and arrested in mitosis by using Colcemid. An extract from these cells was immunoprecipitated, and the proteins were separated on the polyacrylamide gels, transferred to nitrocellulose paper, and processed either for autoradiography or for antibody reactivity by indirect immunoperoxidase stain ing. Fig. 5 (lanes 1 and 2) shows that the 182-kDa, 118-kDa, and 70-kDa polypeptide antigens were ^{32}P-labeled. Other ^{32}P-labeled nonhistone proteins did not react with the antibody. Antigens precipitated from an extract of mitotic cells were also ^{32}P-labeled (Fig. 5, lanes 3 and 4). The sensitivity of the antigenic reactivity to digestion with alkaline phosphatase was studied also. Treatment of an extract with alkaline phosphatase in solution removed detectable antigenic sites (Fig. 5, lanes 5 and 6). No significant proteolysis of the phosphatase-digested samples was detected on amido black-stained immunoblots, nor did phosphatase digestion have any effect on immunostained bands recognized by an unrelated antibody specific for nucleoli. The susceptibility of the immunoreactivity to phosphatase digestion was further tested by the following experiment. Proteins from mitotic cells were separated on polyacrylamide gels and transferred to nitrocellulose paper; then the immunoblot was incubated in the presence or absence of bacterial alkaline phosphatase (10 units/ml). The immunoblots were stained by the indirect immunoperoxidase method. Phosphatase treatment completely removed the antigenic reactivity of the 182-kDa polypeptide and reduced the reactivity of other bands as shown in Fig. 5 (lanes 7 and 8). In control experiments, a similar treatment of immunoblots with phosphatase had no effect on indirect immunoperoxidase staining by an antibody specific for nucleoli.

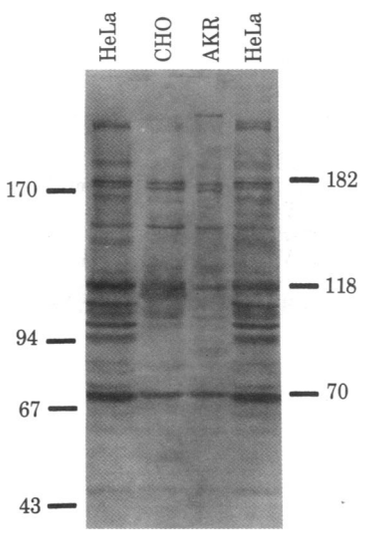

FIG. 4. Reactivity of the antibody MPM-1 with mitotic cells of different species. HeLa cells, Chinese hamster ovary (CHO) cells, and mouse (AKR) cells were synchronized in mitosis with 0.1 μg of Colcemid per ml. The polypeptides were separated and stained as described in the legend to Fig. 3. Approximately 25 μg of protein per slot was analyzed.

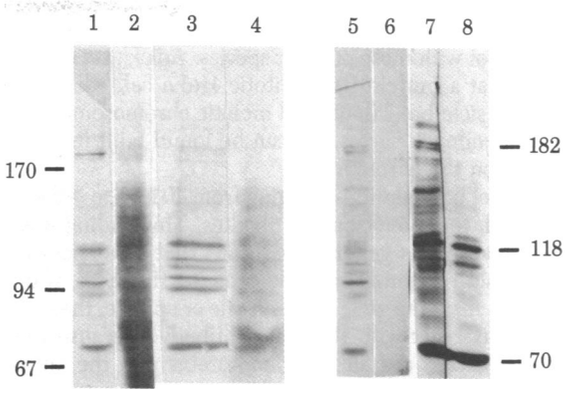

FIG. 5. Synchronized HeLa cells were labeled with [^{32}P]orthophosphate during the G_2 phase, and cells were arrested in mitosis with 0.05 μg of Colcemid per ml. An extract was prepared from one aliquot of the cells by sonication in a buffer consisting of 0.5% Nonidet P-40, P_i/NaCl, and 1 mM phenylmethylsulfonyl fluoride. After immunoprecipitation or digestion with alkaline phosphatase, the polypeptides were separated by electrophoresis and stained by the indirect immunoperoxidase procedure with antibody MPM-1 as described in the legend to Fig. 3. Proteins of whole mitotic HeLa cells were stained by the indirect immunoperoxidase procedure (lane 1; lane 2 is an autoradiograph of lane 1). Antigenic proteins from an extract of mitotic HeLa cells were precipitated with antibody MPM-2 and then separated on the polyacrylamide gel and stained by the indirect immunoperoxidase procedure (lane 3; lane 4 is an autoradiograph of lane 3). The sensitivity of the antigens to alkaline phosphatase was investigated by incubating an extract from mitotic HeLa cells in the absence (lane 5) or the presence (lane 6) of alkaline phosphatase before gel electrophoresis and staining by the indirect immunoperoxidase procedure. Nitrocellulose transfers of separated proteins of whole mitotic cells were incubated also in the absence (lane 7) or presence (lane 8) of alkaline phosphatase before staining by indirect immunoperoxidase. Approximately 25 μg of protein per lane was loaded. Molecular sizes are shown in kDa for marker proteins on the left and major antigens on the right.

DISCUSSION

The two monoclonal antibodies, MPM-1 and MPM-2, raised to extracts from mitotic HeLa cells, reacted specifically with mitotic and meiotic cells. Because the reactivity of the interphase cells is <10% of that detected in mitotic cells, the differences we have observed may be quantitative rather than qualitative. Indirect immunofluorescence studies demonstrated that the antigenic determinant recognized was found both on the chromosomes and in the cytoplasm of mitotic cells of all the animal species tested. In contrast, the rabbit antibodies to mitotic HeLa cells we described earlier (16) reacted specifically with the chromosomes but not with the cytoplasm of mitotic cells. Fur-

thermore, the rabbit antibodies reacted with human chromosomes but not with those of other species. Adlakha *et al.* (6) have reported that a protein from mitotic HeLa cells that induces germinal vesicle breakdown and meiotic chromosome condensation in *Xenopus* oocytes also can be found both in the cytoplasm and on the chromosomes.

A family of polypeptides, ranging from 40 kDa to >200 kDa, reacted with the monoclonal antibodies. This finding was surprising because it is generally assumed that a monoclonal antibody should recognize only one protein. However, if different proteins share the same antigenic site or epitope, then several proteins may react with the same antibody. Because the antigens recognized are all phosphoproteins and the antigenic reactivity is lost when the phosphate groups are removed by alkaline phosphatase digestion, it seems likely that the family of polypeptides recognized share a common or similar phosphorylated site. Both antibodies also recognized the same family of polypeptides, which suggested that the multiplicity of bands recognized did not result from the impurity of the hybridoma clones or from multiple fusion.

The three major antigens detected in mitotic HeLa cells were polypeptides of 182 kDa, 118 kDa, and 70 kDa, and detection of these antigens after polyacrylamide gel electrophoresis and transfer to nitrocellulose sheets was cell cycle dependent. These data are consistent with earlier observations, which suggested the presence of specific, mitosis-related proteins (7). However, the molecular sizes of the major antigens recognized in these studies exceeded those of the nine polypeptides in the 40–50 kDa range that appeared to be necessary for the G_2 to mitotic transition as reported by Al-Bader *et al.* (7).

Although the superphosphorylation of histone H1 has been correlated with chromosome condensation (17), Krystal and Poccia (18) have shown that a high level of phosphorylation of H1 does not necessarily lead to chromosome condensation. Moreover, Tanphaichitr *et al.* (19) have shown that chromosome decondensation can still occur when dephosphorylation of histones is inhibited. These studies suggest that factors other than H1 histone phosphorylation may be involved in chromosome condensation. Our study indicates that phosphorylation of nonhistone proteins also may be needed for mitosis. The antigens recognized by the monoclonal antibodies were shown to be phosphopeptides by ^{32}P-labeling during G_2 and by digestion with alkaline phosphatase. This finding is consistent with earlier reports by Adlakha *et al.* (6) and Wu and Gerhart (20) on meiotic maturation of frog oocytes induced by extracts from mitotic HeLa cells or mature frog oocytes, respectively, that showed that maturation-promoting activity was stabilized by the addition of phosphatase inhibitors.

Dephosphorylation of proteins in extracts from mitotic cells destroyed their reactivity with the antibodies used in our studies. Thus, these antibodies recognized the antigens only when they were phosphopeptides. Although this finding may be surprising, Levine *et al.* (21) raised antibodies that reacted only with methylated cytosine and not with cytosine. Moreover, Hansen and Beavo (22) have described a conformation-specific monoclonal antibody to a cyclic nucleotide phosphodiesterase with a 100-fold greater affinity in the presence of Ca^{2+}. However, that the polypeptides must be phosphorylated in order to be antigenically reactive suggests that the polypeptides also may be present during interphase, albeit in a dephosphorylated state. This possibility is attractive in view of the rapid turnover of the antigens in dividing cells. Cells in early nematode embryos divide every hour during cleavage, yet only the mitotic cells are reactive with the antibodies.

Antigenic polypeptides with apparent molecular weights identical to those of the major antigenic polypeptides in HeLa cells were identified in mouse and hamster. The specificity of the antibodies for mitotic cells over the broad range of species tested, including human, rodent, chicken, frog, insect, and nematode, demonstrated that the antigenic determinant is highly conserved and, therefore, may be functionally important in the mitotic process.

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