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Intranuclear appearance of the phosphorylated form of cytoskeleton-associated 350-kDa proteins in U1-ribonucleoprotein regions after growth stimulation of fibroblasts

(growth factors/microtubule-associated protein/c-*myc*/actinomycin D/messenger RNA)

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ABSTRACT **Cytoskeleton-associated 350-kDa and 80-kDa polypeptides, which were immunoprecipitated with polyclonal antibody against microtubule-associated protein 1 (MAP-1), were rapidly phosphorylated on mitogenic stimulation of quiescent fibroblasts with serum or growth factors. The enhanced phosphorylation was evident within 5 min and reached a maximum 2 hr after the stimulation. Phosphorylated MAP-1 analogues were first detected in the cytoplasm around the microtubule-organizing center and then in the nucleus by immunofluorescent staining with a monoclonal antibody that recognized the phosphorylated form of MAP-1. The monoclonal antibody reacted with the 350-kDa protein in immunoblot analysis and immunostained intranuclear speckles; both immunoreactions were abolished by treatment with alkaline or acid phosphatase. The nuclear speckles stained by the monoclonal antibody were also stained by anti-U1 small nuclear ribonucleoprotein antibodies on double immunofluorescence, suggesting that the stained regions are sites of maturation of messenger RNA. These results support the idea that part of the cytoskeleton-associated 350-kDa protein is phosphorylated and transferred to the nuclear region of mRNA modification as a common early process after growth stimulation.**

Growth factors bind to their specific plasma membrane receptors and stimulate a series of biochemical events that lead to proliferation of target cells. Activation of protein kinases and enhanced phosphorylation of receptors and other proteins are among the earliest responses to the binding of growth factors (1, 2). Enhanced phosphorylation of cytoskeletal proteins has been regarded as a subsequent process to transmit the initial transmembrane signal to the nucleus. The phosphorylations of vinculin (3, 4), myosin light chain (5, 6), 80- to 81-kDa protein (7–9), 35- to 36-kDa proteins (10–14), tubulin (15, 16), and microtubule-associated proteins (MAPs) (9, 15, 16) have been reported to be early events after stimulation by Rous sarcoma virus or growth factors. But although many other cytoskeletal proteins are phosphorylated *in vitro* or in detergent-treated cells, little is known about their increases in phosphorylation in intact cells after growth stimulations.

We have reported calcium-dependent phosphorylations of cytoskeleton-associated 350- and 80-kDa polypeptides in rat embryo fibroblasts after growth stimulations (9). These polypeptides were not phosphorylated appreciably in cells in the quiescent G_0 phase but were phosphorylated 20 min after addition of fresh serum, epidermal growth factor, phorbol ester, insulin, or transferrin. All of these agents also induced incorporation of [^{3}H]thymidine into DNA. Thus, we suggested that the phosphorylations of 350-kDa and 80-kDa polypeptides represent common early events after stimulation with different growth factors (9).

In the present study, we examined the time course of phosphorylation shortly after stimulation and the intracellular location of phosphorylated MAP-1 analogues. Monoclonal antibodies against MAP-1 caused bright immunostaining of intranuclear flecks as well as the centrosome and cytoskeletal network (17–20). The nuclear immunofluorescence disappeared when growth was inhibited and reappeared before the resumption of DNA synthesis in response to growth stimulations (18, 21). The immunoreactive molecules in these diverse sites were suggested to be the 350-kDa and related proteins that shared some common peptides with brain MAP-1 (22). However, the molecular mechanism of the change in the staining pattern was unknown. In the present study, results of phosphatase treatment showed that the monoclonal antibody specifically recognized the phosphorylated form of MAP-1 analogues. Thus, combined results by immunoprecipitation and immunofluorescent staining indicated rapid phosphorylation of cytoskeleton-associated 350-kDa and 80-kDa proteins and the subsequent appearance of their phosphorylated forms in the nucleus.

Assuming that the phosphorylated MAP-1 analogues in the nucleus function in activated transcription of proliferation-related genes, we carried out preliminary studies on the time-dependence of the synthesis of messenger RNA of the c-*myc* gene and the topological relation of phosphorylated MAP-1 analogues with U1 ribonucleoprotein (U1-RNP).

MATERIALS AND METHODS

Cells and Cultures. Clone 1-6 of 3Y1-B cells derived from Fischer rat embryo fibroblasts was used (23). Growth of this cell line is inhibited by cell contact or by serum deprivation (24). The culture medium was Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% or 1% fetal calf serum.

Antibodies. Rabbit antiserum and a monoclonal antibody against brain MAP-1 were raised in our laboratory as reported (9, 17). The antibodies reacted in immunoblots with three polypeptides of MAP-1 species of brain microtubule proteins (9, 17) and with 350-kDa proteins and related polypeptides in fibroblasts (9, 22). The autoantibody against U1-RNP in four patients with mixed connective tissue disease was determined by double immunodiffusion with standard reference sera (25).

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Abbreviations: MAP, microtubule-associated protein; U1-RNP, U1 ribonucleoprotein.

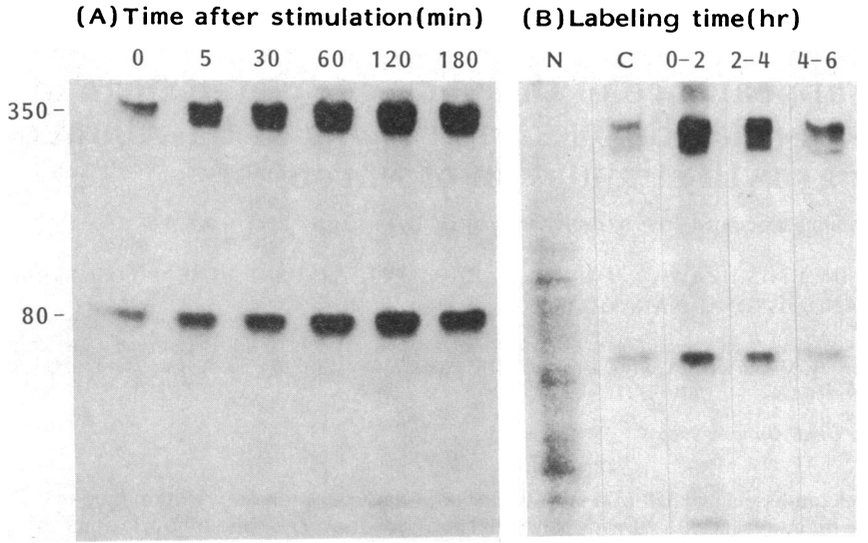

FIG. 1. Autoradiographs of [^{32}P]phosphoproteins immunoprecipitated with polyclonal anti-MAP-1 antibody. Quiescent rat fibroblasts (3Y1-B) were stimulated to grow by adding fresh serum after incubation with 40 μCi (1 Ci = 37 GBq) of ^{32}P per ml of P-free medium (*A*) or were labeled with ^{32}P after growth stimulation (*B*). Sizes are shown in kDa.

Immunoprecipitation. We used a method of indirect immunoprecipitation (9, 26). Briefly, $\approx 10^6$ ^{32}P-labeled cells in phosphate-free medium were lysed with 200 μl of lysis buffer (1% Triton X-100/0.5% sodium deoxycholate/0.1% NaDodSO$_4$/0.2 M NaCl/0.05 M Tris·HCl, pH 8.0) containing protease inhibitors (leupepsin at 10 μg/ml, pepstatin at 10 μg/ml, phenylmethylsulfonyl fluoride at 1 mM, and aprotinin at 10 μg/ml), and the lysate was clarified by centrifugation at 14,000 rpm for 20 min. Antigenic proteins in the supernatant were bound with anti-MAP-1 antiserum, adsorbed by *Staphylococcus aureus* Cowan 1, and then processed for electrophoresis on a linear gradient of 4–15% polyacrylamide gel with 0.1% NaDodSO$_4$. The gel was stained, dried, and autoradiographed with Kodak X-Omat AR film.

Immunofluorescence Microscopy. Cultured cells on glass coverslips were fixed in 10% formalin for 5 min at room temperature, made permeable in 95% ethanol, incubated with the first antibody, washed, and then stained with affinity-purified fluorescein-conjugated second antibody as described (18). For double immunofluorescence staining, monoclonal antibody against MAP-1 and anti-U1-RNP autoantibodies from patients with mixed connective tissue disease were applied simultaneously as the first antibody, and μ chain-specific rhodamine-conjugated goat IgG anti-mouse IgG (Zymed Laboratories, San Francisco) and γ chain-specific fluorescein isothiocyanate-conjugated goat IgG anti-human IgG (Zymed Laboratories) were used as the second antibodies. The affinity-purified second antibodies did not cross-react with the first antibodies.

Treatment with Phosphatases. Fixed cells on a coverslip for immunofluorescence staining, or cellular proteins on a nitrocellulose membrane for immunoperoxidase staining or autoradiography, were pretreated with 1–200 units of alkaline phosphatase from calf intestine (grade I, Boehringer Mannheim) per ml of 50 mM Tris·HCl buffer (pH 8.0) containing 1 mM MgSO$_4$ or with 0.1–100 units of acid phosphatase from potato (grade I, Boehringer Mannheim) per ml of 50 mM Tris·HCl buffer (pH 4.8).

RESULTS

Time Course of Phosphorylation After Stimulation. Fig. 1 shows the time courses of phosphorylation of the 350- and 80-kDa proteins in 3Y1-B cells after mitogenic stimulation

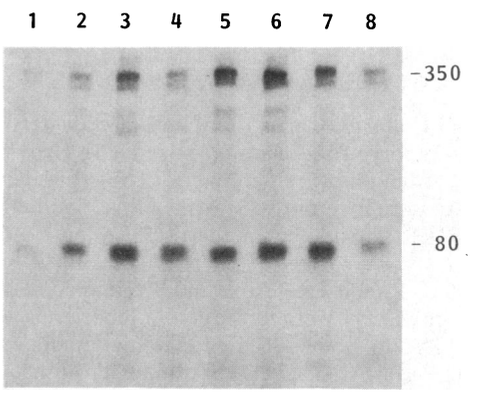

FIG. 2. Autoradiographs of [^{32}P]phosphoproteins immunoprecipitated with polyclonal anti-MAP-1 antibody. Quiescent 3Y1-B cells (lane 1) were stimulated by 10 ng of phorbol 12-tetradecanoate 13-acetate per ml (lanes 2 and 3), 30 ng of epidermal growth factor per ml (lanes 4 and 5), 10% fresh serum (lane 6), or 1 μg of insulin per ml (lanes 7 and 8). The labeling period with 40 μCi of ^{32}P per ml was 0–2 hr (lanes 2, 4, 6, and 8) or 3–5 hr (lanes 3, 5, and 7) after the stimulation. Sizes are shown in kDa.

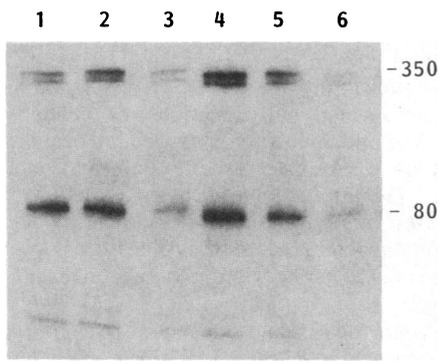

FIG. 3. Release of ^{32}P from ^{32}P-labeled 350-kDa and 80-kDa proteins by phosphatase treatment. Immunoprecipitated [^{32}P]phosphoproteins were resolved by 4–15% polyacrylamide gel electrophoresis and were transferred to a nitrocellulose membrane. Slices of the blotted membrane were incubated in alkaline buffer (pH 8.5) without enzyme (lane 2) or with 20 units of alkaline phosphatase per ml (lane 3) or in acidic buffer (pH 4.8) without enzyme (lane 4) or with 1 unit (lane 5) or 10 units (lane 6) of acid phosphatase per ml at 37°C for 1 hr. After the incubation or after no treatment (lane 1), the membrane slices were autoradiographed to detect the residual ^{32}P.

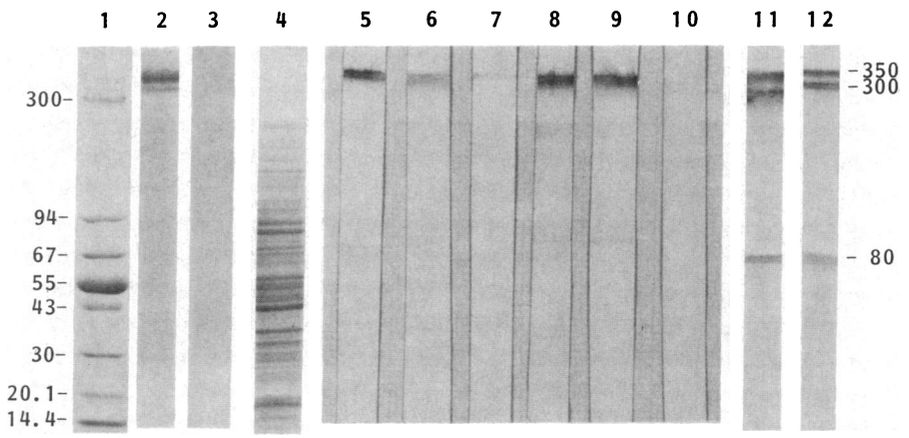

FIG. 4. Effect of phosphatases on immunoblotting. Microtubule proteins of brain (lanes 1–3) and a whole-cell extract of exponentially growing 3Y1-B cells (lanes 4–12) were subjected to gel electrophoresis and immunoblot analysis. Slices of the blotted membrane were not treated (lane 8) or were incubated in buffer without enzyme (lanes 2, 5, and 9) or with phosphatases before immunoperoxidase staining with monoclonal anti-MAP-1 antibody (lanes 2, 3, and 5–10) or with polyclonal anti-MAP-1 antiserum (lanes 11 and 12). Treatments with 20 units of alkaline phosphatase (lanes 3 and 10), with 1 unit of acid phosphatase (lane 6), or with 10 units of acid phosphatase (lane 7) per ml abolished the reaction of the 350-kDa protein with monoclonal anti-MAP-1 antibody but not with polyclonal antibody. Lane 1 shows microtubule proteins and standard proteins of known molecular mass in kDa, and lane 4 shows proteins in the whole-cell extract, stained with Coomassie brilliant blue.

with fresh serum. We used two experimental procedures to detect rapid phosphorylation as reported (9). One involved previous incorporation of ^{32}P into quiescent cells for 5 hr to prelabel the ATP pool and subsequent addition of 10% fresh serum. By this procedure, increases in phosphorylations of the 350- and 80-kDa proteins were evident within 5 min and reached plateau levels after stimulation with serum for 2 hr (Fig. 1*A*). The second procedure was incubation of cells with ^{32}P for 2-hr periods after stimulation. Fig. 1*B* indicates that phosphorylations of these proteins were maximal during the first 2 hr and decreased thereafter. Similar marked phosphorylations of the 350- and 80-kDa proteins during the first 2 hr were seen on stimulation of quiescent 3Y1-B cells with purified growth factors [i.e., epidermal growth factor (Toyobo, Osaka, Japan) at 30 ng/ml, insulin (Fluka) at 1 μg/ml, and 10 ng/ml phorbol 12-tetradecanoate 13-acetate (Sigma) as shown in Fig. 2.

Effect of Phosphatases on [^{32}P]Phosphoproteins. Phosphoproteins were immunoprecipitated with polyclonal anti-MAP-1 antibody after incorporation of ^{32}P for 4 hr, resolved by polyacrylamide gel electrophoresis, and transferred electrophoretically to a nitrocellulose membrane. Slices of the blotted membrane were treated with 20 units of alkaline phosphatase or 10 units of acid phosphatase per ml at 37°C and then were exposed to x-ray film to detect the residual ^{32}P by autoradiography. Treatment with either phosphatase for 1 hr resulted in about 90% loss of ^{32}P from the 350- and 80-kDa phosphoproteins (Fig. 3). Incubation of the blotted membrane with alkaline or acid buffer had no effects on their ^{32}P content.

Effects of Phosphatases on Immunoblotting. Whole-cell proteins were extracted from exponentially growing 3Y1-B cells with 10 mM Pipes buffer containing 1% Triton X-100, 0.4 M NaCl, 0.3 M sucrose, 3 mM $MgCl_2$, 2 mM EGTA, and protease inhibitors; resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis; and subjected to immunoblotting (9, 17, 22). The immunoreactive proteins were stained with immunoperoxidase. A biotin-avidin system was used for the monoclonal antibody to amplify the reaction. Binding of the 350-kDa protein to the monoclonal antibody was abolished by its previous treatment with alkaline phosphatase (20 units/ml) or acid phosphatase (10 units/ml) for 1 hr (Fig. 4). In contrast, the bindings of 350- and 80-kDa proteins to the polyclonal antibody were not substantially affected by their treatment with phosphatase. These results suggest that phosphatase treatment did not release 350-kDa and 80-kDa proteins from the blotted membrane but abolished the binding with the monoclonal antibody, thus showing that the monoclonal antibody recognized phosphorylated sites.

Effects of Phosphatases on Immunofluorescence. 3Y1-B cells in the G_1 phase were stained with the monoclonal antibody against MAP-1. Previous treatment of the cells with alkaline phosphatase (20 units/ml) or acid phosphatase (10 units/ml) for 1 hr at 37°C abolished the nuclear immunofluorescence (Fig. 5). Treatment of the cells with alkaline buffer without the enzyme did not have this effect, while their

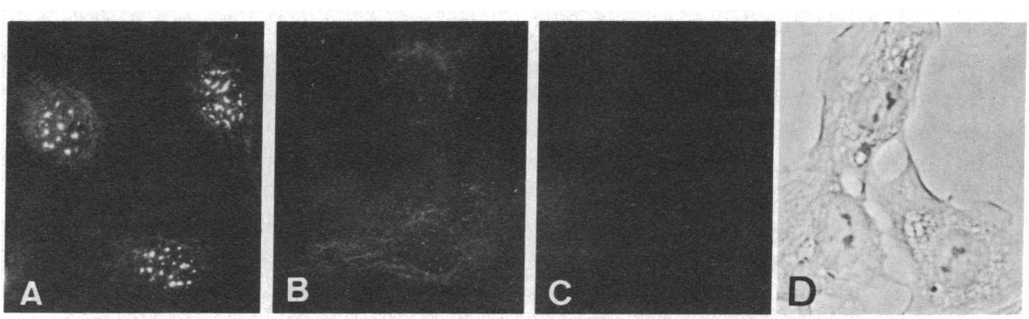

FIG. 5. Indirect immunofluorescent staining of G_1-phase 3Y1-B cells by monoclonal anti-MAP-1 antibody. (*A*) Untreated cells. (*B*) Cells pretreated with 20 units of alkaline phosphatase per ml for 1 hr. (*C*) Cells pretreated with 10 units of acid phosphatase per ml for 1 hr. (*D*) Phase-contrast microscopic appearance of *C*. The immunofluorescence of the nuclear region was decreased uniformly by phosphatase treatment to less than 5% of that of untreated cells as shown by densitometry of the film. The exposure time was the same (10 sec) for *A*, *B*, and *C*.

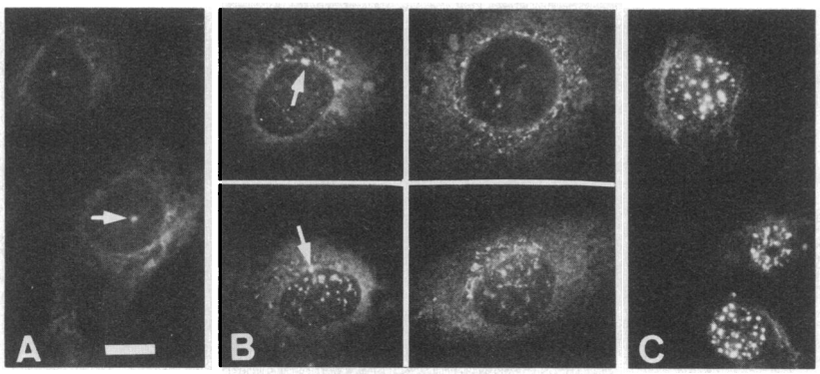

FIG. 6. Indirect immunofluorescent staining by monoclonal anti-MAP-1 antibody of quiescent 3Y1-B cells (*A*) and of cells after stimulation with insulin (1 μg/ml) and fibroblast growth factor (0.1 μg/ml) for 2 hr (*B*) and 5 hr (*C*). Fluorescent dots were not detectable in quiescent cells but were first seen around the centrioles (indicated by arrows) in the perinuclear cytoplasm or in the nucleus in 1.4–6.2% of the cells. After growth stimulation for 5 hr, 54% of the cells showed nuclear immunofluorescence. The exposure time was the same (10 sec) for all photographs.

treatment with acidic buffer diminished slightly the immunofluorescence (data not shown). Polyclonal antibody against MAP-1 mainly stained the cytoplasmic network and the centrosome, and this staining pattern was not affected by pretreatment of the cells with either phosphatase (data not shown). These results suggest that the monoclonal antibody specifically recognized the phosphorylated form of MAP-1 analogues and that these are abundant in the nucleus, although most of the MAP-1 analogues are present in the cytoplasm.

On staining with monoclonal antibody, no speckled nuclear immunofluorescence was detectable in quiescent 3Y1-B cells (Fig. 6*A*), but it was seen in cells after a 5-hr stimulation with insulin (1 μg/ml) and fibroblast growth factor (0.1 μg/ml) (Fig. 6*C*) as reported (18, 21, 22). During the transitional period between 2 and 4 hr after the stimulation, some localized fluorescent dots were seen in 1.4–6.2% of the cells (Fig. 6*B*). These fluorescent dots were often seen around the centrioles, possibly in the Golgi area. Since the immunofluorescent dots observed on staining with monoclonal antibody represented phosphorylated MAP-1 analogues, their transitional localization suggests that a portion of the MAP-1 analogues in the cytoplasm is phosphorylated, assembled around the centrioles, and then transferred to the nucleus.

Colocalization with U1-RNP. Fig. 7 shows the results of double staining with monoclonal anti-MAP-1 antibody (mouse IgM) and anti-U1-RNP autoimmune serum (human IgG). Anti-MAP-1 antibody (Fig. 7*A*) and anti-U1-RNP autoantibody (Fig. 7*B*) produced 20–50 large flecks in identical sites in the same cell. The large flecks represented accumulations of small dots. Double immunofluorescent staining with anti-U1-RNP antisera of four different patients gave similar results. In the immunoblot analysis, the anti-MAP-1 antibodies did not cross-react with polypeptides obtained by immunoaffinity column chromatography with anti-U1-RNP IgG (data not shown). Anti-U1-RNP antisera did not react with the 350-kDa protein or MAP-1 in the immunoblot analysis (data not shown). Incubation of cells with actinomycin D (5 μg/ml) for 4 hr resulted in the aggregation of 20–50 nuclear flecks into several large flecks, which were similarly stained by both the monoclonal anti-MAP-1 antibody (Fig. 7*C*) and anti-U1-RNP antiserum (Fig. 7*D*).

Transcription of the c-*myc* Gene. When quiescent 3Y1-B cells were stimulated with serum, mRNA that hybridized with ^{32}P-labeled c-*myc* cDNA appeared after 2 hr (data not shown) as observed in lymphocytes and 3T3 cells after mitogenic stimulation (27).

DISCUSSION

U1-RNP are thought to participate in the splicing of RNA polymerase II transcripts (28, 29) and in polyadenylylation (30). Therefore, the intranuclear speckles that are immunostained by anti-U1-RNP antiserum are regarded as the regions where the premessenger RNA is processed. U2-RNP colocalizes with U1-RNP on the speckles (31). The interchromatin regions of RNP are resistant to extraction with buffers containing detergent and a high concentration of salt (32). These regions have been proposed to represent the sites for packaging of heterogenous RNA, including pre-mRNA, posttranscriptional modification, and transport of mRNA. Many enzymes and regulatory proteins seem to be assembled on discrete domains of the speckles for these functions. Two proteins were reported to be colocalized with U1-RNPs on the nuclear speckles: these are the nuclear matrix-associated 107-kDa protein (33) and the 30-kDa nonhistone protein BA (34). The 107-kDa protein was suggested to be associated with the transcriptionally active conformation of chromatin (33).

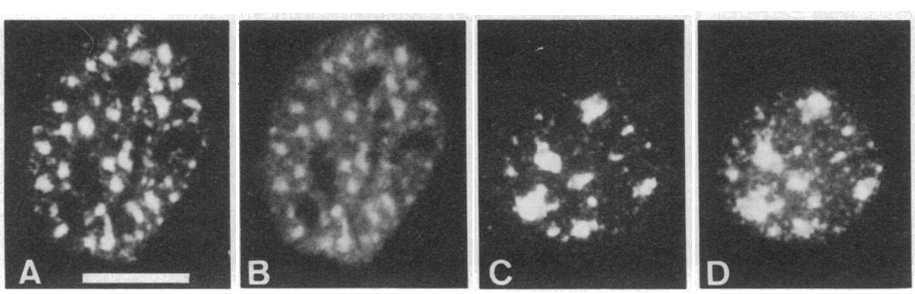

FIG. 7. Double immunofluorescent staining of the same 3Y1-B cell by monoclonal anti-MAP-1 antibody (*A* and *C*) and by anti-U1-RNP antiserum (*B* and *D*). Incubation of cells with actinomycin D (5 μg/ml) for 4 hr caused aggregation of nuclear flecks stained by the two antibodies (*C* and *D*).

The present results showed that phosphorylated 350-kDa and related MAP-1 analogues were also constituents of U1-RNP regions. A certain fraction of the MAP-1 analogues remains attached to the nuclear skeleton after extraction with detergent and a high concentration of salt (17). The nucleoskeleton-bound form of the large tumor (T) antigen of simian virus 40 and p53 was shown to be colocalized with MAP-1 antigen by double immunofluorescence (35). Brain MAP-1 binds to DNA *in vitro* (36), and we observed that the 350-kDa protein bound to either single- or double-stranded DNA (data not shown). Although the function of phosphorylated MAP-1 analogues in RNA metabolism is unknown, their rapid phosphorylation after growth stimulation and their subsequent appearance in the U1-RNP region of the nucleus concomitant with elevated mRNA synthesis of the c-*myc* gene suggest that they have a role in transcriptional activation of proliferation-related genes.

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