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Cell cycle phase-dependent changes of localization and oligomerization states of nucleophosmin / B23

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SUMMARY : Nucleophosmin / B23, an abundant nucleolar phosphoprotein, accumulates in the nucleoplasm of cells during the stationary phase of growth or after exposure to selected cytotoxic drugs [Chan, P.K. (1992) Exp. Cell Res. 203, 174-181]. Monomeric and hexameric forms of nucleophosmin / B23 are present in cells [Yung, B.Y.M. and Chan, P.K. (1987) Biochim. Biophys. Acta. 925,74-82]. Using indirect immunofluorescence, here we show that there are changes in nucleophosmin / B23's cellular localizations throughout the cell cycle. The alternation of the nuclear and nucleolar localizations of nucleophosmin / B23 is most frequently observed in cells of G_1 and G_1/S phases. The incidence of the changes of localizations of nucleophosmin / B23 decreases as cells enter into S and G_2 phases. In parallel, using Western blotting, the reversible change of oligomerization states between the hexameric and monomeric forms of nucleophosmin / B23 is also found to occur most frequently in cells of G_1 and G_1/S phases. As cells progressed into S, G_2 and M phases, the frequency of the reversible change of hexameric and monomeric forms of nucleophosmin / B23 decreases. These findings suggest that nucleophosmin / B23 being possibly involved in rRNA processing and transport, is highly active at G_1 and G_1/S phases as demonstrated by the dynamic, reversible changes of localization and oligomerization states of nucleophosmin / B23.

The nucleolus, which contains rDNA, rRNA, ribosomal proteins and nucleolar proteins, is the site for ribosome synthesis (1,2). During ribosome synthesis, the pre-rRNA is processed and is packaged with the ribosomal

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proteins to form ribosomes. Nucleolar proteins are believed to assist in the ribosome assembly and transport. Generally, the nucleoli are formed in early telophase and are functionally active throughout the interphase (3). The activity of nucleoli ceases normally in late G_2 when r-chromatin is condensed at the nucleolar organiser region (NOR) of "nucleolar" chromosomes (3). Previous studies have shown that the nucleolar volume doubles during S phase and remains unchanged during G_2 phase (4). Both fibrillar and granular components seem to expand and the changes in nucleolar volume are correlated with increased rates of ribosome formation, as well as of protein synthesis during S and G_2 phases (3,5). However, the heterogeneity of the cell populations used in these biochemical investigations does not allow conclusions on the relationship between their rate of ribosome biogenesis and position of the cell cycle. A common observation is that a highly active interphase nucleolus more closely reflects the metabolic state of the cell than its position in the cell cycle (3).

Nucleophosmin / B23 (protein B23, NO38, or Numatrin) is a major nucleolar phosphoprotein which is significantly more abundant in tumor and growing cells than in normal resting cells (6,7). Its localization in the granular regions of the nucleolus (8) along with the association with preribosomal particles (9,10) suggests that it may be involved in ribosome assembly or processing. Our previous studies indicate that nucleophosmin / B23 translocates from nucleoli to nucleoplasm during the stationary phase of growth (11) or during treatment with certain anti-tumor drugs, particularly the intercalators, such as actinomycin D (12,13), luzopeptins (14), doxorubicin (15) and toyocamycin (9). Other nucleolar proteins, such as protein C23 and fibrillarin, do not translocate under the same conditions (15). Recent reports suggest that nucleophosmin / B23 shuttles between the nucleus and the cytoplasm (16) and it binds to Rev protein, a HIV regulatory protein that transports HIV RNA from nucleoli to the cytoplasm (17). Nucleophosmin / B23 binds to single- and double-stranded DNA as well as RNA (18). Amino acid sequence analysis (6,19) indicates that the N-terminal half of nucleophosmin / B23 has 50% sequence identity with nucleoplasmin, a nucleosome assembly factor (20). The middle portion is highly acidic and

contains a stretch of 28 acidic amino acids (6) which may serve as the binding domain for basic ribosomal proteins and Rev protein. The major phosphorylation site is identified in the acidic region of the molecule (21) and is phosphorylated by a nuclear kinase NII (22). Nucleophosmin / B23 forms oligomers (23) which may be essential for its function (24). Studies using deletion mutants indicate that both the N- and C-terminal regions are essential for the oligomer formation (25).

Though many of its chemical and physical properties are known, the function of nucleophosmin / B23 is not well defined. Little information has so far been obtained on the mechanisms controlling cell cycle-dependent changes in subcellular distribution and oligomerization states of nucleophosmin / B23. Our attempt in the present study is to elucidate if there is cell cycle phase-dependent changes in localization and oligomerization states of nucleophosmin/B23. To see whether the change in the localization and oligomerization states of nucleophosmin/B23 is most or least frequently observed in one particular phase of cell cycle, we analyzed the localization and oligomerization states of nucleophosmin/B23 at some time intervals (10 min) in every phase of cell cycle. Here we report the alternation of nucleophosmin/B23's nucleolar and nuclear localizations as well as the reversible change between hexameric and monomeric forms of nucleophosmin / B23 , that are cell cycle phase-dependent.

MATERIALS AND METHODS

Cell culture and antibody

HeLa cells were grown as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 0.5% antibiotics in a 5% CO_2 humidified incubator at 37°C. For immunofluorescence studies, cells were subcultured over coverglasses in a 10-cm Petri dish. The monoclonal antibody (MAb) to nucleophosmin / B23 (37/5.1) was kindly provided by Dr. P.K. Chan, Dept. of Pharmacology, Baylor College of Medicine, Houston, Texas. Characterization of nucleophsomin / B23 MAb has been reported previously (8).

Synchronization of cells

Synchronization of HeLa cells was accomplished by growth for 24 h in medium containing 3 mM thymidine, followed by growth for 10 h in the absence

of thymidine and then an additional 24 h with thymidine. Cells before release from the second thymidine block were considered to be arrested in G_1/S phase. Cells after release from double thymidine block were pulsed with 0.5 μCi/ml of ^{3}H-thymidine 1 h prior to the end of the incubation time. Incorporation of ^{3}H-thymidine into DNA and increase of cell number were used to define the different cell cycle stages.

Immunofluorescence staining

Immuofluorescence staining was performed as previously described (11). Briefly, HeLa cells grown on coverglasses were fixed in 2% EM-grade formaldehyde in PBS (8.5 mM Na_2HPO_4 / 1.6 mM NaH_2PO_4 / 0.145 M NaCl / pH 7.2) for 20 min at room temperature. The cells were permeabilized with acetone at -20°C for 3 min. After a wash with PBS, the fixed cells were incubated with the monoclonal antibody (diluted 1:30) at 37°C for 1 h. Then cells were washed four times for 15 min each in PBS and incubated with fluorescein-conjugated affinity-purified goat antimouse IgG (diluted 1:20 with PBS) at 37°C for 35 min. The cells were then washed four times for 15 min each with PBS and mounted in 10% glycerol in PBS (pH 9). All immunofluorescence examinations were done by one investigator (Y.-H.Chou), who was blinded to the status of the coverglasses being examined.

Western blotting

Cell lysate (protein sample) was boiled in SDS sample buffer (62.5 mM Tris, pH 6.8, 5% β-mercaptoethanol, 10% glycerol, 2.0% SDS, 0.001% bromophenol blue) and was subjected to SDS-PAGE. The separated proteins were then electrotransferred (30 Volts for 16h) to a Hybond-PVDF membrane (Amersham). Proteins, even those with molecular weight higher than 200 Kd were efficiently transferred. Gels after the electrotransfer were silver-stained, showing negligible amounts of proteins remained in the gels. The PVDF membrane was then soaked in a blocking solution (10 mM Tris-HCl, pH 7.5, 3% bovine serum albumin, 10% chicken serum, 0.9% NaCl) for 30 min at room temperature. The PDVF membrane was then incubated with monoclonal antibody to nucleophosmin / B23 (diluted 1:2,000 in blocking solution) overnight in the refrigerator, washed (3 x 15 min) with TBS buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.1% Triton X-100), and incubated for 1 h in horse-radish peroxidase conjugated goat anti-mouse antibody (diluted 1:2000 in TBS buffer) at room temperature. The membrane was then washed (3x 10 min) in TBS buffer before incubation with peroxidase substrates for color development.

RESULTS

To ensure that the cells were synchronously re-entering the cell cycle after release from the double thymidine block, the ^{3}H-thymidine incorporation and

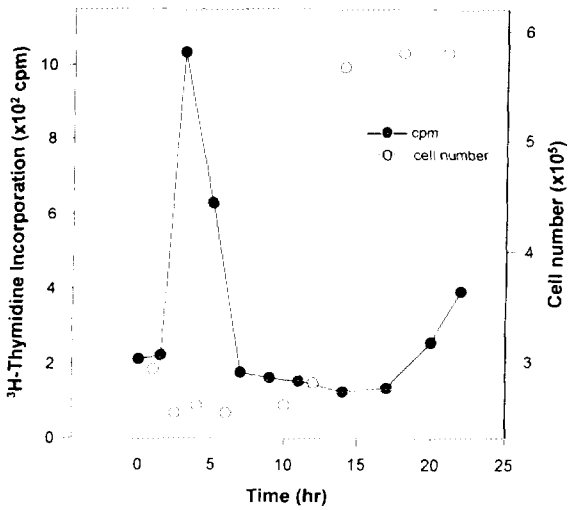


Fig. 1. Determination of cell cycle phases. Synchronization of HeLa cells was accomplished by growth for 24 h in medium containing 3 mM thymidine, followed by growth for 10 h in the absence of thymidine and then an additional 24 h with thymidine. Cells released from the second thymidine block were further incubated in medium at 37°C. One hour prior to the incubation period, the cells were pulsed with 0.5 μCi/ml of ^{3}H-thymidine. Incorporation of radioactive materials into DNA was assayed. Cell numbers were obtained by counting cell suspensions with a hemocytometer. The figure is the representative of the results obtained in at least three independent experiments.

cell growth (cell number increase) were determined. As shown in Fig. 1, DNA synthesis increased and reached to its maximum within 3 h after the release from the double thymidine block. The rate of DNA synthesis then declined back to its lowest in another 3 h. After that, cell number started to increase and doubled at 13 h after the release. The G_1/S- and S- phases could then be confined to 0-2 h and 2-6 h respectively after the release from the double thymidine block; G_2-phase was confined to 6-12 h while M-phase was between 12 and 13 h. Finally, G_1-phase was confined to 13-20 h after the release from the double thymidine block. Flow cytometric analysis confirmed that more than 85 % [except the cells of M phase (60%)] of the cells were at each of the defined synchronized phase of the cell cycle (data not shown).

Cytotoxic agents have been employed for the study of the function of nucleophosmin / B23 (11-15). Certain cytotoxic drugs temporarily block the transport of nucleophosmin / B23 and the treatments result in its redistribution in

the nucleoplasm. Nucleophosmin / B23 of the exponentially growing cells is found to be mainly localized in the nucleolus. However, this protein translocates from nucleolus to nucleoplasm in cells maintained at the stationary phase (11). Such translocation may be related to its functional roles since it has been suggested that nucleophosmin / B23 is a dynamic molecule involved in assembly and/or transport of ribosomes in the nucleus (9,10,15). In order to explore the possible functional role of nucleophosmin / B23 in cell proliferation, we further investigated the localizations of nucleophosmin / B23 in every phase (G_1/S, S, G_2, M and G_1) of the cell cycle. At 10 minute intervals in every phase of the cell cycle, the localization of nucleophosmin / B23 was examined using indirect immunofluorescence. Two populations of cells were detected regarding the localizations of nucleophosmin / B23. Cells at some stages of cell cycle showed large and pleomorphic nucleoli with bright fluorescence (Fig. 2A and 2C), and the nucleoplasmic fluorescence was relatively weak. On the other hand, cells at other stages of cell cycle showed an relatively uniform nuclear (both nucleolar and nucleoplasmic) fluorescence (Fig. 2B and 2D) which indicated that the nucleophosmin / B23 had translocated from nucleoli to nucleoplasm. As shown in Fig. 3A, alternation of the nuclear and nucleolar localizations of nucleophosmin / B23 was most frequently observed in cells of G_1 and G_1/S phases. The incidence of the changes of nucleophosmin / B23's localizations decreased as cells entered into S and G_2 phases. Fig. 3B summarizes the study of the localizations of Nucleophosmin / B23 at 10 min intervals in every phases of cell cycle except M phase. Nucleophosmin/B23 was distributed evenly in cells at M phase (data not shown).

A hexameric form of nucleophosmin / B23 has been identified in cells (23). Chan (24) has also reported that tumor or proliferating cells have a higher concentration of hexameric form of nucleophosmin / B23. Since both monomer and hexamer are present in cells (23), there may be an equilibrium between these two forms of nucleophosmin / B23, one form possibly serving as the reservoir for the other active form of nucleophosmin / B23. To understand further the relationship between the oligomerization of nucleophosmin / B23 and cell proliferation, we analyzed the cells in every phase of cell cycle for the

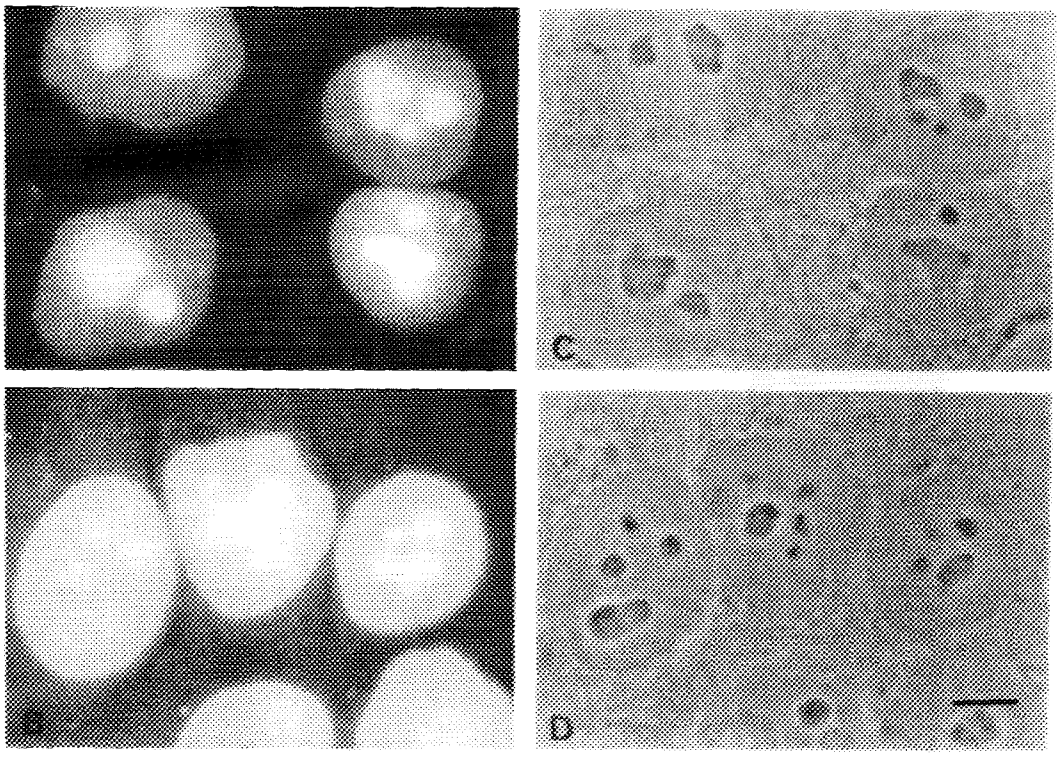
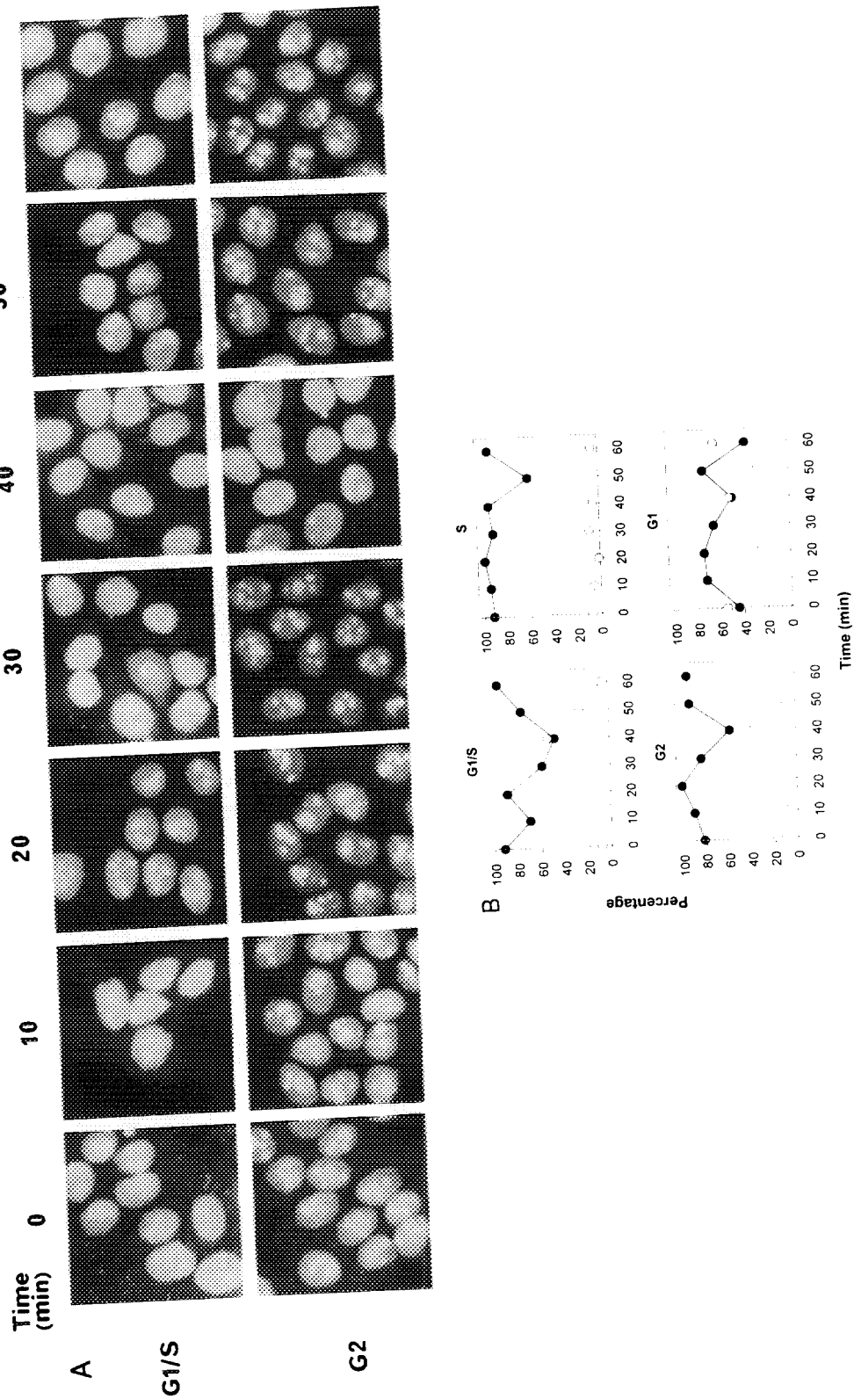


Fig. 2. Immunofluorescence localization of nucleophosmin / B23. At 10-min intervals in every phase of cell cycle, the localizations of nucleophosmin / B23 were examined using indirect immunofluorescence. Cells were fixed with 2 % formaldehyde and permeabilized with acetone. Monoclonal antibody to nucleophosmin / B23 and fluorescein-labeled secondary antibody were used to detect nucleophosmin / B23. Cells at some stages of cell cycle showed large and pleomorphic nucleoli with bright fluorescence (A, C), and the nucleoplasmic fluorescence was relatively weak. Cells at some other stages of cell cycle showed an relatively uniform, both nucleolar and nucleoplasmic fluorescence (B, D),which indicated that nucleophosmin / B23 had translocated from nucleoli to nucleoplasm. A and B, immunofluorescence; C and D, phase contrast. Bar in D, 5 μm.

immunobands of nucleophosmin / B23 with molecular weights of 230,000 and 37,000. At 10 minute intervals in every phase of cell cycle, the immunobands were examined with Western-blot assay. As shown in Fig. 4A, ratio of the hexameric and monomeric forms of nucleophosmin / B23 changed most frequently in cells of G_1 and G_1/S phases. As cells progressed into S, G_2 and M phases, the frequency of the reversible change of hexameric and monomeric forms of nucleophosmin / B23 decreased. Fig. 4B summarizes the changes of


Time (min)
0
10
20
30
40
50
60
A
G1/S
G2
B
G1/S
S
G2
G1
Percentage
Time (min)

the oligomerization states of nucleophosmin/B23 at 10 min intervals in every phases of cell cycle.

These results indicated that the dynamic changes of the localization and the oligomerization states of nucleophosmin/B23 are most frequently observed in cells of G_1 and G_1/S phases. It is previously known that rRNA processing occurs mostly at G_1 and G_1/S phases (3). These results thus lead to a tentative conclusion that nucleophosmin / B23 being possibly involved in rRNA processing and transport, is highly active at G_1 and G_1/S phases as demonstrated by the dynamic, reversible changes of localization and oligomerization states of nucleophosmin / B23.

DISCUSSION

Intricate mechanisms that mobilize hundreds of proteins, RNAs, and other elements in the nucleolus are orchestrated in a concerted manner to make ribosomes. These mechanisms are dependent on a balanced supply of rRNA and ribosomal proteins (26) and a proper conformation of rRNA (27). The biosynthesis of ribosomes involves multiple steps, such as transport of ribosomal proteins to the nucleolus and binding to the nascent pre-rRNA, splicing of the 45S RNA, packaging of ribosomal proteins into ribosome subunits, and delivery of the pre-ribosomal RNP through nucleoplasm to the cytoplasm. Even though the function of nucleophosmin / B23 has not been

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Fig. 3. The alternations of nuclear and nucleolar localizations of nucleophosmin / B23 occur most frequently in cells of G_1 and G_1/S phases. At 10-min intervals in every phase [G_1/S (0.5 h after release from double thymidine block), S (2.5 h after the release), G_2 (9.5 h after the release), M (12.5 h after the release), G_1 (15 h after the release)] of cell cycle, the localizations of nucleophosmin / B23 were examined using indirect immunofluorescence as described in Figure legend 2. (A) Immunofluorescence patterns of cells at 10-min intervals in G_1/S and G_2 phases of cell cycle. (B) Abscissa, 10-min intervals in every phase (G_1/S, S, G_2, M and G_1) of cell cycle. Ordinate, percentages of cells with different fluorescence patterns. ●----●, cells with bright nucleolar fluorescence ; 0------0, cells with relatively uniform nuclear (both nucleolar and nucleoplasmic) fluorescence. Triplicate 100-cell counts from each coverglass sample were performed. The figure is the representative of the results obtained in at least three independent experiments.

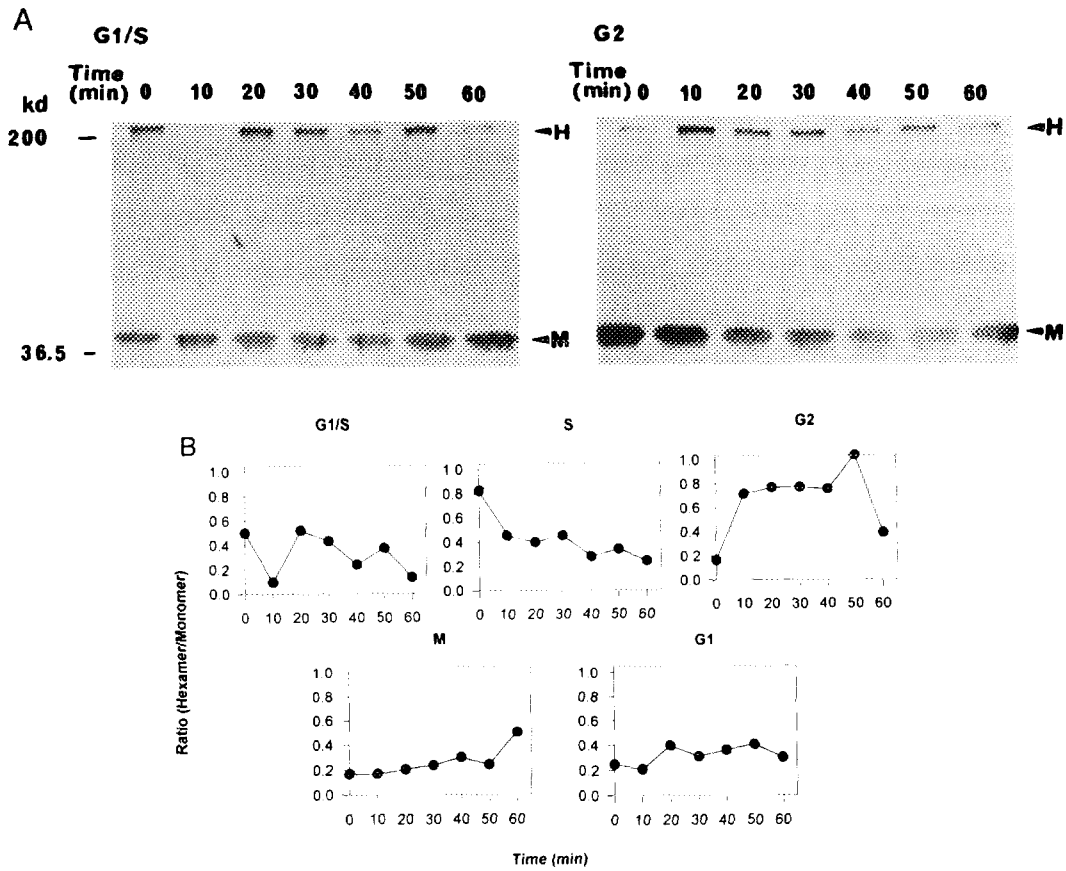

Fig. 4. The reversible changes of hexameric and monomeric forms of nucleophosmin / B23 occur most frequently in cells of G_1 and G_1/S phases. Immunobands of nucleophosmin / B23 at Mr=230,000 and Mr=37,000 from cells at 10-min intervals in various phases [G_1/S , S, G_2, M, G_1]of cell cycle were analyzed by Western-blot assay. Western blots of whole HeLa cellular lysates were tested with anti-nucleophosmin / B23 monoclonal antibody. Cellular proteins (30 µg) were separated by SDS-PAGE and transferred to Hybond-PDVF membrane. Anti-nucleophosmin / B23 antibody was detected with horse-radish peroxidase conjugated antibody and peroxidase substrates were used for color development. (A) Western blots of whole HeLa cells at 10-min intervals in G_1/S and G_2 phases of cell cycle tested with anti-nucleophosmin / B23 monoclonal antibody. Arrows indicate the immunostained hexameric (H) and monomeric (M) form of nucleophosmin / B23. (B) Abscissa, 10-min intervals in every phase (G_1/S, S, G_2, M and G_1) of cell cycle. Ordinate, ratio of hexameric to monomeric form of nucleophosmin / B23. The relative levels of hexameric and monomeric form of nucleophosmin / B23 were determined by densitometry. Background levels of optical density were subtracted. The figure is the representative of the results obtained in at least three independent experiments.

defined, there is ample evidence to suggest that nucleophosmin / B23 is involved in the assembly of ribosomes (9,10,28). Electron microscopic studies have indicated that nucleophosmin / B23 is concentrated in the granular region of the nucleolus where the processing of preribosomal particles may take place.

Generally, the nucleoli are active in rRNA synthesis and processing throughout G_1 and G_1/S phases. The volume of the nucleolus doubles and protein synthesis increases during S and G_2 phases while the activity of nucleoli ceases in late G_2. Our current results show that the alternation of the nucleophosmin / B23's nucleolar and nuclear localizations is most frequently observed in cells during G_1 and G_1/S phases. In parallel to this, the reversible change between hexameric and monomeric forms of nucleophosmin / B23 also occurs most frequently in cells during G_1 and G_1/S phases. Nucleophosmin / B23 being possibly involved in rRNA processing and transport, is highly active in G_1 and G_1/S phases as demonstrated by the dynamic, reversible changes of localization and oligomerization states of nucleophosmin / B23. Since the changes of the localization and oligomerization states of nucleophosmin/B23 could be observed at 10 to 20 min intervals in G_1 and G_1/S phases, it is possible that the biological process that nucleophosmin/B23 is involved may take place in a synchronous pattern with a period of 10 to 20 min. These studies also indicate that it is the frequency of the dynamic changes in oligomerization and subcellular localization states of nucleophosmin / B23 rather than its particular stationary oligomerization or localization state reflects the state of the activity in the nucleolus.

Nucleophosmin / B23 has been shown to shuttle between the cytoplasm and the nucleolus (16). The interactions of nucleophosmin / B23 to nucleolar proteins like Rex protein of human T-cell leukemia virus-I (29), Rev protein of human immunodeficiency virus-I (17), and cellular protein p120 protein (30) support the notion that nucleophosmin / B23 is a nucleolar shuttle protein that may help proteins having nucleolar localization signals to be properly targeted to the nucleolus. Oligomerization of nucleophosmin / B23 may be important for its function (binding to other nucleolar proteins, association with pre-ribosomal particles). In the absence of nucleolar membrane, nucleophosmin / B23 being

involved in rRNA processing and transport, may probably be part of the machinery which imports proteins to the nucleolus, exports the pre-ribosomal particles and shuttles back to the extranucleolar portion of the nucleus.

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