Ultrastructural Localization and Fluctuation in the Level of the Proliferating Cell Nuclear Antigen and myc Oncoproteins in Synchronized Neuroblastoma Cells

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ABSTRACT

A method for rapid synchrony of neuroblastoma cells was developed using the thymidine block to arrest cells in the G1-S boundary. Following release from the thymidine block, cells traversed to G2-M in 7-8 h with 85% cell synchrony. Determination of the steady-state level of proliferating cell nuclear antigen (PCNA) mRNA and protein by Northern and Western blots revealed an accumulation of the PCNA messenger RNA transcripts and PCNA protein at G1-S and a rapid decrease when cells entered S phase. The level of both the messenger RNA transcripts and protein increased as the cells moved to late-S and G2-M. Similarly, the steady-state level of c-myc and N-myc messenger RNA transcripts and proteins increased during the G1-S block, decreased when the cells entered S, and increased as the cells moved through S phase to G2-M. However, immunofluorescence staining for PCNA and myc protein indicated a low level of staining for all three proteins at G1-S and a significant increase in staining intensity during S phase. Similarly, immunoelectron microscopy revealed low levels of N-myc and c-myc staining during G1-S and increased staining during mid-S and late S phase of the cell cycle. These results suggest differential cell cycle-dependent accessibility of myc protein and PCNA to staining in the intact cells compared to the whole cell extract.

Furthermore, using immunofluorescence staining, confocal microscopy, and immuno-electron microscopy, we demonstrate for the first time that myc proteins are associated with the chromosomes during mitosis.

INTRODUCTION

myc proteins have been implicated in the regulation of growth, differentiation, and malignant transformation of many mammalian cells. Overexpression of N-myc in NB cells has been correlated with adverse prognosis of NB patients. The c-myc protein level was shown to increase following the addition of serum to quiescent fibroblasts (1); however, human leukemic cells enriched for G1, S, or G2-M phases of the cell cycle by the counter flow elutriation technique, when analyzed for c-myc expression, did not show cell cycle-dependent differences in the rate of synthesis or in the half life of c-myc protein (1). Little is known in mammalian cells concerning the levels, distribution, and exact ultrastructural localization within the nucleus of myc proteins during the various phases of the cell cycle. Spector et al. (2) microinjected Q8 quail cells with v-myc and c-myc and demonstrated by electron microscopy (using immunoperoxidase-stained sections) the migration of the myc protein to the nucleus and its localization adjacent to ribonucleoprotein particles. No association of c-myc was found with the nucleolus, nucleopore-lamina complex, or nucleoloplas (2).

In a recent study, Henriksson et al. (3) transfected fibroblasts with N-myc or c-myc sequences and were able to demonstrate different effects of c-myc or N-myc proteins on the shape and fine structure of the chromatin within the nucleus of transfected fibroblasts. They reported that overexpressed c-myc protein was localized in the nucleus by fluorescence microscopy, and examination by transmission electron microscopy of the fine structure of nuclei detected no changes in the chromatin. On the other hand, N-myc overexpression induced the formation of chromatins loops in the nucleus and the formation of cells with irregularly shaped nuclei. From these studies it was concluded the c-myc and N-myc bind to different nuclear structures and may have different functions (3).

Winquist et al. (4) studied the distribution of v-myc protein in Q8 quail cells during mitosis by immunofluorescence staining and reported that myc protein is not associated with the chromatin during mitosis (4). More recently, Waitz and Loidl (5) studied the localization and fluctuation of c-myc in the lower organism Physarum polycephalum in plasmidia at various phases of the cell cycle, using immunoelectron microscopy. In their studies, Waitz et al. did not find fluctuations in the level of c-myc during the naturally occurring synchronous cycle of this organism. However, the nuclear-matrix-bound portion of myc protein was higher in S phase than in G2. The localization of c-myc within the nuclear matrix varied during the cell cycle and was localized more to the periphery of the nucleus during S phase.

Recently, we have described a novel anti-N-myc antibody and used it, in combination with anti-c-myc antibody, to develop a two color immunofluorescence method for quantitation and localization of c-myc and N-myc proteins in neuroblastoma cells (6, 7). Moreover, a methodology was developed for the immunolocalization of c-myc and N-myc based on postembedding staining with gold-labeled secondary antibodies and analysis of stained sections by transmission electron microscopy (6, 7). We have shown by both IF staining and immunoelectron microscopy that c-myc is localized exclusively in the nucleus, whereas N-myc is localized to the nucleus but is found also in the cytoplasm (6, 7).

In this paper, we utilized both techniques to stain and localize c-myc and N-myc in synchronized neuroblastoma cells at various phases of the cell cycle and during mitosis. We also quantitated the steady-state level of myc protein and mRNA transcripts in NB cells at various phases of the cell cycle. Fluctuations in myc proteins and mRNA transcripts were compared to the cell cycle-dependent changes of the PCNA.

MATERIALS AND METHODS

Cell Culture

Cultures of newly established cell lines were carried out in DMEM plus 20% FCS (both from Gibco, Grand Island, NY) in T-75 flasks. After reaching confluency, cells were diluted 1:5 with fresh medium. Cells were dislodged for cell transfer by brief exposure to trypsin-EDTA (Gibco). The established NB cell line [CHP 126, 30 copies of the N-myc gene (6)] was cultured in DMEM medium containing 10% FCS (Gibco) in T-75 flasks. Confluent cultures were transferred at 1:8 dilution following dislodging of cells with trypsin-EDTA.
Antibodies

Anti-c-myc antibody (IgG,) was obtained from hybridoma tissue culture of a National Cancer Institute clone specific for amino acid peptide residues 171-188 of the c-myc oncoprotein.

Anti-N-myc antibody (IgM) was generated in our laboratory by immunization of BALB/c mice with N-myc-specific amino acid peptide residues 336-348 of the N-myc protein. The specificity of the antibody was determined by Western blot analysis and by positive staining of established NB cell lines with a known amplification of N-myc oncogene and negative staining of leukemic lines (6, 7).

Immunofluorescence Staining

Staining for N-myc and c-myc Oncopeptides. Staining of NB cells was carried out as described (6, 7). Cells were dislodged from the flasks by quick (1-5 min) treatment with 5 ml of trypsin-EDTA (Gibco) and washed twice with PBS containing 4% FCS; then the cells were fixed with 1 ml of 1% paraformaldehyde for 20 min. Cells were washed and permeated with 0.1% Triton X-100 in PBS containing FCS for 20 min. Staining was performed on 0.1-0.5 × 10^6 cells/tube with 100 μl hybridoma supernatant. Following a 30-min incubation on ice, tubes were washed once with the same buffer and secondary antibody [sheep anti-mouse IgG (Fc)-specific-FITC (for c-myc antibody) or sheep anti-mouse IgM-phycocyanin specific (for N-myc antibody)] was added for 30 min on ice. Unbound secondary antibody was removed by washing as before. Both secondary antibodies were affinity purified Fab fragments absorbed on animal sera (Jackson Immunchemicals, Mountain View, CA). Mouse IgG and mouse IgM controls at 5-fold excess were used for each staining combination to determine nonspecific binding. All cell staining was done at antibody-saturating concentrations (6).

Staining of NB Cells Fixed in Petri Dish. Cells growing in a Petri dish were fixed by brief exposure to 1% paraformaldehyde followed by permeation with 0.1% Triton X-100. Primary antibodies (anti-c-myc/N-myc) were added as described above for cells in suspension. Cells stained by the immunofluorescence method were photographed for green fluorescence (FITC filter) and red fluorescence (phycoerythrin filter) using a Nikon fluorescence microscope equipped with FX-35 camera and AFX-II automatic exposure device.

Confocal Microscopy

Confocal images were obtained using the BioRad MRC 600 system equipped with a krypton-argon laser.

Staining with Immunogold-labeled Antibodies and Electron Microscopy: Postembedding Labeling

Cells were harvested and washed by centrifugation and resuspended in a mixture of 0.1% glutaraldehyde, 4% freshly prepared paraformaldehyde, and 50 mM lysine (free base) in PBS and placed in an ice bath for 30 min. The cells were washed for 3 cycles of 15 min in PBS on ice and dehydrated and embedded in LR Gold according to the supplier’s instructions (Polysciences, Inc., Warrington, PA). Thin sections were cut and mounted on Piolof orm-coated nickel grids and incubated overnight at 4°C with hybridoma supernatant (anti-N-myc/c-myc or IgM/ IgG isotopic controls) diluted 1:1 in 0.5 M NaCl-0.02 M Tris-HCl (pH 7.2)-0.1% Tween 20. Grids were washed twice on 0.5 M NaCl-0.02 M Tris-HCl (pH 7.2)-0.1% Tween 20 and once with PBS prior to incubation with 15 nm gold-labeled goat anti-mouse IgG (E-Y Laboratories, San Mateo, CA), for 1 h at room temperature. The grids were then washed twice in PBS and once in distilled water. They were observed unstained or lightly stained with uranyl acetate and lead citrate.

Quantification of the number of gold particles was performed by using a grid of 1 in² and counting 5-6 squares in each electronic micrograph. At least 4 electron micrographs of similar magnification were used for each time point. The background staining with the IgM and IgG isotopic controls was very low (<10% of specific staining).

Cell Synchronization and Determination of G1, S, and G2 + M Phases of the Cell Cycle

Cell synchronization was performed by a modification of the procedure described before for leukemic cells (8). Stationary phase NB cells were transferred to T-25 flasks at a density of 1 × 10^6 cells/flask in DMEM medium containing 10% FCS. Flasks were cultured for 24 h, after which thymidine (Sigma Chemical Co., St. Louis, MO) was added (2 μm final concentration), and flasks cultured for an additional 24 h. At this point, over 90% of the cells were at the G1-S boundary and viability of over 90%. Spent medium was removed and fresh medium without thymidine was added. Samples of cell cultures were detached mechanically and removed every 3–4 h for a period of 12 h for determination of cell viability and cell cycle progression. Cell cycle parameters were determined by staining for DNA with propidium iodide as described in Ref. 8. Briefly, aliquots of approximately 10^6 cells were removed and centrifuged, and the supernatant fluid was discarded. Cells were lysed, treated with RNase A to prevent staining of RNA, and stained with propidium iodide in a single-step procedure as follows. The cell pellets were suspended in 1 ml of a solution containing 50 μg of propidium iodide (Sigma) dissolved in 10 mM Tris-HCl (pH 7.5), 15 μg RNase A (Sigma), and 5 mM MgCl2. The cells were kept at 4°C for 2–3 h before flow cytometry measurements. Coulter (EPICS Profile II) was used to quantitate the number of cells in each phase of the cell cycle by the cytology program, using second degree polynomial distribution for S phase, according to the method of Dean (9). Ten thousand cells were analyzed in each run at 800 V.

Flow Cytometry

A Coulter EPICS (Profile II Hialeah, FL) was used to analyze stained cells. Stained samples with green fluorescence (c-myc) or for red fluorescence (N-myc) were run against their isotopic control at 800 V. Log fluorescence was used and 5000 cells were analyzed. For two color immunofluorescence, 10% compensation of red over green was usually used and 4% compensation was used to correct spill of green to red fluorescence. All samples were run within the same day. The average background staining of the IgG isotopic control was less than 1% with a mean peak channel of 1–2. The average background staining for IgM Isotopic control is 1–2% at a mean peak channel of 2.5. Analysis of two color staining (green plus red) was obtained by using an adequate built in program.

Western Blot Analysis

CHP-126 cells (5 × 10^7) were washed with PBS (3 times), and the cell pellet was digested with 10 μM Tris-HCl buffer (pH 7.4) containing 5 mM MgCl2, 0.5% SDS, 0.5% deoxycholate, 0.5% Nonidet P-40, 10 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Protein content was determined by the BCA method (Pierce, Rockford, IL), and 50 μg of protein were run on a 9% SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (10). Proteins were transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA) using the Bio-Rad Transblot-SBD semidry electrophoretic transfer cell (Richardson, CA) at 25 V for 45 min in Bjerrum and Schafer-Nielson transfer buffer (48 mM Tris:39 mM glycine:1.3 mM SDS:20% methanol). Transfer paper was then blocked for 1 h with 4% BSA + 0.5% Tween 20 in 50 mM Tris-HCl buffer (pH 8.0). Hybridoma culture supernatant with anti-N-myc or c-myc specificity or mouse IgG/IgM control was added overnight at 4°C with gentle rocking. Unbound antibody was then washed with 3 changes of PBS containing 0.5% BSA and 0.5% Tween 20. Biotin-goat anti-mouse IgG was then added for 2 h followed by avidin:peroxidase (ABC kit; Vector Laboratories, Inc., Burlingame, CA), with 4-chloro-i-naphtol reagent as a substrate. Biotinylated standard protein markers were included in each run (BSA, M, 68,000; ovalbumin, M, 43,000; carboxy anhydride, M, 30,000). Nontransfected gels were stained with Coomassie Brilliant Blue and destained with 10% acetic acid.

Northern Blot Analysis of mRNA for myc and PCNA Transcripts

RNA was extracted from NB cells by a modification of the guanidine thiocyanate method described by Xie and Rothblum (11). RNA was electrophoresed in denaturing agarose gels, transferred to Immobilon-N nylon membrane (Millipore, Bedford, MA) and probed according to the method of Maniatis (12). Probes for c-myc, N-myc and actin were from Oncor (Gaithersburg, MD). The probe for PCNA was obtained from Dr. Marietta Lee (University of Miami). All the probes were labeled with 32P by the random primer method using the BRL labeling kit (BRL, Bethesda, MD). Each blot was probed with 10 ng of the probe, and filter strips were exposed to X-ray film at -70°C for 4–18 h.
NB. CELL SYNCHRONY, myc FLUCTUATION AND LOCALIZATION

LFL3

Fig. 1. DNA content of synchronized NB cells in various phases of the cell cycle. A, histogram of cells in G1-S boundary following a 24-h block with thymidine. Peak 1, G1 phase (90% of the cells); Peak 2, S-phase peak (4% of cells); Peak 3, G2-M region (2.4% of cells). Region 4, dead cells (<5%). B, histogram of cells in mid-S phase (3 h postrelease from the block). Greater than 80% of cells are in S phase and 11% are in G1. C, cells in late S phase (6 h postrelease) with 46% of cells in late S, 34% in G2-M, and 20% of cells in G1. D, log phase cells with 41% of cells in G1, 39% of cells in S, and 20% of cells in G2 + M. Cells were synchronized, DNA was stained, and cells analyzed by flow cytometry as described in “Materials and Methods.”

Table 1 Immunoßuorescence staining of myc oncoproteins and PCNA in synchronized neuroblastoma cells

<table>
<thead>
<tr>
<th>Cell cycle position</th>
<th>N-myc</th>
<th>c-myc</th>
<th>PCNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1-S (“time 0”)</td>
<td>39.1 (78)</td>
<td>21.0 (84)</td>
<td>3.6 (59)</td>
</tr>
<tr>
<td>Mid-S (3.0 h)</td>
<td>152.0 (93)</td>
<td>106.0 (95)</td>
<td>12.0 (71)</td>
</tr>
<tr>
<td>Late S-G2 (6 h)</td>
<td>130.5 (89)</td>
<td>61.3 (88)</td>
<td>6.2 (57)</td>
</tr>
<tr>
<td>Nonsynchronized</td>
<td>60.4 (90)</td>
<td>39.4 (89)</td>
<td>4.3 (62)</td>
</tr>
</tbody>
</table>

Table 1 depicts the relative immunofluorescence intensity of NB cells stained for N-myc, c-myc, and PCNA. The values obtained with IgM/IgG isotypic controls were in the range of 1–5 mean peak channels and 2–5% positive cells.

RESULTS

Distribution of PCNA and myc Proteins and mRNA Transcripts in Synchronized NB Cells. Cell synchrony in NB cells was achieved by the thymidine block method and the results are depicted in Fig. 1. Following a 24-h block, over 90% of the cells were arrested at the G1-S boundary (Fig. 1A, Peak 1). Three h after release from the block, the cells traversed to S and over 80% of cells were in S (Fig. 1B, Peak 2). Three h later, 34% of the cells were in G2-M (Fig. 1C, Peak 3), 46% were in late S (Fig. 1C, Peak 2), and 20% entered the subsequent G1 (Fig. 1C, Peak 1). The distribution of cells in log phase growth is shown in Fig. 1D for comparison. Forty % of the cells were in G1 and 60% were in S + G2-M. Peak 4 represents the proportion of dead cells. The highest proportion of dead cells was detected 6 h after release from the block and reached 15% (Fig. 1C). Table 1 depicts the relative immunofluorescence intensity of NB cells stained for N-myc, c-myc, and PCNA. The results indicate about 4-fold increase in the mean peak channel for all three proteins as the cells traversed from G1-S to mid-S. Intermediate fluorescence intensity for all three proteins was obtained for cells in the log phase of the cell cycle (Table 1). The fluctuations in the level of PCNA protein are depicted in the Western blot shown in Fig. 2. The level of PCNA is relatively high in G1-S (Fig. 2, left Lane B), decreases 9–10-fold during mid-S (Fig. 2, Lane C, 3 h postrelease), and increases about 2-fold as the cells progress to late S (3 h later). Further increase in the level of PCNA protein was observed 2 h later as the majority of cells were in late-S and G2/M (Fig. 2, Lane E) and was comparable with the amount found in unsynchronized, log phase cells (Fig. 2, Lane A). The Coomassie Blue stain for this gel is shown on the right, indicating similar amounts of protein in each lane.

The fluctuations in the levels of PCNA mRNA transcripts are shown in Fig. 3. The amount of transcripts was relatively high in G1-S (Fig. 3, right Lane A) and decreased 8–9 fold in mid-S (Fig. 3, right Lane B), followed by a 3–4-fold increase in the mRNA level of PCNA transcripts (Fig. 3, right Lane C). Cells in late-S and G2-M and showed further increase (2–3-fold) in mRNA transcripts for PCNA (Fig. 3, right Lane D). Only small fluctuations in the amount of total was hybridized, developed, stripped, rehybridized, and redeveloped to make sure the radioactive bands were real.

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Fig. 4. Western blot analysis of the level of N-myc protein in synchronized NB cells. Lane I, control nonsynchronous cells; Lane 2, cells in G1-S; Lane 3, cells in mid-S; Lane 4, cells in late-S; Lane 5, cells in late S-G2 + M (9 h postrelease). Total cell extract proteins (50 μg) were applied onto each slot. Proteins were transferred to Immobilon-P membrane. Blots were developed with anti-N-myc antibodies (A). B, Coomassie Brilliant Blue stain of a duplicate gel. Further experimental details are given in “Materials and Methods.” kd, molecular weight in thousands.

Fig. 5. Northern blot analysis of myc mRNA transcripts in synchronized NB cells. Lane 1, control nonsynchronous cells; Lane 2, cells in G1-S; Lane 3, cells in mid-S; Lane 4, cells in late-S; Lane 5, cells in late S-G2 + M (9 h postrelease). Twenty μg of total RNA were run in each lane. Lane A, cells in G1-S; Lane B, cells in mid-S; Lane C, cells in late S; Lane D, cells in late S-G2 + M (9 h postrelease). Lane E, RNA extract from the leukemic AGF line which coexpresses N-myc and c-myc used and is used as a control for comparison.

**DISCUSSION**

**Fluctuation of PCNA and myc Proteins in Various Phases of the Cell Cycle.** In this paper, we have shown that arrest of NB cells in the G1-S boundary results in a good cell synchrony with over 85% cell viability. Synchronized cells traverse through S phase with 80% cell synchrony in 6-7 h. Analysis of PCNA proteins and mRNA transcripts in the various phases of the cell cycle revealed an accumulation of PCNA protein and mRNA transcript in G1-S followed by a decrease during mid-S and subsequent increase during late late S-G2. Similar results were obtained with c-myc and N-myc proteins and mRNA transcripts (Figs. 2-5). Previous studies by Morris and Mathews (13) indicated that the rate of synthesis of the PCNA protein is higher (2-3-fold) in S phase elutriated cell fraction compared to the G1 cell fraction. However, the amount of PCNA relative to other cellular proteins and estimated by Western blot did not increase. Also, the stability of the PCNA protein did not differ from that of the rest of cellular proteins in various phases of the cell cycle (13). Our results for myc proteins seems to be different from the results obtained by Hann et al. (1) in which c-myc protein and mRNA transcripts appeared to be independent of the cell cycle in K-562 and various avian cells. In their study, Hann et al. actually measured the rate of c-myc synthesis ([15S]-methionine incorporation) and used cells enriched, to some extent, for the various phases of the cell cycle by elutriation. In contrast, in our study, the steady-state level of the proteins and mRNA transcripts was determined in a population of

microscopy revealed fluctuations in the level of myc proteins, with minimal immunogold staining in cells at G1-S (11 + 4 gold particles/in², Lane A) and increased staining during mid-S (3 h after release from thymidine block) to 31 + 7 gold particles/in² (Lane B). A further increase in the number of gold particles was observed 2 h later (38 + 6 particles/in², Lane D). In log phase, nonsynchronous cultures, the labeling was relatively low with large variations between cells analyzed (43 + 32 particles/in²).

**Association of myc Proteins with Chromosomes during Mitosis.** Two approaches were used to prove that myc proteins are associated with the chromosomes during mitosis. As shown in Fig. 7, NB cells stained for c-myc exhibit bright staining of phycoerythrin in a prometaphase cell (Fig. 7B) and in metaphase cell (Fig. 7C). On the other hand, cells in G1 (Fig. 7A) or immediately after cell division (Fig. 7D) show diffuse nuclear staining of c-myc. Similar results were obtained with cells stained for N-myc (results not shown). Furthermore, synchronized cells stained for N-myc postembedding by immunogold labeled secondary antibodies localize the N-myc protein during prometaphase to the condensing chromosomes (Fig. 8C), while in metaphase cell, N-myc is localized exclusively to the chromosomes (Fig. 8D). Diffuse pattern of association with the chromatin in interphase and early prophase cells is shown in Fig. 8, A and B, respectively. c-myc was also associated with chromosomes during mitosis, as was the case for N-myc (results not shown).
Fig. 6. Expression of N-myc protein in synchronized NB cells. N, nucleus; arrowheads, nuclear membrane. A, cell in G1-S; B, cell in mid-S; C, control nonsynchronous cell; D, cell in late S (6 h postrelease). For experimental details regarding staining and quantitation of the myc protein, see “Materials and Methods.”

Fig. 7. Immunofluorescence staining and confocal microscopy-localization of c-myc in mitotic NB cells. A, cell in G1 with condensed nucleus (right); B, cell in prometaphase (the chromosomes are stained bright); C, cell in metaphase with typical ring form chromosomes; D, two dividing cells with diffuse chromatin. Cells are magnified × 600 and zoomed × 2. For experimental details, see “Materials and Methods.”
cells highly synchronized by the thymidine block. Interestingly, in one experiment where Hann et al. used the double thymidine block for cell synchrony, they observed fluctuation in myc protein, similar to the fluctuation observed in our study [Fig. 2d of Hann et al. (1)]. Interestingly, both Hann et al. (1) and Jhiang et al. (14) have shown that growth stimulation of quiescent cells by serum (1) or by phytohemagglutinin (14) results in a substantial increase in the level of both mRNA transcripts and protein coinciding with an early increase in p120, a nucleolar protein associated with cell proliferation (14). Our results are in good agreement with the results of Cosenza et al. (15) who showed recently that mRNA transcripts for c-myc are high in G1 and are markedly reduced during S phase in mitotically selected 3T3 cells.

The biological significance of the observed accumulation of myc and PCNA proteins at the G1-S block is not clear at this point; however, a recent publication links apoptosis to growth arrest in cells overexpressing the myc protein (16). Indeed, we observed increased cell death in cells arrested in G1-S for a long period of time with morphological and ultrastructural changes, similar to those described during apoptosis or “programmed cell death.”

Interestingly, our studies, using quantitative immunofluorescence staining for myc and PCNA proteins and semi-quantitative immunoelectron microscopy for myc proteins, indicate low level of myc protein and PCNA protein in NB cells at G1-S. The results obtained in our study for PCNA (by IF staining and flow cytometry) were identical to recent results reported by Bolton et al. (17) for CHO cells synchronized by the mitotic selection method.

The discrepancy between the results obtained by the biochemical approach and the immunocytochemical approach could be explained by assuming differences in accessibility of the myc and PCNA proteins to the antibodies in the intact cell, or sequestering of myc and PCNA proteins in various compartments of the nucleus during the G1-S block. Indeed, cell cycle-dependent association of c-myc protein with the nuclear matrix was shown before for the lower organism P. polycephalum (5). This hypothesis is also supported by the finding that about 30% of PCNA protein is tightly associated with the nucleolus (13). Furthermore, it was shown recently for PCNA that antibody binding and intensity of IF staining depend greatly on the mode of cell fixation and preparation prior to IF staining (18). Also, the fact that IF and immunoelectron microscopy result in the same pattern of staining support the hypothesis of differential cell cycle-dependent nuclear sequestering. If this hypothesis is substantiated, then it could shed more light on the role of myc proteins and PCNA in the regulation of the cell cycle. In support of this hypothesis is the very recent publication by Vriz et al. (19) who showed by immunofluorescence staining that both c-myc and PCNA are present exclusively in the nucleus in log phase cells and translocate to the cytoplasm following growth arrest of 3T3 cells.

**Association of myc Proteins with Chromosomes during Mitosis.**

Confocal microscopy and immunoelectron microscopy studies revealed specific association of both c-myc and N-myc with chromosomes in prometaphase and metaphase cells (Figs. 7 and 8). These results do not agree with previous results published by Winqvist et al. (4) which concluded, using IF staining and regular fluorescence microscopy, that c-myc protein is not associated with chromatin in mitotic Q8 quail cells. Although it is possible that quail cells differ from mammalian cells, we believe that the resolution of the conventional fluorescence microscope is not enough to distinguish the clear association of c-myc with the chromosomes that we observed in mitotic NB cells (and AGF leukemic cells). The clustering of immunogold particles associated with N-myc, in mitotic NB cells (Fig. 8) clearly corroborates the confocal microscopy results. At this point, it

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4 Y. Gazit et al., unpublished observation.
is not clear whether myc proteins segregate to daughter cells by way of binding to the chromosomes during mitosis, or if this binding reflects a special role for myc proteins during mitosis.

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