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# Influence of Different Metal Ions on the Ultrastructure, Biochemical Properties, and Protein Localization of the K562 Cell Nuclear Matrix

**Luca M. Neri,[1,2] Roberta Bortul,[3] Marina Zweyer,[3] Giovanna Tabellini,[3] Paola Borgatti,[1] Marco Marchisio,[1] Renato Bareggi,[3] Silvano Capitani,[1] Alberto M. Martelli[3]***

[1]Dipartimento di Morfologia ed Embriologia, Sezione di Anatomia Umana Normale, Università di Ferrara, Italy 44100
[2]Istituto di Citomorfologia Normale e Patologica del C.N.R., c/o I.O.R., Bologna, Italy 40137
[3]Dipartimento di Morfologia Umana Normale, Università di Trieste, Trieste, Italy 34138

**Abstract** The higher order of chromatin organization is thought to be determined by the nuclear matrix, a mainly proteinaceous structure that would act as a nucleoskeleton. The matrix is obtained from isolated nuclei by a series of extraction steps involving the use of high salt and nonspecific nucleases, which remove chromatin and other loosely bound components. It is currently under debate whether these structures, isolated in vitro by unphysiological extraction buffers, correspond to a nucleoskeleton existing in vivo. In most cell types investigated, the nuclear matrix does not spontaneously resist these extractions steps; rather, it must be stabilized before the application of extracting agents. In this study nuclei, isolated from K562 human erythroleukemia cells, were stabilized by incubation with different metal ions ($Ca^{2+}$, $Cu^{2+}$, $Zn^{2+}$, $Cd^{2+}$), and the matrix was obtained by extraction with 2 M NaCl. By means of ultrastructural analysis of the resulting structures, we determined that, except for $Ca^{2+}$, all the other metals induced a stabilization of the matrix, which retained the inner fibrogranular network and residual nucleoli. The biochemical composition, analyzed by two-dimensional gel electrophoresis separation, exhibited a distinct matrix polypeptide pattern, characteristic of each type of stabilizing ion employed. We also investigated to what extent metal ions could maintain in the final structures the original distribution of three inner matrix components, i.e. NuMA, topoisomerase IIα, and RNP. Confocal microscopy analysis showed that only NuMa, and, to a lesser extent, topoisomerase IIα, were unaffected by stabilization with divalent ions. On the contrary, the fluorescent RNP patterns detected in the resulting matrices were always disarranged, irrespective of the stabilization procedure. These results indicate that several metal ions are powerful stabilizing agents of the nuclear matrix prepared from K562 erythroleukemia cells and also strengthen the concept that NuMA and topoisomerase IIα may act as structural components of the nuclear matrix. J. Cell. Biochem. 73:342–354, 1999. 

**Key words:** nuclear matrix; divalent cations; ultrastructure; protein composition; protein distribution

The nuclear matrix is a mainly proteinaceous insoluble framework remaining after isolated nuclei are treated with nonionic detergents, nucleases (DNase, RNase A) and solutions of high ionic strength [Berezney et al., 1995; Martelli et al., 1996]. Evidence has demonstrated that this insoluble network could be involved in DNA replication, RNA synthesis and processing, anchoring of DNA loops through the interaction of specific sequences, regulation of gene expression, and a plethora of other functions [for a review, see Berezney, 1991]. The nuclear matrix is composed mostly of nonhistone proteins, of which many have been extensively characterized at the molecular level during recent years [Berezney et al., 1995; Martelli et al., 1996]. Ultrastructural studies have shown that the matrix contains three distinct domains: an outer lamina, an inner fibrogranular network, and residual nucleoli [Berezney et al., 1995]. Even if a nuclear matrix has been prepared from many tissues and cell lines, its existence in vivo is still under debate. This is mainly because isolated nuclei are subjected to extensive manipulations before the final structure is obtained and

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*Correspondence to: Alberto M. Martelli, Dipartimento di Morfologia Umana Normale, Università di Trieste, via Manzoni 16, I-34138 Trieste, Italy. E-mail: martelli@univ.trieste.it

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there is a substantial risk of creating in vitro artifacts [Cook, 1988; Jack and Eggert, 1992; Pederson, 1998]. In particular, depending on the cell type, as well as the isolation procedure, the inner network and/or nucleolar remnants can be absent, while the outer lamina is always present [Stuurman et al., 1992a]. Therefore, in some cases, to preserve the inner fibrogranular network and the nucleolar remnants, it is necessary to "stabilize" isolated nuclei by either physical (mild heat exposure) or chemical (sodium tetrathionate, $Ca^{2+}$, $Cu^{2+}$) treatment [Evan and Hancock, 1985; Martelli et al., 1991; Stuurman et al., 1992b; Mattern et al., 1996; Lebkowski and Laemmli, 1982a; 1982b; Lewis et al., 1984]. Since we have demonstrated that incubation of isolated nuclei at 37°C induces changes in the immunofluorescent pattern of some nuclear proteins [Neri et al., 1994], we have begun to investigate systematically how nuclear matrix preparations in the final structures can maintain the spatial distribution of nuclear matrix polypeptides as seen in whole cells [Neri et al., 1995] and we have shown that also chemical stabilizing procedures (i.e., exposure to $Cu^{2+}$ ions) cause marked rearrangements of some proteins [Neri et al., 1997a,b]. Thus, we undertook a comprehensive study in which isolated K562 erythroleukemic nuclei were exposed to millimolar concentrations of metal ions such as $Ca^{2+}$, $Cu^{2+}$, $Cd^{2+}$, and $Zn^{2+}$, all of which are supposed to exert stabilizing effects on nuclear structure. We focused our attention on three polypeptides (240-kDa NuMA, topoisomerase IIα, and an RNP component) thought to play an important structural role at the matrix level [Mattern et al., 1996; Neri et al., 1997b,c]. We have found $Ca^{2+}$ to be ineffective in regard to stabilization of the nuclear matrix, while each of the other ions produced a distinctive stabilizing effect in maintaining the inner fibrogranular network and residual nucleoli. Metal ion influence on K562 cell nuclear matrix was evaluated by ultrastructural analysis, two-dimensional gel separations of nuclear matrix proteins, and immunofluorescent studies carried out on the spatial distribution of those proteins.

## MATERIALS AND METHODS

### Cell Culture

K562 human erythroleukemia cells were grown in RPMI-1640 medium supplemented with 10% newborn calf serum. They were seeded at a density of $10^5$/ml and used 4 days later, when they reached a density of $10^6$/ml.

### Isolation of Nuclei and Preparation of Nuclear Matrix

Cells were washed once in phosphate-buffered saline (PBS) and resuspended to $1.5 \times 10^7$/ml in 10 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 1.0 mM PMSF, 1 µg/ml each of aprotinin and leupeptin (TM-2 buffer, at 10°C). After 5 min at 0°C, Triton X-100 was added to 0.5% (w/v), and cells were sheared by one passage through a 22-gauge needle fitted to a 30-ml plastic syringe. Nuclei were sedimented at 400*g* for 8 min and washed twice in TM-2 buffer. They were resuspended to 2 mg DNA/ml in 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 5 mM $MgCl_2$ plus protease inhibitors as above (STM-5 buffer). They were stabilized for 30 min at 0°C in STM-5 buffer containing 2 mM $CaCl_2$, $CuSO_4$, $ZnCl_2$, or $CdCl_2$. In some cases, nuclei were incubated in STM-5 for 30 min at either 0°C or 37°C. After two washes in STM-5 buffer, they were digested for 60 min at 0°C with 50 IU DNase I/mg DNA (Sigma Chemical Co, St. Louis, MO). An equal volume of 4 M NaCl in 10 mM Tris-HCl, pH 7.4, 0.2 mM $MgCl_2$, 1 mM PMSF (LM buffer) was added, followed by 8 vol of 2 M NaCl in LM buffer. Structures were sedimented at 1,500*g* for 10 min and washed once in LM buffer.

### Transmission Electron Microscope Analysis

Transmission electron microscope (TEM) analysis of isolated nuclei, nuclear scaffolds, and matrices was performed as previously described, using a JEOL-JEM 100S electron microscope [Martelli et al., 1991].

### Protein and DNA Recovery

Assays were performed as described by Bradford [1976] and Munro and Fleck [1965], respectively.

### Two-Dimensional Gel Electrophoresis

Nuclear matrix protein was suspended to a concentration of 7 µg/µl in lysis buffer [O'Farrell, 1975] and incubated for 3 h at room temperature. Insoluble material was removed by centrifugation at 10,000*g* for 5 min; the supernatant was layered on the first dimension gel. Nonequilibrium pH gradient electrophoresis (NEPHGE) was carried out as reported by

O'Farrell et al. [1977] in 9.2 M urea, 2% Nonidet P-40 (NP-40), 4% polyacrylamide, 2% ampholytes (Bio-Lyte pH 3–10, Bio-Rad, Hercules, CA). First dimension gels (10 cm long, with a diameter of 3 mm) were run for 16 h at 400 V (constant). Second dimension gels were 8% polyacrylamide-0.1% sodium dodecyl sulfate (SDS) slabs. Gels were stained with Coomassie Blue R-250. The pH gradient was calibrated using carbamylated glyceraldehyde-3-phosphate dehydrogenase standards (Amersham Pharmacia Biotech, Uppsala, Sweden). Gels representative of three different preparations are shown.

### Source of Antibodies

Monoclonal antibody to 240-kDa NuMA protein (an IgG, employed at 1:100 dilution) was purchased from Oncogene Science (Cambridge, MA). Monoclonal antibody to 170-kDa topoisomerase IIα (clone Ki-S1, an IgG, employed at 1:100 dilution) was purchased from Boehringer, Mannheim (Germany). Monoclonal antibody recognizing the RNP polypeptide (an IgM, used at 1:20 dilution) (Clevenger and Epstein, 1984) was purchased from Chemicon International (Temecula, CA).

### SDS-PAGE and Western Blotting Analysis

Protein from $1 \times 10^7$ nuclei or from an equivalent number of nuclear matrices was dissolved in electrophoresis sample buffer [Laemmli, 1970], separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and stained with Coomassie Brilliant Blue R-250. In some cases, proteins were transferred to 0.2-μm nitrocellulose sheets for Western blotting analysis. After saturation with 4% bovine serum albumin (BSA) and 5% normal goat serum (NGS) in PBS for 1 h at 37°C, the sheets were incubated overnight at 4°C with the primary antibodies in PBS containing 4% BSA, 5% NGS. After washing in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 (TBST buffer), the sheets were incubated for 30 min at 25°C with peroxidase-conjugated anti-mouse IgG (Sigma) diluted 1:3,000 in TBST buffer. After washing as above, antibody binding was revealed by enhanced chemiluminescence (ECL) (Boehringer, Mannheim).

### Immunofluorescent Staining

Cells in PBS, nuclei in STM-5, and matrices in TM-2, respectively, were plated onto 0.1% poly-L-lysine-coated glass slides; adhesion was allowed to proceed for 30 min at 37°C for cells or at room temperature for nuclei and nuclear matrices. Whole cells were fixed in freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature and then permeabilized for 10 min at room temperature in PBS containing 0.5% Triton X-100. Isolated nuclei and matrix samples were fixed without any additional treatment. Isolated nuclei were fixed with the same concentration of paraformaldehyde as above, prepared in STM-5 buffer, while fixation buffer for matrix preparations was TM-2. These buffers were supplemented with 50 mM sodium cacodylate, pH 7.4. After several washes with PBS, nonspecific binding of antibodies was blocked by a 30 min incubation at 37°C with PBS, 2% BSA, 5% NGS. Slides were incubated for 3 h at 37°C with the appropriate primary antibody diluted in PBS, 2% BSA. Slides were then washed three times in PBS and reacted with the appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (from Sigma), diluted 1:50 in PBS, 2% BSA, 5% NGS for 1 h at 37°C, and mounted as previously described [Neri et al., 1994].

### Confocal Laser Scanning Microscope Analysis

Samples were imaged by a LSM410 (Zeiss, Oberkochen, Germany). This confocal system (CLSM) was coupled with a 25-mW multiline Argon ion laser as the light source. To reveal an FITC signal, a 488-nm wavelength was selected with a bandpass filter. The laser power was tuned at 10 mW to obtain the highest light stability, and the laser beam was attenuated to 30% of transmission with a neutral density filter to limit bleaching of FITC fluorescence. Samples were observed with a ×100, 1.4 numerical aperture (NA) planapochromat objective lens. In the detection path, the emitted fluorescent light was focused on a back pinhole in front of the detector, a photomultiplier tube (PMT). To block any unwanted contribution signal, when observing FITC, a 515-nm OG longpass filter was inserted before the PMT, as a barrier filter. The PMT was set at 864 mV. Images were acquired, frame by frame, with a scanning mode format of 512 × 512 pixels. Pixel values were recorded in the range of 0–255 (8 bits), as previously described [Neri et al., 1997b].

### Image Processing Analysis

Digitalized optical sections were transferred from the CLSM to the graphics workstation

Indigo Irix XS24 (Silicon Graphics, Mountain View, CA) and stored on the graphics workstation with a scanning mode format of 512 × 512 pixels and 256 gray levels. Image processing was performed using the ImageSpace software (Molecular Dynamics, Sunnyvale, CA). To reduce the unwanted background noise generated by the photomultiplier signal amplification, all the image stacks were treated with a three-dimensional filter (Gaussian filtering) that was carried out on each voxel, with a mask of 3 pixels in the x, y, and z directions (3 × 3 × 3). Photographs were taken by a digital video recorder Focus ImageCorder Plus (Focus Graphics, Foster City, CA), using 100 ASA TMax black and white film (Kodak Limited, Rochester, NY).

### Statistical Analysis

Data are the mean from three different experiments, expressed as mean ± SE. The asterisk indicates significant differences ($P < 0.001$) in a Student's paired t-test. All other differences were found to be not significant, at $P > 0.05$.

## RESULTS

### TEM Analysis of Isolated Nuclei and Nuclear Matrices

We first examined by TEM the morphology of isolated nuclei and nuclear matrices prepared from K562 erythroleukemia cells. A shown in Figure 1A, isolated nuclei kept for 30 at 0°C in STM-5 buffer devoid of stabilizing cations (control nuclei) showed a peripheral ring and variable spots of heterochromatin, electrondense nucleoli, and several large clusters of interchromatin granules. If 2 mM $CaCl_2$ was present during the incubation, the appearance was quite similar to control nuclei (Fig. 1B). Nuclei exposed to $Cu^{2+}$, $Zn^{2+}$, and $Cd^{2+}$ displayed fewer clusters of interchromatin granules, while interchromatin spaces were larger (Fig. 1C–E).

We found that $Ca^{2+}$ ions did not stabilize nuclei from K562 cells, so that after DNase I digestion and 2 M NaCl extraction, the resulting structures only displayed a peripheral lamina surrounding an amorphous inner material [data not shown and Martelli et al., 1991]. By contrast, $Cu^{2+}$ ions were very effective, and the resulting nuclear matrix structures appeared well maintained with an extensive fibrogranular network (Fig. 1F). Residual nucleoli of these matrices appeared quite characteristic, in that they were constituted by a network of thick threaded structures delimiting small spaces containing fibrogranular material (Fig. 1G). Nuclear matrices prepared from $Zn^{2+}$-exposed nuclei looked emptier than $Cu^{2+}$-stabilized structures (Fig. 1H). The peripheral lamina and nucleolar remnants were well preserved. Finally, in matrix structures obtained from $Cd^{2+}$-exposed nuclei the inner material appeared homogeneously fibrogranular, while the peripheral lamina and residual nucleoli were not easily recognizable (Fig. 1I).

### Chemical Composition of Isolated Nuclear Matrices

In Table I, we summarize the protein and DNA recovery in isolated nuclear matrices. It is evident that $Ca^{2+}$-treated nuclear matrices retained a lower amount of both DNA and protein in comparison to the other three types of structures, consistent with their morphology. Indeed, both recoveries were similar to those of matrices not stabilized by any treatment (i.e., obtained from nuclei incubated for 30 min in STM-5 buffer at 0°C, a condition that does not result in stabilization of the inner network and residual nucleoli [Martelli et al., 1991]). In the matrices stabilized with 37°C, $Zn^{2+}$, $Cu^{2+}$, or $Cd^{2+}$, the DNA recovery was similar. By contrast, $Cu^{2+}$-stabilized nuclear matrices retained a percentage of nuclear higher than matrices exposed to other stabilizing treatments.

### Protein Composition of Isolated Nuclear Matrices

To determine which proteins are common to all three types of matrix preparations, we used two-dimensional gel electrophoresis separations. As shown in Figure 2A ($Zn^{2+}$), B ($Cu^{2+}$), C ($Cd^{2+}$), the overall pattern was quite similar, although there were some differences at the single spot level. In general, we can say that low-molecular-weight proteins (<50 kDa) were more abundant in $Zn^{2+}$-stabilized nuclear matrices, followed by those exposed to $Cu^{2+}$ and $Cd^{2+}$, respectively. Differences in high-molecular-weight proteins (>66 kDa) were less striking, except for two acidic spots with a molecular mass of approximately 105 kDa, which were more represented in $Cu^{2+}$-stabilized matrices. Finally, in $Cd^{2+}$-stabilized samples, we detected a larger quantity of basic proteins with a molecular weight of >66 kDa.

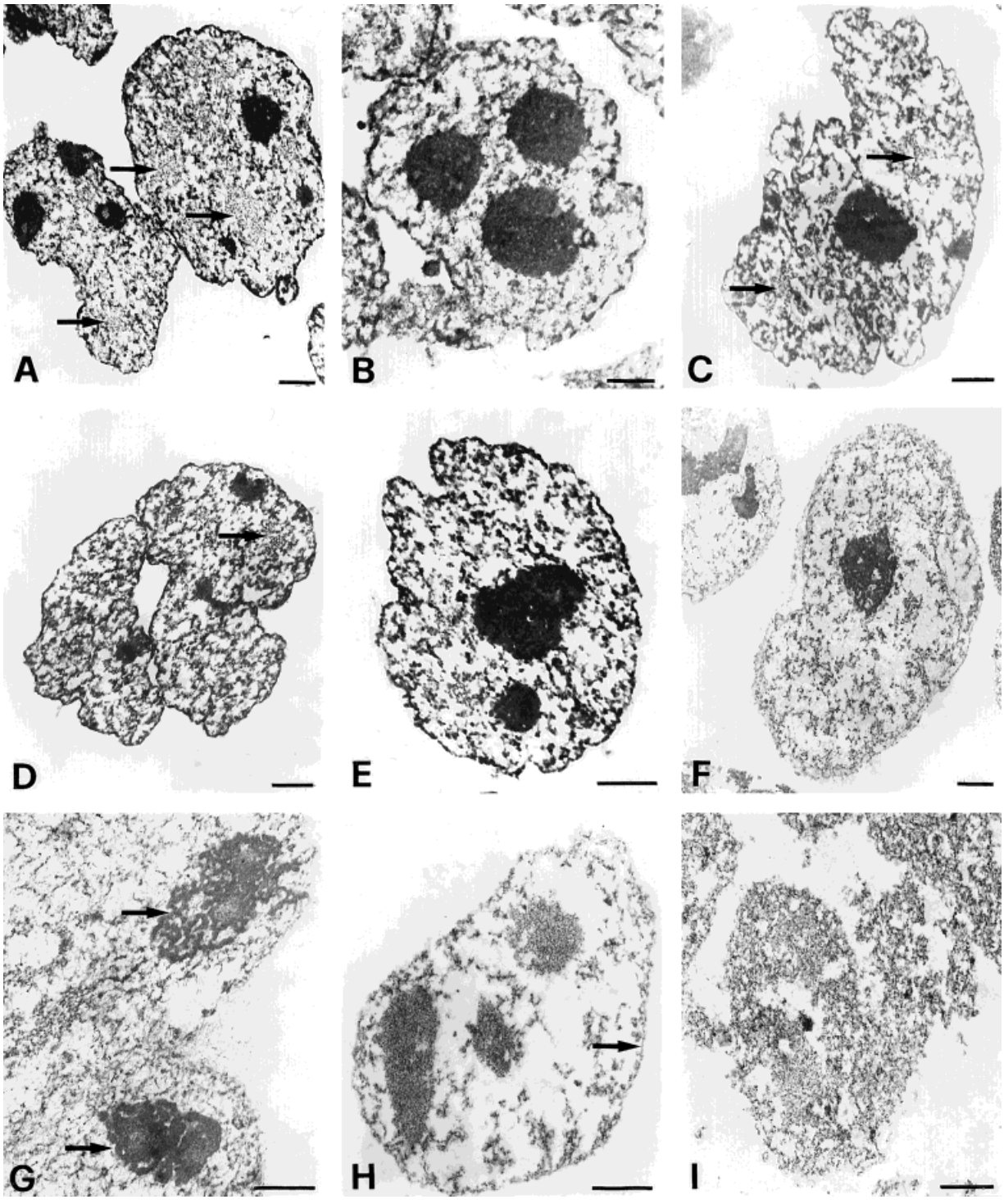


**Fig. 1.** Micrographs of TEM analysis of K562 cell nuclei and nuclear matrices subjected to different treatments. **A:** Nuclei kept for 30 min at 0°C in STM-5 buffer; arrows, clusters of interchromatin granules. **B:** Nuclei stabilized with $Ca^{2+}$. **C:** Nuclei with $Cu^{2+}$; arrows, clusters of interchromatin granules. **D:** Nuclei stabilized with $Zn^{2+}$; arrow, cluster of interchromatin granules. **E:** Nuclei stabilized with $Cd^{2+}$. **F,G:** $Cu^{2+}$-stabilized nuclear matrix. G: Arrows, residual nucleoli, exhibiting threaded structures. **H:** $Zn^{2+}$-stabilized nuclear matrix; arrow, peripheral lamina. **I:** $Cd^{2+}$-stabilized nuclear matrix. Scale bars = 1 μm.

**TABLE I. Percentage of Nuclear DNA and Protein Recovered in the Various Types of Nuclear Matrices**

| Treatment | DNA recovered (%) | Protein recovered (%) |
|---|---|---|
| None | 1.6 ± 0.5 | 5.9 ± 1.7 |
| 37°C | 5.9 ± 1.5 | 20.7 ± 3.9 |
| $Ca^{2+}$ | 1.7 ± 0.4 | 6.2 ± 1.9 |
| $Zn^{2+}$ | 5.8 ± 1.2 | 20.4 ± 3.4 |
| $Cu^{2+}$ | 6.1 ± 1.4 | 25.9 ± 3.6 |
| $Cd^{2+}$ | 5.7 ± 1.6 | 18.3 ± 2.7 |

### Western Blotting Analysis

By Western blotting analysis, we determined whether the antigens studied in this communication were retained in the nuclear matrices prepared by the different stabilizing procedures. Monoclonal antibody to NuMA protein stained a band with an apparent molecular mass of 240 kDa. The immunoreactivity present in isolated nuclei (Fig. 3, lane 1) was completely recovered in all matrix preparations, which were indeed enriched in comparison with nuclei (Fig. 3, lanes 2–4). Similar results were observed for topoisomerase IIα, which appeared as a single band at 170 kDa (Fig. 3, lanes 1–4).

The monoclonal antibody to the RNP antigen did not work on Western blots [Clevenger and Epstein, 1984] and was therefore used only for in situ analysis. Although we cannot be sure that all of the antigen was retained in the final matrices in this case, we would like to emphasize that the intensity of the immunofluorescent staining in the matrix was comparable to that seen in isolated nuclei, suggesting that most of the antigen was still present at the end of the extraction procedures.

### Immunofluorescent Staining

**240-kDa NuMA protein.** The protein NuMA showed, in intact K562 cells, a fine granular distribution of tiny dots all over the nucleoplasm, except for very evident negative nucleolar areas, as detected by phase-contrast microscope (data not shown) (Fig. 4A). The immunolabeling was distributed homogeneously in the nuclear interior and periphery. A similar pattern was observed in all types of nuclei and nuclear matrices (Fig. 4B–I).

**Topoisomerase IIα.** In intact cells, the antibody to topoisomerase IIα displayed a granular distribution characterized by a fine punctate and regularly ordered staining. Negative nucleoli were clearly distinguishable (Fig. 5A). All nuclear and nuclear matrix preparations showed the same staining pattern, independent of the various stabilizing procedures (Fig. 5B–H). The only exception was represented by $Cd^{2+}$-stabilized nuclear matrices, in which the structures were decorated homogeneously by slightly more loosely spaced granules, so that the negative nucleolar regions were no longer recognizable (Fig. 5I).

**RNP component.** In intact cells, in nuclei kept at 0° and in nuclei incubated with either $Ca^{2+}$ or $Cd^{2+}$, the fluorescent signal was mainly located in the inner part, while the periphery was barely labeled together with negative nucleoli (Fig. 6A–C,F). Exposure of isolated nuclei to $Cu^{2+}$ caused a very slight increase in the peripheral immunostaining (Fig. 6D). The most dramatic changes at the nuclear level were induced by $Zn^{2+}$. In this case, the signal dispersed into numerous brilliant dots, and negative nucleolar areas were significantly reduced. The nuclear edge exhibited a sharp profile of fluorescent structures ordered like beads on a string (Fig. 6E). All types of nuclear matrices, independently of the stabilizing procedure, showed a staining pattern very similar to $Zn^{2+}$-stabilized nuclei. Furthermore, the nucleolar negative areas completely disappeared (Fig. 6G–I).

### Quantitative Analysis

Table II reports the results of quantitative analysis performed on the various samples we analyzed, immunostained for the three antigens studied. For each condition, 400 cells, nuclei, and nuclear matrices were manually counted and the staining patterns examined, using three different experiments. A statistical test was performed, comparing the number of structures exhibiting the typical immunofluorescent pattern identified in intact cells with the number of those showing a different pattern. The data revealed that highly significant differences were detected in several samples following different treatments, demonstrating that the changes in the distribution of antigens illustrated in the section on CLSM analysis

**Fig. 2. (overleaf)** Two-dimensional gel electrophoresis analysis of proteins recovered in the nuclear matrix prepared from nuclei ($8 \times 10^8$) stabilized by various treatments. **A:** $Zn^{2+}$-stabilized samples. **B:** $Cu^{2+}$-stabilized samples. **C:** $Cd^{2+}$-stabilized samples. Migration in the first dimension was from left to right. Dashed line, pH 7.0. Molecular-weight standards are indicated on the left.

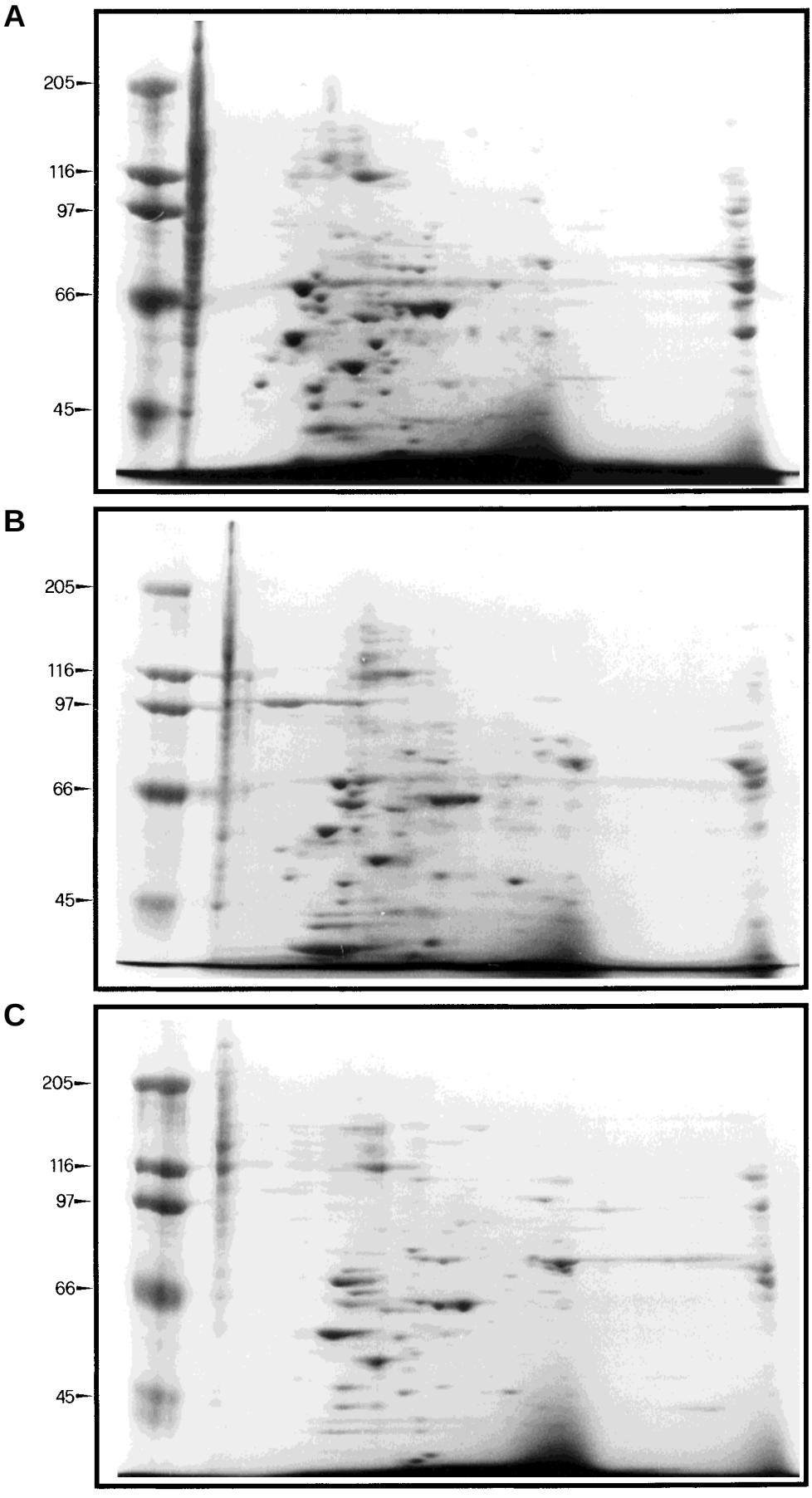


**Figure 2.**

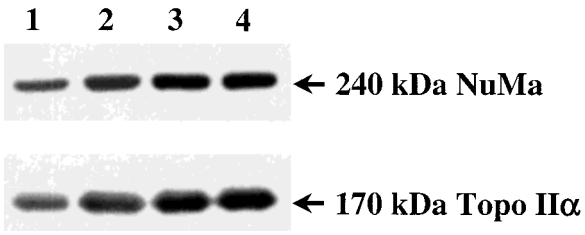


**Fig. 3.** Western blotting analysis of 240-kDa NuMA and topoisomerase IIα. **Lane 1,** isolated nuclei; **lane 2,** $Zn^{2+}$-stabilized matrix; **lane 3,** $Cu^{2+}$-stabilized matrix; **lane 4,** $Cd^{2+}$-stabilized matrix.

were indeed present in the great majority of isolated nuclei and matrices.

## DISCUSSION

Laemmli and coworkers first suggested that metalloprotein interactions could be important in maintaining the organization of mammalian cell nuclei, similar to chromosome scaffold [Lebkowski and Laemmli, 1982a; 1982b; Lewis and Laemmli, 1982; Lewis et al., 1984]. Indeed, when HeLa cell nuclei were exposed to metal chelators during histone extraction carried out by 2 M NaCl, empty structures (referred to as type II) were generated, which retained only 3–5% of nuclear protein, almost exclusively represented by the three nuclear lamins. From a morphological point of view, type II structures showed the peripheral lamina and some residual nucleolar material, but no inner fibrogranular network. On the other hand, the type I structures (obtained from nuclei not exposed to metal chelators) retained 10–15% of nuclear protein and displayed all three canonical matrix domains. The addition of micromolar concentrations of either $Cu^{2+}$ or $Ca^{2+}$ (this latter only if coupled to a 37°C incubation) to metal-depleted nuclei restored the capacity to generate type I structures.

This finding is at variance with the work of Eberharter et al. [1993], who found that a clear-cut discrimination between type I and II matrices was not possible. Their analysis using either one- or two-dimensional gel electrophoresis was carried out on nucleoskeletal structures stabilized either with $Ca^{2+}$ or $Cu^{2+}$ and derived from agarose-encapsulated nuclei prepared from the mammalian renal epithelial cell line LLC-$PK_1$. Rzeszowska-Wolny et al. [1988] also came to the same conclusions studying the effects of $Cu^{2+}$ stabilization in avian erythroblasts nuclei and nuclear matrix.

These observations suggested to us that the stabilizing effect of metal ions could be different, depending on the cell line studied. We decided to test the effect of several different divalent cations on the nuclear matrix of K562 erythroleukemia cells, as this cell line requires some type of stabilization if the internal network and residual nucleoli need to be preserved [Neri et al., 1997c].

Our TEM data showed that $Ca^{2+}$ ions did not affect the ultrastructure of isolated nuclei that was identical to control nuclei. On the contrary, $Cu^{2+}$, $Zn^{2+}$, and $Cd^{2+}$ ions caused a reduction in the number of interchromatin granules and an enlargement of interchromatin spaces. These changes had been previously observed in heat-exposed mouse and human erythroleukemic nuclei [Martelli et al., 1991; Neri et al., 1997c]. $Cu^{2+}$, $Zn^{2+}$, and $Cd^{2+}$ ions caused an overall stabilization of the matrix structures, as a 37°C incubation does in K562 cells [Neri et al., 1997c]. Therefore, the reduction of interchromatin granules and the enlargement of interchromatin spaces are features peculiar to physical and chemical stabilizing agents, except for sodium tetrathionate [Neri et al., 1997c]. Nevertheless, every metal ion induced a characteristic ultrastructural aspect of the matrices.

Even though we did not intend to perform a detailed analysis of single matrix proteins, the use of two-dimensional gel analysis allowed us to detect significant differences between the different types of matrix stabilization. This might reflect the different molecular targets of the metal ions and could also explain the different ultrastructural aspects we detected in the final structures. For example, previous observations by Chiu et al. [1993] showed that $Cu^{2+}$-exposure of isolated nuclei caused additional DNA, and especially newly replicated DNA, to become matrix associated. Furthermore, the stabilizing treatment enhanced radiation-produced DNA-protein cross-linking sensitive to OH radical scavengers, and to be more sensitive to EDTA and catalase than in untreated nuclei. It was therefore proposed that excess DNA-protein crosslinking in $Cu^{2+}$-treated nuclei occurs preferentially at the sites of $Cu^{2+}$ binding to chromatin where hydroxyl radicals are produced repeatedly through the Fenton reaction. The stabilizing effect of $Zn^{2+}$ might be related to the presence of nuclear proteins with zinc-finger domains. Several studies have demonstrated the existence of nuclear matrix pro-

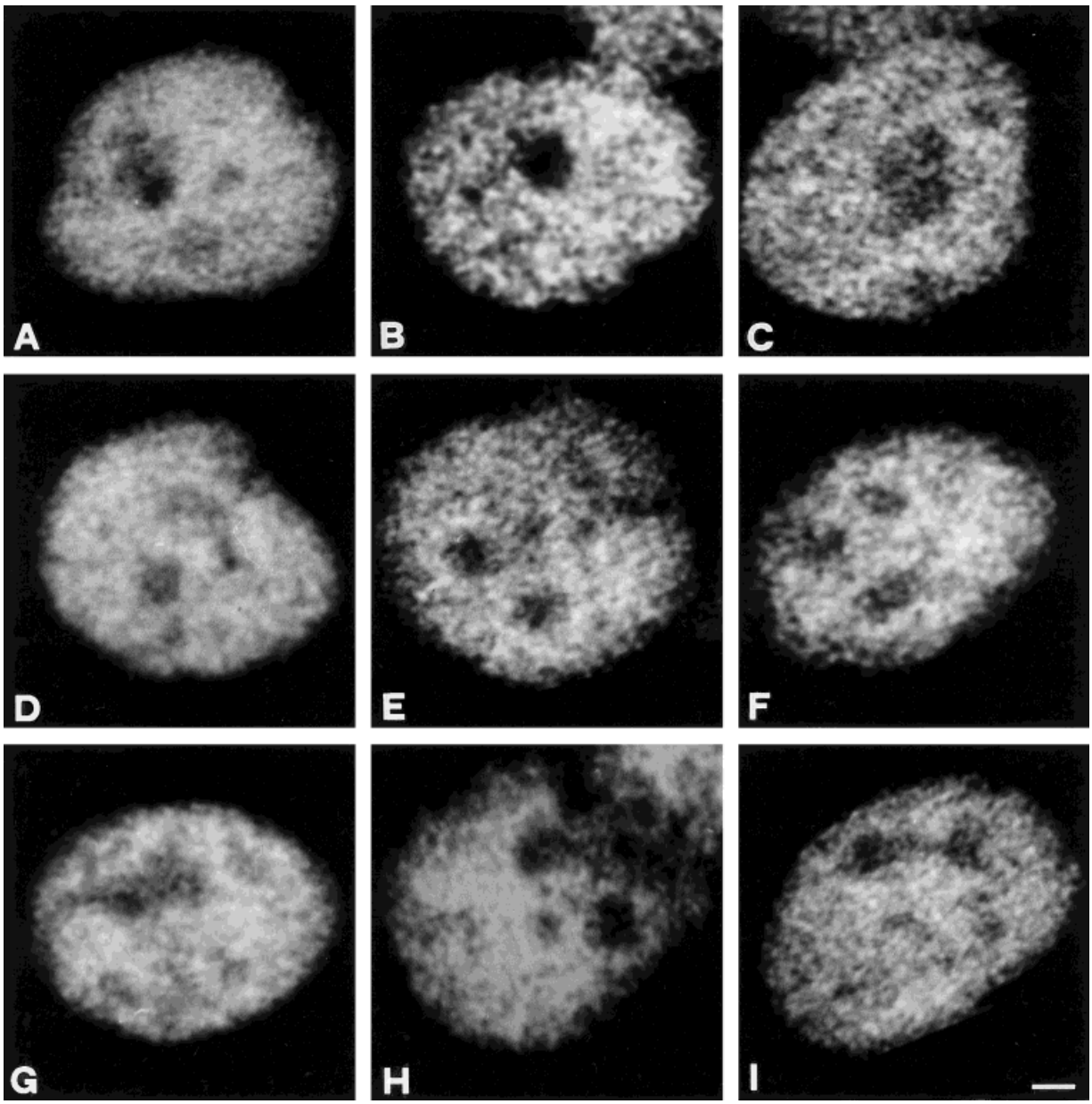


**Fig. 4.** Single optical section taken at the equatorial plane of intact cell (**A**), isolated nuclei (B–F) and nuclear matrices (G–I) immunodecorated with anti-NuMA protein antibody. Nuclei in STM-5 buffer were incubated at 0°C (**B**), at 0°C in the presence of $Ca^{2+}$ (**C**), at 0°C in the presence of $Cu^{2+}$ (**D**), at 0°C in the presence of $Zn^{2+}$ (**E**), and at 0°C in the presence of $Cd^{2+}$ (**F**). Nuclear matrices were stabilized by $Cu^{2+}$ (**G**), $Zn^{2+}$ (**H**), or $Cd^{2+}$ (**I**). Scale bar = 1 μm.

teins with zinc-finger motifs, including the multifunctional transcription factor YY1 [McNeil et al., 1998; Bushmayer and Atchison, 1998], ZNF74 (an RNA-binding protein belonging to a large subfamily containing a Kruppel-associated box repressor motif [Grondin et al., 1997], and AKAP 95 (a protein that interacts with the regulatory subunit of type II cAMP-dependent protein kinase [Coghlan et al., 1994]. It is of interest to recall that Muller et al. [1989] showed that $Zn^{2+}$ or $Cu^{2+}$ stabilized the association of matrix host cell and viral mRNA with the matrix. The same investigators [Muller et al., 1990] demonstrated that HIV-1 Tat protein bound to the nuclear matrix displays its characteristic bimodal function only in the presence of $Zn^{2+}$ ions, suggesting yet another function of this metal at the matrix level.

The potential molecular targets of $Cd^{2+}$ ions at the nuclear matrix level remain to be ex-

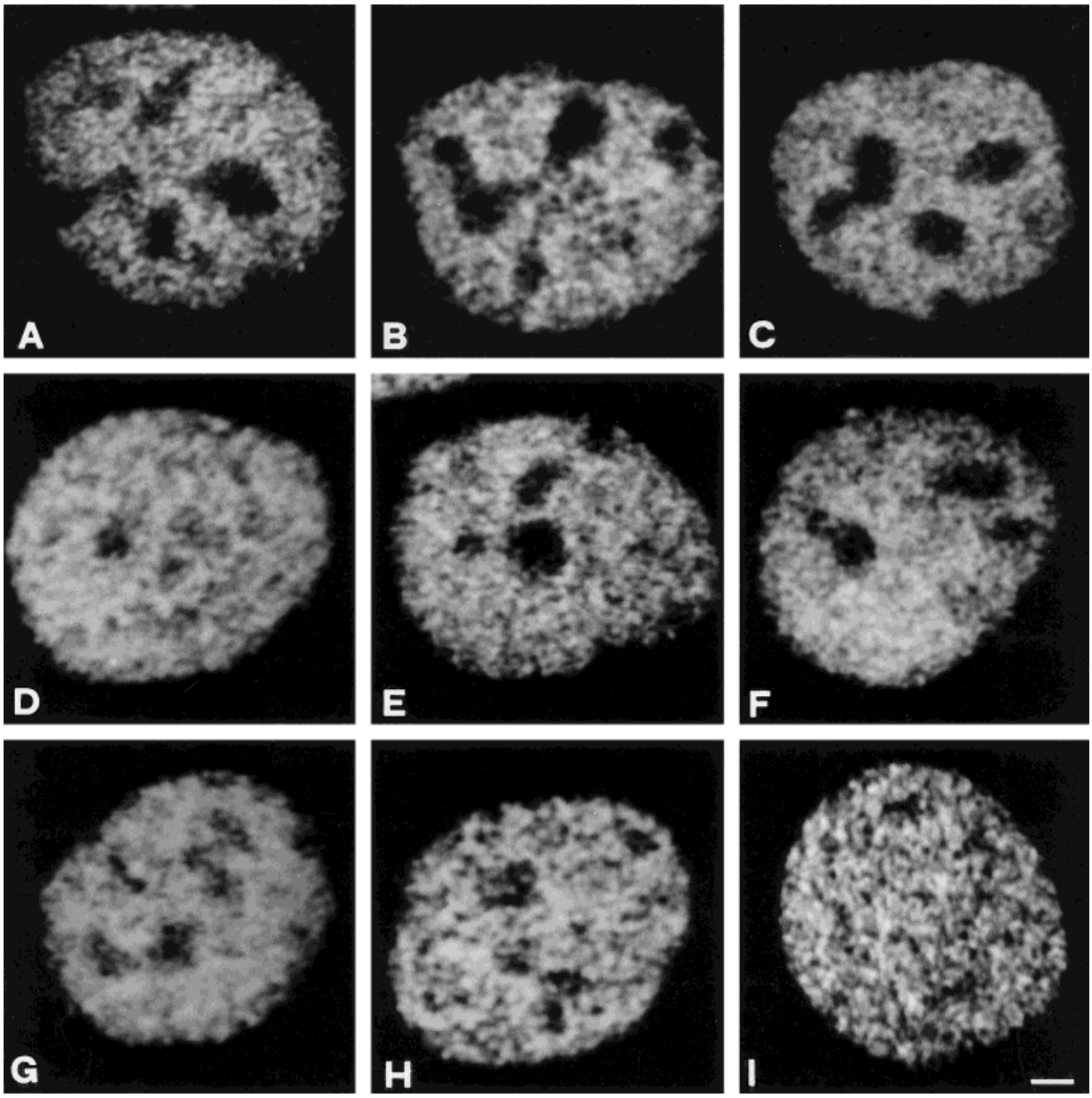


**Fig. 5.** Single optical section taken at the equatorial plane of intact cell (**A**), isolated nuclei (B–F), and nuclear matrices (G–I) immunodecorated with anti-topoisomerase IIα antibody. Nuclei in STM-5 buffer were incubated at 0°C (**B**), at 0°C in the presence of $Ca^{2+}$ (**C**), at 0°C in the presence of $Cu^{2+}$ (**D**), at 0°C in the presence of $Zn^{2+}$ (**E**), and at 0°C in the presence of $Cd^{2+}$ (**F**). Nuclear matrices were stabilized by $Cu^{2+}$ (**G**), $Zn^{2+}$ (**H**), or $Cd^{2+}$ (**I**). Scale bar = 1 μm.

plored. Nevertheless, it should be borne in mind that $Cd^{2+}$ ions, when employed in vivo at cytotoxic levels, induce a response similar to that of heat shock [Hung et al., 1998]. Interestingly, heat shock also causes a stabilization of the nuclear matrix [e.g., Martelli et al., 1991]. Alternatively, it could be envisioned that $Cd^{2+}$ ions are nearly equivalent to $Zn^{2+}$ ions in promoting protein–protein interactions at the nuclear level, in agreement with the proposal by Block et al. [1992].

Of the three proteins we investigated by immunofluorescent staining, NuMA and topoisomerase IIα were not significantly affected by metal ion stabilization, except for topoisomerase IIα in $Cd^{2+}$-stabilized nuclear matrices. Indeed, in this case, we observed a dispersion of the immunoreactivity which covered the nucleolar areas. This correlates well with the ultrastructural observations. Since several reports indicate that NuMA could oligomerize through coiled-coil in-

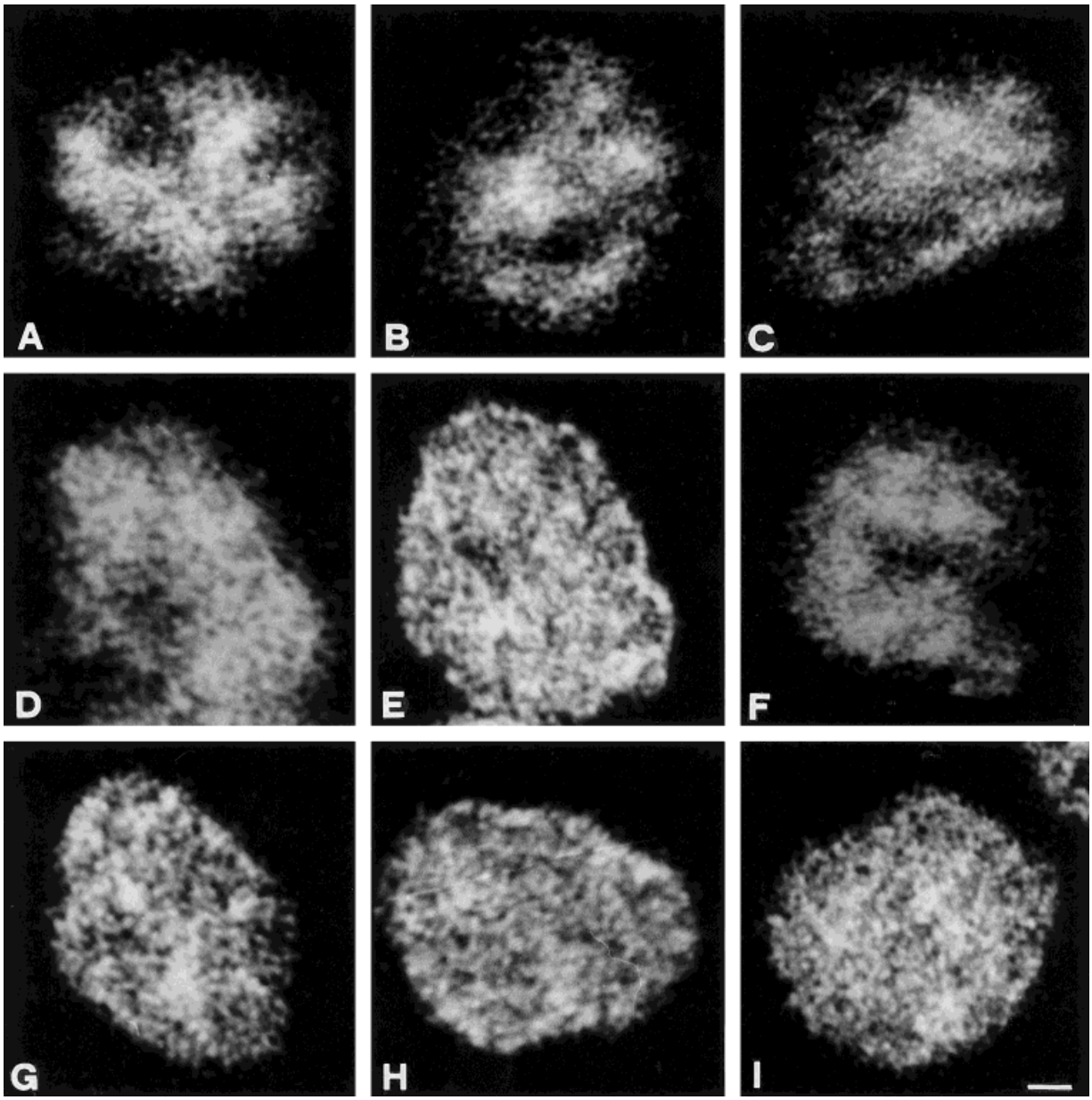


**Fig. 6.** Single optical section taken at the equatorial plane of intact cell (**A**), isolated nuclei (B–F), and nuclear matrices (G–I) immunodecorated with anti-RNP antibody. Nuclei in STM-5 buffer were incubated at 0°C (**B**), at 0°C in the presence of $Ca^{2+}$ (**C**), at 0°C in the presence of $Cu^{2+}$ (**D**), at 0°C in the presence of $Zn^{2+}$ (**E**), and at 0°C in the presence of $Cd^{2+}$ (**F**). Nuclear matrices were stabilized by $Cu^{2+}$ (**G**), $Zn^{2+}$ (**H**), or $Cd^{2+}$ (**I**). Scale bar = 1 μm.

teractions to create an intranuclear filamentous network [e.g., Zeng et al., 1994; Saredi et al., 1996], it seems important that this protein did not change its intranuclear spatial distribution, independent of the stabilization and the extraction procedures [see also Neri et al., 1997b]. Therefore, NuMA protein might be a true structural component of a nuclear framework, possibly corresponding to the core filaments of the nuclear matrix, of which NuMA is a component [Zeng et al., 1994]. Our results strengthen the hypothesis that NuMA may be a structural constituent of the matrix. This finding is in agreement with very recent data reported by Gueth-Hallonet et al. [1998], who transiently overexpressed NuMa in HeLa cells. The overexpression of wild-type protein resulted in ordered lattices that filled the nucleus and were stable to detergent extraction. By contrast, overexpression of NuMA constructs

**TABLE II. Percentage of Cells, Isolated Nuclei, and Matrices Displaying an Immunofluorescent Staining Different from the Typical Pattern Described for Intact Cells[a]**

| | NuMA (%) | Topoisomerase IIα (%) | RNP (%) |
|---|---|---|---|
| Cells | 0.5 (0.1)** | 2.5 (1.0)** | 3.4 (0.6)** |
| Nuclei 0°C | 2.3 (0.5)** | 3.7 (1.5)** | 4.1 (0.8)** |
| Nuclei 0°C + $Ca^{2+}$ | 3.3 (0.5)** | 5.5 (1.4)** | 4.8 (0.7)** |
| Nuclei 0°C + $Cu^{2+}$ | 3.2 (0.6)** | 6.3 (1.6)** | 81.9 (2.9)* |
| Nuclei 0°C + $Zn^{2+}$ | 3.4 (0.6)** | 5.6 (1.6)** | 84.4 (3.1)* |
| Nuclei 0°C + $Cd^{2+}$ | 3.8 (0.6)** | 7.3 (1.8)** | 5.8 (1.5)** |
| Matrix + $Cu^{2+}$ | 4.1 (0.8)** | 6.2 (1.2)** | 75.4 (2.6)* |
| Matrix + $Zn^{2+}$ | 85.2 (2.8)* | 6.5 (1.7)** | 79.7 (3.4)* |
| Matrix + $Cd^{2+}$ | 4.3 (0.9)** | 77.8 (2.2)* | 86.0 (3.3)* |

[a]See Results section for each of the antibodies employed in this study. Data are the mean from three different experiments. Standard error is shown in parentheses. A total of 400 cells, isolated nuclei, or matrices were manually counted and examined for each condition in each experiment. For each of the antibodies, the staining patterns of isolated nuclei and matrices were compared with the immunofluorescent staining displayed by intact cells that served as a control.
*Highly significant differences ($P < 0.001$).
**Not significant, $P > 0.05$.

truncated at different levels in the tail domain caused a drastic reorganization of nuclear components such as DNA, histone H1, and nucleoli, which relocated to the nuclear rim.

With regard to DNA topoisomerase IIα, its role in acting as a structural linkage anchoring the basis of chromatin loops to the nuclear matrix or chromosome scaffold has been repeatedly proposed in mammalian cells [e.g., Gasser and Laemmli, 1986].

As far as the third antigen was concerned, Clevenger and Epstein [1984] demonstrated its association with 20- to 80-nm electron-dense granules, conceivably representing RNPs. Some investigators consider the internal network of the matrix an aggregate of RNA–protein complexes, important in determining higher chromatin order [Berezney et al., 1995; Mattern et al., 1996]. Nevertheless, our results showed this antigen to be the most sensitive to the stabilizing effect of metal ions. Of the different stabilizing procedures investigated so far, only in vitro cross-linking with sodium tetrathionate preserved almost completely the distribution of the immunofluorescent signal due to this antigen [Neri et al., 1997c]. Taken together, our results point to a structural role of NuMA and topoisomerase IIα, and against one of RNP.

Thus, we can infer from these data that $Cu^{2+}$, $Zn^{2+}$, and $Cd^{2+}$ ions are effective stabilizing agents for the nuclear matrix from K562 cells. They may represent a useful tool to investigate the functions of different nuclear matrix proteins, some of which could be structural components, such as NuMA and topoisomerase IIα, while others, such as RNPs, may be bound to the inner matrix fibrous network, without structural relevance.

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