

# Association of Chromosome Territories with the Nuclear Matrix: Disruption of Human Chromosome Territories Correlates with the Release of a Subset of Nuclear Matrix Proteins

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**Abstract.** To study the possible role of the nuclear matrix in chromosome territory organization, normal human fibroblast cells are treated in situ via classic isolation procedures for nuclear matrix in the absence of nuclease (e.g., DNase I) digestion, followed by chromosome painting. We report for the first time that chromosome territories are maintained intact on the nuclear matrix. In contrast, complete extraction of the internal nuclear matrix components with RNase treatment followed by 2 M NaCl results in the disruption of higher order chromosome territory architecture. Correlative with territorial disruption is the formation of a faint DNA halo surrounding the nuclear lamina and a dispersive effect on the characteristically discrete DNA replication sites in the nuclear interior. Identical results

were obtained using eight different human chromosome paints. Based on these findings, we developed a fractionation strategy to release the bulk of nuclear matrix proteins under conditions where the chromosome territories are maintained intact. A second treatment results in disruption of the chromosome territories in conjunction with the release of a small subset of acidic proteins. These proteins are distinct from the major nuclear matrix proteins and may be involved in mediating chromosome territory organization.

**Key words:** chromosome territory • chromosome painting • fluorescence in situ hybridization • DNA-rich nuclear matrix • nuclear matrix proteins

DEVELOPMENTS in visualization approaches have stimulated a renewed interest in understanding the functional organization of the eukaryotic cell nucleus. Discrete domains of DNA replication, transcription, and RNA splicing factors have been defined by numerous studies (Spector, 1993; Berezney et al., 1995; Jackson and Cook, 1995; Nickerson et al., 1995; van Driel et al., 1995; Berezney and Wei, 1998; Ma et al., 1998; Wei et al., 1998, 1999). The corresponding association of these functional domains with the nuclear matrix has led to the view that the cell nucleus may have an underlying structural architecture as a basis for form and function (Hoffman, 1993; Spector, 1993; Baskin, 1995; Berezney et al., 1995; Nickerson et al., 1995; van Driel et al., 1995; Berezney and Wei, 1998).

Progress in elucidating higher order structure in the cell nucleus is not limited to strictly functional domains, but applies to the very genome itself. It is now clear that the

chromatin in the interphase cell nucleus is arranged in spatially separate, chromosome specific territories (Stack et al., 1977; Zorn et al., 1979; Cremer et al., 1982, 1993, 1995; Manuelidis, 1985, 1990; Schardin et al., 1985; Licher et al., 1988; Pinkel et al., 1988; Leitch et al., 1990; Schwarzacher, 1994; Zink et al., 1998). Initial results further indicate a high degree of organization of specific subchromosomal regions within individual chromosome territories (Manuelidis, 1990; Dietzel et al., 1998).

While the question remains as to what mediates organization into chromosome territories, numerous studies have demonstrated that chromatin is arranged into repeating loop domains of 50–200 kb in the interphase nucleus and mitotic chromosomes (Cook and Brazell, 1975; Cook et al., 1976; Paulson and Laemmli, 1977; Pardoll et al., 1980; Vogelstein et al., 1980). These chromatin loops are believed to be anchored to components of the nuclear matrix or chromosome scaffold by S/MARs (scaffold/matrix attachment regions)<sup>1</sup>, which presumably bind to specific components on the nuclear matrix/scaffold (Goldberg et al., 1983; Pienta and Coffey, 1984; Nelson and Coffey, 1987; Chai and Sandberg, 1988; Laemmli et al., 1992; Roberge

This article is dedicated to Professor Donald S. Coffey on the occasion of his 40th year at The Johns Hopkins University School of Medicine.

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1. *Abbreviations used in this paper:* BrdU, 5-bromo-2-deoxy-uridine; CTAPs, chromosome territory anchor proteins; FISH, fluorescence in situ hybridization; MARs, matrix attachment regions.

et al., 1992; Hiraoka et al., 1993). Several MAR binding proteins have been identified in association with the nuclear matrix (von Kries et al., 1991; Romig et al., 1992; Dickinson et al., 1992, 1997; Tsutsui et al., 1993; Fackel-mayer et al., 1994; Dickinson and Kohwi-Shigematsu, 1995; Wang et al., 1995; Liu et al., 1997; de Belle et al., 1998). Moreover, DNA topoisomerase II (Gasser and Laemmli, 1987; Adachi et al., 1989, 1991) and ScII (Saitoh et al., 1994) are two major proteins of the chromosome scaffold that have been proposed to be involved in chromatin loop organization.

Studies of chromosome architecture (Pienta and Coffey, 1984; Saitoh and Laemmli, 1994) have led to the proposal that the organization of chromatin into higher order chromosome bands is mediated by the corresponding clustering of repeating loops attached to the nuclear matrix/scaffold. This higher order clustering of loop domains may also be a fundamental feature of functional units of chromatin in the cell nucleus. It has long been recognized that DNA replication occurs in clusters of replicons (Hand, 1978). Models of DNA replication associated with the nuclear matrix have proposed that the replicon clusters correspond to the 50–200-kb repeating DNA loop domains that are attached to the nuclear matrix (Dijkwel et al., 1979; Pardoll et al., 1980; Vogelstein et al., 1980; Berezney and Buchholtz, 1981; Berezney, 1991, 1992; Cook, 1991). Consistent with this view, discrete replication sites corresponding to replicon clusters have been visualized in the nucleus of intact cells and are strikingly maintained after extraction of cells for nuclear matrix (Nakayasu and Berezney, 1989; Neri et al., 1992; Berezney et al., 1995). Similarly, individual transcription sites in the cell nucleus are believed to be composed of clusters of transcriptional units attached to the nuclear matrix (Jackson and Cook, 1995; Wei et al., 1999).

These previous findings have prompted us to investigate the possible role of the nuclear matrix in the territorial organization of human chromosomes during interphase. We demonstrate for the first time that chromosome territories are maintained intact after extraction for the nuclear matrix. Virtually complete extraction of the internal nuclear matrix with RNase A and 2.0 M NaCl leads to a corresponding disruption of the chromosome territories. A small subset of nuclear matrix proteins are released in conjunction with territorial disruption and may play a role in mediating chromosomal organization.

## Materials and Methods

### Cell Culture and In Situ DNA-rich Nuclear Matrix Extraction

Three human cell lines (WI-38, NHF-1, and HeLa S3) were used for these studies. WI-38 and HeLa S3 cells (American Type Culture Collection) were grown as monolayers in MEM (GIBCO BRL) plus 10% FBS (Summits Biotechnology) supplemented with sodium pyruvate acid (GIBCO BRL) and MEM nonessential amino acids (GIBCO BRL). NHF-1 (normal human fibroblast) cells were grown as monolayers in MEM (GIBCO BRL) plus 10% FBS (Summits Biotechnology) supplemented with sodium pyruvate acid (GIBCO BRL) and MEM nonessential amino acids (GIBCO BRL), 1% L-glutamine (GIBCO BRL), and 50 µg/ml gentamicin (Sigma Chemical Co.).

For in situ nuclear matrix preparation, cells plated on coverslips were permeabilized with CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM MgCl<sub>2</sub>, 10 µM leupeptin, 1 mM EGTA, 1.2 mM

PMSF, 0.5% Triton X-100, with or without 4 mM vanadyl complex) followed by extraction with 0.25 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.65 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, or 2.0 M NaCl, with or without RNase A (200 µg/ml) digestion for 1 h on ice (Fey et al., 1986; He et al., 1990).

### Chromosome Painting and Replication Site Labeling

Human chromosome painting probes, which were directly labeled with Spectrum green or orange, were obtained from GIBCO BRL and Vysis. Experiments were performed after standard fluorescence in situ hybridization (FISH) procedures, modified with instructions from GIBCO BRL and Vysis. In brief, the cells were fixed with 4% formaldehyde and denatured with 50% formamide/2× SSC by heating in an 80°C waterbath for 30 min. 1 µl chromosome painting probe with 7 µl hybridization buffer and 2 µl double distilled H<sub>2</sub>O was denatured by heating in a 75°C waterbath for 10 min, and then placed immediately on ice. The final concentration of hybridization buffer was 50% formamide, 2× SSC, and 10% dextran sulfate. The probe in hybridization buffer was added to the coverslips, placed cell-side down on a glass microscope slide, and sealed with rubber cement. After incubation in a 37°C incubator overnight, the cell samples were washed three times (10 min each) in 50% formamide in 2× SSC at 45°C, once for 10 min in 2× SSC at 45°C, once for 5 min in 2× SSC with 1% NP-40, and then air dried.

DNA replication sites were labeled after a 5 min in vivo incorporation of 5-bromo-2-deoxy-uridine (BrdU; 10 µM) in NHF-1 cells grown on coverslips, according to the instructions of the BrdU labeling and detection kit I (Boehringer Mannheim Corp.) as previously described (Ma et al., 1998) with mouse anti-BrdU mAbs and Texas red-conjugated anti-mouse IgG secondary antibodies (Boehringer Mannheim Corp.).

### Immunofluorescence Labeling of Matrin 250 and Lamin A/C

Double labeling of nuclear matrix associated proteins was performed in NHF-1 cells after appropriate extractions and fixation with 4% formaldehyde. Antibody reactions were carried out successively with mAb anti-matrin 250, also known as nuclear matrix associated RNA polymerase II<sub>0</sub> (B3, mouse IgM, diluted 50-fold with PBS; Mortillaro et al., 1996), and rabbit antilamin A/C polyclonal antibodies (diluted 20-fold with PBS) for 1 h at 37°C. FITC and Texas red secondary antibodies (both diluted 50-fold with PBS), respectively, were used for detection. In experiments involving propidium iodide staining of the DNA, the nuclear lamins were detected with FITC-conjugated antibodies.

### Electron and Confocal Microscopy

Samples from HeLa S3 cells were prepared for thin section EM (Belgrader et al., 1991). Cells were fixed in 2.5% glutaraldehyde, 100 mM sodium cacodylate, pH 7.4, and 3 mM MgCl<sub>2</sub> for at least 2 h at 4°C. The cells were rinsed with cacodylate buffer without glutaraldehyde, post-fixed with 1% OsO<sub>4</sub> for 30 min on ice, dehydrated (graded 30–100% ethanol and 100% acetone), and infiltrated with Epon-Araldite resin (Electron Microscopy Sciences). Cured blocks were thin sectioned and stained with lead citrate and uranyl acetate. Sections were examined on a Hitachi H-500 electron microscope.

The confocal imaging was performed on a Bio-Rad MRC-1024 three channel laser scanning confocal imaging system equipped with a Nikon Optiphot 2 microscope, a Nikon 60×, 1.4 NA objective, and an argon/krypton laser (λ = 488/565 nm). Optical sections of 512 pixels × 512 pixels × 8 bits/pixel were collected through the samples at 0.5 µm intervals.

### Fractionation of Nuclear Matrix Proteins, Biochemical Analysis, and Two-dimensional PAGE

For biochemical studies, HeLa S3 cells were used. Nuclei were isolated from disrupted cells using the syringe technique in Belgrader et al. (1991). Our strategy of identifying proteins released in correlation with chromosome disruption is shown in Fig. 4. It involves consecutive extraction of isolated HeLa cell nuclei with CSK buffer, RNase A digestion, 0.65-M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 2.0-M NaCl extractions. For total nuclear matrix proteins, HeLa S3 cells were permeabilized by CSK buffer, followed by DNase I digestion and 0.25-M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> extraction.

Protein was determined using the BCA kit (Pierce Chemical Co.). DNA was measured after incorporation of [<sup>3</sup>H]thymidine (1 µCi/ml) into HeLa S3 cells for 48 h of culture and RNA by alkaline hydrolysis methods

(Berezney and Buchholtz, 1981). The data were averaged from four individual experiments.

For two-dimensional PAGE, proteins were run on a nonequilibrium pH gradient (first dimension) and on SDS-PAGE in the second dimension (Belgrader et al., 1991). The proteins were stained with Coomassie blue (0.2%). Total nuclear matrix protein was prepared after treatment of isolated nuclei with DNase I and 0.25-M  $(\text{NH}_4)_2\text{SO}_4$  extraction (Belgrader et al., 1991).

## Results

### ***Chromosome Territories Remain Intact After Extraction for Nuclear Matrix***

Previous studies have shown that human interphase chromosomes are confined to discrete regions in the cell nucleus termed chromosome territories (Manuelidis, 1985, 1990; Schardin et al., 1985; Licher et al., 1988; Pinkel et al., 1988; Leitch et al., 1990; Cremer et al., 1993; Schwarzscher, 1994). To study the possible role of the nuclear matrix in chromosome territory organization, we extracted normal diploid human fibroblasts cells (NHF-1 or WI-38) grown on coverslips for DNA-rich nuclear matrix (Berezney and Buchholtz, 1981). In DNA-rich nuclear matrix preparations, the nuclear DNA is not cleaved by either endogenous or exogenously added nucleases. The protein components in DNA-rich nuclear matrix, however, are virtually identical to DNA-depleted matrix in recovery of total nuclear protein and overall polypeptide composition on SDS-PAGE (Berezney and Buchholtz, 1981). Moreover, the DNA in such structures is maintained predominantly inside the nuclear structures in a highly supercoiled state (Cook and Brazell, 1975; Cook et al., 1976; Warren and Cook, 1978; Berezney and Buchholtz, 1981).

We find that the chromosomes are maintained in the DNA-rich nuclear matrices (Fig. 1, B and E) as separate territories that are indistinguishable from those in intact cells (Fig. 1, A and D). Similar observations were made with all human chromosomes examined (numbers 1, 2, 4, 7, 9, 11, 14, and 22) and different two paint combinations. There was no chromosome hybridization signal in DNA-depleted nuclear matrix (results not shown).

These experiments were performed using moderate ionic strength for salt extraction (0.25 M  $(\text{NH}_4)_2\text{SO}_4$ ; Belgrader et al., 1991). We next used a higher salt concentration (2.0 M NaCl and the same ionic strength 0.65 M  $(\text{NH}_4)_2\text{SO}_4$ ) to extract the cells. The chromosome territories remain intact after these higher salt extractions (Fig. 2, A and B), and were indistinguishable from those observed in nuclear matrix prepared with moderate salt levels (Fig. 1 B) or intact cells (Fig. 1 A). Greater than 90% of the total histone proteins and other soluble nuclear proteins are removed under these salt extraction conditions (Berezney and Buchholtz, 1981; results not shown). Chromosome territories are, therefore, maintained in association with the nuclear matrix without the bulk of the histone proteins.

### ***Disruption of Internal Nuclear Matrix with RNase A and 2.0 M NaCl, but Not 0.65 M $(\text{NH}_4)_2\text{SO}_4$ , Results in the Corresponding Disruption of Chromosome Territories***

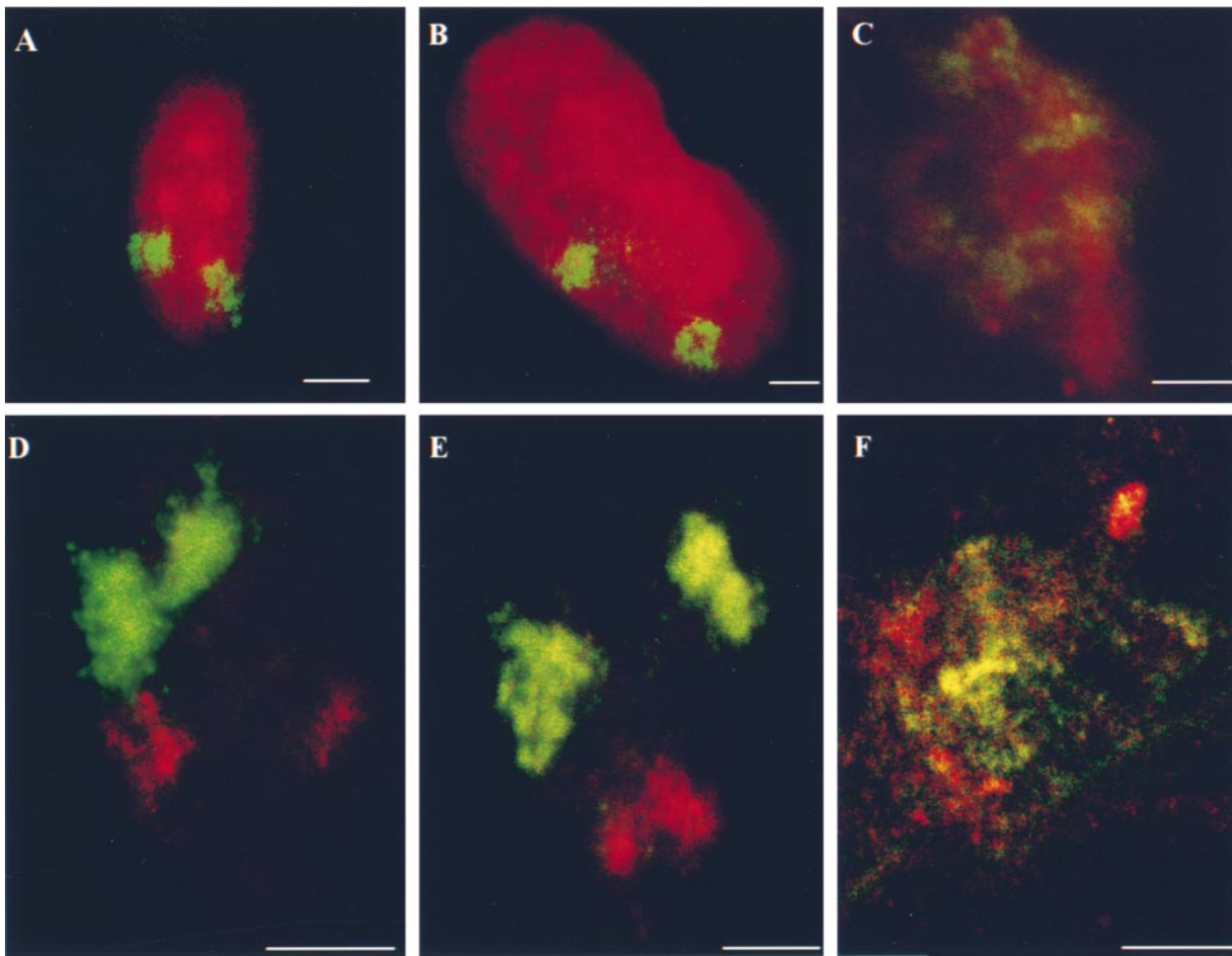
Since the structural integrity of the nuclear matrix is dependent on RNA and intermolecular disulfide bonds

(Kaufman et al., 1981; Belgrader et al., 1991), salt extractions were also performed after 200  $\mu\text{g/ml}$  RNase A digestion, in the presence of 20 mM DTT or a combination of these two treatments. Surprisingly, chromosome territories are highly disrupted after RNase A and 2.0-M NaCl treatment (Figs. 1 C and 2 D), but extraction with similar ionic strength 0.65 M  $(\text{NH}_4)_2\text{SO}_4$  after RNase treatment had no visible effect (Fig. 2 C). Identical results were obtained in eight separate experiments using eight different human chromosome paints (numbers 1, 2, 4, 7, 9, 11, 14, and 22) examined in three human cell lines (WI-38, NHF-1, and HeLa S3). 82% of the cells (>500 cells counted in each experiment) had disrupted chromosome territories when averaged among all the experiments (results not shown). In contrast, >90% of the cells had intact territories after all other nuclear matrix preparations (Fig. 2, A–C), including the cells extracted with 20 mM DTT (results not shown). This compares with intact cells, in which nearly 100% of the chromosome territories were intact even under conditions where a small percentage of the nuclei (~20%) showed visible breakage or leakage of chromatin. We conclude that intermolecular disulfide bonds, which form between nuclear matrix proteins (Kaufman et al., 1981; Belgrader et al., 1991), do not play a significant role in maintaining chromosome territory organization. RNA and/or RNP interactions, however, are crucial.

While disrupted chromosome territories display diffuse staining inside the nucleus (Fig. 2 D), it could be argued that the individual chromosomes maintain a degree of their territorial organization despite unraveling into more diffuse structures. Double labeling experiments (e.g., chromosomes 1 and 11; Fig. 1 F), however, clearly show a high degree of mixing (yellow coloration) of the two chromosome pairs and confirm the loss of territorial arrangement.

The difference in chromosome territory organization between 2.0-M NaCl extraction and its same ionic strength 0.65 M  $(\text{NH}_4)_2\text{SO}_4$  after RNase A digestion led us to study the nuclear matrix organization after these salt extractions. In the same preparations, matrin 250 and lamin A/C were used as markers for the internal nuclear matrix and the peripheral nuclear lamina, respectively, by double immunofluorescence labeling (see Materials and Methods). Cells extracted with 0.65 M  $(\text{NH}_4)_2\text{SO}_4$  had a matrin 250 staining intensity and pattern identical to intact cells (Fig. 2 E; 89% of 464 cells counted). Extraction with 2.0 M NaCl, however, resulted in significant aggregation of the structures (Fig. 2 F; 76% of 480 cells). Matrix preparations obtained with RNase A after 0.65 M  $(\text{NH}_4)_2\text{SO}_4$  are depleted in matrin 250, but it is still detectable (Fig. 2 G; 81% of 447 cells). There is no detectable matrin 250 staining inside of the nuclei after RNase A digestion and 2.0-M NaCl extraction (Fig. 2 H; 80% of 367 cells). Virtually identical results were obtained when the internal nuclear matrix was decorated with matrin CYP (Mortillaro and Berezney, 1998) instead of matrin 250 (results not shown). In contrast, there is no detectable effect on the nuclear lamina structure by any of the extraction conditions (Fig. 2, E–H).

The overall nuclear morphology of the extracted cell samples were then studied by thin sectioning EM. The results are in close agreement with the double immunofluorescence analysis of nuclear matrix components. An elabo-



**Figure 1.** Chromosome territories are maintained after extraction of WI-38 cells for DNA-rich nuclear matrix, but are disrupted when RNase A digestion precedes 2.0-M NaCl extraction. A and D, intact cells; B and E, DNA-rich nuclear matrix (0.25 M  $(\text{NH}_4)_2\text{SO}_4$ ); C and F, DNA-rich nuclear matrix (2.0 M NaCl after RNase A digestion; nuclear matrix 2 of Fig. 4). A–C are counter-stained with propidium iodide. A, Chromosome 1; B, chromosome 4; C, chromosome 1; D, chromosomes 4 and 9; E, chromosomes 1 and 9; F, chromosomes 1 and 11. Bars, 5  $\mu\text{m}$ .

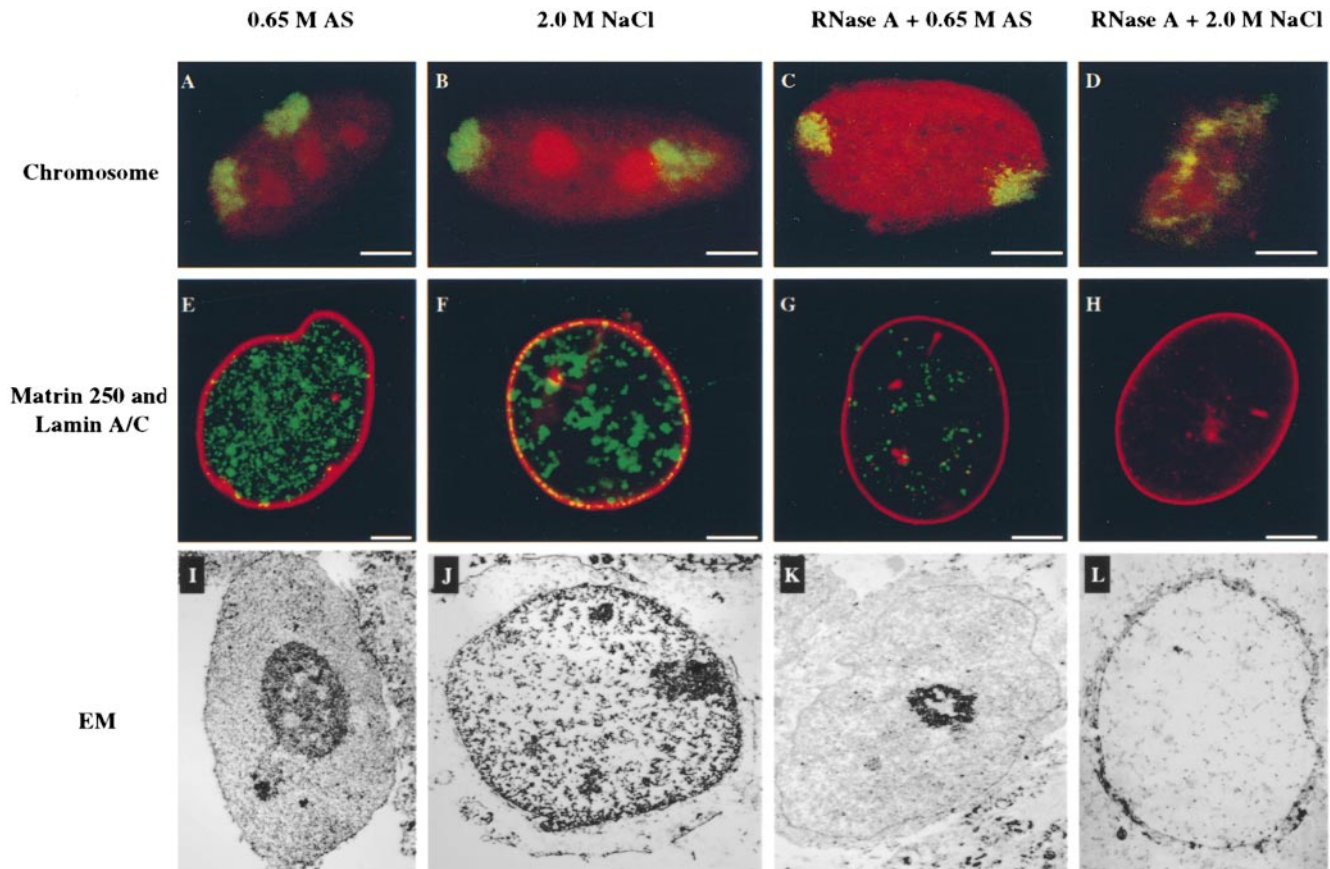
rate structure is seen in the nuclear matrix after 0.65-M  $(\text{NH}_4)_2\text{SO}_4$  extraction (Fig. 2 I). Nuclear matrix structure is significantly aggregated by 2.0-M NaCl extraction (Fig. 2 J). After RNase A and 0.65-M  $(\text{NH}_4)_2\text{SO}_4$  treatments, the DNA-rich nuclear matrices are depleted of internal structure, although it is still detectable (Fig. 2 K). The interior of the nuclear matrix, however, is virtually devoid of structure when extracted by 2.0 M NaCl after RNase A digestion (Fig. 2 L).

Extraction of internal nuclear matrix components with RNase and 0.65 M  $(\text{NH}_4)_2\text{SO}_4$  had no visible effect on the relative intactness of the resulting nuclear matrix structures, as evaluated by fluorescence microscopy or EM (Fig. 2). Similarly, the majority of nuclear matrix structures observed after chromosome disruption (RNase and 2-M NaCl extraction) were indistinguishable in terms of overall nuclear shape from those following other extractions (Fig. 2, H and L). Significantly, chromosome territo-

ries were routinely disrupted irrespective of the degree of intactness of overall nuclear shape (Figs. 1 C and 2 D).

#### ***DNA Halos and DNA Replication Sites after Extraction of Cells for Nuclear Matrix***

When DNA-rich nuclear matrices are prepared under conditions where cleavage or nicking of DNA is avoided, the DNA remains inside the nuclear matrix structure and is highly supercoiled (Cook and Brazell, 1975; Cook et al., 1976; Berezney and Buchholtz, 1981). Progressive relaxation of this supercoiled DNA with intercalating agents, such as ethidium bromide, leads to the corresponding formation of a halo of relaxed DNA loops surrounding the overall nuclear matrix structure (Cook et al., 1976; Warren and Cook, 1978; Vogelstein et al., 1980). Some preparations of DNA-rich nuclear matrix, however, are designed for studying DNA halos (Gerdes et al., 1994; de Belle et



**Figure 2.** Relationship of nuclear matrix structure to chromosome territory disruption in NHF-1 cells. Four different conditions for preparing DNA-rich nuclear matrix are analyzed for chromosome 1 territories by chromosome painting (A–D), with propidium iodide counterstaining and nuclear matrix architecture as defined by immunofluorescence of nuclear matrix associated proteins (E–H), matrin 250, also known as nuclear matrix associated RNA polymerase II<sub>0</sub> large subunit (FITC as the secondary antibody) and lamin A/C (Texas red as secondary antibody), and thin section EM (I–L). The preparation conditions include: A, E, and I, 0.65 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> with ribonucleoside vanadyl complex (4 mM); B, F, and J, 2.0 M NaCl with ribonucleoside vanadyl complex (4 mM); C, G, and K (nuclear matrix 1 of Fig. 4), 0.65 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> after RNase A (200 μg/ml) digestion; D, H, and L (nuclear matrix 2 of Fig. 4), 2.0 M NaCl after RNase A (200 μg/ml) digestion. Bars, 5 μm.

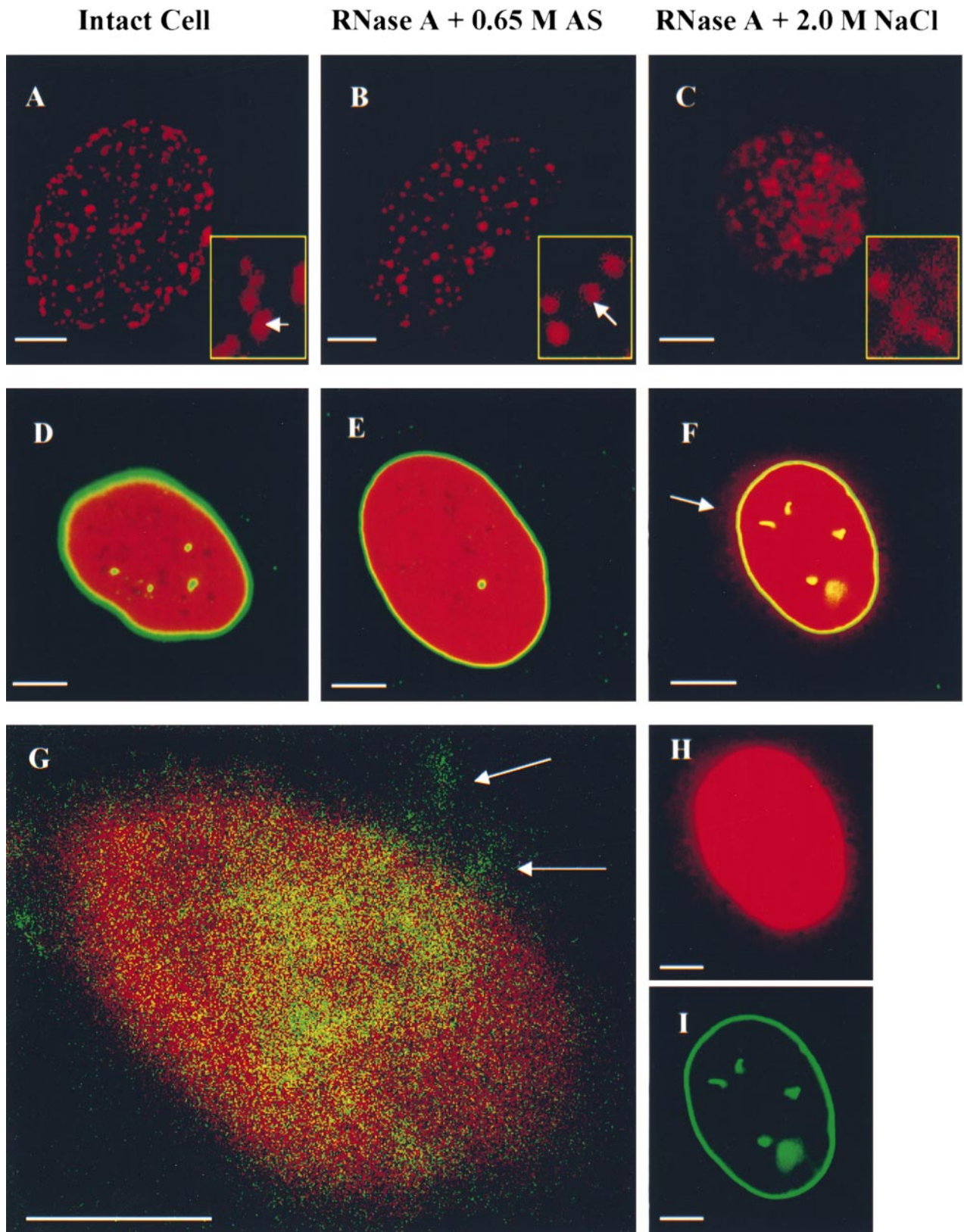
al., 1998) and include the ethanol dehydration and air drying steps that are used in electron microscopic spreading techniques for visualizing DNA loops in halos (Paulson and Laemmli, 1977; McCready et al., 1979).

These differences in DNA halo formation led us to examine this property more closely in our preparations. Using the nuclear lamina as a marker for the periphery of the overall nuclear structure (Fig. 3 I), we determined whether or not significant levels of DNA were detected in regions that extended beyond the nuclear lamina border (i.e., a DNA halo). Under conditions where chromosome territories remained intact, virtually all of the DNA was detected inside the nuclear matrix structure in a manner indistinguishable from unextracted cells. This is demonstrated in Fig. 3, D and E, for untreated cells and RNase A–0.65-M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> extracted cells, respectively. In contrast, in DNA-rich nuclear matrix, where the chromosome territories are routinely disrupted (RNase A–2-M NaCl treated), faint DNA halos were observed surrounding the nuclear lamina (Fig. 3, F–H). Although a vast majority of the DNA was still contained inside the nuclear matrix struc-

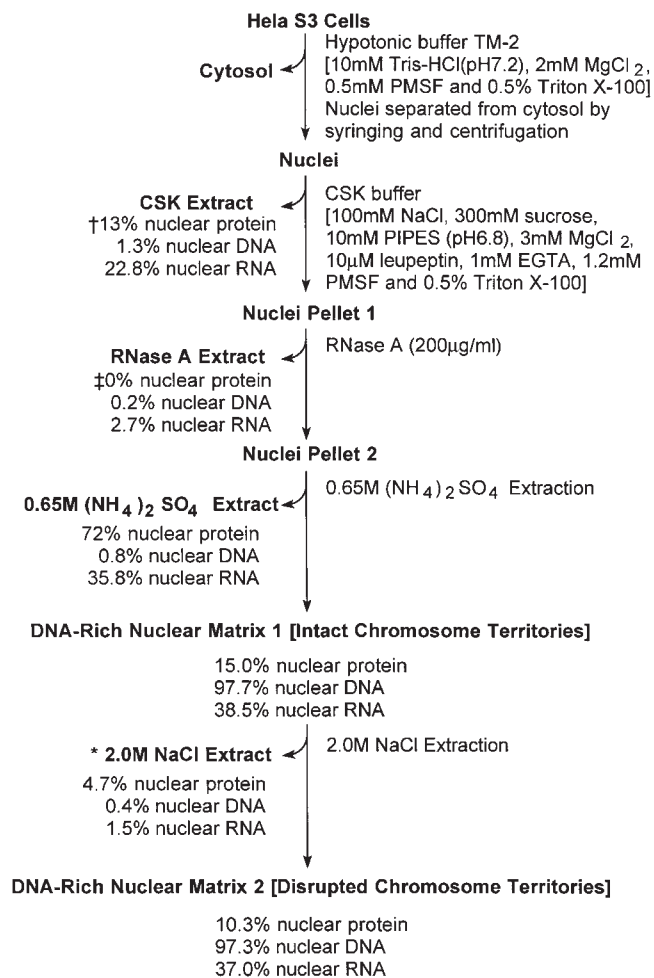
ture (Fig. 3, F–I), small amounts of both total DNA and chromosome specific territories extend beyond the border of nuclear matrices with disrupted chromosome territories (Figs. 1 C, 2 D, and 3 G), but not in whole cells (Fig. 1 A) or other nuclear matrix preparations where the chromosome territories remain intact (Fig. 2, A–C).

Since the formation of a faint DNA halo in these DNA-rich nuclear matrices with disrupted chromosome territories might be the result of the unpacking of multiple regions of DNA loops from within the nuclear matrix structure, we decided to examine DNA replication sites under these conditions. Individual sites of replication in mammalian cells are discrete and contain an average of ~1 mbp DNA per site during early S-phase (Nakayasu and Berezney, 1989; Jackson and Pombo, 1998; Ma et al., 1998). Thus, each early S replication site likely contains at least several typically sized DNA loops (replicons) of 50–200 kbp packaged together (Jackson and Pombo, 1998; Ma et al., 1998).

The question we could then address is whether chromosome territory disruption leads to a corresponding disruption at the level of clusters of DNA loops that compose



**Figure 3.** DNA halos and replication sites in relationship to chromosome territory disruption in NHF-1 cells. A and D, intact cells; B and E, DNA-rich nuclear matrix 1 (RNase + 0.65 M  $(\text{NH}_4)_2\text{SO}_4$ ); C, F, G, H, and I, DNA-rich nuclear matrix 2 (2.0 M NaCl following RNase A digestion). The presence of a DNA halo was evaluated by dual staining of the nuclear periphery with nuclear lamin antibodies (green) and total DNA with propidium iodide (red). All preparations of DNA-rich nuclear matrix were devoid of DNA halos (E), as were control cells (D), except under conditions that led to chromosome territory disruption (F, see arrow). H and I show the single channel images of DNA and lamin staining, respectively, that correspond to the image in F. G shows portions of a disrupted chromo-



**Figure 4.** Protocol for releasing nuclear matrix associated proteins that correlate with disruption of chromosome territories. Under these described fractionation conditions, 4.7% of the total nuclear protein (~0.56% of total cellular protein) was released in conjunction with the chromosome territory disruption. 97.3% of the total nuclear DNA, and near one third of the nuclear RNA are associated with the final extracted nuclear matrix pellet. \*Protein subfraction that is released during chromosome territory disruption. †Percent recovery of total protein, DNA, and RNA from HeLa nuclei. ‡Protein amount in this subfraction was corrected for the added RNase A.

each replication site. We found that replication sites characteristic of early S phase in unextracted cells (Fig. 3 A) are maintained in DNA-rich nuclear matrix, as long as the chromosome territories are intact (Fig. 3 B and results not shown). After territory disruption, however, the replication sites were still identifiable but highly diffuse in structure (Fig. 3 C). This indicates a change in the organization (relaxation) of DNA loops or their higher order packing in correlation with territorial disruption.

some territory (chromosome 1, green) extending into the faint DNA halo (see arrows). DNA replication sites are labeled in vivo with BrdU (A, B, and C; see Materials and Methods) followed by extraction for DNA-rich nuclear matrix. Boxed inserts show replication sites at higher magnification (see arrows for individual sites). The replication sites are identical to those of control cells (A) in all DNA-rich nuclear matrix preparations (e.g., B), except where chromosome territories are disrupted (C). Bars (A–G, 5 μm; H and I, 4 μm).

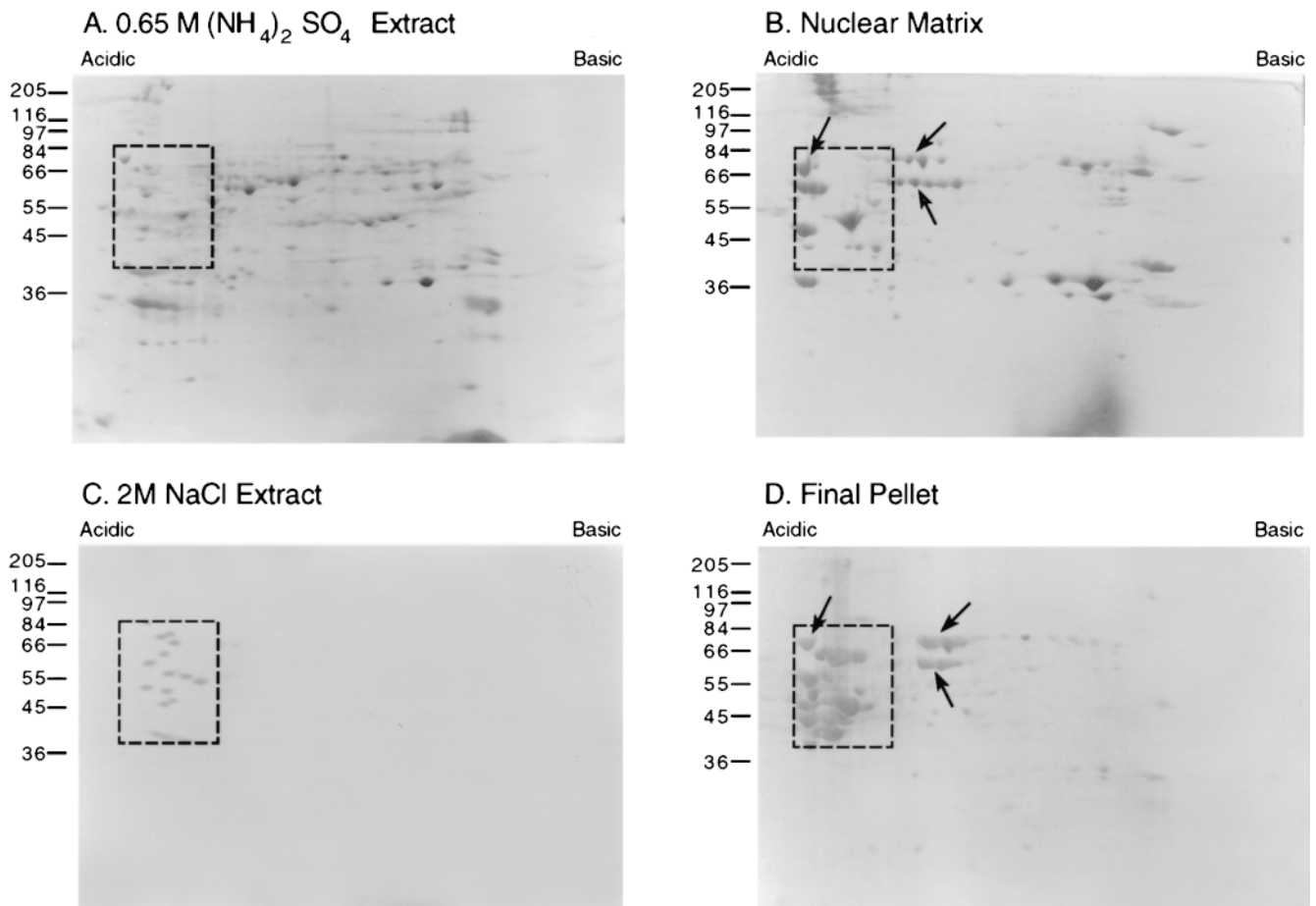
### **A Small Subset of Acidic Nuclear Matrix Proteins Are Released in Conjunction with Chromosome Territory Disruption**

The differential effect of 2.0-M NaCl vs. 0.65-M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> salt extraction after RNase A treatment suggests a strategy (described in Materials and Methods and shown in Fig. 4) for identifying proteins that may play a role in chromosome territory organization. In brief, the bulk of nuclear matrix proteins are released under conditions where the chromosome territories are maintained intact (0.65 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> after RNase A, nuclear matrix 1 of Fig. 4). Then, a second salt extraction treatment (2.0 M NaCl) results in disruption of the chromosome territories (nuclear matrix 2 of Fig. 4). Proteins released into the second extraction are candidates for chromosome territory anchor proteins (CTAPs) and, therefore, we term this fraction the CTAPs extract.

Under these described fractionation conditions, 4.7% of the total nuclear protein (~0.56% of total cellular protein) was released in conjunction with the chromosome territory disruption. 97.3% of the total nuclear DNA, and nearly one third of the nuclear RNA, remain associated with the final extracted nuclear matrix pellet (Fig. 4). The CTAPs extract was composed predominantly of protein (>95%) with only trace amounts of RNA (1.5% of total nuclear RNA) and DNA (0.4% of total nuclear DNA).

Two-dimensional SDS-PAGE was then performed in an attempt to identify the specific proteins released in conjunction with chromosome territory disruption. The CTAPs 2-M NaCl extract (Fig. 5 C) was highly depleted in the major proteins that constitute a typical nuclear matrix fraction (Fig. 5 B; Belgrader et al., 1991; Nakayasu and Be-rezney, 1991). As anticipated, the major nuclear matrix proteins were released after the 0.65-M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> extraction, along with numerous other proteins (Fig. 5 A). Over 95% of the lamin proteins remain in the final pellet (nuclear matrix 2 of Fig. 4) after extraction for CTAPs (Fig. 5 D). Small amounts of lamins are occasionally found in the CTAPs extract and likely represent contamination from the final pellet fraction.

A relatively simple constellation of polypeptides was found in the CTAPs extract that migrated predominantly between 40 and 90 kD in the acidic region of the gel (Fig. 5 C). As indicated by the corresponding boxed areas in Fig. 5, these proteins were found in trace amounts or were not detectable in the two-dimensional gel of total nuclear matrix proteins (Fig. 5 B), the 0.65-M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> extract (Fig. 5 A), or the final pellet (Fig. 5 D). The final pellet, however, contained numerous proteins in the boxed region corresponding to lamin B (arrow) and a cluster of presumptive cytokeratin proteins that do not correspond to those in the CTAPs extract. Taken together, our results indicate that the proteins released in conjunction with chromosome territory disruption are a minor acidic subset of the total nuclear matrix proteins.



**Figure 5.** Two-dimensional PAGE of nuclear matrix proteins released during disruption of chromosome territories. Proteins are separated on two-dimensional PAGE and stained with 0.2% Coomassie blue (see Fig. 4 for details of the fractions). A, 0.65-M  $(\text{NH}_4)_2\text{SO}_4$  extract (500  $\mu\text{g}$ ); B, control, total HeLa nuclear matrix proteins (500  $\mu\text{g}$ ); C, 2.0-M NaCl extract (proteins released during chromosome territories disruption, 100  $\mu\text{g}$ ); D, final pellet (500  $\mu\text{g}$ ). The small subset of total nuclear matrix proteins that constitutes the released proteins is indicated by the boxed area. Arrows indicate the positions of nuclear lamins A, B, and C. Molecular markers are indicated in kD.

## Discussion

In this paper we have examined the possible role of the nuclear matrix in the territorial organization of human chromosomes in the interphase cell nucleus. DNA-rich nuclear matrix are prepared by in situ extraction of human cells grown on coverslips. Previous studies have demonstrated that nuclear DNA anchored to these structures is highly supercoiled and present predominantly inside the nuclear structures (Cook and Brazell, 1975; Cook et al., 1976; Warren and Cook, 1978; Berezney and Buchholtz, 1981). Since DNA halos can be observed after relaxation of the supercoiled DNA loops (Vogelstein et al., 1980) or after spreading techniques on electron microscopic grids (Paulson and Laemmli, 1977; McCready et al., 1979), it is likely that recent studies demonstrating DNA halos in cells extracted for DNA-rich nuclear matrix are due to unwinding of the supercoiled DNA during the preparative steps and/or spreading induced by ethanol dehydration and air drying of the specimens (Gerdes et al., 1994; de Belle et al., 1998).

We reasoned that specific nuclear matrix proteins likely interact with chromatin at matrix attachment sites. More-

over, there may be higher levels of organization of these interactions ranging from the chromatin loop clusters to the level of the chromosome territory. We report a striking maintenance of chromosome territory organization despite the extraction of >90% of the histones and other soluble nuclear proteins in these DNA-rich nuclear matrix preparations (Berezney and Buchholtz, 1981). Our results further suggest that nuclear matrix components are involved not only in the anchoring of chromatin in repeating domains, but also in constraining the overall architecture of the chromosomes.

We next examined the role of the nuclear lamina versus the internal nuclear matrix components in chromosome territory organization. Previous studies have demonstrated that chromatin is attached to both nuclear lamina and internal nuclear matrix components (Lebkowski and Laemmli, 1982; Smith et al., 1984; Luderus et al., 1992, 1994). Extraction of the bulk of the internal nuclear matrix with RNase treatment followed by 0.65 M  $(\text{NH}_4)_2\text{SO}_4$  (nuclear matrix 1, Fig. 4; Belgrader et al., 1991) had no effect on the chromosome territory organization. While this might argue for a critical role of the nuclear lamina in ter-



itory organization, it was also possible that a minimal internal matrix structure that resisted extraction was also involved. Indeed, examination by immunofluorescence microscopy and EM confirmed a minimal internal matrix structure in these preparations. In contrast, extraction with RNase treatment followed by 2 M NaCl (nuclear matrix 2, Fig. 4) resulted in a dramatic disruption of the chromosome territories and a corresponding complete extraction of the internal matrix as evaluated by the same microscopic criteria.

Despite disruption of chromosome territories in conjunction with the removal of the internal matrix, the DNA remained predominantly inside the residual nuclear structures. A small amount of DNA, however, extended past the nuclear lamina boundary to form a faint DNA halo (Fig. 3, F, H, and I). This phenomenon was also observed with individual chromosome territories: the vast majority of the disrupted territories remained inside the nuclear matrix structure while a small but discrete amount extended past the nuclear border in DNA loop-like fashion (Fig. 3 G). Thus, an important role in the overall anchoring of the chromosomal DNA is attributable to the nuclear lamina, but specific territorial arrangements requires the additional participation of a component(s) of the internal nuclear matrix.

To study the possible relationship of chromosome territories to higher order chromatin loop organization further, we examined DNA replication sites under conditions of intact and disrupted chromosome territories. Recent studies suggest that individual replication sites contain ~1 mbp of DNA arranged in a cluster of repeating DNA loops or replicons (Jackson and Pombo, 1998; Ma et al., 1998). Correlative with the disruption of chromosome territories, we observed a corresponding disruption of individual replication sites from discrete to markedly diffuse structures (Fig. 3, A–C). These findings support the view that destabilization of DNA loop anchoring sites or their higher order arrangement after extraction of nuclear matrix components leads to a corresponding disruption of the chromosome territories. Of course, we cannot rule out the possibility that these structural relationships are purely coincidental and that other features of higher order chromatin organization other than nuclear matrix association of DNA loop domains are mediating these interactions. The overall intactness of the nuclear matrix shape, however, did not correlate with territorial disruption.

It is also possible that while RNase–2-M NaCl extraction has a destabilizing effect on chromosome territories, it is actually the harsh FISH procedure that leads to disruption, rather than the extractions themselves. The effect, however, is specific for RNase–2-M NaCl treatment and, in the absence of FISH, results in the virtual complete emptying of the nuclear matrix and a corresponding formation of a faint DNA halo and dispersion of DNA replication sites. This argues for a direct dispersive effect of the extractions on higher order chromatin architecture.

Our findings also provide a potentially powerful approach for elucidating the proteins and other factors that are involved in higher order chromosome territory organization. We have identified a small constellation of proteins whose release from the nuclear matrix correlates with the disruption of human chromosome territories. Work is in

progress to further identify these proteins and their possible relationship to S/MARs binding proteins (von Kries et al., 1991; Dickinson et al., 1992; Romig et al., 1992; Tsutsui et al., 1993; Fackelmayer et al., 1994; Dickinson and Kohwi-Shigematsu, 1995; Wang et al., 1995; Dickinson et al., 1997; Liu et al., 1997; de Belle et al., 1998), proteins of the human SWI/SNF complex that associate with both chromatin and nuclear matrix (Reyes et al., 1997), the mitotic scaffold associated proteins Sc II (Saitoh et al., 1994) and XCAP (Hirano and Mitchison, 1994), which are believed to play a major role in maintaining the condensed state of chromosomes.

The nuclear matrix associations that we demonstrate in this study further suggest that the chromosome territories may be highly constrained in the cell nucleus. Initial studies of territories by an *in vivo* labeling approach in living mammalian cells supports this conclusion, but also demonstrates a limited degree of mobility and putative shape changes (Zink et al., 1998). Recently, Abney et al. (1997) directly measured chromatin mobility in the nuclei of living cells using the FRAP (fluorescence recovery after photobleaching) technique. They found that the overall chromatin in the nucleus is highly immobile at the level of 0.6–0.8- $\mu$ m diam spots. This is a size considerably larger than the ~0.5- $\mu$ m diam replication sites that have been estimated to contain ~1 mb DNA (Jackson and Pombo, 1998; Ma et al., 1998). Since recent results suggest that large molecules, up to 500 kD, can freely diffuse in the nucleus of living cells (Seksek et al., 1997), Abney et al. (1997) concluded that the chromatin in living cells is likely constrained by attachment to a nuclear substructure. On the other hand, several investigations have revealed significant mobility of subchromosomal regions in the interphase nucleus, especially involving the centromeric and telomeric regions of chromosomes (De Boni, 1986; Ferguson and Ward, 1992; Funabiki et al., 1993; Janevski et al., 1995; Pluta et al., 1995; LaSalle and Lalande, 1996; Bass et al., 1997). In the interphase nucleus of living HeLa cells, however, centromeres are generally motionless (Shelby et al., 1996).

Taken together, these results suggest that chromatin in the nucleus of living cells may be highly constrained, but there is a degree of plasticity that allows limited motion and potentially dynamic shape changes (Shelby et al., 1997; Zink et al., 1998) while maintaining an overall high degree of organization of the chromosome territories (Dietzel et al., 1995, 1998; Nagele et al., 1995; Elis et al., 1996; Zink and Cremer, 1998; Zink et al., 1998). In this regard, it has been proposed that specific positional movements of chromosomal regions are regulated by the transcriptional state of the cell (Janevski et al., 1995; Park and De Boni, 1998) and further postulate that this might be mediated by an underlying dynamic nuclear matrix structure (De Boni, 1994). Indeed, there is growing awareness of the dynamics of nuclear architecture (Berezney and Coffey, 1977; Berezney, 1979, 1984; Baskin, 1995; Berezney et al., 1995; Cremer et al., 1995; Berezney and Wei, 1998).

It is important to stress that disruption of the human chromosome territories, while related to complete extraction of the internal nuclear matrix structure, also requires digestion of RNA with RNase. This is consistent with the previous findings (Fey et al., 1986; Belgrader et al., 1991)

that treatment of HeLa matrices and their intermediate filament-like core filaments (He et al., 1990) with RNase A results in destabilization of internal matrix structure and further implies an important role of RNA and/or RNPs in stabilizing chromosome territories. In this regard, the perichromosomal layer, which is likely involved in chromosome organization (Hernandez-Verdun and Gautier, 1994), contains several classes of proteins and RNPs, including nuclear matrix associated proteins (Verheijen et al., 1989; He et al., 1991).

The findings of Clemson et al. (1996) demonstrating a role of XIST RNA in maintaining the territorial organization of the inactive X chromosome may be a specialized example of what we report for chromosome territories in general. Significantly, the XIST RNA is a component of the underlying nuclear matrix structure (Clemson et al., 1996). Moreover, SAF-A, a presumptive MAR binding protein associated with the nuclear matrix, binds both DNA (single and double strands) and RNA, and is identical to hnRNP-U, which is involved in packaging of hnRNA into RNP particles (Fackelmayer et al., 1994). All of these studies suggest a closer interplay than generally acknowledged between the organization of chromosomes and RNP in the cell nucleus.

We are extremely grateful to Dr. R. Summers for his valuable advice with confocal microscopy and to Dr. J. Samarabandu, R.S. Devdhar, S. So-manathan, and J. Stamos for their assistance with computer analysis, illustrations, and photography. We are very thankful to Dr. D. Kaufman for contributing the NHF-1 cell line and Drs. N. Chaudhary and G. Blobel for the lamin A/C and lamin B polyclonal antibodies.

The experiments were funded by the National Institutes of Health grant GM 23922 to R. Berezney and a grant from the Mark Diamond Fund of SUNY Buffalo Graduate School to H. Ma (27-S-98).

Submitted: 3 August 1998

Revised: 17 June 1999

Accepted: 2 July 1999

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