A Major 62-kD Intranuclear Matrix Polypeptide Is a Component of Metaphase Chromosomes

Alan P. Fields and Joel H. Shaper

Department of Pharmacology and Molecular Sciences and The Oncology Center, The Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205

Abstract. We have isolated and partially characterized a major intranuclear matrix polypeptide from rat liver. This polypeptide, which is reversibly stabilized into the intranuclear matrix under conditions which promote intermolecular disulfide bond formation, has a M_r of 62,000 and pI of 6.8–7.2 as determined by two-dimensional IEF/SDS–PAGE. A chicken polyclonal antiserum was raised against the polypeptide purified from two-dimensional polyacrylamide gels. Affinity-purified anti-62-kD IgG was prepared and used to immunolocalize this polypeptide in rat liver tissue hepatocytes. In interphase hepatocytes the 62-kD antigen is localized in small, discrete patches within the nucleus consistent with the distribution of chromatin. The staining is most prominent at the nuclear periphery and somewhat less dense in the nuclear interior. Nucleoli and cytoplasm are devoid of staining. During mitosis the 62-kD antigen localizes to the condensed chromosomes with no apparent staining of cytoplasmic areas. The chromosomal staining during mitosis is uniform with no suggestion of the patching seen in interphase nuclei. Fractionation and immunoblotting studies using rat hepatoma tissue culture cells blocked in metaphase with colcemid confirm the chromosomal localization of this 62-kD intranuclear protein during mitosis. The 62-kD polypeptide fractionates completely with metaphase chromosome scaffolds generated by sequential treatment of isolated chromosomes with DNAse I and 1.6 M NaCl, suggesting that this major 62-kD intranuclear protein may be involved in maintaining metaphase chromosomal architecture.

NUMEROUS studies have reported the isolation of a residual proteinaceous nuclear matrix or scaffold from interphase nuclei from a variety of sources (reviewed in 3, 26, 38, 39). These structures retain many of the morphological features of the intact nucleus and have been implicated in a number of nuclear functions including the structural organization of interphase DNA. Residual structures have also been isolated by sequential treatment of isolated chromosomes with nucleases and polyanions or high salt buffers (2, 11, 32, 36). Termed metaphase chromosome scaffolds, these structures are thought to be involved in the topological organization of DNA into the metaphase morphology (33). Consistent with the view that both the interphase nuclear matrix and metaphase chromosome scaffold is involved in the organization of DNA is the recent finding that DNA topoisomerase II, a nuclear enzyme which is capable of regulating DNA topology, is a major component of the interphase nuclear matrix (4) and metaphase chromosome scaffold (12, 15). In addition, several workers have identified a class of nuclear proteins which appear to be constituents of both the interphase nuclear matrix and metaphase chromosome scaffold (1, 9, 18, 37). These common constituents are of interest as they may play a role in DNA organization throughout the cell cycle. However, with the exception of topoisomerase II, little is known about the specific role any of these polypeptides plays within these structures.

In the present study we describe the isolation and characterization of a major polypeptide from the rat liver intranuclear matrix. The polypeptide, which has a relative molecular mass of 62,000 and pI of 6.8–7.2, localizes to the nucleus of interphase cells. During mitosis, this 62-kD polypeptide distributes exclusively with the condensing chromosomes. Fractionation studies on isolated metaphase chromosomes from rat hepatoma tissue culture (HTC)[1] cells indicate that the 62-kD polypeptide is a major component of metaphase chromosome scaffolds. Since this 62-kD polypeptide is a constituent of both the interphase nuclear matrix and metaphase chromosome scaffold it may play a structural role in the organization of DNA throughout the cell cycle.

Materials and Methods

Animals and Cell Culture

Male Sprague-Dawley rats (300–350 g, Harlan, Indianapolis, IN) were used for isolation of rat liver nuclei. HTC cells were maintained in monolayer culture in DME medium supplemented with 10% (vol/vol) heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate.

1. *Abbreviation used in this paper*: HTC, hepatoma tissue culture.

Isolation and Fractionation of Rat Liver Nuclei

Rat liver nuclei were isolated as previously described (28). All steps were performed at 4°C and all buffers contained 1 mM phenylmethylsulfonyl fluoride (PMSF) added just before use from a 100 mM stock in anhydrous isopropanol.

Freshly isolated nuclei were treated sequentially with the sulfhydryl cross-linking reagent sodium tetrathionate (NaTT), DNase I and RNase A and then extracted with 1.6 M sodium chloride (NaCl) to yield nuclear matrix structures as previously described (25). The resulting nuclear matrix structures contained residual nucleoli, extensive intranuclear material and a nuclear envelope (25). The intranuclear, disulfide-crosslinked polypeptides were solubilized by treatment of the structures with 1.0 M NaCl containing 20 mM dithiothreitol (DTT) for 20 min. Nuclear envelopes were removed by sedimentation at 3,000 *g* for 20 min and the supernatant stored at −20°C. This supernatant was used for isolation of the 62-kD polypeptide.

Isolation and Characterization of the 62-kD Polypeptide

Solubilized intranuclear polypeptides were reduced and alkylated with iodoacetamide and resolved by two-dimensional IEF/SDS-PAGE as previously described (23). Gels were stained for 30 min with 0.25% (wt/vol) Coomassie Brilliant Blue R-250 (C. I. 42660), 50% (vol/vol) methanol, 12% (vol/vol) acetic acid and destained in 7% (vol/vol) methanol-12% (vol/vol) acetic acid for 30 min. Destained gels were washed with distilled water and gel spots containing the resolved 62-kD polypeptide were excised from the gels. Excised spots were washed extensively with distilled water and stored at −70°C.

For some experiments, the 62-kD polypeptide was electroeluted from polyacrylamide gel spots using the procedure and apparatus described by Hunkapiller et al. (23). Purity of the electroeluted protein was confirmed by one-dimensional (29) and two-dimensional IEF/SDS-PAGE.

Preparation and Characterization of a Polyclonal Antiserum Against the 62-kD Polypeptide

Female white Leghorn chickens (6–8-wk old; Truslow Farms, Chestertown, MD) were injected with small polyacrylamide gel fragments containing the reduced and alkylated 62-kD polypeptide (~40 μg protein/injection) emulsified in sterile Ca^{+2}, Mg^{+2}-free PBS and complete Freund's adjuvant (1:1, vol/vol). A second injection using polyacrylamide gel fragments containing antigen emulsified in complete Freund's was given 4 wk later. Blood was obtained from a wing vein beginning 2 wk after the second injection. Booster injections using antigen emulsified in incomplete Freund's adjuvant were given as necessary to maintain titers. Preimmune and potential immune sera were screened for anti-62 kD antibodies by immunoblotting as described below.

Immunoblot Analysis

Intranuclear polypeptides, after reduction and alkylation, were resolved by one-dimensional SDS-PAGE or two-dimensional IEF/SDS-PAGE and electrophoretically transferred to nitrocellulose as described by Burnette (5). The nitrocellulose sheets were stained with 0.1% (wt/vol) Fast Green FCF (C. I. 42053) in 20% (vol/vol) methanol, 5% (vol/vol) acetic acid for 5 min, destained in 20% (vol/vol) methanol, 5% (vol/vol) acetic acid and washed with PBS. Protein-binding sites were blocked by incubation in PBS containing 0.05% (vol/vol) Tween 20, 5% (wt/vol) non-fat dry milk, 0.2 mM sodium azide (PBS/Tween/milk/azide) for 12–18 h at room temperature. Nitrocellulose strips were incubated with immune serum at a 1:100 dilution in PBS/Tween/milk/azide for 1 h at room temperature, washed three times in PBS containing 0.05% Tween (PBS/Tween) for 10 min each and incubated with peroxidase-labeled affinity-purified goat anti-chicken IgG (1 μg/ml; Kirkegaard and Perry, Gaithersburg, MD) in PBS/Tween/milk for 1 h at room temperature. The strips were washed in PBS/Tween as described above and the antigen-antibody complex visualized by incubation of the washed strips with 0.5 mg/ml diaminobenzidine in PBS containing 0.006% (vol/vol) H_2O_2 for 1–2 min. In some experiments, the antigen-antibody complex was detected by incubating the nitrocellulose replicas with ^{125}I-labeled goat anti-chicken IgG or goat anti-rabbit IgG (10 μCi/replica; labeled to a specific activity of 10 μCi/μg by the procedure of Fraker and Speck [14]). The sheets were then washed as described above, dried, and exposed to Kodak X-Omat AR film at −70°C.

Affinity-Purification of Anti-62 kD Specific IgG

Monospecific antibodies were prepared by affinity-purification on nitrocellulose strips containing the immobilized 62-kD polypeptide. The 62-kD polypeptide, purified from two-dimensional IEF/SDS-PAGE gels, was subjected to re-electrophoresis into one-dimensional slab gels and electrophoretically transferred to nitrocellulose. The nitrocellulose sheet was stained with Fast Green FCF as described above and the area of the nitrocellulose containing the immobilized 62-kD polypeptide was excised and used for affinity-purification. Excess protein-binding sites on the nitrocellulose strip were blocked with 3% BSA in PBS containing 0.2 mM sodium azide 12–18 h at room temperature. The coated strip was incubated with a 1:100 dilution of the anti-62-kD serum in 3% BSA/PBS for 1 h at room temperature. The strip was washed two times with PBS for 10 min total, four times with PBS containing 0.05% (wt/vol) Nonidet P-40 for 60 min total and then two times with PBS for 10 min total. Bound anti-62-kD IgG was eluted from the strip by incubation with 6 M deionized urea in PBS containing 1 mg/ml BSA at 4°C for 5 min. The urea elution step was repeated, the eluates combined and dialyzed at 4°C against multiple changes of PBS containing 0.2 mM sodium azide. As a control, the above procedures were performed using strips of nitrocellulose containing an irrelevant protein (lamin C isolated from two-dimensional gels) or unmodified nitrocellulose. Lamin C was chosen as a control because it exhibits an electrophoretic mobility similar to the 62-kD polypeptide (see Fig. 6) and the anti-62-kD antiserum and the affinity-purified IgG do not recognize this protein (nor other matrix proteins) as determined by immunoblotting. The undiluted affinity-eluates were used for immunoblotting as described above. For immunolocalization, the affinity-purified anti-62-kD IgG solution was concentrated in a Centricon concentrator unit (Amicon, Danvers, MA; 30 kD molecular mass cut-off) to a final concentration of ~2 μg/ml IgG.

Nonimmune chicken IgG was prepared from serum of a nonimmune animal by sodium sulfate precipitation (20). Nonimmune IgG concentration was determined by the Bradford protein assay using bovine gamma globulin as a standard (Bio-Rad Laboratories, Richmond, CA). Affinity-purified anti-62-kD IgG concentrations were estimated by a dot blot immunoassay procedure in which known concentrations of nonimmune chicken IgG and serial dilutions of the affinity-purified anti-62-kD IgG were spotted onto nitrocellulose sheets. The sheets were blocked with PBS/Tween/milk/azide for 12–18 h at room temperature, washed three times with PBS/Tween, and then incubated with affinity-purified peroxidase-labeled anti-chicken IgG (1 μg/ml) in PBS/Tween/milk for 1 h at room temperature, washed with PBS/Tween three times for 30 min total and developed as described above for immunblotting analysis.

Comparative Peptide Mapping

Comparative peptide mapping was performed on polypeptides excised from two-dimensional IEF/SDS gels as described above. Approximately equal amounts of each polypeptide (as judged by Coomassie Brilliant Blue-staining intensity) were subjected to comparative peptide mapping using *Staphylococcus* V-8 protease (50 ng/lane) according to Cleveland et al. (7). Peptides were resolved by one-dimensional SDS-PAGE (15%) and visualized using the silver nitrate method of Morrissey (34).

Immunolocalization of the 62-kD Polypeptide

Immunolocalization was performed on sections of rat liver using the avidin-biotin-horseradish peroxidase system (ABC Vectastain Kit, Vector Labs, Burlingame, CA). Fresh rat liver was cut into 1-mm cubes, fixed in Bouin-Hollande's fixative for 4 h, embedded in paraffin and cut in 5-μm sections. The sections were deparaffinized in xylene, rehydrated through a graded ethanol series, washed twice with PBS for 10 min each, and incubated with a 1:100 dilution of normal goat serum for 1 h at room temperature. The normal goat serum was removed by rinsing with PBS and the sections incubated overnight at 4°C in a humidified chamber with either affinity-purified anti-62-kD IgG (2 μg/ml), nonimmune chicken IgG (2 μg/ml), affinity-eluate from unmodified nitrocellulose or affinity-eluate from nitrocellulose containing an irrelevant protein. The sections were washed three times with PBS for 15 min total, incubated with biotinylated anti-chicken IgG (5 μg/ml) in PBS containing 1% (wt/vol) BSA for 1 h at room temperature, washed with PBS for 15 min total and incubated with ABC reagent (Avidin-Biotin-horseradish peroxidase complex) in PBS containing 1% (wt/vol) BSA for 1 h. The sections were washed three times with PBS as described above and developed in 0.5 mg/ml diaminobenzidine in PBS containing 0.02% (vol/vol) hydrogen peroxide for 2–5 min. The stained sections were washed

briefly with PBS, counterstained with 0.001% (wt/vol) Fast Green FCF in 1% acetic acid for 5 min, rinsed with PBS, and dehydrated through a graded ethanol series and xylene. The dehydrated sections were mounted and viewed under a Zeiss microscope using bright-field illumination.

Isolation and Fractionation of HTC Cell Nuclei and Metaphase Chromosomes

HTC cell nuclei and metaphase chromosomes were isolated by the method of Earnshaw and Heck (10) modified by the inclusion of 100 mM iodoacetamide in all isolation buffers (added under subdued light just before use from a freshly prepared 1 M stock). For isolation of nuclei, cells grown in 100-mm culture dishes were scraped into their growth medium, pelleted at 65 *g* for 5 min, resuspended in reticulocyte standard buffer (RSB:10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$) and immediately pelleted at 65 *g* for 5 min. The cells were resuspended in RSB and after 5 min at room temperature, pelleted at 65 *g* for 5 min, resuspended in lysis buffer (15 mM Tris-HCl, pH 7.4, 2 mM potassium-EDTA, 80 mM KCl, 0.2 mM spermine, 0.5 mM spermidine, 0.1% digitonin), homogenized in a Dounce homogenizer with twelve strokes of a "B" pestle and nuclei pelleted at 800 *g* for 10 min at 4°C. The isolated nuclei appeared intact and relatively free from cytoplasmic contamination as judged by light microscopic inspection.

Chromosomes were isolated from HTC cells blocked in metaphase by treatment with 0.1 μg/ml colcemid for 18 h. Mitotic cells were selected by shake-off and collected by centrifugation at 65 *g* for 5 min. The mitotic index was routinely between 90 and 95%. The cells were hypotonically swollen in RSB and homogenized in lysis buffer as described above for interphase cells. Nuclei and cellular debris were removed by centrifugation at 250 *g* for 1 min followed by 1,500 *g* for 4 min. The supernatant was transfered to a fresh tube and pelleted at 1,500 *g* for 20 min. Chromosomes isolated by this procedure appeared intact and free from contamination by nuclei and cytoplasmic debris.

Nuclei were resuspended in RSB buffer (1×10^7 nuclei/ml) containing 25 μg/ml DNAse I and 25 μg/ml RNase A, incubated for 1 h at 4°C and sedimented at 1,500 *g* for 20 min. Chromosomes were incubated in RSB buffer (1×10^7 nuclei equivalents/ml) containing 25 μg/ml DNase I for 1 h at 4°C and sedimented at 1,500 *g* for 20 min. Chromosomes were resuspended in 10 mM Tris-HCl, pH 7.4, (LS) buffer, and 4 vol of 10 mM Tris-HCl, pH 7.4, 2 M NaCl (HS) buffer were added dropwise. After a 20-min incubation at 4°C, chromosome scaffolds were sedimented at 3,000 *g* for 20 min.

Cell fractions from interphase and metaphase cells were precipitated in 10% (wt/vol) TCA for 45 min on ice. TCA-precipitable material was sedimented at 1,500 *g* for 15 min and the pellet washed once with 10% TCA and twice with −20°C acetone. The final acetone pellet was dried under a stream of nitrogen and resolubilized in 0.5 M Tris-HCl, pH 7.4, 2% (wt/vol) SDS, 140 mM 2-mercaptoethanol at room temperature for 4 h. The solubilized samples were alkylated for 1 h at room temperature in the presence of 133 mM iodoacetamide and dialyzed against multiple changes of 0.1% (wt/vol) SDS. The samples were prepared for gel electrophoresis by the addition of one-fourth volume of 4× stock SDS sample buffer and heating for 30 min at 60°C. The samples were subjected to electrophoresis and electrophoretically transferred to nitrocellulose sheets for immunoblotting using affinity-purified anti-62-kD IgG as described above.

Results

Isolation and Partial Characterization of a Major Intranuclear Matrix 62-kD Polypeptide

Previous work from our laboratory has focused on the role of intermolecular disulfide bonds in stabilizing the intranuclear protein components of the nuclear matrix (25). The reversible nature of these disulfide bonds allows the selective solubilization of these nonlamin, nonhistone, intranuclear matrix polypeptides. Two-dimensional IEF/SDS-PAGE of the solubilized intranuclear matrix (Fig. 1 *A*) reveals prominent polypeptides in the 60–70-kD range. Among these is a major polypeptide with an apparent molecular mass of 62 kD and exhibiting a series of charge isoforms ranging in pI from 6.8–7.2 (arrowhead in Fig. 1 *A*). This polypeptide, which is similar in size and electrophoretic mobility to lamin C, has previously been shown to be both structurally and immunologically unrelated to the lamins (see Fig. 2 *E*, peptide 3 in reference 25). The 62-kD polypeptide was purified from preparative two-dimensional gels of intranuclear polypeptides and recovered by electroelution. The isolated polypeptide migrates as a single band with an apparent molecular mass of 62 kD in one-dimensional SDS gels (arrowhead in Fig. 1 *B*) and with a pI in the 6.8–7.2 range by two-dimensional IEF/SDS-PAGE (data not shown).

Generation and Characterization of a Polyclonal Antiserum Against the 62-kD Polypeptide

Acrylamide gel spots containing the resolved 62-kD poly-

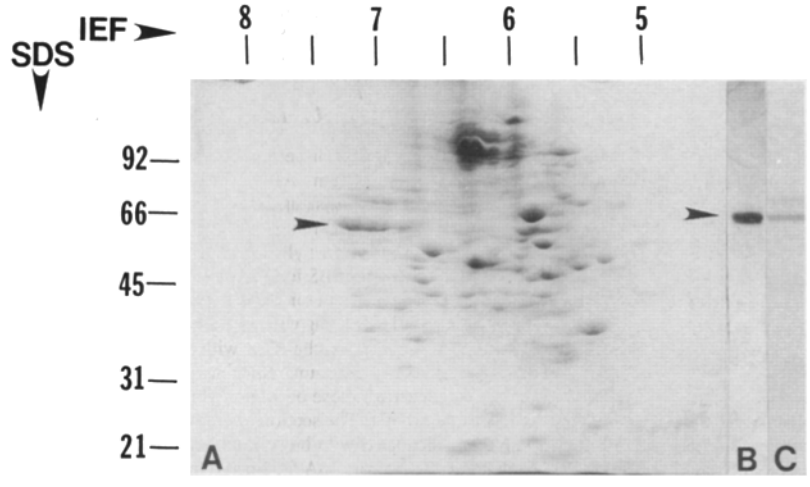

Figure 1. Isolation of the 62-kD polypeptide and characterization of an anti-62-kD antibody. (*A*) Two-dimensional IEF/SDS-PAGE separation of the intranuclear matrix polypeptides from rat liver. Nuclei (1×10^8) were treated with 2 mM NaTT for 1 h followed by digestion with DNAse I and RNAse A and extraction with 1.6 M NaCl as described in Materials and Methods. The intranuclear matrix polypeptides were solubilized by treatment with 1.0 M NaCl containing 20 mM DTT and after alkylation resolved by two-dimensional IEF/SDS-PAGE and stained with Coomassie Brilliant Blue. The position of the 62-kD polypeptide is indicated by the arrowhead. (*B*) Silver stained one-dimensional SDS-PAGE gel of the purified 62-kD polypeptide. The peptide (*arrowhead*) was purified from two-dimensional IEF/SDS-PAGE gels as described in Materials and Methods. (*C*) Immunoblot analysis of the intranuclear polypeptides using affinity-purified anti-62-kD IgG. Proteins from nuclei (5×10^6) were transferred from a one dimension gel to nitrocellulose, sequentially incubated with affinity-purified anti-62-kD IgG and a peroxidase-labeled conjugate. The immune reaction was visualized with diaminobenzidine. Note the presence of a minor, high molecular mass immunologically related polypeptide above the major staining band (*arrowhead*) which is detected by immunoblot analysis (lane *C*) but not by silver staining (lane *B*).

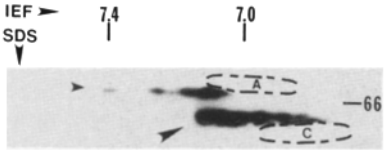

Figure 2. Identification of a minor 69-kD polypeptide from rat liver nuclear matrix which is immunologically related to the 62-kD polypeptide. Nuclear matrix polypeptides (1×10^8 nuclei) were resolved on two-dimensional IEF/SDS-PAGE gels and transferred to nitrocellulose. The nitrocellulose sheet was stained with Fast Green to visualize the protein pattern and was then subjected to immunoblot analysis using anti-62-kD antibody and ^{125}I-labeled conjugate. The antibody recognizes the 62-kD antigen (*large arrowhead*) as well as a quantitatively minor 69-kD polypeptide (*small arrowhead*). The positions of the major Fast Green staining polypeptides in the vicinity of the antigens (lamins *A* and *C*) are indicated by broken circles.

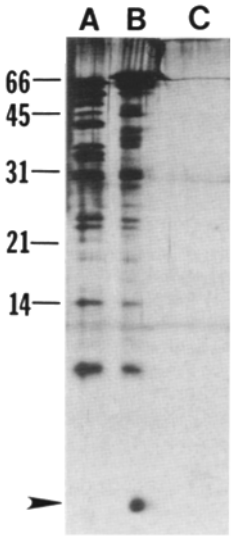

Figure 3. Comparative peptide mapping of the 62- and 69-kD polypeptides. Equal amounts (~2 μg) of the two polypeptides isolated from preparative two-dimensional IEF/SDS-PAGE gels were subjected to comparative peptide mapping with *Staphylococcus* V-8 protease (50 ng) as described in Materials and Methods. The peptides were resolved by one-dimensional SDS-PAGE (15%) and visualized by silver staining. (Lane *A*) 62-kD polypeptide; (lane *B*) 69-kD polypeptide; (lane *C*) V-8 protease only (50 ng). The similarity of the peptide patterns indicates the structural relatedness of the two polypeptides. Note the presence of a low molecular weight peptide which is specific for the 69-kD polypeptide (arrowhead in lane *B*).

peptide were used to immunize chickens. Immunoglobulins specific for the antigen were obtained from the immune serum by affinity-purification using nitrocellulose strips containing the immobilized antigen. Fig. 1 *C* shows the results of immunoblotting of intranuclear polypeptides using affinity-purified anti-62-kD IgG (0.2 μg/ml) and a horseradish peroxidase-labeled anti-chicken IgG conjugate. A prominent band with a relative molecular mass of 62 kD corresponding to the antigen is detected as well as a second, faint band with a relative molecular mass of 69 kD. This second band is reproducibly seen and is not due to nonspecific adsorption of antibody. The relationship of this minor 69-kD band to the 62-kD polypeptide is addressed below. Both one- and two-dimensional immunoblotting studies demonstrate that the polyclonal serum and the affinity-purified anti-62-kD IgG specifically recognize the 62-kD antigen and the 69-kD polypeptide, but not purified lamins (including lamin C), nor any of the other polypeptides of the intranuclear matrix.

Identification of a Polypeptide from Rat Liver Nuclei Which is Immunologically and Structurally Related to the 62-kD Polypeptide

Immunoblotting experiments using the affinity-purified anti-62-kD IgG demonstrated the presence of an immunologically related polypeptide with a relative molecular mass of 69 kD in the intranuclear matrix extracts (Fig. 1 *C*). To more fully characterize this immunologically-related polypeptide, immunoblotting was performed on nuclear matrix polypeptides separated by two-dimensional IEF/SDS-PAGE. Fig. 2 shows the results of this analysis. In addition to the 62 kD/pI 6.8-7.2 antigen (*large arrowhead*) an additional band with a relative molecular mass of 69 kD and pI of 7.0-7.4 (*small arrowhead*) is detected. The 69-kD polypeptide exhibits a pattern of charge isoforms similar to those of the 62-kD antigen, but slightly more basic. Furthermore, the 69-kD polypeptide does not correspond to any of the major Fast Green-staining polypeptides in the extract (lamins A and C, the positions of which are indicated) but rather is only a minor component.

To determine whether the 62- and 69-kD polypeptides are structurally related, comparative one-dimensional peptide mapping was performed according to the method of Cleveland et al. (7). The peptides generated from these two peptides are shown in Fig. 3 after silver staining. The peptide maps of the 62- and 69-kD polypeptides are similar (compare lanes *A* and *B*) indicating the structural relatedness of these two polypeptides. Below 35 kD, the maps are virtually identical while above this molecular mass, the peptides are analogous in pattern but show the expected difference in molecular mass of the parent polypeptides. In addition, a unique peptide of low molecular mass is generated from the 69-kD polypeptide (Fig. 3, arrowhead in lane *B*). Fig. 3, lane *C* shows the peptide pattern of the protease only, indicating that the peptides in lanes *A* and *B* are not protease derived.

Immunolocalization of the 62-kD Polypeptide in Interphase and Mitotic Rat Hepatocytes

The subcellular distribution of the 62-kD polypeptide was determined by immunohistochemistry using an avidin-biotin-horseradish peroxidase detection system as detailed in Materials and Methods. In interphase cells a pattern of staining is observed which is predominantly at the nuclear periphery and less dense throughout the nuclear interior (Fig. 4 *A*). Cytoplasm and nucleoli are devoid of staining. At higher magnification, the nuclear staining is resolved as discrete patches along the nuclear periphery and interior, consistent with the distribution of chromatin within the nucleus. The same pattern of distribution is observed when the immune chicken serum is used in place of the affinity-purified IgG (data not shown).

During metaphase, the 62-kD polypeptide is localized to the condensed chromosomes; there is no apparent staining of the cytoplasmic areas (arrowhead in Fig. 4 *A*). The staining pattern appears uniform across the longitudinal section of the chromosome. Results using nonimmune chicken IgG (2 μg/ml) are shown in Fig. 4 *B*. In addition, the following controls were used: (*a*) preimmune serum at the same dilution as the immune serum, (*b*) affinity-eluate from unmodified nitrocellulose strips, and (*c*) affinity-eluate from nitrocellulose strips containing an irrelevant protein (lamin C). In all cases the results were similar to those shown in Fig. 4 *B* using nonimmune IgG, in which no staining was detected.

The localization of the 62-kD polypeptide was followed in cells in various stages of mitosis (Fig. 5). During prophase (Fig. 5 *A*) when the nuclear envelope has begun to break-

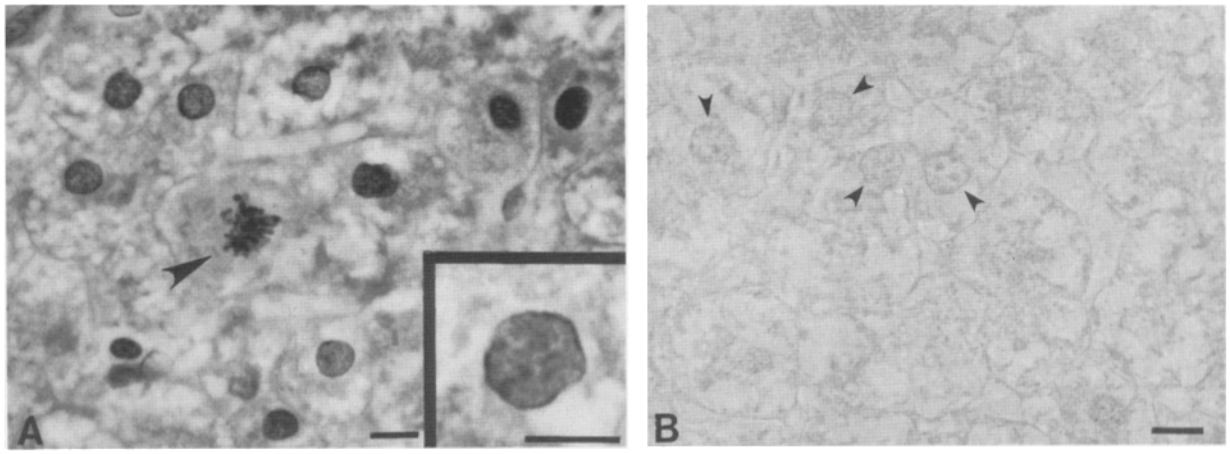

Figure 4. Immunohistochemical localization of the 62-kD polypeptide in rat liver. Sections of rat liver were incubated with affinity-purified anti-62-kD IgG (*A*) or nonimmune IgG (*B*) at 2 μg/ml followed by biotinylated conjugate and avidin-biotin-peroxidase complex. Reaction with diaminobenzidine in the presence of H_2O_2 allowed visualization of any antigen-antibody complexes. The sections were counterstained with Fast Green. The antigen is distributed as small, discrete patches within the interphase nucleus (*A*), a pattern which is more evident at higher magnification (*inset*). During mitosis the 62-kD polypeptide appears to be exclusively localized with the condensed chromosomes (*arrowhead* in *A*); there is no apparent staining of the cytoplasm. Nonimmune IgG shows no staining of the tissue sections (*B*); nuclei are indicated with small arrowheads. Bars, 10 μm.

down and the chromosomes to condense, the antigen localizes predominantly to the chromosomal masses. By prometaphase (Fig. 5 *B*) the chromosomes have condensed into discrete structures to which the 62-kD antigen localizes exclusively. The antigen continues to localize to the chromosomes as they align along the equatorial plane of the spindle during metaphase (Fig. 5 *C*) and begin to migrate to the spindle poles during anaphase (Fig. 5 *D*).

The 62-kD Polypeptide is a Component of Isolated Metaphase Chromosomes and the Chromosome Scaffold

Recent studies from several laboratories have demonstrated that metaphase chromosomes can be fractionated into a residual structure termed the metaphase chromosome scaffold by sequential treatment with DNAse and high salt (2, 33). This scaffold structure is thought to function in maintaining DNA topology during mitosis. To determine if the 62-kD polypeptide fractionates with both the metaphase chromosomes and the scaffold, rat HTC cells were used to obtain sufficient quantities of isolated metaphase chromosomes for analysis. Initially, we determined the distribution of the 62-kD polypeptide in interphase HTC cells. A nuclear and a cytosolic fraction were prepared as described in Materials and Methods. The proteins from each subcellular fraction were resolved by one-dimensional SDS-PAGE and either visualized directly with Coomassie Brilliant Blue (Fig. 6, lanes *1* and *2*) or transferred to nitrocellulose and analyzed by immunoblotting (Fig. 6, lanes *1'* and *2'*). As shown in Fig. 6, the 62-kD polypeptide fractionates exclusively with the nuclear fraction (arrowhead in lane *2*) but is not detectable in the cytoplasmic extract (lane *1'*) from interphase cells.

In metaphase-arrested cells (Fig. 7 *B*), the 62-kD antigen fractionates exclusively with the metaphase chromosomes (lane *2'*); none is detectable in the cytoplasmic fraction (lane *1'*). Metaphase chromosomes were fractionated to yield chromosomal scaffolds by a modification of the method of Earnshaw and Heck (10). The polypeptide profile from each frac-

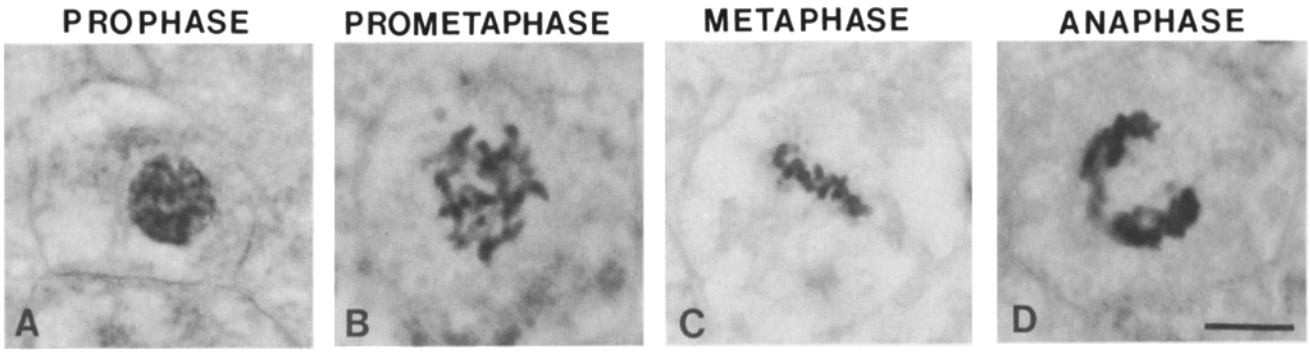

Figure 5. Immunohistochemical localization of the 62-kD polypeptide during mitosis in rat liver. Sections of rat liver were incubated with affinity-purified anti-62-kD IgG followed by biotinylated conjugate and avidin-biotin-peroxidase complex as described in Materials and Methods. The distribution of the 62-kD antigen was observed in prophase (*A*), prometaphase (*B*), metaphase (*C*), and anaphase (*D*). Note the exclusive chromosomal localization of the 62-kD polypeptide throughout mitosis. Bar, 10 μm.

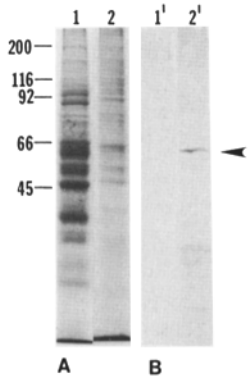

Figure 6. Distribution of the 62-kD polypeptide in interphase HTC cells. Nuclei and a cytoplasmic fraction were prepared. The polypeptides from each subcellular fraction were resolved by one-dimensional SDS-PAGE and either stained with Coomassie Brilliant Blue (*A*) or analyzed by immunoblot analysis (*B*) for the presence of the 62-kD polypeptide as described in Materials and Methods. (*A*) Protein profiles of cytoplasmic (lane *1*) and nuclear (lane *2*) fractions from HTC interphase cells (5×10^6) after staining with Coomassie Brilliant Blue. (*B*) Immunoblot analysis of replica lanes (*A*) using affinity-purified anti-62-kD IgG (lanes *1'* and *2'*). Arrowhead indicates the position of the 62-kD polypeptide.

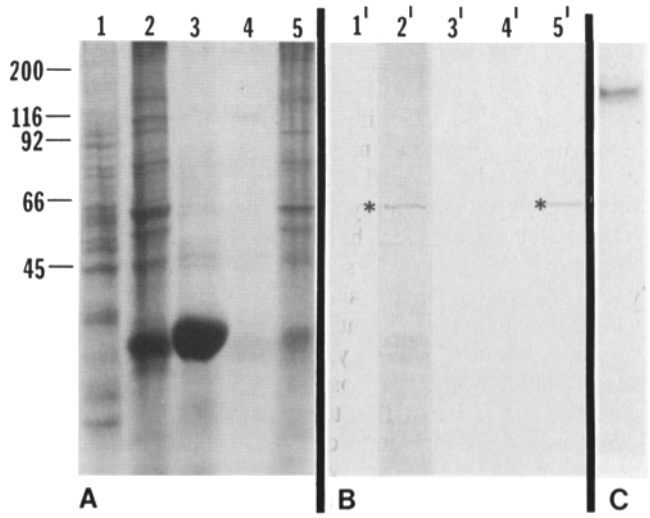

Figure 7. Distribution of the 62-kD polypeptide in metaphase HTC cells and fractionated metaphase chromosomes. HTC cells blocked in metaphase with colcemid were fractionated to yield chromosomes and a cytosolic extract. Metaphase chromosome scaffolds were isolated by sequential extraction of isolated chromosomes with DNAse I and 1.6 M NaCl. Polypeptides from each fraction (from 5×10^6 cells) were resolved by one-dimensional SDS-PAGE and visualized directly with Coomassie Brilliant Blue (*A*) or electrophoretically transferred to nitrocellulose and immunoblotted for the presence of the 62-kD antigen (*B*) or topoisomerase II (*C*). (*A*) Protein patterns of fractionated metaphase cells, fractionated chromosomes and the chromosomal scaffold after staining with Coomassie Brilliant Blue: (Lane *1*) cytoplasm; (lane *2*) isolated chromosomes; (lanes *3* and *4*) proteins released from isolated chromosomes by sequential treatment with DNAse (lane *3*) and with 1.6 M NaCl (lane *4*); (lane *5*) chromosome scaffolds. (*B*) Nitrocellulose replicas of *A* after immunoblot analysis using affinity-purified anti-62-kD IgG and peroxidase-labeled conjugate (lanes *1'-5'*). The position of the 62-kD polypeptide is indicated by the asterisk. (*C*) Replica of chromosome scaffold extract (lane *5* or *5'*) after transfer to nitrocellulose and immunoblot analysis using a rabbit anti-topoisomerase II antibody and an ^{125}I-labeled conjugate. Fluorography was for 48 h at −70°C using Kodak XRP film.

tionation step was analyzed by SDS-PAGE (Fig. 7 *A*, lanes *2-5*). The polypeptide patterns are similar to those obtained by Earnshaw and Heck (10) using a chicken (MSB-1) tissue culture cell line. When replica lanes are subjected to immunoblot analysis, it is observed that the 62-kD polypeptide is not extracted from chromosomes by treatment with 25 μg/ml of DNAse I nor 1.6 M NaCl (lanes *3'* and *4'*, respectively) but remains as a component of the metaphase chromosome scaffold (lane *5'*).

As a control, a replica lane containing the resolved polypeptides from our scaffold preparation was analyzed for the presence of topoisomerase II by immunobot analysis using a specific rabbit polyclonal antiserum (19, kindly provided by Dr. L. F. Liu, Johns Hopkins Medical School). As shown in Fig. 7 *C*, a single band of 170-kD corresponding to the molecular size of topoisomerase II is detected in our scaffold preparation. Thus, studies using rat hepatoma cells in culture indicate that the 62-kD polypeptide fractionates with nuclei in interphase and with chromosomes in cells blocked in metaphase. These results are consistent with the immunolocalization of the 62-kD polypeptide in rat liver tissue presented in Figs. 4 and 5.

Discussion

We have recently described a procedure by which the intranuclear components of the rat liver nuclear matrix can be isolated from the nuclear envelope. This procedure takes advantage of the observation that the intranuclear matrix is resistant to high salt extraction when intermolecular disulfide bonds are induced before nuclear matrix isolation (25). The reversible nature of this resistance (lability to reducing agents) formed the basis for the identification of a distinct class of nonhistone intranuclear matrix proteins (25). Using this fractionation procedure a major acidic nucleolar phosphoprotein, B-23, was identified as a prominent intranuclear matrix component (13). Furthermore, the use of sodium tetrathionate to introduce limited intramolecular disulfide bond cross-linking has been shown to be a useful approach in determining nearest neighbor relationships between intranuclear matrix polypeptides, including B-23 (13) and the glucocorticoid receptor (27).

In the present study, we have developed a polyclonal antiserum against a major, nonlamin intranuclear matrix polypeptide isolated from rat liver. This polypeptide has a relative molecular mass of 62,000 and pI of 6.8-7.2 as determined by two-dimensional IEF/SDS-PAGE. An immunologically related polypeptide with a relative molecular mass of 62 kD has been identified by immunobotting in rat spleen, kidney, and prostate nuclear matrix (data not shown) suggesting that this antigen is not an organ-specific protein but rather may play a general, structural role in nuclear organization.

We have used both indirect immunofluorescence and indirect immunoperoxidase staining to demonstrate the exclusive nuclear localization of the 62-kD polypeptide in HTC tissue culture cells and rat liver tissue sections respectively. By indirect immunofluorescence, this 62-kD antigen appears to be uniformly distributed throughout the nuclear interior with the notable exception of the nucleolus; the cytoplasm is devoid of staining (data not shown). With immunoperoxidase detection the antigen distribution is resolved into discrete patches which are readily observed throughout the nuclear interior but appear to be concentrated in the area immediately underlying the nuclear envelope (Fig. 4). There is no apparent staining of the nucleoli or the cytoplasm. During mitosis the antigen localizes to the condensed chromosomes

with no apparent staining of the cytoplasm. This distribution during mitosis is in contrast to that of the nuclear envelope lamin proteins which are redistributed uniformly throughout the cytoplasm during mitosis (17). Concomitant with this redistribution of the lamins during mitosis is an increase in the phosphorylation level of the individual lamin polypeptides (16, 35). Preliminary results indicate that the 62-kD polypeptide described in this study is also a phosphoprotein (data not shown). Current studies are focused on whether a similar reversible posttranslational modification cycle, possibly involving phosphorylation, may play a role in the redistribution of the 62-kD polypeptide during mitosis. Of interest in this regard are reports that both nuclear matrix and chromosome-associated proteins undergo cell cycle–dependent posttranslational modifications, including phosphorylation (21, 41) and ADP-ribosylation (22, 24).

Fractionation studies on chromosomes isolated from metaphase-blocked HTC cells indicate that the 62-kD polypeptide is a major component of metaphase chromosome scaffolds as defined by its resistance to extraction with DNAse I and 1.6 M NaCl. Interestingly the 62-kD polypeptide is resistant to high salt extraction of chromosomes even when isolated in the presence of iodoacetamide, whereas, in interphase nuclei, the 62-kD polypeptide is high salt soluble when disulfide bonding is prevented. It is possible that the 62-kD polypeptide may be associated with the chromosome scaffold by intermolecular disulfide bonds which are present in vivo. Alternatively, this association may be mediated by copper ion complexation between the 62-kD polypeptide and other chromosome scaffold polypeptides as has been suggested for other scaffold proteins (32).

These residual chromosome structures have been implicated in the maintenance of metaphase architecture by providing a proteinaceous framework upon which the highly condensed chromosomal DNA can be organized into supercoiled loops (2, 32). Suggestive evidence for the involvement of scaffold structures in the organization of metaphase DNA is the recent identification of DNA topoisomerase II as a major structural component of chromosome scaffolds (12, 15). The presence of the 62-kD polypeptide in scaffolds suggests that it may also function in maintaining metaphase morphology. Several laboratories have identified a subset of proteins, including species in the 60-kD range, which fractionate with both interphase nuclear matrices and metaphase chromosome scaffolds (1, 9, 18, 37); however, direct confirmation of their association with these structures will require immunocytochemical localization in situ to eliminate the possibility that they are cytoplasmic contaminants (for example, intermediate filament proteins [32]) which can nonspecifically associate with these structures during isolation. Similarly, Chaly et al. (6) have localized several nuclear matrix antigens during the cell cycle by indirect immunofluorescence. While some of these antigens localized to chromosomes during mitosis, fractionation studies are necessary to determine whether any of these polypeptides are chromosome scaffold components.

Immunoblotting studies indicate the presence in rat liver nuclear matrix of a 69-kD polypeptide which is immunologically related to the 62-kD antigen. The two forms of the antigen give similar peptide maps indicating their structural relatedness. Though the possibility remains that the 69-kD form of the antigen may arise due to a posttranslational modification of the 62-kD polypeptide, the shift in median pI is inconsistent with the more common posttranslational modifications of nuclear proteins (such as phosphorylation, ADP-ribosylation and glycosylation) all of which would be expected to give rise to a modified polypeptide with a more acidic pI. A precursor/product relationship between the two polypeptides seems more likely and this possibility is currently under investigation. Of interest in this regard are the recent reports that lamin A is synthesized as a higher molecular weight, more basic precursor (8, 31, 35; for alternative view see reference 30) which is subsequently proteolytically processed. A similar posttranslational processing of the nuclear lamin of *Drosophila* has also been described (40).

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References

1. Adolph, K. W. 1984. Conservation of interphase chromatin nonhistone antigens as components of metaphase chromosomes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 165:211–215.
2. Adolph, K. W., S. M. Cheng, and U. K. Laemmli. 1977. Role of nonhistone proteins in metaphase chromosome structure. *Cell.* 12:805–816.
3. Barrack, E. R., and D. S. Coffey. 1982. Biological properties of the nuclear matrix: steroid hormone binding. *Rec. Prog. Hormone Res.* 38:133–195.
4. Berrios, M., N. Osheroff, and P. A. Fisher. 1985. *In situ* localization of DNA topoisomerase II, a major polypeptide component of the *Drosophila* nuclear matrix fraction. *Proc. Natl. Acad. Sci. USA.* 82:4142–4146.
5. Burnette, W. N. 1981. "Western blotting": Electrophoretic transfer of proteins from sodum dodecylsulfate-polyacrylamide and detection with antibody and radio-iodinated Protein A. *Anal. Biochem.* 112:195–203.
6. Chaly, N., T. Bladon, J. Setterfield, J. E. Little, J. G. Kaplan, and D. L. Brown. 1984. Changes in distribution of nuclear matrix antigens during the mitotic cell cycle. *J. Cell Biol.* 99:661–671.
7. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecylsulfate and analysis by gel electrophoresis. *J. Biol. Chem.* 252:1102–1106.
8. Dagenais, A., V. Bibor-Hardy, J. F. Laiberte, A. Royal, and R. Simard. 1985. Detection in BHK cells of a precursor form for lamin A. *Exp. Cell Res.* 161:269–276.
9. Detke, S., and J. M. Keller. 1982. Comparison of the proteins present in HeLa cell interphase nucleoskeletons and metaphase chromosome scaffolds. *J. Biol. Chem.* 257:3905–3911.
10. Earnshaw, W. C., and M. M. S. Heck. 1985. Localization of topoisomerase II in mitotic chromosomes. *J. Cell Biol.* 100:1716–1725.
11. Earnshaw, W. C., and U. K. Laemmli. 1983. Architecture of metaphase chromosomes and chromosome scaffolds. *J. Cell Biol.* 96:84–93.
12. Earnshaw, W. C., B. Halligan, C. A. Cooke, M. M. S. Heck, and L. F. Liu. 1985. Topoisomerase II is a structural component of mitotic chromosome scaffolds. *J. Cell Biol.* 100:1706–1715.
13. Fields, A. P., S. H. Kaufmann, and J. H. Shaper. 1986. Analysis of the internal nuclear matrix: oligomers of a 38 kD nucleolar polypeptide stabilized by disulfide bonds. *Exp. Cell Res.* 164:139–153.
14. Fraker, P. J., and J. C. Speck, Jr. 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-Tetrachloro-3a,6a-Diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80:849–857.
15. Gasser, S. M., T. Laroche, J. Falquet, E. Boy de la Tour, and U. K. Laemmli. 1986. Metaphase chromosome structure: involvement of topoisomerse II. *J. Mol. Biol.* 188:613–629.
16. Gerace, L., and G. Blobel. 1980. The nuclear envelope lamina is reversibly depolymerized during mitosis. *Cell.* 19:277–287.
17. Gerace, L., A. Blum, and G. Blobel. 1978. Immunocytochemical localization of the major polypeptides of the nuclear pore complex-lamina fraction. *J. Cell Biol.* 79:546–566.
18. Gooderham, K., and P. Jeppesen. 1983. Chinese hamster metaphase chro-

mosomes isolated under physiological conditions: a partial characterization of associated nonhistone proteins and protein cores. *Exp. Cell Res.* 144:1–14.
19. Halligan, B. D., K. A. Edwards, and L. F. Liu. 1985. Purification and characterization of calf thymus DNA topoisomerase II. *J. Biol. Chem.* 260: 2475–2482.
20. Heide, K., and H. G. Schwick. 1973. Salt fractionation of immunoglobulins. *In* Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell, Oxford, Chapter 6.
21. Henry, S. M., and L. D. Hodge. 1983. Nuclear matrix: a cell cycle-dependent site of increased intranuclear protein phosphorylation. *Eur. J. Biochem.* 133:23–29.
22. Holtlund, J., T. Kristensen, A. C. Ostvold, and S. G. Laland. 1983. ADP-ribosylation in permeable HeLa S3 cells. *Eur. J. Biochem.* 130:47–51.
23. Hunkapiller, M. W., E. Lujan, F. Ostrander, and L. E. Hood. 1983. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. *Meth. Enzymol.* 91:227–235.
24. Kanai, Y., S. Tanuma, and T. Sugimura. 1981. Immunofluorescent staining of poly(ADP-ribose) *in situ* in HeLa cell chromosomes in the M phase. *Proc. Natl. Acad. Sci. USA.* 78:2801–2804.
25. Kaufmann, S. H., and J. H. Shaper. 1984. A subset of nonhistone intranuclear proteins reversibly stabilized by the sulfhydryl crosslinking reagent tetrathionate: fractionation of the nuclear matrix. *Exp. Cell Res.* 155:477–495.
26. Kaufmann, S. H., A. P. Fields, and J. H. Shaper. 1986. The nuclear matrix: current concepts and unanswered questions. In *Meth. Achiev. Exp. Pathol. G.* 12:141–171.
27. Kaufmann, S. H., S. Okret, A-C. Wikstrom, J-A. Gustafsson, and J. H. Shaper. 1986. Binding of the glucocorticoid receptor to the rat liver nuclear matrix: the role of disulfide bond formation. *J. Biol. Chem.* 261: 11962–11967.
28. Kaufmann, S. H., W. Gibson, and J. H. Shaper. 1983. Characterization of the major polypeptides of the rat liver nuclear envelope. *J. Biol. Chem.* 258:2710–2717.
29. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
30. Lebel, S., and Y. Raymond. 1987. Lamin A is not synthesized as a larger precursor polypeptide. *Biochem. Biophys. Res. Commun.* 149:417–423.
31. Lehner, C. F., G. Fursteinberger, H. M. Eppenberger, and E. A. Nigg. 1986. Biogenesis of the nuclear lamina: *in vivo* synthesis and processing of nuclear protein precursors. *Proc. Natl. Acad. Sci. USA.* 83:2096–2099.
32. Lewis, C. D., and U. K. Laemmli. 1982. Higher order metaphase chromosome structure: evidence for metalloprotein interactions. *Cell.* 29:171–181.
33. Marsden, M. P. F., and U. K. Laemmli. 1979. Metaphase chromosome structure: evidence for a radial loop model. *Cell.* 17:849–858.
34. Morrissey, J. H. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* 117:307–310.
35. Ottaviano, Y., and L. Gerace. 1985. Phosphorylation of the nuclear lamins during interphase and mitosis. *J. Biol. Chem.* 260:624–632.
36. Paulson, J. R., and U. K. Laemmli. 1977. The structure of histone-depleted metaphase chromosomes. *Cell.* 12:817–828.
37. Pieck, A. C. M., H. M. W. vander Velden, A. A. M. Rijken, J. M. Neis, and F. Wanka. 1985. Protein composition of the chromosomal scaffold and interphase nuclear matrix. *Chromosoma.* 91:137–144.
38. Pogo, A. D., and R. Procyk. 1985. Nonhistone proteins and nuclear matrix structures. *In* Progress in nonhistone protein research. I. Bekhor, editor. CRC Press, Boca Raton, Florida. 2:131–150.
39. Razin, S. V., V. V. Chernokhvostov, O. V. Yarovaya, and G. P. Georgiev. 1985. Organization of the sites of DNA attachment to the nonhistone proteinaceous nuclear skeleton. In: Progess in nonhistone protein research. I. Bekhor, editor. CRC Press, Boca Raton, Florida. 2:91–114.
40. Smith, D. E., Y. Gruenbaum, M. Berrios, and P. A. Fisher. 1987. Biosynthesis and interconversion of *Drosophila* nuclear lamin isoforms during normal growth and in response to heat shock. *J. Cell Biol.* 105:771–790.
41. Song, M. K. H., and K. W. Adolph. 1983. Phosphorylation of nonhistone proteins during the HeLa cell cycle: relationship to DNA synthesis and mitotic chromosome condensation. *J. Biol. Chem.* 258:3309–3318.