Stepwise Reassembly of the Nuclear Envelope at the End of Mitosis

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Abstract. The nuclear envelope consists of three distinct membrane domains: the outer membrane with the bound ribosomes, the inner membrane with the bound lamina, and the pore membrane with the bound pore complexes. Using biochemical and morphological methods, we observed that the nuclear membranes of HeLa cells undergoing mitosis are disassembled in a domain-specific manner, i.e., integral membrane proteins representing the inner nuclear membrane (the lamin B receptor) and the nuclear pore membrane (gp210) are segregated into different populations of mitotic vesicles. At the completion of mitosis, the inner nuclear membrane-derived vesicles associate with chromatin first, beginning in anaphase, whereas the pore membranes and the lamina assemble later, during telophase and cytokinesis. Our data suggest that the ordered reassembly of the nuclear envelope is triggered by the early attachment of inner nuclear membrane-derived vesicles to the chromatin.

BASED on ultrastructural and biochemical evidence, at least three domains are distinguishable within the eukaryotic interphase nuclear envelope, and specific proteins have been found to be associated with each of these domains. The outer nuclear membrane domain is continuous with the RER and contains resident proteins characteristic of the RER, such as cytochrome p450 (Matsuura et al., 1981). The inner nuclear membrane domain is tightly associated with the nuclear lamina, a fibrous proteinaceous meshwork located between the membrane and chromatin (Aaronson and Blobel, 1974; Dwyer and Blobel, 1976). The major proteins of the nuclear lamina of mammalian cells are lamins A (M_r 70 kD), B (M_r 67 kD), and C (M_r 60 kD) (Gerace et al., 1978), all of which share structural homology with intermediate filament proteins (Fisher et al., 1986; McKeon et al., 1986; Höger et al., 1988; Pollard et al., 1990). Other variants of lamins have also been described (Nigg, 1989; Höger et al., 1991b). A specific integral membrane protein of the inner nuclear membrane, called the lamin B receptor, has been proposed to serve in anchoring the nuclear lamina to the membrane bilayer (Gerace and Blobel, 1982; Worman et al., 1988). Based on a chicken cDNA derived sequence, the lamin B receptor is a 73-kD protein with eight potential transmembrane domains, but the avian protein and its mammalian homolog migrate as ∼60-kD proteins on SDS-gels (Worman et al., 1990; Courvalin et al., 1990c). The pore membrane forms at the junction between the inner and the outer nuclear membranes and is as-

sociated with the pore complex. The nuclear pore complex is a large supramolecular assembly of ∼120 × 10⁶ D (Reichelt et al., 1990) through which the regulated nucleocytoplasmic exchange of macromolecules takes place (reviewed in Miller et al., 1991). A 210-kD integral membrane glycoprotein (gp210) has been shown to be a resident protein of the nuclear pore complex (Gerace et al., 1982; Wozniak et al., 1989; Courvalin et al., 1990b). The COOH-terminal tail of gp210 faces the pore complex and is postulated to anchor the pore complex to the membrane.

The nuclear envelope of mammalian cells undergoes dramatic structural changes during cell division. During prophase and metaphase of mitosis, the nuclear membranes vesiculate and many peripheral proteins associated with the nuclear membranes become dissociated and disperse in the cytoplasm (Robbins and Gonatas, 1964; Zatsepina et al., 1977; Zeligs and Wollman, 1979; Gerace et al., 1978, 1982; Gerace and Blobel, 1980; Davis and Blobel, 1986). Disassembled components of the nuclear membranes are morphologically indistinguishable from most other membranes in the mitotic cell. The lamins become phosphorylated and the lamina depolymerizes into soluble (lamins A/C) and membrane-associated (lamin B) forms (Gerace and Blobel, 1980). Components of the nuclear pore complex also disassemble and are dispersed throughout the cytoplasm (Gerace et al., 1982; Davis and Blobel, 1986; Snow et al., 1987). As mitosis nears completion, the preexisting pools of nuclear envelope precursors are used in the process of nuclear envelope reassembly (Conner et al., 1980). Membrane vesicles bind to the surface of daughter chromosomes and fuse into flat cisternae (Zeligs and Wollman, 1979), the pore complexes reassemble (Maul, 1977) and the lamina repolymerizes (Gerace et al., 1978).

Nuclear envelope reformation has been studied in cell-free systems derived from either mammalian somatic cells or amphibian eggs. From these studies, two models of nuclear envelope reassembly have been proposed (reviewed in Lohka, 1988). In the first model, the early binding to chromosomes of peripheral proteins of the nuclear envelope, such as lamins (Burke and Gerace, 1986; Glass and Gerace, 1990; Burke, 1990; Höger et al., 1991a; Ulitzur et al., 1992) or pore components (Sheehan et al., 1988), is a prerequisite for the subsequent binding of membrane vesicles to chromatin. In the second model, a lamin-independent targeting of membrane vesicles to chromosomes has been proposed as the initial event of nuclear envelope reconstruction (Wilson and Newport, 1988; Newport et al., 1990; Benavente et al., 1989), after which the pore complexes and the lamina reassemble. A major difficulty in reconciling the data of cell-free experiments originates in the fact that a detailed chronology of the in vivo nuclear envelope assembly which accounts for the lamina, the pore complex and the membrane vesicles has not been described.

In this study, we have used specific antibody probes against marker proteins of the different nuclear membrane domains to investigate, by biochemical and morphological methods, the fate of the nuclear envelope in dividing HeLa cells. We have observed that, in vivo, the reconstruction of the nuclear envelope at the completion of mitosis appears to occur by the sequential targeting of envelope components to the chromatin surface, beginning with the attachment of vesicles that contain the lamin B receptor, an inner membrane protein.

Materials and Methods

Cells and Cell Cycle Analysis

HeLa cells were grown in spinner culture or in monolayers as described (Dignam et al., 1983). Cells grown in spinner culture at 37°C were fractionated into populations enriched in different phases of the cell cycle (G1, S, and G2/M) by centrifugal elutriation (Roberts et al., 1991). To obtain a synchronized population of cells arrested in the mitotic phase, the cells at S phase were incubated with 60 ng/ml nocodazole (Aldrich Chemical Co., St. Louis, MO) (Zieve et al., 1980). After a 12-h incubation, at least 90% of the cells were arrested at the metaphase stage of mitosis, as determined by staining of cells with the DNA-binding dye Hoechst-33258 (Molecular Probes, Inc., Eugene, OR) and examining the samples with a Zeiss Axiophot microscope equipped for fluorescence microscopy (Carl Zeiss Inc., Thornwood, NY).

Subcellular Fractionation

The mitotic cells were collected by centrifugation at 1,500 g_{av} for 10 min, at room temperature and then resuspended in ice-cold PBS containing 20 μM cytochalasin B (Sigma Chemical Co., St. Louis, MO) for 20 min on ice (Burke and Gerace, 1986). All subsequent fractionation and centrifugation steps were carried out at 0–4°C unless otherwise specified. Cells were centrifuged again at 1,500 g_{av} and then resuspended (5 ml/10⁹ cells) in hypotonic buffer (buffer A) composed of 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM DTT, 20 μM cytochalasin B, protease inhibitor mixture (Aris and Blobel, 1989), 2.5 mM sodium pyrophosphate, 0.1 mM orthovanadate, 2 mM sodium fluoride and 5 μM aluminum ammonium sulfate. The cells were allowed to swell for 15 min and then homogenized 10–15 times with a Dounce "B" homogenizer (Wheaton, Millville, NJ). The homogenate was layered above a 3 ml cushion of 30% sucrose (wt/vol) in buffer A and spun at 2,000 g_{av} for 10 min. The supernatant containing the membranes and soluble components of cells was removed, leaving the pellet containing the chromosomes of mitotic cells, the nuclei of nonmitotic cells, and remnants of partially homogenized cells. Centrifugation of the supernatant at 140,000 g_{av} for 30 min in a fixed angle roto yielded a pellet containing mitotic membranes and supernatant containing the soluble components of mitotic cells. Subcellular extracts were either used immediately, or frozen in liquid nitrogen and stored at −80°C.

Sucrose Gradient Centrifugation

The soluble components of mitotic cells were separated on a sucrose velocity gradient. A 0.4-ml aliquot of 140,000 g_{av} supernatant (derived from ~8 × 10⁷ cells) was layered above a 5–20% (wt/vol) sucrose gradient (12 ml) made in 15 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, and 0.5 mM PMSF. The tube was centrifuged at 180,000 g_{av} for 16 h in a swinging-bucket rotor and then 12 fractions were collected from the top using a Buchler fraction collector (Labconco, Lenexa, KS). Proteins in each fraction were precipitated by the addition of TCA (to 15%) and incubation on ice for 10 min. The precipitate was collected by centrifugation at 15,000 g_{av} for 10 min, resuspended in SDS-sample buffer and analyzed by SDS-PAGE and immunoblotting as previously described (Courvalin et al., 1990a). Density markers for sedimentation gradients (bovine erythrocyte carbonic anhydrase, 3.2S; BSA, 4.2 S; sweet potato beta amylase, 8.9 S) were centrifuged on separate but identical gradients, and their contents were fractionated and processed as described above.

The membrane-bound components of the mitotic cells were separated by a sucrose step-gradient. The 2,000 g_{av} supernatant from mitotic cells (see above) was adjusted to 50% sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, plus the same protease and phosphatase inhibitors as in buffer A, the sample was transferred to a swinging bucket ultracentrifuge tube (Beckman SW40), overlaid successively with 3 ml of 35% sucrose in buffer B (10 mM Tris-HCl, pH 7.5, 10 mM NaCl), 3 ml 20% sucrose in buffer B, and filled to the top with 10% sucrose in buffer B. The tube was centrifuged for 16 h at 140,000 g_{av}, after which bands of membranes could be seen at the 35–50% interface (heavy membranes) and 20–35% interface (light membranes), with a negligible amount at the 10–20% interface. The gradient contents were fractionated using a syringe with 18 gauge needle so that the material at each interface could be collected in a single tube. The sucrose concentration was adjusted to ~15% with buffer B and the membranes were collected by centrifugation at 200,000 g_{av} for 30 min. The membrane pellets were resuspended in SDS-sample buffer, and proteins resolved on SDS-polyacrylamide gels followed by immunoblotting with antibodies against the various nuclear envelope proteins (see figure legend).

Antibodies

Human autoimmune antibodies against lamin B, the lamin B receptor, and the nuclear pore protein gp210 have been previously described (Guilly et al., 1987; Courvalin et al., 1990b,c). These antibodies were affinity purified
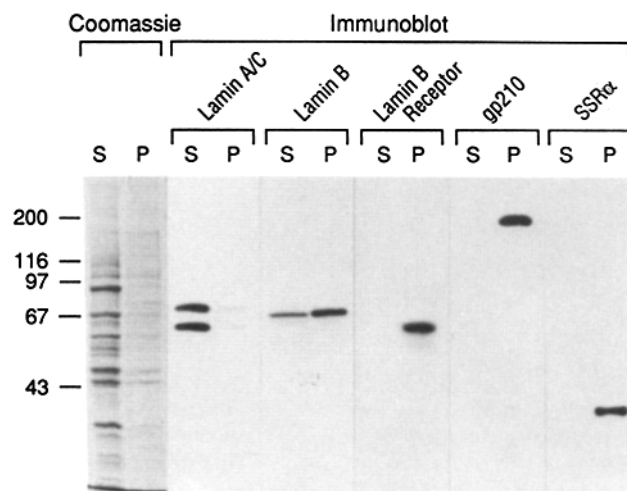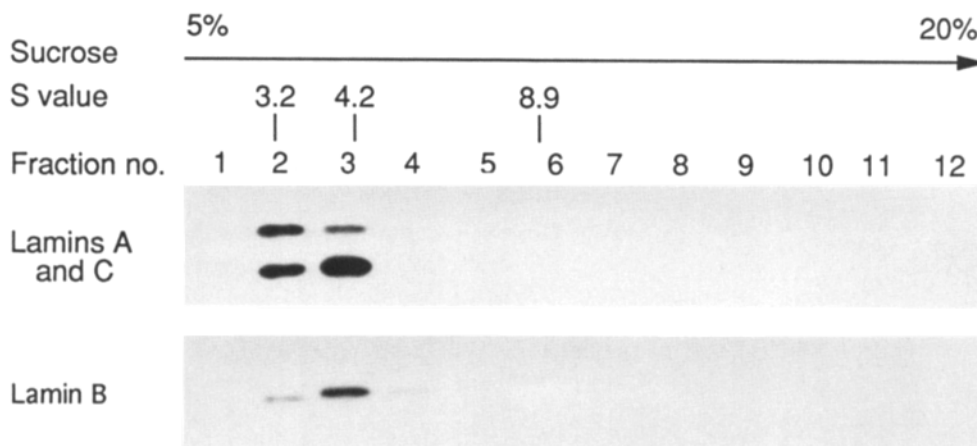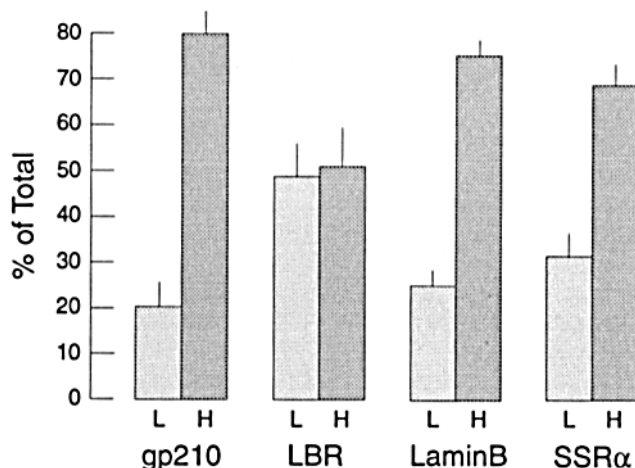

Figure 1. The distribution of nuclear envelope proteins in extracts of mitotic HeLa cells. Postchromosomal supernatant from cells arrested in metaphase was centrifuged at 140,000 g_{av}, and proteins in the resulting supernatant (S) and pellet (P) fractions were analyzed by SDS-PAGE and immunoblotting. Coomassie blue stained gel of proteins in the S and P fractions (derived from ~10⁵ mitotic cells). The size of molecular mass markers ($\times 10^{-3}$) are indicated at left. Separate blots of S and P fractions were probed with antibodies against lamins A/C, lamin B, lamin B receptor, gp210, and the α subunit of the signal sequence receptor (*SSRα*), followed by incubation with ^{125}I-protein A and autoradiography.

A

B

Figure 2. Sucrose gradient analysis of nuclear envelope proteins in mitotic HeLa cells. (*A*) Sedimentation analysis of nuclear lamins in soluble mitotic extracts. The 140,000 g_{av} supernatant of mitotic cells was layered on a 5–20% sucrose gradient and centrifuged for 16 h at 180,000 g_{av}. Twelve fractions were collected (fractions 1–12 from top to bottom) and the proteins in each fraction were analyzed by immunoblotting with anti-lamin antibodies, followed by autoradiography (see Materials and Methods). Density marker proteins were centrifuged on parallel gradients, fractionated similarly, and the proteins in each fraction were analyzed by SDS-PAGE, followed by staining with Coomassie blue (gel not shown). (*B*) Sucrose gradient analysis of nuclear envelope proteins that are membrane associated during mitosis. Mitotic membranes were separated into 'heavy' (*H*; membranes at the 35–50% interface) and 'light' (*L*; membranes at the 20–35% interface) fractions by flotation on sucrose step-gradient (see Materials and Methods), and the proteins in the two membrane fractions were analyzed by immunoblotting. The blot was probed, sequentially, with antibodies against the lamin B receptor (*LBR*), gp210, lamin B, and *SSRα*, and finally with ^{125}I-protein A. The bound radioactivity was quantified and used to determine the percentage of each protein in 'H' or 'L' membranes (shown as histograms). The data are the average of three measurements, with the SEM being <10% of the indicated amount.

by the method of Smith and Fisher (1984). Antibodies against synthetic peptides derived from the deduced amino acid sequence of human lamins A and C (GSVTKKRKLESTESC; Fisher et al., 1986; McKeon et al., 1986) or lamin B (TTRGKRKRVDVEESC; Höger et al., 1988; Pollard et al., 1990) were generated in rabbits and then affinity purified, essentially as described (Chaudhary et al., 1991). The cysteine at the carboxyl terminus was added to the above peptides for purposes not discussed here. An affinity purified antipeptide antibody against the α subunit of the signal sequence receptor, a 35-kD integral membrane protein of the ER (Wiedmann et al., 1987; Migliaccio et al., 1992), was a gift of Drs. Giovanni Migliaccio and Christopher Nicchitta (Rockefeller University, New York). mAb against β-tubulin (clone TUB 2.1) was obtained from Sigma Chemical Co. Fluorochrome-conjugated antibodies were used for indirect immunofluorescence studies. FITC-conjugated goat anti-human antibody and Texas red-conjugated goat anti-rabbit were from Vector Laboratories Inc. (Burlingame, CA), Texas red-conjugated goat anti-mouse was from Molecular Probes, Inc., and FITC-conjugated goat anti-rabbit antibodies were from Cappel Laboratories (Organon Teknika Corp., West Chester, PA).

Immunoblots

Proteins were separated by SDS-PAGE on 10% polyacrylamide gels and then electrophoretically transferred from gels to nitrocellulose (Schleicher and Schuell, Keene, NH) as previously described (Courvalin et al., 1990a). Nitrocellulose blots were probed with the various antibodies followed by ^{125}I-protein A (New England Nuclear, Wilmington, DE), essentially as described (Courvalin et al., 1990a). The amount of radioactivity bound to nitrocellulose was quantified using the Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Immunofluorescence

Monolayer cultures of HeLa cells were grown on coverslips and then processed for indirect immunofluorescence studies as described (Chaudhary et al., 1991). Identical results were obtained with paraformaldehyde fixation (Davis and Blobel, 1986) or methanol fixation at −20°C (Courvalin et al., 1990a) and only the observations made on methanol-fixed cells are reported. Briefly, the fixed cells were probed either with antibodies against a single protein, or with a mixture of antibodies directed against two different proteins (see appropriate figure legends). For indirect immunofluorescence imaging, an appropriate mix of fluorescein isothiocyanate-conjugated or Texas red-conjugated second antibodies were used to visualize the primary antibodies. The dye Hoechst-33258 (Molecular Probes, Inc.) was added at a concentration of 0.5 μg/ml to the second antibody solution to visualize the DNA. Control experiments showed that in double im-
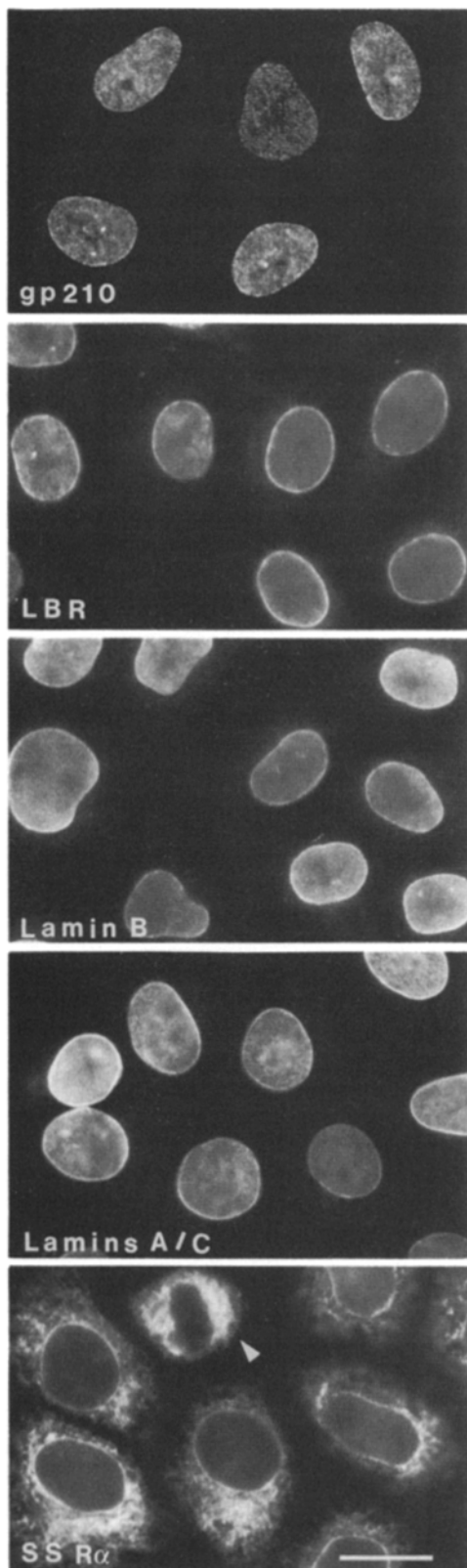
Figure 3. Indirect immunofluorescence analysis of HeLa cells probed with antibodies against different nuclear envelope proteins. HeLa cells were probed with antibodies against the nuclear envelope proteins gp210, lamin B receptor, lamin B, lamins A/C, and the signal sequence receptor (*SSRα*). All antibodies reacted with proteins located at the nuclear periphery. Note that the pore component gp210 had a distinctly punctate localization. SSRα is found in the nuclear envelope as well as the ER. Arrowhead points to a mitotic cell. Bar, 10 μm.

Results

Biochemical Analysis of Nuclear Envelope Components in Mitotic HeLa Cell Extracts

Mitotic HeLa cells arrested in metaphase were obtained by nocodazole treatment of cells in spinner culture. Gentle homogenization of mitotic cells followed by low speed centrifugation (to remove chromosomes) yielded a cell extract containing both the membrane-bound and soluble nuclear envelope components. The post-chromosomal supernatant was recentrifuged to separate membranes from cytosol. Proteins in the two fractions were separated by SDS-PAGE and either stained with Coomassie blue or transferred to nitrocellulose sheets for immunodetection of the components of the nuclear envelope (Fig. 1). Replicate blots of soluble versus insoluble proteins were then incubated separately with each antibody probe. Lamins A and C, which are peripheral proteins, were found almost entirely in the mitotic cytosol fraction, whereas lamin B, also a peripheral protein, was detected in both the membrane and soluble fractions (Fig. 1). When the mitotic cytosol (140,000 g supernatant) was further fractionated on a velocity sucrose gradient, the three lamins were detected in the ∼4S fraction (Fig. 2 *A*).

The integral membrane proteins gp210, lamin B receptor, and signal sequence receptor, representing the three domains of the nuclear envelope, were all found in the membrane fraction of mitotic cell extracts (Fig. 1). The mitotic membranes were further fractionated by a sucrose density step-gradient into 'light' and 'heavy' membranes (see 'Methods'). Immunoblot analysis followed by quantitation of the bound radioactivity (from ^{125}I-protein A bound to the primary antibody) showed that the marker for the inner nuclear membrane (lamin B receptor) was almost evenly distributed between the two membrane fractions, whereas the marker for the nuclear pore membrane (gp210) and the marker for the ER (SSRα) were enriched in high density membranes (Fig. 2 *B*). These fractionation data suggested that the integral membrane proteins lamin B receptor and gp210 might segregate into different sets of mitotic vesicles. In addition, the results indicated that the membrane-bound mitotic lamin B was enriched in the heavy membrane fraction, i.e., in association with a different set of membranes than its putative receptor.

Nuclear Membrane Reassembly at the End of Mitosis

Immunofluorescence experiments were carried out in HeLa cells in order to document, in vivo, the localization of the nuclear envelope-associated marker proteins during mitosis. Monolayer cultures of unsynchronized cells were labeled with affinity purified antibodies against the lamins A/C and B, the lamin B receptor, gp210, and SSRα, and then scanned for cells fixed at the various stages of the cell cycle. The stage of mitosis was deduced by monitoring (*a*) the shape of the cell by phase contrast microscopy (Mazia, 1961), (*b*) the degree of separation of the two sets of daughter chromosome visualized by counterstaining with Hoechst dye, and (*c*) the

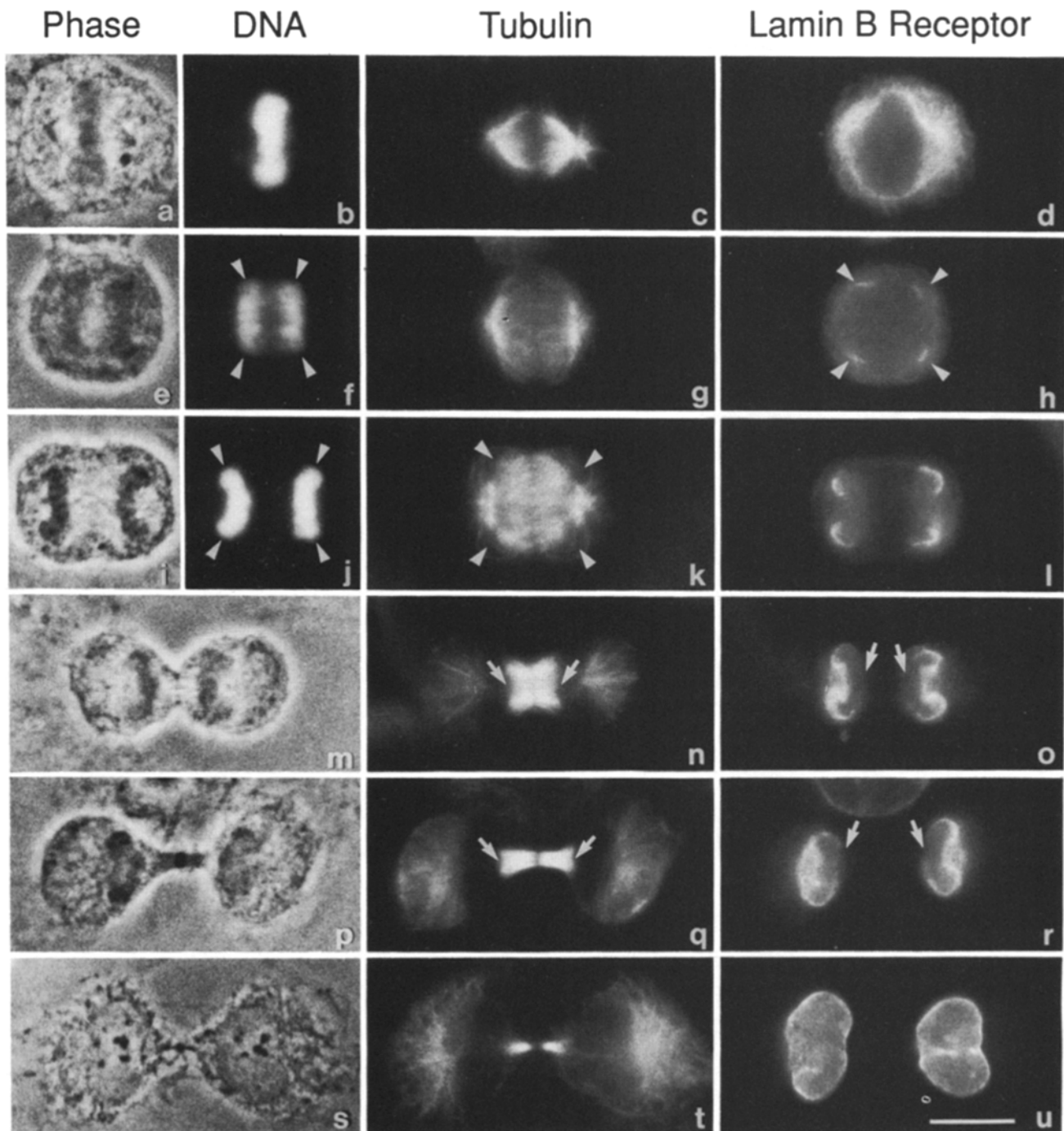| Phase	DNA	Tubulin	Lamin B Receptor

Figure 4. Mitotic distribution of the lamin B receptor. Immunofluorescence of HeLa cells labeled with anti-lamin B receptor antibodies, a mouse anti-tubulin mAb, and the DNA-binding dye Hoechst-33258. The pictures are arranged to show the progression of mitosis from metaphase to cytokinesis (from top to bottom). (*a–d*) metaphase; (*e–h*) anaphase-A; arrowheads in *h* point to the regions where the lamin B receptor-containing membranes reassemble. Arrowheads in *f* point to the corresponding chromatin areas. (*i–l*) Late anaphase-early telophase; arrowheads in *j* point to the chromatin surfaces where the binding of lamin B receptor-containing membranes observed in *l* takes place. Arrowheads in *k* point to the corresponding areas of the mitotic apparatus. (*m–o*) Late telophase; arrows in *n* and *o* point to the areas of chromatin adjacent to densely packed microtubules that are devoid of lamin B receptor-containing membranes. (*p–r*) Early cytokinesis; arrows in *q* and *r* indicate the same phenomenon as observed in *m–o*. (*s–u*) late cytokinesis. Bar, 10 µm.

shape of the mitotic apparatus by double-labeling with an-titubulin antibodies.

The immunofluorescence staining patterns of interphase cells labeled with antibodies against nuclear envelope proteins are shown in Fig. 3. The anti-gp210 antibodies specifically reacted with the nuclear pore and gave the punctate staining of the nuclear surface characteristic of nuclear

pore components (Fig. 3, *top*; Davis and Blobel, 1986; Courvalin et al., 1990*b*). In addition, each of the antibodies stained the nuclear rim exclusively (Fig. 3, *top four panels*), with the anticipated exception of antisignal sequence receptor, which bound to the cytoplasmic network of the ER along with the outer nuclear membrane (Fig. 3, *bottom*). The strong cytoplasmic staining observed with anti-SSRα made
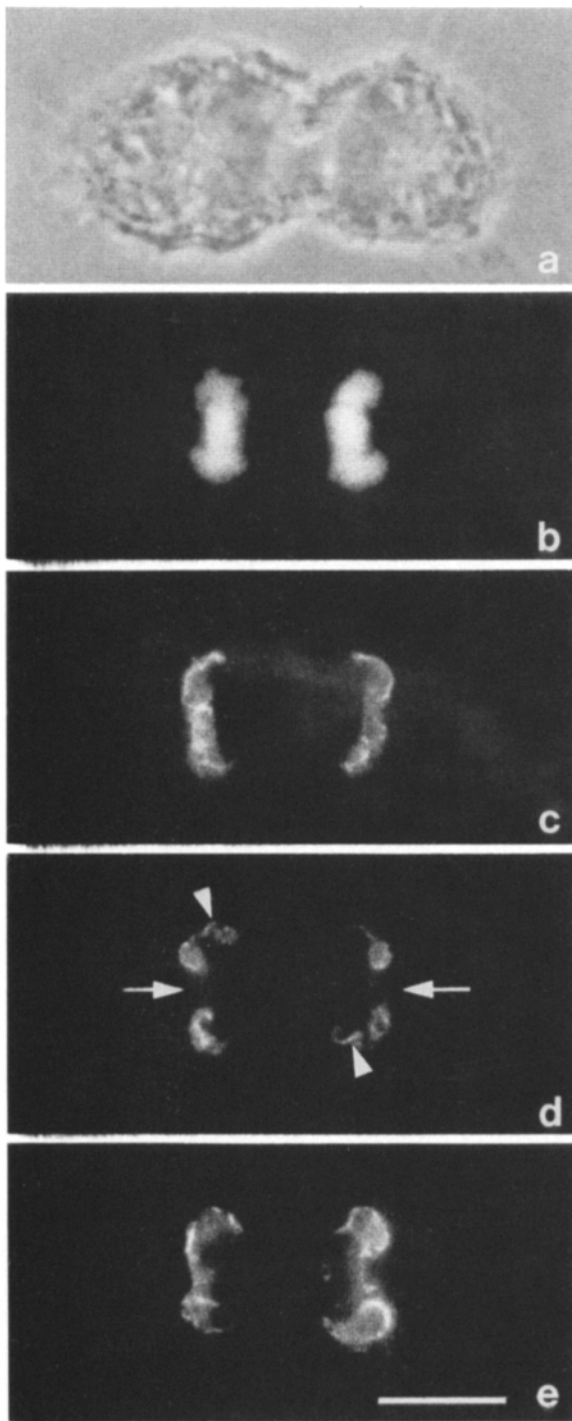
Figure 5. The discontinuous attachment of lamin B receptor-containing membranes to chromosomes. The pictures show views of three different focal planes of a HeLa cell in early telophase, stained with anti-lamin B receptor antibodies. (*a*) Phase contrast. (*b*) DNA staining with Hoechst 33258 dye. (*c-e*) Immunofluorescence localization of lamin B receptor in a dividing cell, as observed in the upper, middle, and lower focal planes, respectively. Arrowheads in *d* point to areas where the contour of individual chromosomes is still visible. Arrows in *d* point to the postulated location of kinetochores. Bar, 10 µm.

it difficult to visualize the reformation of the nuclear envelope at the end of mitosis, thus this antibody was not used in the immunofluorescence studies of mitotic cells.

In Fig. 4, the sequence of events associated with postmitotic reassembly of the lamin B receptor around chromosomes is shown. The pictures are arranged to show the progression of mitosis from metaphase (*upper*) to late cytokinesis (*bottom*). At metaphase (Fig. 4, *a-d*) antibodies to the lamin B receptor only reacted with structures in the cytoplasm, outside the spindle region (Fig. 4 *d*).

The first association of the lamin B receptor-containing membranes with chromatin was detected during anaphase-A (Fig. 4, *e-h*, arrowheads in *h*), although most of the lamin B receptor-containing membranes still remained in the cytoplasm. In late anaphase and early telophase (Fig. 4, *i-l*), the binding of lamin B receptor-containing membranes to chromatin continued to extend from the lateral surfaces and covered progressively greater areas of the chromosome mass (Fig. 4, *l*). Initially, these membranes covered only a small region of the lateral chromatin surface (the regions radially furthest from the pole-to-pole axis; Fig. 4, arrowheads in *f* and *j*). The labeling was absent from central chromatin surfaces (i.e., outer regions of daughter chromosomes nearest to the poles). Labeling was also absent from the chromatin surfaces that face each other (i.e., regions near the center of the mitotic apparatus). In late telophase (Fig. 4, *n-o*), and early cytokinesis (Fig. 4, *p-r*), those same chromatin surfaces were the only ones not yet coated by lamin B receptor-containing membranes. These regions were finally covered by the lamin B receptor-containing membranes during cytokinesis (Fig. 4, *s-u*), when the daughter cells had separated and formed a decondensed nucleus. No discernible pool of the lamin B receptor-containing membranes remained in the cytoplasm at the end of cytokinesis. By juxtaposing the tubulin labeling with the anti-lamin B receptor labeling, it was evident that the chromosomal surfaces covered by lamin B receptor-containing membranes coincided with regions containing a low density of microtubules. This was most noticeable during telophase and cytokinesis but also observable at earlier stages of mitosis (Fig. 4, compare the labeling in *l* with corresponding regions of the mitotic apparatus indicated by arrowhead in *k*). In contrast, the chromatin surfaces devoid of membranes were adjacent to the densely packed microtubules (Fig. 4, compare tubulin distribution in *n* and *q* with chromatin-bound lamin B receptor in *o* and *r*, arrows).

The images shown in Fig. 5 provide more detail about the three-dimensional location of the chromosomal sites that bind to lamin B receptor-containing membranes. A cell in early telophase (Fig. 5, *a* and *b*) was stained with lamin B receptor antibodies and pictures were taken in three different focal planes (Fig. 5, *c-e*). First, the figure shows that the association of lamin B receptor vesicles with chromatin surfaces begins whereas the chromosomes are still distinct and not fused into a chromatin mass (Fig. 5 *d*, arrowheads, the contours of some chromosomes are still visible). Second, there was a total absence of lamin B receptor-membranes from the inner chromosomal surfaces (regions facing the center of the dividing cell, and facing each other; Fig. 5, *c-e*). Finally, the outer chromosomal surfaces were intensely stained in all sections, with the marked exception of a central area likely to correspond to the sites of insertion of microtubules onto kinetochores (Fig. 5 *d*, arrows).
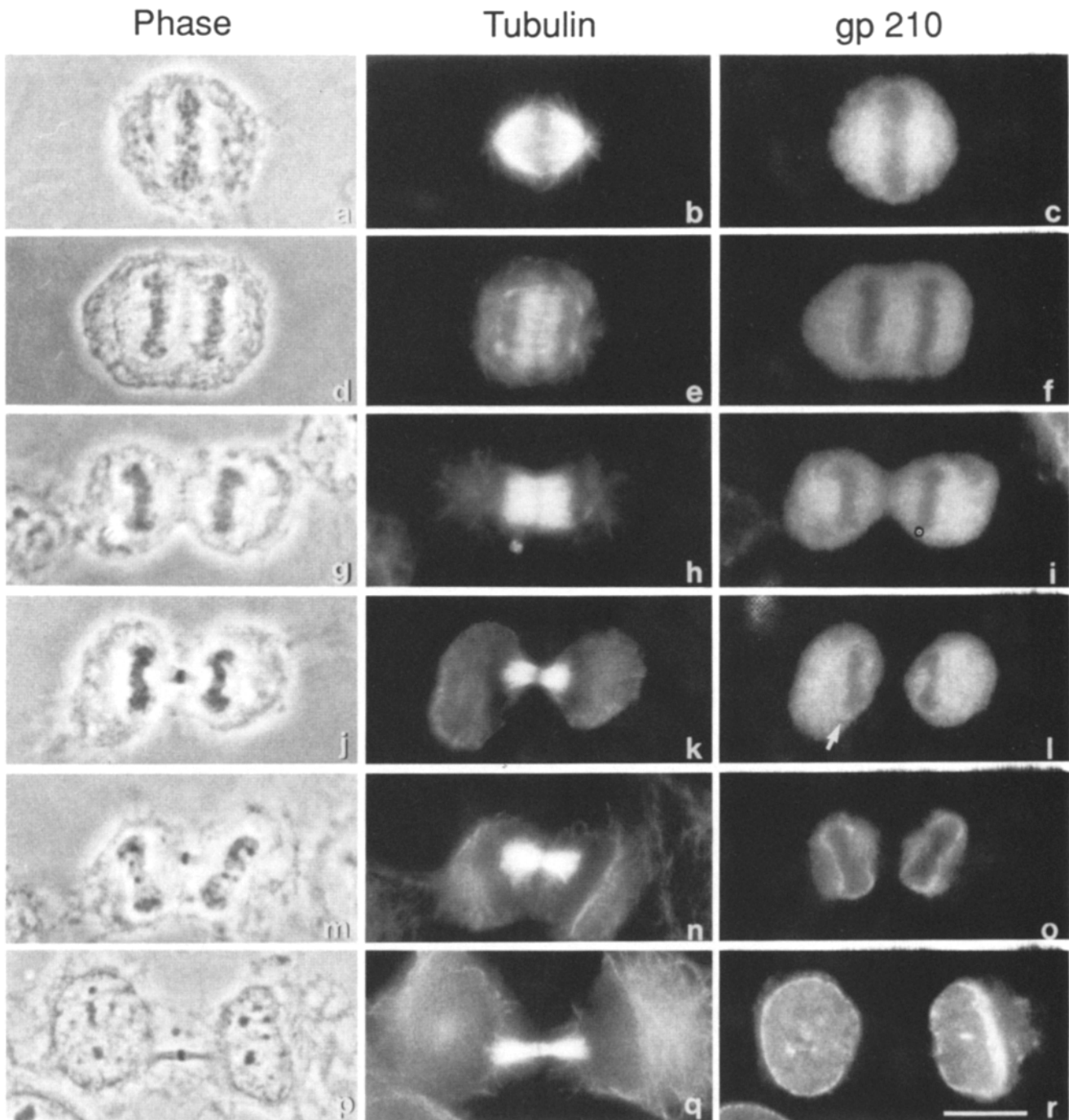
Phase   Tubulin   gp 210

Figure 6. Mitotic distribution of the nuclear pore membrane glycoprotein gp210 in HeLa cells. The experiment is similar to that in Fig. 4 except that anti-gp210 antibodies were used instead of anti-lamin B receptor antibodies. Cells are shown in metaphase (*a–c*), anaphase (*d–f*), telophase (*g–i*), late telophase (*j–l*), early cytokinesis (*m–o*), and late cytokinesis (*p–r*), respectively. The earliest association of gp210 with the outer chromatin surface is discernible during late telophase (*arrow in l*), and much of the remaining cytoplasmic gp210 is targeted to the nuclear envelope during cytokinesis. Bar, 10 μm.

The localization of gp210-containing membranes, observed by indirect immunofluorescence staining of mitotic cells, is shown in Fig. 6. The labeling of metaphase cells (Fig. 6, *a–c*) with the gp210 antibodies was entirely cytoplasmic, and in contrast to the labeling with anti-lamin B receptor (Fig. 4 *d*), was not excluded from the spindle (Fig. 6 *c*). As mitosis progressed into anaphase (Fig. 6, *d–f*) and

telophase (Fig. 6, *g–i*), the antibody labeling remained cytoplasmic. The earliest labeling of the chromatin surface was observed during late telophase (Fig. 6, *j–l*, *arrow*). An abrupt increase in membrane binding took place during cytokinesis, as the nuclei began to decondense and nucleoli reappeared (Fig. 6, *m–o*). However, a significant amount of cytoplasmic staining was present even at this late stage of di-
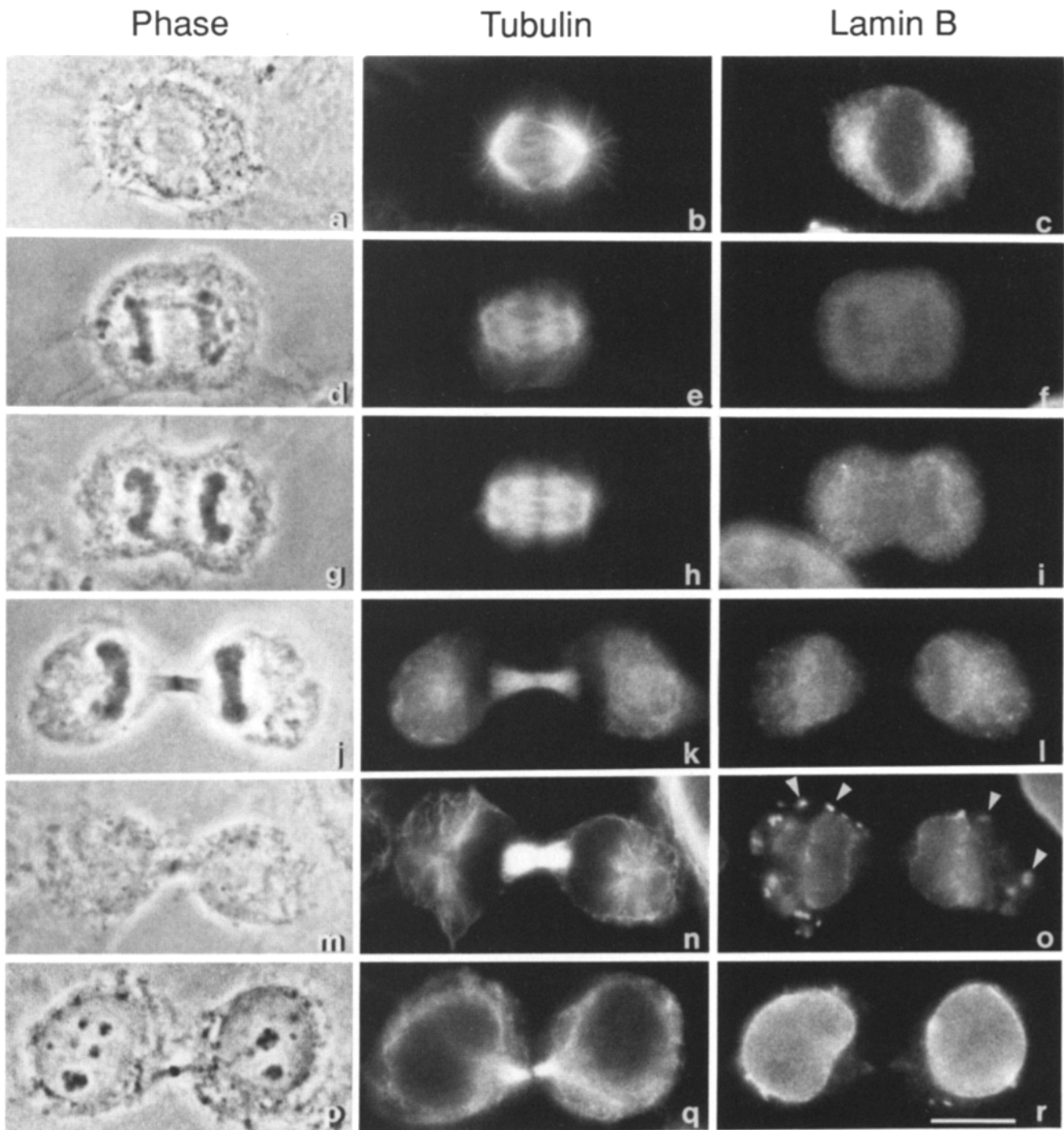
Phase	Tubulin	Lamin B

Figure 7. Mitotic distribution of lamin B in HeLa cells. The experiment is similar to that in Fig. 4, except that human anti-lamin B antibodies were used as probes. Cells are shown in metaphase (*a–c*), anaphase (*d–f*), early telophase (*g–i*), late telophase (*j–l*), cytokinesis (*m–o*), and early G1 (*p–r*), respectively. Note that the bulk of the mitotic lamins remains cytoplasmic until telophase, when the protein begins to associate with the chromosomal surface. Arrowheads in *o* point to some of the numerous lamin-containing deposits that are present during cytokinesis and early G1. Bar, 10 μm.

vision. The remaining gp210-containing membranes were reattached to the nascent nucleus as it acquired the interphase structure (Fig. 6, *p–r*).

Reassembly of the Nuclear Lamina

To compare the time course of lamin reassembly to that of the nuclear membrane, an immunofluorescence study of nuclear lamina reformation was also carried out. The time course of reassembly of lamin B (Fig. 7) and lamins A/C (data not shown) was identical. In metaphase, lamin B antibody staining was cytoplasmic, but enriched outside the spindle (Fig. 7, compare *b* with *c*). In anaphase and early telophase, the labeling remained cytoplasmic (Fig. 7, *f* and *i*). During later stages of telophase and the beginning of cytokinesis, the cytoplasmic labeling was still strong, but at that time a trace amount of lamins could be detected in as-
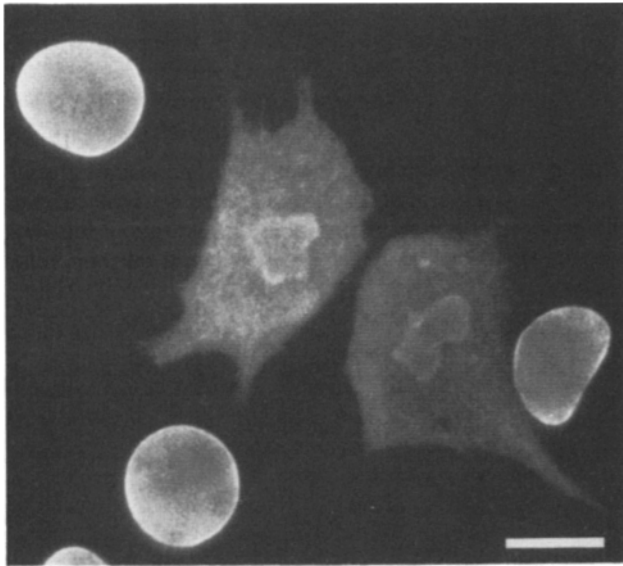
Figure 8. Lamin B distribution in the nontransformed chick embryo fibroblast cells, during telophase. The cell was fixed and stained for immunofluorescence with anti-lamin B antibody (as in Fig. 7). A portion of the lamins are associated with the nuclear periphery while the remaining mitotic lamins are distributed diffusely throughout the cytoplasm. Note the absence of cytoplasmic lamin aggregates. Bar, 10 μm.

sociation with the nuclear envelope (Fig. 7 *l*). The bulk of the lamins repolymerized into the lamina when the daughter chromosomes decondensed and the nucleoli reappeared (Fig. 7, *o* and *r*). Also at this stage, a variable number of discrete cytoplasmic compartments, of heterogeneous size and shape that stained very brightly with lamin antibodies could be discerned (Fig. 7 *o*, *arrowheads*). Double immunofluorescence experiments indicated that these cytoplasmic deposits contained lamins A, B, and C, but did not contain lamin B receptor, gp210, or nucleoporins (double immunofluorescence data not shown). The cytoplasmic deposits of lamins completely disappeared as the daughter cells matured (see Fig. 3, panels stained with anti-lamin antibodies). In contrast, when a nontransformed cell line (chick embryo fibroblast cells) was probed with the lamin antibodies, a homogeneous labeling of the cytoplasm was observed in late telophase and cytokinesis, in the absence of any significant cytoplasmic aggregation of lamins (Fig. 8). The presence of the lamin aggregates in HeLa cells was not a function of the source of the antibodies because rabbit antibodies and human autoantibodies both reacted with identical structures (data not shown).

Discussion

Two Distinct Sets of Vesicles That Originate from Nuclear Membrane Domains during Cell Division are Sequentially Targeted to Chromatin at the End of Mitosis

Morphological and biochemical evidence indicates that the nuclear envelope is organized into three distinct membrane domains consisting of the outer membrane, the inner membrane and the pore membranes. Specific resident proteins are associated with each type of membrane. The evidence obtained in this study suggests that the distinctness of the inner and pore membranes is retained during cell division, when the nuclear membranes separate from the chromosomes and disassemble into vesicles.

By density equilibrium sedimentation analysis on sucrose gradients of membranes from cells in metaphase, we found that the sedimentation pattern of the marker of the inner nuclear membrane (lamin B receptor) and of the marker for the pore membrane (gp210) were different. This result suggested that the proteins may partition into separate sets of membrane vesicles. The existence of two distinct sets of mitotic membranes originating from the nuclear envelope was further supported by immunolocalization experiments which showed that the lamin B receptor and gp210 were segregated in different compartments of the mitotic cell and were targeted sequentially to the chromosomal surface during the reformation of the nuclear envelope. The binding to chromosomes of the inner nuclear membrane-derived vesicles begins as early as anaphase A. The gp210-containing membranes bind to chromosomes mostly during telophase and cytokinesis.

Double labeling experiments with antibodies to tubulin and the lamin B receptor revealed that the lamin B receptor-containing membranes are largely excluded from the mitotic apparatus at all stages of mitosis. It is possible that the mitotic fragments of the inner nuclear membrane are too large to penetrate the dense microtubule network. Consequently, the reassociation of the lamin B receptor-containing membranes with chromatin begins only when the chromosomal surfaces emerge free of the microtubule network. This results in a complex, discontinuous pattern of nuclear envelope formation at the end of mitosis. In contrast, the gp210-containing vesicles seem to be able to penetrate the mitotic apparatus, suggesting that these vesicles have a smaller dimension or different properties compared to the lamin B receptor-containing membranes. The time course and topology of nuclear envelope reassembly reported here closely corresponds to the ultrastructural observations made by several groups on nuclear envelope reformation in mitotic cells (Robbins and Gonatas, 1964; Zatsepina et al., 1977; Zeligs and Wollman, 1979). In these studies, the earliest attachment of membranes to chromatin was observed to occur in a discontinuous manner along the outer surface of daughter chromosomes, during the middle of anaphase. Furthermore, the membrane-chromatin associations were restricted to regions of low microtubule density.

The possibility that different populations of nuclear envelope precursor vesicles may be required for the proper assembly of the nuclear envelope was recently examined in the *Xenopus* egg (Vigers and Lohka, 1991). In an in vitro nuclear envelope reassembly assay consisting of detergent-treated *Xenopus* sperm, *Xenopus* egg cytosol, and egg membranes, it was shown that the proper reconstitution of the nuclear envelope requires the sequential addition of two types of precursor vesicles. Thus, from these in vitro studies and our own observations on intact mitotic cells, a similar picture of nuclear envelope assembly emerges.

What is the mechanism of reassembly of the inner and pore membranes onto chromatin? Because the first detectable event of the nuclear envelope reconstruction is the binding of lamin B receptor-containing membranes to chroma-

tin, we suggest that these vesicles play a pivotal role in the subsequent assembly of other envelope components. Only a few resident proteins of the inner membrane have been identified to date, and the structure and function for most have not yet been elucidated (Richardson and Maddy, 1982; Worman et al., 1988; Senior and Gerace, 1988; Harel et al., 1989; Padan et al., 1990; Bailer et al., 1991). The best characterized inner nuclear membrane protein is the lamin B receptor, which was identified by its ability to bind to lamin B in vitro (Worman et al., 1988). Our data suggest that the lamin B receptor might also be a receptor for a chromatin component other than lamin B. This is consistent with a recent report showing that the lamin B receptor may have a role in the targeting of nuclear membrane vesicles to chromatin (Soullam and Worman, 1993). The lamin B receptor has recently been shown to be specifically phosphorylated during mitosis by a p34^{cdc2}-type protein kinase (Courvalin et al., 1992), suggesting that a function(s) of this protein may be regulated during mitosis. Along these lines, integral membrane proteins regulated by a phosphatase/kinase system have been postulated to assist in the in vitro reconstitution of membranes around chromosomes in *Xenopus* (Wilson and Newport, 1988; Pfaller et al., 1991; Vigers and Lohka, 1992).

The receptor for the inner membrane on the chromatin surface has not been identified, though certain histone variants and nuclear 'scaffold' proteins have been proposed to carry out this function (Newport, 1987). Another possibility is that a nonchromosomal protein is necessary for linking the inner membrane vesicles to chromatin. On the basis of cell-free reconstitution experiments, lamins, and in particular the A-type lamins, have been proposed to mediate the attachment of chromatin with nuclear envelope precursor vesicles (Burke and Gerace, 1986; Glass and Gerace, 1990; Burke, 1990; Höger et al., 1991a; Ulitzur et al., 1992). Our immunolocalization data do not show the early binding of lamins to chromatin. However, it is possible that the high background of lamin B staining makes it difficult to see the earliest time that lamin B associates with the chromosome surface. Thus, the possibility remains that a portion of the mitotic lamins (or a lamin variant) binds to chromosomes very early, but in quantities not detectable by our experimental procedures (Dabauvalle and Scheer, 1991).

Regarding the targeting of the pore membrane vesicles, it remains to be shown whether these membranes have the intrinsic capacity to bind to chromatin or whether they recognize and fuse with the inner membrane vesicles. The glycoprotein gp210 is the only integral membrane protein of the pore domain to be characterized (Gerace et al., 1982; Wozniak et al., 1989; Courvalin et al., 1990b; Greber et al., 1990). Although gp210 is believed to have a role in anchoring the pore complex to the membrane, it may also function in membrane fusion during pore assembly (Wozniak et al., 1989; Greber et al., 1990), which is a topologically distinct process from the fusion of membranes exposed to the cytoplasm (White, 1992). Other, as yet uncharacterized components of gp210-containing vesicles are likely to mediate vesicle recognition and fusion events.

Disassembly and Reassembly of the Nuclear Lamina

The nuclear lamina has been previously shown to disassemble during prophase (Gerace and Blobel, 1980). In cell-free

extracts, lamins A and C were found to be soluble whereas lamin B was associated with mitotic membranes (Gerace and Blobel, 1980; Stick et al., 1988; Kitten and Nigg, 1991). Here we report similar data, except that a significant amount of lamin B is also present in soluble extracts of mitotic cells. In addition, our membrane flotation experiments showed that the membrane-bound form of mitotic lamin B is not enriched in the same membrane fractions as the lamin B receptor. This suggests that during metaphase, the membrane-bound lamin B is not associated with its receptor but rather with membranes that do not derive from the inner nuclear membrane. This attachment may be mediated through the isoprenyl moiety that is covalently attached to the carboxyl terminus of lamin B (Beck et al., 1988; Wolda and Glomset, 1988). The fact that a mutation that prevents the farnesylation of avian lamin B completely abolishes the association of the protein with membranes during mitosis supports this hypothesis (Kitten and Nigg, 1991). The separation of lamin B from its receptor may be promoted by mitosis-specific modifications of these two proteins, such as phosphorylation (Gerace and Blobel, 1980; Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990; Courvalin et al., 1992).

We observed that lamins A, B, and C reassemble into the nuclear lamina during late telophase and cytokinesis. The late mitotic reassembly of the nuclear lamina is now well documented (Gerace et al., 1978; Bailer et al., 1991; Yang et al., 1992). Because the lamin B receptor is targeted to chromatin in anaphase and lamin B during telophase and cytokinesis, it is apparent that lamin B has not reassociated with its receptor during the immediate postmetaphase stages of mitosis. This time course of lamina reassembly is compatible with the hypothesis that the lamina may reform in late telophase by import of the lamins into the nucleus through the nuclear pore, and subsequent polymerization in contact with the inner membrane (Wilson and Newport, 1988; Benavente et al., 1989; Newport et al., 1990; Goldman et al., 1992).

An unexpected observation in the present study is the finding of transient lamin deposits (or 'compartments') in the cytoplasm of HeLa cells during cytokinesis and early G1 phase of the cell cycle. This phenomenon was not observed in the cytoplasm of untransformed cells. The time course of appearance of these lamin compartments in the cytoplasm is simultaneous with the repolymerization of lamina that occurs around the daughter chromatin. In contrast, the annulate lamellae (cytoplasmic deposits of pore-complex containing membranes) are present throughout interphase in oocytes, many transformed cells, and several normal cell lines (Kessel, 1989). Annulate lamellae do not contain lamins (Chen and Merisko, 1988; Dabauvalle et al., 1991), therefore, the lamin deposits in the cytoplasm of fast growing cells represent a potentially interesting new type of structure or compartment.

In conclusion, our observations in intact mammalian cells indicate that biochemically distinct sets of vesicles/membranes fuse to form the nuclear envelope. The integral membrane protein components of the nuclear envelope are not randomly distributed at any time in the cell cycle. The mechanism of biogenesis of these different membrane systems, and the signals that are necessary for their proper assembly into the nuclear envelope, remain to be investigated.

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