

# Involvement of Nuclear Lamins in Postmitotic Reorganization of Chromatin As Demonstrated by Microinjection of Lamin Antibodies

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**Abstract.** The nuclear lamins are major components of a proteinaceous polymer that is located at the interface of the nuclear membrane and chromatin; these lamins are solubilized and dispersed throughout the cytoplasm during mitosis. It has been postulated that these proteins, assembled into the lamina, provide an architectural framework for the organization of the cell nucleus. To test this hypothesis we microinjected lamin antibodies into cultured PtK<sub>2</sub> cells during mitosis, thereby decreasing the soluble pool of lamins. The antibody injected was identified, together with the lamins, in cytoplasmic aggregates by immunoelectron

microscopy. We show that microinjected cells are not able to form normal daughter nuclei, in contrast to cells injected with other immunoglobulins. Although cells injected with lamin antibodies are able to complete cytokinesis, the chromatin of their daughter nuclei remains arrested in a telophase-like configuration, and the telophase-like chromatin remains inactive as judged from its condensed state and by the absence of nucleoli. These results indicate that lamins and the nuclear lamina structure are involved in the functional organization of the interphase chromatin.

**T**HE nuclear lamina is a karyoskeletal structure that forms a fibrillar meshwork of proteinaceous material subjacent to the inner nuclear membrane and is tightly associated with the nuclear pore complexes (Aaronson and Blobel, 1975; Scheer et al., 1976; for reviews see Franke et al., 1981; Gerace et al., 1984; Benavente et al., 1984). Both the nuclear lamina and the pore complexes are resistant to nucleases, as well as to buffers that contain high salt concentrations and non-ionic detergents, and both are the major components of the residual "pore complex-lamina fraction" (Aaronson and Blobel, 1975; Scheer et al., 1976; Gerace et al., 1978; Krohne et al., 1978a).

Depending on the cell type and the species, the nuclear lamina contains one, two, or three major polypeptides with molecular weights in the range of 60,000 to 80,000 (for review see Krohne and Benavente, 1986a), named "lamins" (Gerace and Blobel, 1980). Nuclear lamins are members of a multigene family of proteins that are expressed—at least in *Xenopus laevis*—in a cell type-specific fashion (Krohne et al., 1981, 1984; Benavente et al., 1985; Benavente and Krohne, 1985; Stick and Hausen, 1985; for review see Krohne and Benavente, 1986a). Moreover, recent sequencing of lamin cDNA clones has demonstrated that they share structural homologies with intermediate filament proteins (McKeon et al., 1986).

The nuclear lamins undergo spectacular changes of their state of assembly during the cell cycle. With the onset of mi-

toisis the lamina is disassembled into soluble subunits that are dispersed throughout the cytoplasm (Gerace et al., 1978, 1984; Krohne et al., 1978b; Gerace and Blobel, 1980). In addition, lamins are disassembled into distinct soluble forms during nuclear envelope breakdown in oocyte meiotic maturation (Benavente et al., 1985; Krohne and Benavente, 1986b). Subsequently, lamins are reassembled and, during telophase, become part of the reforming daughter nuclei. A model has been proposed in which the depolymerization-polymerization cycle of the nuclear lamina is related to a cycle of phosphorylation-dephosphorylation of lamins (Gerace and Blobel, 1980; for a recent review see Gerace et al., 1984).

In contrast to our growing knowledge of the biochemistry and intracellular distribution of lamins, little is known at present about the function of the nuclear lamina. It has been proposed that these karyoskeletal proteins are involved in the organization of the nuclear envelope and the chromatin of the interphase nucleus (for reviews see Hancock, 1982; Gerace et al., 1984). Recent experiments using cell-free extracts of mitotic cells have shown that nuclear envelope formation *in vitro* is significantly inhibited when extracts are depleted of lamins (Burke and Gerace, 1986). However, it is not known whether such results can be extrapolated to the living cells.

Therefore, we have designed experiments that allow the *in vivo* analysis of the involvement of lamins in postmitotic reorganization of the interphase chromatin and the nucleus.

Our approach was to capture soluble lamins by the microinjection of cultured dividing cells with antibodies to lamins and thereby to interfere with their polymerization during telophase.

## Materials and Methods

### Cells and Microinjection

PtK<sub>2</sub> (rat kangaroo kidney epithelial) cells were grown on coverslips as previously described (Franke et al., 1978). Microinjection was performed as described (Ansoorge, 1982; Kreis and Birchmeier, 1982) using an Eppendorf Microinjector 5242 (Eppendorf Gerätebau, Hamburg, FRG) with glass capillaries (GC150; Clark Electromedical Instruments, Reading, UK). Antibodies were used at concentrations from 3 to 15 mg/ml in PBS (137 mM NaCl, 2.7 mM KCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>). Preferably, cells undergoing mitosis (prometaphase and metaphase) were microinjected.

### Antibodies

A nuclear lamina fraction from the livers of eight adult rats (~250 g) was prepared essentially as described (Krohne and Franke, 1983; Kaufmann et al., 1983), and polypeptides were separated by one-dimensional SDS PAGE essentially according to Laemmli (1970). Gels were stained with 4 M sodium acetate (Higgins and Dahmus, 1979) and bands corresponding to lamins were excised. Protein was eluted from gel slices by shaking for 24 h in 0.01% SDS, lyophilized, and then resuspended in PBS. The suspension was mixed

(1:1) with complete Freund's adjuvant. Guinea pigs were injected subcutaneously and boosted 3 wk later with the same amount of protein mixed with incomplete adjuvant. Blood was collected 1 wk later by heart puncture. The serum obtained contained antibodies specific for nuclear lamins of rat liver and PtK<sub>2</sub> cells and reacted with lamins A and C as well as with lamin B, as demonstrated by two-dimensional immunoblotting (for methods see O'Farrell et al., 1977; Gigi et al., 1982) of nuclear lamina and of total cytoskeletal fractions (Fig. 1). Immunoglobulins specific for lamins were affinity purified by the nitrocellulose blot method as described by Krohne et al. (1982).

Monoclonal murine antibody L<sub>0</sub>46F7, specific for *Xenopus* lamins L<sub>III</sub> and L<sub>IV</sub>, was described previously (Benavente et al., 1985; Benavente and Krohne, 1985). IgG was purified from ascites fluid by ammonium sulfate precipitation, followed by DE52 chromatography (Johnstone and Thorpe, 1982). Monoclonal murine antibody PKB8 (a lamin antibody reacting with somatic cells of all vertebrates; see Krohne and Benavente, 1986a) has been described elsewhere (Krohne et al., 1984). Monoclonal murine antibodies RSI-105 (IgM) against ribosomal protein S1 (Hügler et al., 1985) and AK30-10 (IgM) against single- and double-stranded DNA (Messner, 1985; Scheer, U., K. Messner, R. Hazan, I. Raska, P. Hausmann, H. Falk, E. Spiess, and W. W. Franke, manuscript in preparation) were used either as ascites fluid or as immunoglobulins purified by gel filtration on Sephacryl S-300.

### Immunofluorescence Microscopy

Microinjected cells were fixed after different incubation times in methanol (-20°C, 10 min) and air dried. Distribution of the injected antibody was visualized by incubating coverslips with secondary antibodies conjugated to fluorescein isothiocyanate or Texas red (Dianova, Herling, FRG) for 10 min. In some cases, double-labeled immunolocalization was performed either with monoclonal antibody PKB8, guinea pig antibodies to lamins, or monoclonal antibody RSI-105. For visualization of nuclei and chromosomes, cells were stained with diamidinophenylindole (DAPI)<sup>1</sup> (Benavente et al., 1985).

### Electron Microscopy and Electron Microscopical Immunolocalization

Cells microinjected with guinea pig immunoglobulins specific for lamins were fixed in 2.5% glutaraldehyde (15 min) and then postfixated with 2% osmium tetroxide (15 min). After overnight staining with 0.5% uranyl acetate, the coverslips with the attached cells were dehydrated in ethanol series and embedded in Epon. Alternatively, microinjected cells were fixed in PBS containing 2% formaldehyde for 30 min. Coverslips were then washed for 30 min in PBS containing 50 mM ammonium chloride and cells were permeabilized in PBS/ammonium chloride containing 0.1% saponin (15 min). After several washes in PBS, coverslips were incubated overnight with secondary antibodies coupled to colloidal gold particles (5 nm; Janssen Pharmaceutica, Beerse, Belgium). After washing in PBS, cells were fixed and embedded as above. Overnight staining with uranyl acetate was omitted. Ultrathin sections were double stained with uranyl acetate and lead citrate and examined at 80 kV in a Siemens Elmiskop 101 electron microscope.

## Results

In our preliminary experiments, microinjection of various immunoglobulins, including lamin antibodies, into the cytoplasm of nondividing cells did not result in the binding of the antibodies to the nuclear lamina, as demonstrated by immunofluorescence microscopy. This seems to be due to the inaccessibility of internal nuclear structures to intact immunoglobulins injected into the cytoplasm (for references see Einck and Bustin, 1984). We also microinjected lamin antibodies into the cell nucleus of PtK<sub>2</sub> cells. After different incubation times (1, 3 and 8 h) significant changes in the nuclear morphology were not found when injected cells were compared with non-injected cells (Fig. 2). Anti-lamin immunoglobulins were seen within the confinements of the nucleus, and a large proportion of them appeared to be away

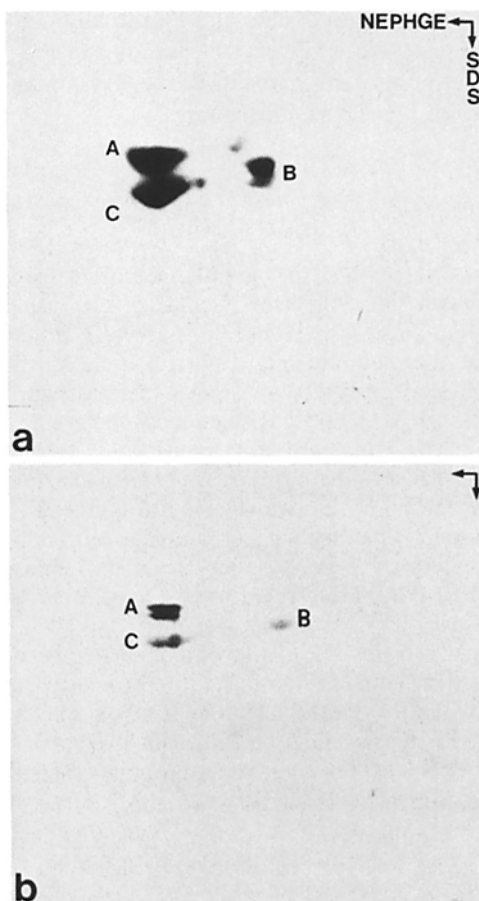
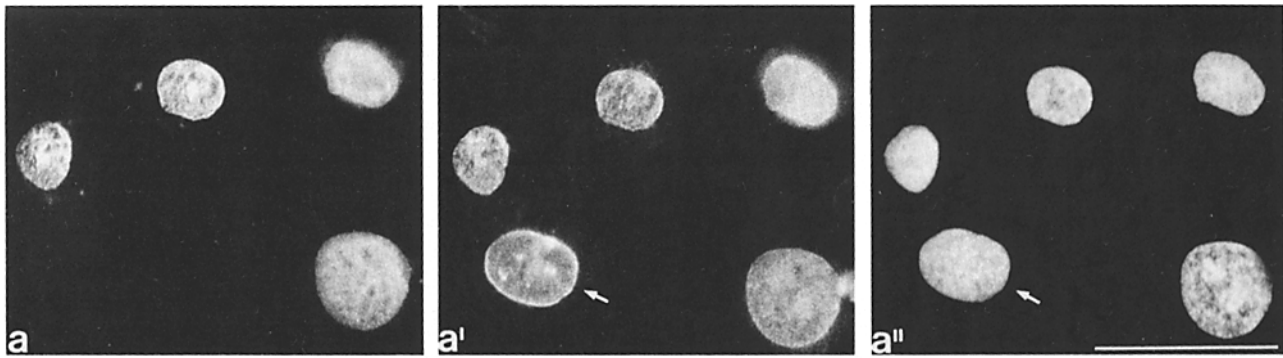


Figure 1. Autoradiographs showing the specific reaction of the guinea pig antibodies with all the three nuclear lamins (A, B, and C) from rat liver (a; nuclear lamina fraction) and PtK<sub>2</sub> cells (b; total cytoskeletal fraction). NEPHGE, non-equilibrium pH gradient gel electrophoresis in the first dimension; SDS, polyacrylamide (10% polyacrylamide) gel electrophoresis in the second dimension.

1. Abbreviation used in this paper: DAPI, diamidinophenylindole.



**Figure 2.** Fluorescence micrographs showing the same PtK<sub>2</sub> cells. (a) Distribution of lamin antibodies (from guinea pig; 10 mg/ml) 3 h after microinjection into interphase nuclei. (a') Double-label fluorescence of cells was done with monoclonal lamin antibody PKB8. (a'') DNA staining with DAPI. A non-microinjected nucleus is indicated by arrows (a'-a''). Bar, 25 µm.

from the lamina. This result may reflect the inaccessibility of the nuclear lamina to the microinjected immunoglobulins, possibly due to the presence of condensed chromatin along the nuclear periphery.

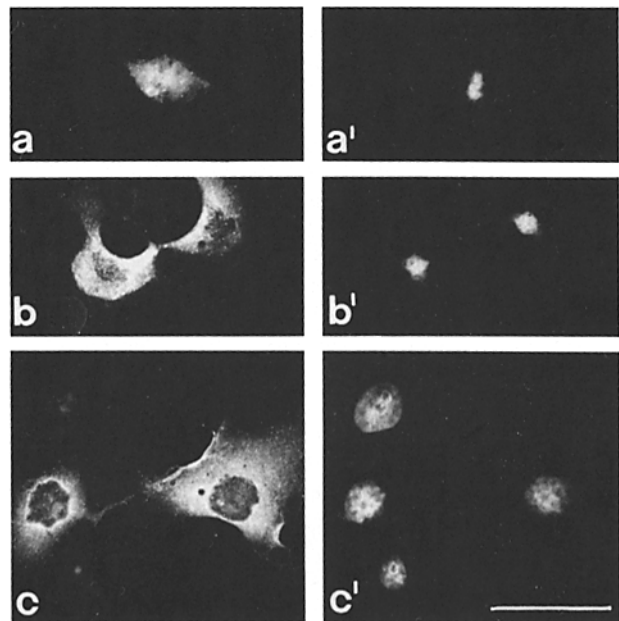
In an effort to circumvent the inaccessibility of nuclear lamins to injected immunoglobulins, we used mitotic PtK<sub>2</sub> cells (prometaphase and metaphase). We reasoned that, at this stage, the "nuclear" components of such cells would be more accessible to microinjected antibodies. Moreover, use of mitotic cells would allow us to closely monitor the behavior of the individual cells and their daughters formed at division. In this way we could control for cell damage resulting from microinjection and establish the time course of post-mitotic events under specific experimental conditions.

In one series of experiments, cells were microinjected with immunoglobulins not reactive with any PtK<sub>2</sub> antigen and were then fixed at different post-injection times. We injected various commercially available antibodies (goat anti-rabbit IgG, rabbit anti-guinea pig IgG) conjugated with fluorescein isothiocyanate or rhodamine (not shown) as well as the monoclonal antibody L<sub>o</sub>46F7 (Fig. 3) which reacts with *Xenopus* lamins L<sub>III</sub> and L<sub>IV</sub> (Benavente et al., 1985; Benavente and Krohne, 1985) but not with lamins of non-amphibian species. All these antibodies gave essentially identical results: upon injection, the immunoglobulins became homogeneously distributed throughout the cytoplasm and did not interfere with the progression of cell division. As judged by morphological criteria, such as nuclear shape, state of chromatin distribution, and nucleolar size and shape, by 2 h after injection, daughter nuclei had formed which were identical to nuclei of non-injected cells. With the onset of telophase, immunoglobulins appeared to be excluded from the nucleus and remained in the cytoplasm.

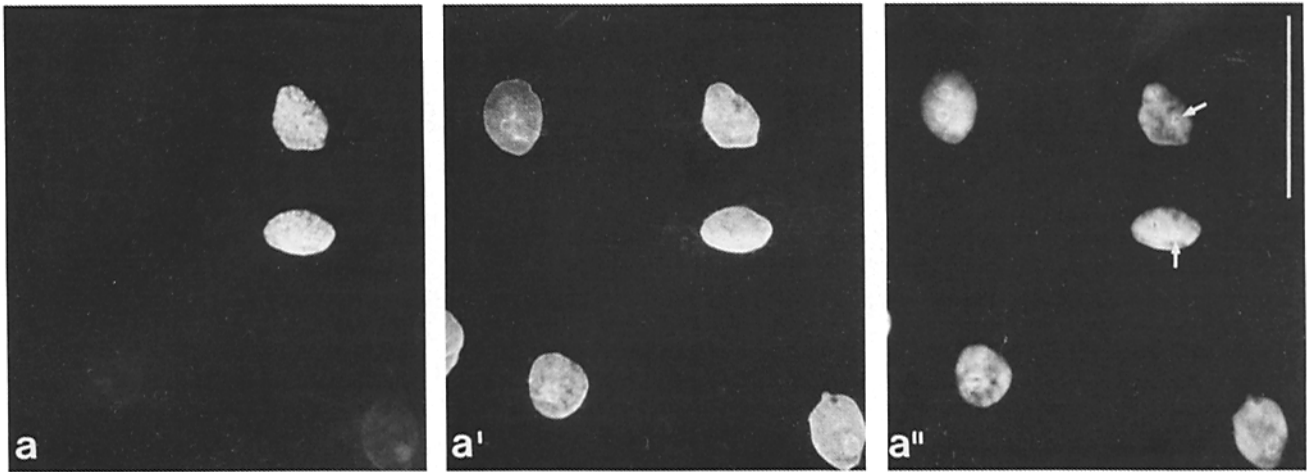
As a positive control, we microinjected mitotic cells with a monoclonal antibody against DNA (AK30-10). 2 h after microinjection, normal-appearing daughter nuclei were formed (Fig. 4) which could not be distinguished from nuclei of non-injected cells. It is noteworthy that in this case, the antibodies accumulated within daughter nuclei, apparently remaining bound to the chromatin (Fig. 4 a). Taken together, these results demonstrate that the experimental conditions used did not interfere with chromosome distribution, nuclear reformation, chromosome decondensation, and cytokinesis.

In contrast, the microinjection of species-competent lamin antibodies into mitotic cells had significant effects on the

progression of mitosis and the development of daughter nuclei. 2 h after microinjection, the chromosomes of the injected cells were still in a telophase-like configuration (Fig. 5, a-a''). Although cytokinesis had been completed (Fig. 6 a), daughter cells carrying lamin antibodies lacked a nuclear lamina as determined by immunofluorescence microscopy. The lamin "pool" of such cells appeared to be distributed throughout the cytoplasm, partly in large aggregates, resulting in a punctate pattern (Fig. 5, a-a''). This telophase-like configuration was maintained for at least 8 h after injection (Fig. 5, b-b'') at which time non-injected PtK<sub>2</sub> cells would have progressed to late G1 or early S phase of the subsequent cell cycle. After prolonged incubation times (24 h), most of



**Figure 3.** Fluorescence micrographs showing the time course of mitosis in microinjected PtK<sub>2</sub> cells as shown by immunofluorescence microscopy (a-c) and DNA staining with DAPI (a'-c'). Distribution of the microinjected control antibody L<sub>o</sub>46F7 (reacting only with amphibian but not with PtK<sub>2</sub> lamins) after 30 (a), 90 (b), and 120 min (c). Note completion of mitosis and formation of daughter cells and the exclusion of antibodies from the daughter cell nuclei. Bar, 50 µm.



**Figure 4.** Fluorescence micrographs showing the same cells. (a) Distribution of DNA antibody AK30-10 in postmitotic daughter cells 2 h after microinjection. (a') Double-label fluorescence of cells with the lamin antibodies (from guinea pig) and (a'') DNA staining with DAPI. Reformed nucleoli are indicated by arrows (a''). Bar, 50  $\mu\text{m}$ .

the injected cells had not recovered from the telophase-like arrest (data not shown).

We also examined the progression of nucleologenesis in mitotic cells microinjected with lamin antibodies. Prometaphase and metaphase cells were microinjected with lamin antibodies and processed 2–8 h later for immunofluorescence microscopy with nucleolus-specific antibodies (Fig. 5, *c–c''*). In this case, we used monoclonal antibody RS1-105 specific for ribosomal protein S1 which localizes to the granular component of nucleoli (Hügler et al., 1985). As shown in Fig. 5 *c'*, aggregates of nucleolar material recognized by monoclonal antibody RS1-105 did not reassociate with nucleolar organizer regions and remained free in the cytoplasm in a pattern similar to that of early telophase of non-injected cells (see Fig. 10 of Hügler et al., 1985). Using such nucleolar markers, reformed nucleoli were not seen up to 8 h after injection. This is in contrast to cells that had been injected with antibodies not reacting with lamins (Fig. 3 and 4; compare also Fig. 5, *c–c''* with Fig. 4 *a''*).

When analyzed by electron microscopy, the chromatin and other nuclear components of cells microinjected with lamina antibodies during mitosis showed aberrations, resulting sometimes in bizarre configurations (Fig. 6, *a–c*). Extended aggregates of chromosomes retained the highly condensed state of metaphase chromatin alternating with regions that were decondensed. In none of these microinjected cells were nucleoli or reorganizing nucleoli observed. Remarkably, these chromatin masses were surrounded by a nuclear envelope; i.e., a cisterna containing typical pore complexes (Fig. 6 *b*). The contours of the envelope appeared to be more irregular than those of nuclei of non-injected cells. In general, observations at the ultrastructural level revealed that the chromatin of injected cells, arrested in a telophase-like morphology, was different from that of normal telophase as well as interphase configurations of PtK<sub>2</sub> cells (Fig. 6 *b*; Roos, 1973).

Microinjected cells could be unequivocally identified at the ultrastructural level by the presence of relatively large (0.2–2  $\mu\text{m}$ ) cytoplasmic aggregates of finely granular material (granule diameter 3–6 nm) which were often closely associated with cisternae or vesicles (Fig. 6 *c* and 7 *a*). The

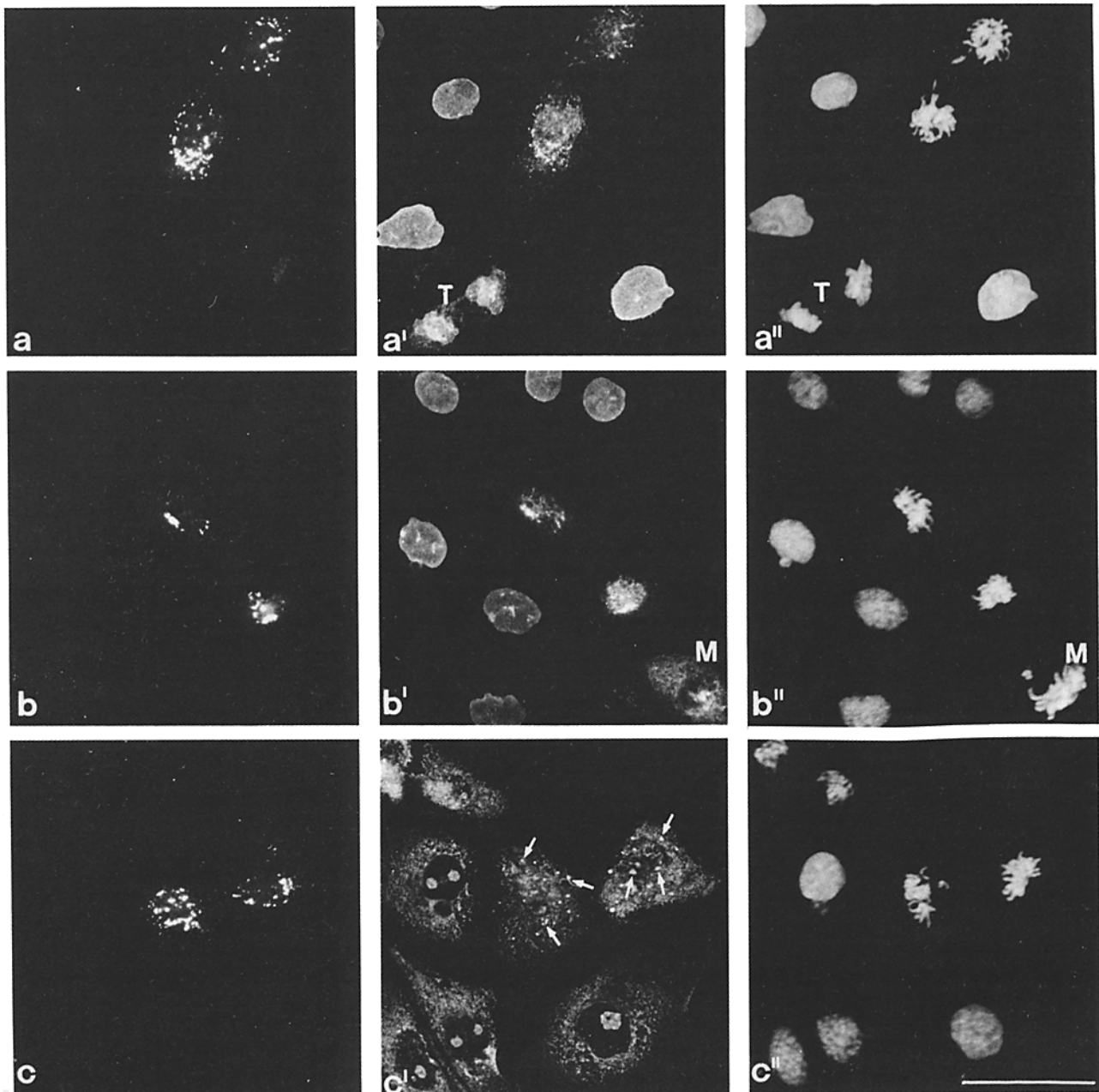
cisternae usually did not possess pore complexes (in only one case was a pore complex observed). The granular aggregates seen with the electron microscope appeared to correspond to the fluorescence-positive material seen in the light microscope (Fig. 5 *a* and *b*), based on their similarity in size and composition (Fig. 7 *a*). In addition, small granular structures containing injected antibodies and lamins were also found scattered throughout the cytoplasm (Fig. 7, *b* and *c*), and these seemed to correspond to the more diffuse staining of microinjected cells seen with fluorescence microscopy (cf. Fig. 5 *a'*). The dispersed small aggregates of lamins and lamin antibodies, which probably represent antigen–antibody complexes, were also associated with the cytoplasmic surface of nuclear envelope-like structures and with cytoplasmic vesicles and cisternae (Fig. 7 *b* and *c*).

## Discussion

The present study provides evidence for the involvement of the nuclear lamins in the postmitotic reconstitution of the interphase nucleus. Our results indicate that a nucleus cannot reform in telophase when the soluble pool of mitotic lamins has been depleted by microinjection of lamin antibodies. Instead, the telophase-like configuration of the chromosomes is maintained, at least for several hours.

Resumption of nuclear function is also perturbed in such lamin-depleted cells as judged by the inability of lamin-depleted cells to disperse chromatin and to reform nucleoli. This seems to reflect a more general inhibition, indicating that the nuclear lamins function in the structural and functional organization of the nuclear interior.

Recently, Burke and Gerace (1986) reported that the reformation of the nuclear envelope *in vitro* was significantly, although not totally, inhibited when the cell extracts used had been previously depleted of lamins by incubation with lamin antibodies. In our *in vivo* experiments, the arrested telophase-like chromatin structures were surrounded by a cisternal system containing pore complexes. Our results are not necessarily in contradiction to the data reported by Burke and Gerace (1986) since we cannot exclude that a small



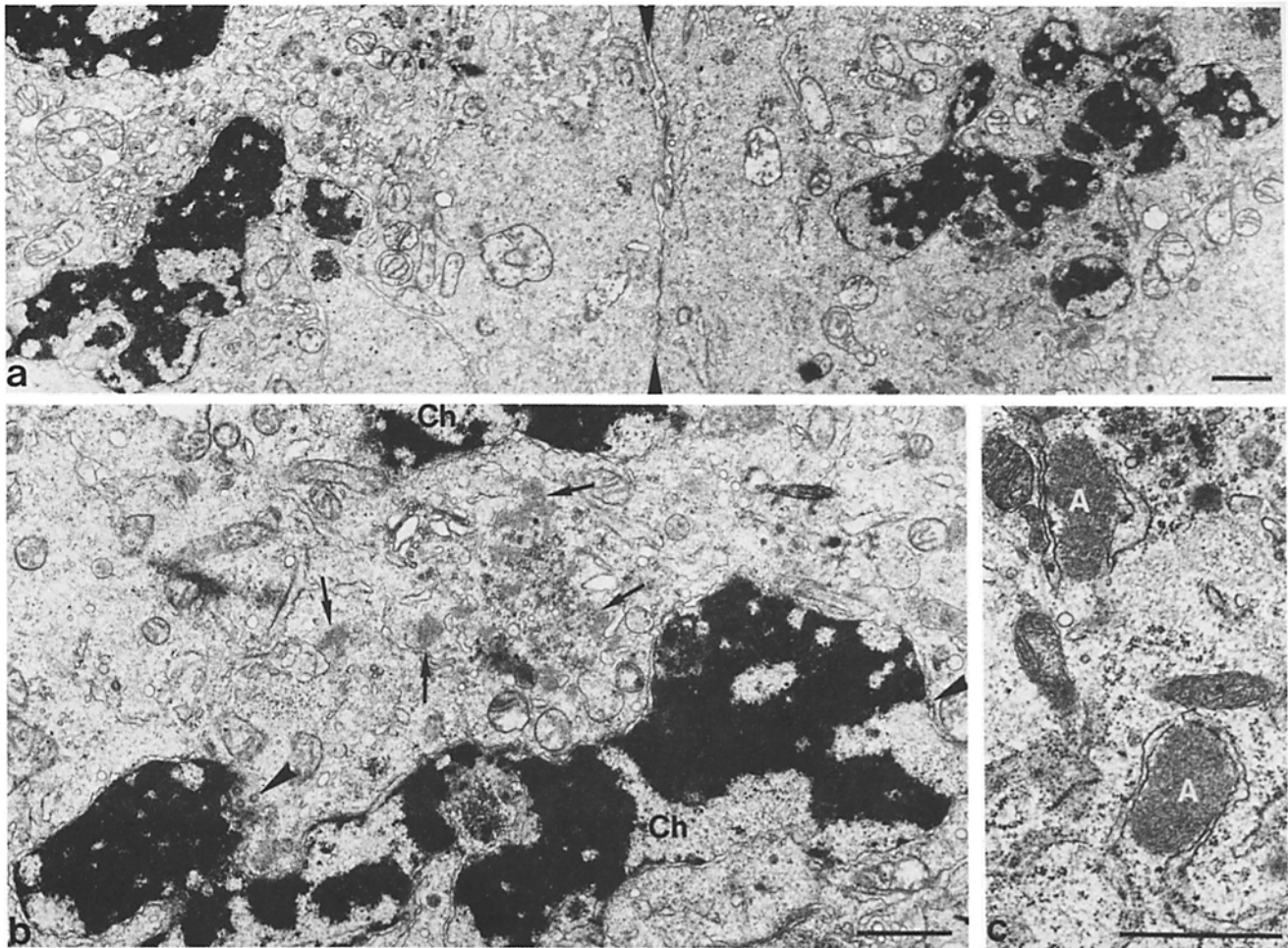
**Figure 5.** Distribution of lamin antibodies (from guinea pig; 10 mg/ml) in postmitotic daughter cells 2 (*a* and *c*) or 8 (*b*) h after microinjection. Double immunofluorescence of cells was done with monoclonal lamin antibody PKB8 (*a'* and *b'*) and antibody RSI-105 against ribosomal protein S1 (*c'*). (*a''-c''*) DNA staining of the same cells with DAPI. Non-injected cells undergoing metaphase (*M*) or telophase (*T*) are indicated. Some of the cytoplasmic aggregates containing nucleolar material recognized by monoclonal antibody RSI-105 are denoted by arrows; the more diffuse fluorescence in the cytoplasm probably corresponds to ribosomes (*c'*; see also Hügle et al., 1985). Bar, 50  $\mu$ m.

proportion of mitotic lamins has not been immobilized by the injected antibodies. These remaining lamins might then be sufficient to allow membrane apposition on the surface of the chromosomes. Nevertheless, we can conclude from our experiments that the membranous components of the nuclear envelope (depleted of most of the lamins) and the pore complexes are not sufficient to induce nuclear reorganization at telophase and the formation of normal daughter nuclei.

From our experiments we cannot distinguish which of the mammalian lamins (A/C or B) are specifically involved in the re-targeting of membranes around chromosomes during

telophase in vivo. In vitro experiments which have addressed this question have shown quantitative but not qualitative differences of lamins A/C and B in their ability to promote nuclear formation (see Table II in Burke and Gerace, 1986). In this respect, it should be also mentioned that in early *Xenopus* embryos, blastomeres are able to form a nuclear envelope with only one lamin species ( $L_{III}$ ). Lamin  $L_{III}$ , as mammalian lamins A/C, but unlike lamin B, is largely soluble during metaphase (Benavente et al., 1985; Krohne and Benavente, 1986b).

The experiments reported here also raise the question of



**Figure 6.** Electron microscopy of daughter cells 2 h after microinjection with lamin antibodies (from guinea pig; 10 mg/ml). Nuclear reformation is severely perturbed (*a* and *b*) whereas cytokinesis has been completed (arrowheads in *a*). The cytoplasm of microinjected cells shows relatively large distinct granular aggregates (some of them are denoted by arrows in *b* or by *A* in *c*) that are associated with cisternae and vesicles. Arrowheads in *b* denote pore complexes. *Ch*, chromosomal material. Bars, 1  $\mu\text{m}$ .

how, in the interphase nucleus, the chromatin is attached to the nuclear lamina. Certain nonlamin proteins have been identified (McKeon et al., 1984; Chaly et al., 1984) such as "perichromin" and the "P1 antigens" which are also located in the nuclear periphery during interphase but, in contrast to the lamins, remain in association with the chromosomal surfaces during mitosis. It has been proposed that these proteins may mediate the association of chromatin with the nuclear lamina (for discussion see McKeon et al., 1984; Chaly et al., 1984) although other possibilities have not been excluded. The possible involvement of these proteins in nuclear organization may be also tested following a strategy similar to that used in the present study; i.e., microinjection of antibodies into dividing cells.

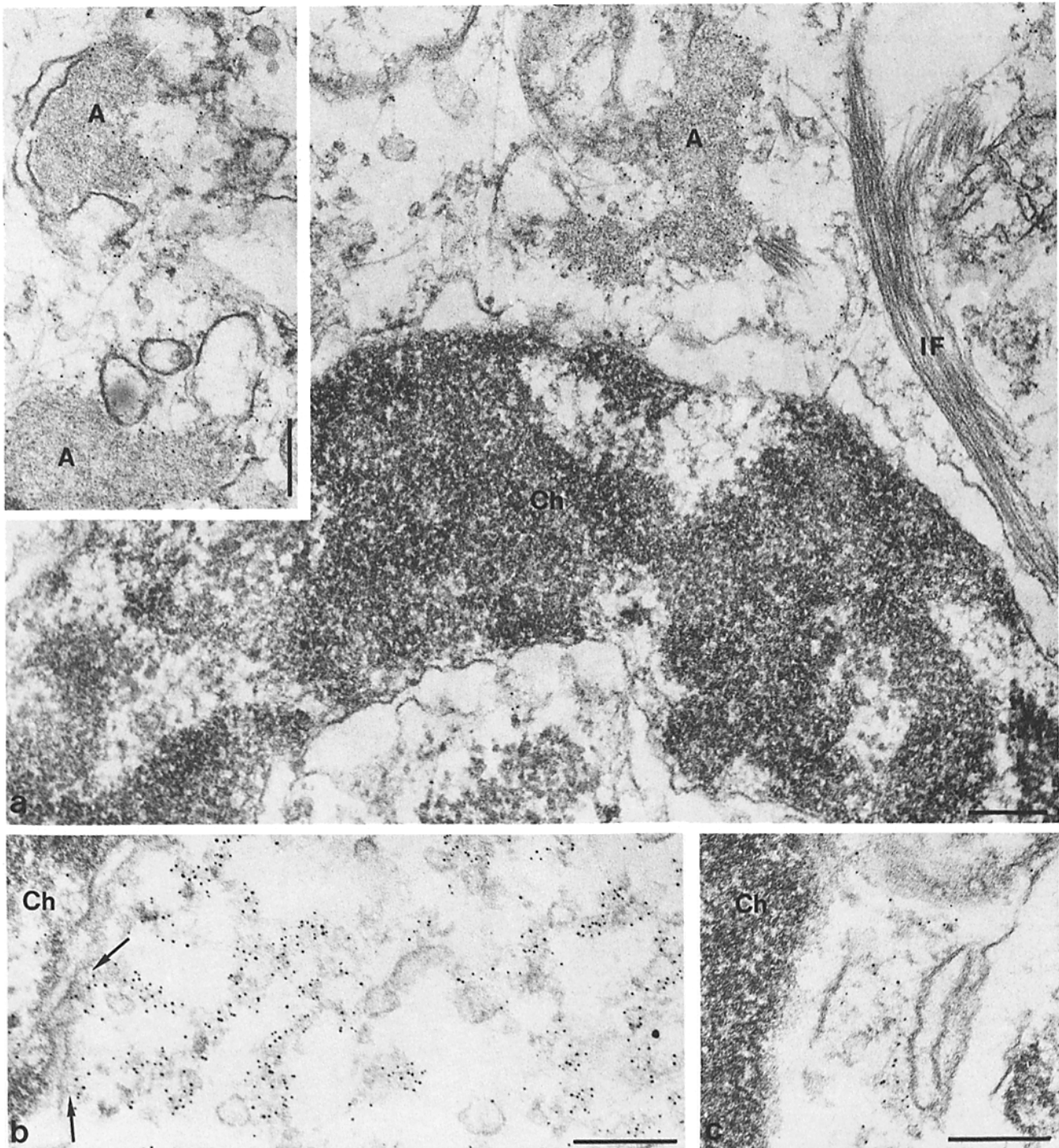
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**Figure 7.** Electron microscopical immunolocalization of microinjected lamin antibodies (from guinea pig; 10 mg/ml) in daughter cells. The large granular aggregates (*A* in *a* and *inset*) are decorated with  $\sim 5$ -nm colloidal immunogold particles. Immunogold particles are also seen in association with smaller aggregates dispersed throughout the cytoplasm, sometimes in association with nuclear envelope-like membranes (arrows in *b*) or certain vesicles and cisternae. *Ch*, chromosomal material; *IF*, intermediate filament bundle probably of cytokeratins (for PK<sub>2</sub> cytoskeleton see Franke et al., 1978). Bars, 1  $\mu$ m.

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