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Nucleocytoplasmic sorting of macromolecules following mitosis: fate of nuclear constituents after inhibition of pore complex function

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PtK_2 cells in which pore complex-mediated transport is blocked by microinjection early in mitosis of a monoclonal antibody (specific for an M_r 68 000 pore complex glycoprotein) or of wheat germ agglutinin (WGA) complete cytokinesis. However, their nuclei remain stably arrested in a telophase-like organization characterized by highly condensed chromatin and the absence of nucleoli, indicating a requirement for pore-mediated transport for the reassembly of interphase nuclei. We have now examined this requirement more closely by monitoring the behavior of individual nuclear macromolecules in microinjected cells using immunofluorescence microscopy and have investigated the effect of microinjecting the antibody or WGA on cellular ultrastructure. The absence of nuclear transport did not affect the sequestration into daughter nuclei of components such as DNA, DNA topoisomerase I and the nucleolar protein fibrillarin that are carried through mitosis on chromosomes. On the other hand, lamins, snRNAs and the p68 pore complex glycoprotein, all cytoplasmic during mitosis, remained largely cytoplasmic in the telophase-arrested cells. Electron microscopy showed the nuclei to be surrounded by a double-layered membrane with some inserted pore complexes. In addition, however, a variety of membranous structures with associated pore complexes was regularly noted in the cytoplasm, suggesting that chromatin may not be essential for the postmitotic formation of pore complexes. We propose that cellular compartmentalization at telophase is a two-step process. First, a nuclear envelope tightly encloses the condensed chromosomes, excluding non-selectively all macromolecules not associated with the chromosomes. Interphase nuclear organization is then progressively restored by selective pore complex-mediated uptake of nuclear proteins from the cytoplasm.

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Introduction

The "open" mitosis of higher eukaryotes is characterized by the breakdown of the nuclear envelope with concomitant loss of nucleocytoplasmic compartmentalization. As the first step in reestablishing the interphase situation, the nuclear envelope reassembles in early telophase around the two sets of daughter chromosomes. Concomitantly with the reformation of the double-layered nuclear membrane, pore complexes become inserted by a hitherto poorly understood mechanism (e.g., [41, 42, 58]; for a discussion of possible modes of pore complex formation see [31]).

During mitosis, maternal nuclear material may be distributed to the daughter nuclei by different pathways. Nuclear constituents either remain bound to the mitotic chromosomes and are thus directly partitioned to the daughter nuclei, or they disperse throughout the cytoplasm after breakdown of the nuclear envelope followed by their sequestration into the newly formed daughter nuclei. In the latter case, a sorting mechanism is required that directs the cytoplasmically located maternal nuclear material back into the daughter nuclei. This nuclear retargeting could be accomplished either by specific binding to telophase chromosomes before completion of nuclear envelope reformation or by selective transport through the newly formed pore complexes after nuclear envelope assembly.

Until recently it was not possible to distinguish between these alternative pathways since no reagents were available that could modulate pore complex function. However, with the demonstration that nuclear uptake of proteins can be inhibited by the lectin wheat germ agglutinin (WGA) [10, 14, 56, 57] as well as by antibodies against specific pore complex proteins [5, 9, 13], it is now feasible to interfere experimentally with pore complex-mediated transport processes in the living cell. Among the group of glycoproteins with cytoplasmically exposed O-linked N-acetylglucosamine (GlcNAc) moieties that have been identified as integral constituents of nuclear pore complexes [9, 11, 12, 21, 23, 38, 45, 48], a major component of M_r 68 000 has been located in the pore channel and implicated in transport processes of karyophilic proteins ([5, 9]; see also [13]).

Recently, we have begun to examine the importance of pore-mediated nuclear protein uptake in the postmitotic nuclear reorganization process [5]. For this purpose we microinjected mitotic PtK_2 cells either with WGA or monoclonal antibody PI1 directed against the M_r 68000 glycoprotein of pore complexes. Our results showed that the injected cells completed cytokinesis but that the daughter cells were unable to proceed into G1. Furthermore, their nuclei remained arrested in a telophase-like state characterized by the presence of highly condensed chromatin masses surrounded by a complete double-layered membrane. While this study provided evidence that early influx of proteins via pore complexes is essential for the structural remodelling process of nuclei during their transition from telophase to interphase, the nature of the proteins involved has not been examined in detail.

In the present communication we report on the distribution of several nuclear proteins during mitosis and the mode by which they are partitioned to the postmitotic nuclei.

Materials and methods

Antibodies

Monoclonal murine antibody $L_0$46F7, specific for Xenopus lamins L_{III} and L_{IV}, has been described previously [1, 3]. IgG was purified from ascites fluid by ammonium sulfate precipitation, followed by DE52 chromatography [24]. Monoclonal murine antibody AK30-10 (IgM) against DNA (for details see [46]) was purchased from Progen (Heidelberg/FRG). Monoclonal antibody PI1 (IgM) against the major GlcNAc-containing pore complex glycoprotein (p68) [5, 7, 9] was purified by hydroxylapatite chromatography, concentrated and lyophilized as previously described [50]. Guinea pig antibodies reacting with lamins from rat liver and PtK_2 cells were characterized elsewhere [2]. Human autoimmune sera directed against the nucleolar protein fibrillarin [39] and DNA topoisomerase I [20, 48] were a generous gift of Dr. Georg Reimer (Dermatology Department, University of Erlangen/FRG). Rabbit IgG recognizing the m_3G-cap of snRNAs [40] was kindly provided by Dr. Reinhard Lührmann (University of Marburg/FRG).

Cells and microinjection

Rat kangaroo kidney epithelial cells (PtK_2) grown on coverslips [17] were microinjected during prometaphase or metaphase as described [2]. WGA conjugated with fluorescein isothiocyanate (FITC; Medac, Hamburg/FRG) and antibodies $L_0$46F7, AK30-10 and PI1 were used at concentrations ranging from 0.5 to 10 mg/ml in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4).

Immunofluorescence microscopy

Microinjected cells were fixed in methanol (−20 °C) for 10 min, dipped for a few seconds in acetone (−20 °C), and then air dried. Alternatively, microinjected cells were directly fixed in PBS containing 3% formaldehyde (prepared from paraformaldehyde) and permeabilized in PBS containing 0.1% Triton X-100. The distribution of microinjected antibodies ($L_0$46F7, AK30-10, and PI1) was visualized by anti-mouse IgG/IgM antibodies (Dianova, Hamburg/FRG) conjugated to Texas red or FITC. The fate of endogenous nuclear constituents was examined in the microinjected cells by double-label immunofluorescence microscopy. For this purpose cells processed as above were subsequently incubated with another antibody (10–30 min) followed by the appropriate secondary antibodies conjugated to FITC or Texas red (Dianova). For visualization of nuclei and chromosomes, cells were stained with diamidinophenylindole (DAPI; for further details see [2, 4]). Micrographs were taken with a Zeiss Axiophot equipped with epifluorescence optics.

Electron microscopy

Cells microinjected with WGA or antibody PI1 were fixed in 2.5% glutaraldehyde for 15 min followed by 2% osmium tetroxide (15 min) and processed for electron microscopy as described [2]. Micrographs were taken with a Zeiss EM 10 electron microscope.

Results

Microinjection of mitotic PtK_2 cells with non-competent antibodies has no noticeable effect on the progression of cell division and reorganization of the daughter nuclei (see also [2, 4]). For example, 2 h after microinjection, monoclonal antibodies $L_0$46F7 (specific for Xenopus lamins L_{III} and L_{IV}, but not for mammalian lamins [26]) were equally partitioned to the cytoplasm of the two resulting daughter cells (Figs. 1a, b). The daughter cells derived from a microinjected mother could be easily identified since they always occurred in pairs and were selectively decorated by secondary antibodies directed against the injected material. By phase-contrast microscopy, the descendants of the injected PtK_2 cells appeared perfectly normal (Figs. 1a″, b″), and this impression was substantiated by double-label immunolocalization using antibodies against mammalian nuclear lamins and the nucleolar protein fibrillarin (Figs. 1a′, b′). Thus, the immunofluorescence pattern obtained was indistinguishable from that of adjacent non-injected cells indicating that nuclear reassembly had proceeded normally (Figs. 1a′, b′).

Interestingly, microinjected antibody $L_0$46F7 (IgG) was excluded from the daughter nuclei (Figs. 1a, b). This cytoplasmic distribution is consistent with previous reports showing that IgG molecules per se lack karyophilic signals (e.g., [52]; which they might, however, acquire by conjugation with karyophilic antigens) and further illustrates that the newly formed daughter nuclei represent a zone of exclusion for cytoplasmic proteins [2] (see also [53]). In contrast, when DNA antibodies were microinjected during mitosis, they were found concentrated in the nuclei of the daughter cells (Fig. 2a). This differential location suggests that binding to the mitotic chromosomes before nuclear envelope reformation might represent an effective mode of postmitotic nuclear retargeting of macromolecules.

In Figures 3 and 4 we have examined the postmitotic fate of several nuclear constituents in PtK_2 cells which were microinjected with antibody PI1 or FITC-conjugated WGA in order to block pore complex-mediated transport. The PI1 injected cells were identified by immunofluorescence microscopy. Antibody PI1 was distributed in the cy-

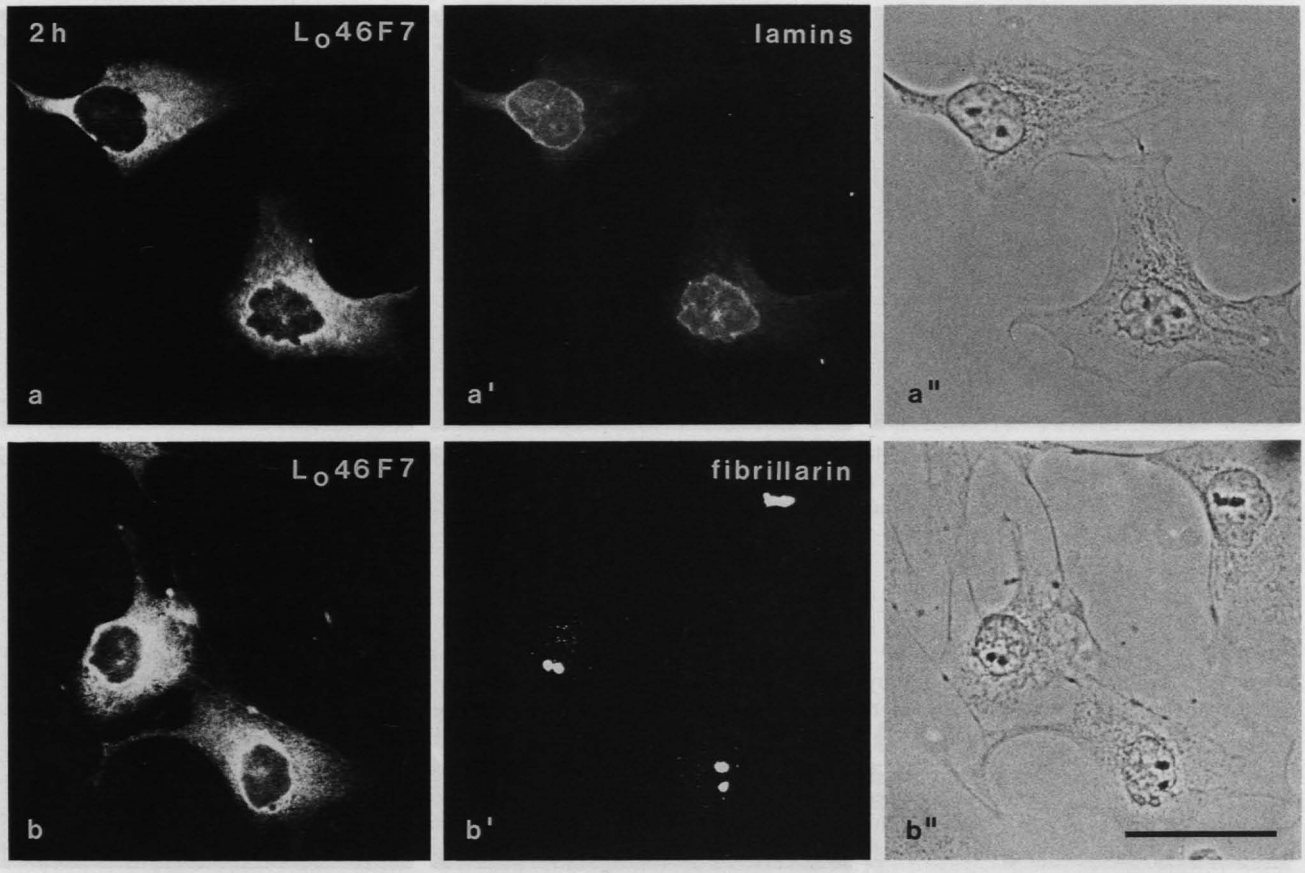


Fig. 1. Postmitotic daughter cells 2 h after microinjection of control antibody $L_0$46F7 into mitotic PtK_2 cells as shown by immunofluorescence (**a, a'; b, b'**) and phase-contrast microscopy (**a", b"**). — **a, b.** Distribution of microinjected antibody $L_0$46F7. Note exclusion of the IgGs from the cell nuclei. — **a', b'.** Double-label immunofluorescence microscopy showing the reorganized nuclear lamina and nucleoli as demonstrated by antibodies against lamins (**a'**) and fibrillarin (**b'**). A non-injected cell is seen in the upper right corner of (**b', b"**). — Bar 50 μm.

toplasm of the daughter cells both diffusely and in the form of numerous dot-like aggregates (Figs. 3a–c). The injected FITC-WGA also formed numerous discrete cytoplasmic entities but was preferentially enriched in the region of the arrested daughter chromosomes (Figs. 4a–d; for the presence of WGA-binding proteins in chromatin see [22]). After injection of antibody PI1 or WGA, PtK_2 cells completed cytokinesis, but, in contrast to the control experiments described above (Figs. 1, 2), the daughter cells were unable to proceed to interphase. As revealed by phase-contrast microscopy, their nuclei remained stably arrested for at least 18 h [5] in a telophase-like organization characterized by a condensed state of the chromatin and absence of nucleoli. Examples of cells 3 h after microinjection are presented in Figures 3 and 4. This behavior illustrates the requirement of nuclear protein influx for transition of the daughter cells from telophase to G1 (see also [5]). Under such conditions the various nuclear constituents examined (Tab. I) revealed different patterns of arrangement. Thus, nuclear envelope components such as lamina proteins (Figs. 3a′, 4a′) and the M_r 68000 pore complex glycoprotein p68 (Fig. 4b′) were largely excluded from the arrested daughter nuclei. Other nuclear components such as the nucleolar protein fibrillarin (Figs. 3b′, 4c′) and DNA topoisomerase I (Fig. 4d′), however, were detected essentially exclusively within the daughter nuclei. Remarkably, in some of the injected cells fibrillarin was found in form of small dots (Figs. 3b′, 4c′) indicating that — at least in part — the formation of prenucleolar bodies had occurred but that they did not coalesce at the site of the nucleolus organizing regions of the chromosomes. This observation confirms our previous data showing that fusion of prenucleolar bodies requires transcriptional activity of the rRNA genes [4]. In contrast to the behavior of fibrillarin and DNA topoisomerase I, snRNAs were excluded from the arrested daughter nuclei as revealed by the m_3G-cap specific antibody (Fig. 3c′).

Electron microscopy confirmed our light microscopic observations. Nuclei of daughter cells arrested by microinjection of antibody PI1 or WGA during the preceding

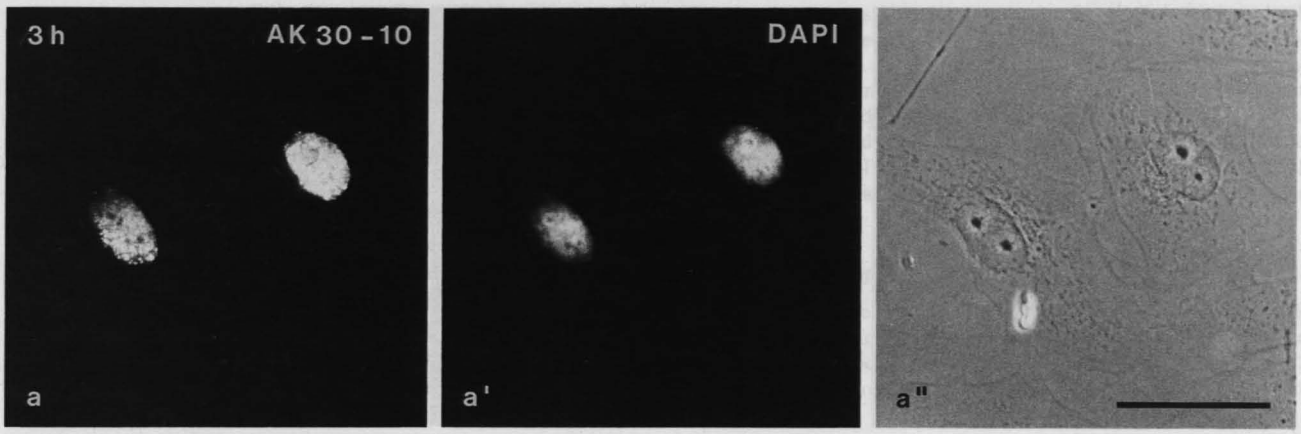


Fig. 2. Daughter cells 2 h after microinjection of a mitotic PtK_2 cell with antibody AK30-10 directed against DNA. — **a.** Distribution of the microinjected antibody as demonstrated by immunofluorescence microscopy. Note the intranuclear location of the antibodies. — **a'.** DNA staining with DAPI. — **a".** Phase-contrast microscopy of the same cells. — Bar 50 μm.

metaphase were characterized by the presence of condensed chromatin masses surrounded by a double-layered nuclear membrane with some inserted pore complexes (Figs. 5, 6; see also [5]). Furthermore, we noted characteristic membranous structures in the cytoplasm adjacent to the arrested nuclei which were never observed in telophasic non-injected cells. In cells injected with antibody PI1, numerous cytoplasmic aggregates of finely granular texture occurred (Fig. 5), most probably corresponding to the antibody-containing aggregates seen by immunofluorescence microscopy (Figs. 3a–c; for similar aggregates found after microinjection of lamin antibodies see [2]). These aggregates were frequently associated with vesicles and flattened membrane cisternae with pore complexes (Figs. 5c, d). In cells microinjected with WGA, the granular aggregates were lacking but numerous elongated membranous cisternae with inserted pore complexes occurred in the cytoplasm (Fig. 6b). Two of these cisternae were often closely apposed and resembled the paired cisternae described in a variety of mitotic cells (e.g., [16, 34, 54]). Such unusual quadruple membrane complexes have been interpreted as remnants of the maternal nuclear envelope which are reutilized by the daughter cells for nuclear envelope formation [34, 54]. In fact, in favorable sections we have observed structural continuities of the nuclear envelope surrounding the condensed chromatin with such elongated and pore-bearing membranes.

Tab. I. Distribution of some nuclear constituents examined in the present study during interphase and mitosis as revealed by immunofluorescence microscopy.

Constituent	Interphase	Mitosis	References
Lamins	nuclear periphery	diffuse in the cytoplasm	[19, 28]
Pore complex Glycoprotein p68	punctate at the nuclear periphery	disperse in the cytoplasm	[5, 7]
Fibrillarin	nucleolus	mainly chromosome surfaces	[39]
snRNAs (U1–U5)	nuclear interior and nucleolus (U3)	diffuse in the cytoplasm	[40]
DNA topoisomerase I	nuclear interior, enriched in the nucleolus	chromosomes and NOR	[20, 43]

Discussion

In the present study we have analyzed the pathways by which reforming daughter nuclei acquire constituents characteristic of interphase nuclei. In order to assess the involvement of the newly formed nuclear pore complexes in such distributive processes, we have microinjected WGA or antibodies to the pore complex glycoprotein p68 into mitotic PtK_2 cells. These conditions are known to inhibit pore-mediated nuclear transport of macromolecules in living cells [9, 10, 13, 56, 57]. As shown in the present and a previous study [5], injected PtK_2 cells completed cytokinesis, but the daughter cells became arrested in a telophase-like configuration and apparently were unable to resume normal nuclear functions as judged by the persistently condensed chromatin and the absence of nucleoli [5]. These results indicated that some essential nuclear component(s) have to be transported from the cytoplasm via the newly formed pore complexes into the newly formed daughter nuclei in order to allow progression of the cells into G1.

Previous immunofluorescence studies have indicated that nuclear constituents behave differently during mitosis. Some of them remain associated with the condensed chromosomes while others disperse in the cytoplasm upon dissolution of the nuclear envelope (for references see [5]). In the present study we have examined the nuclear proteins

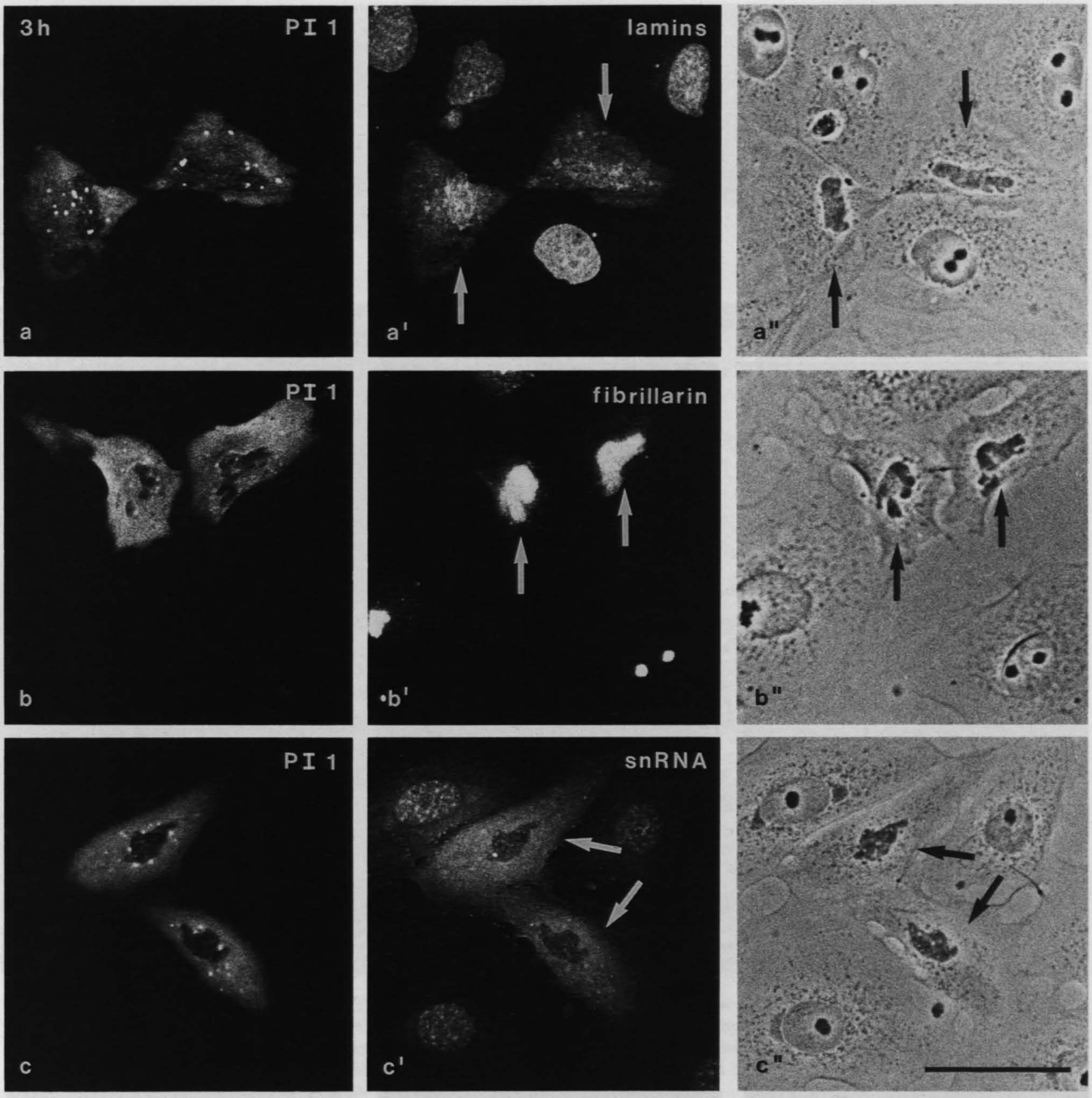
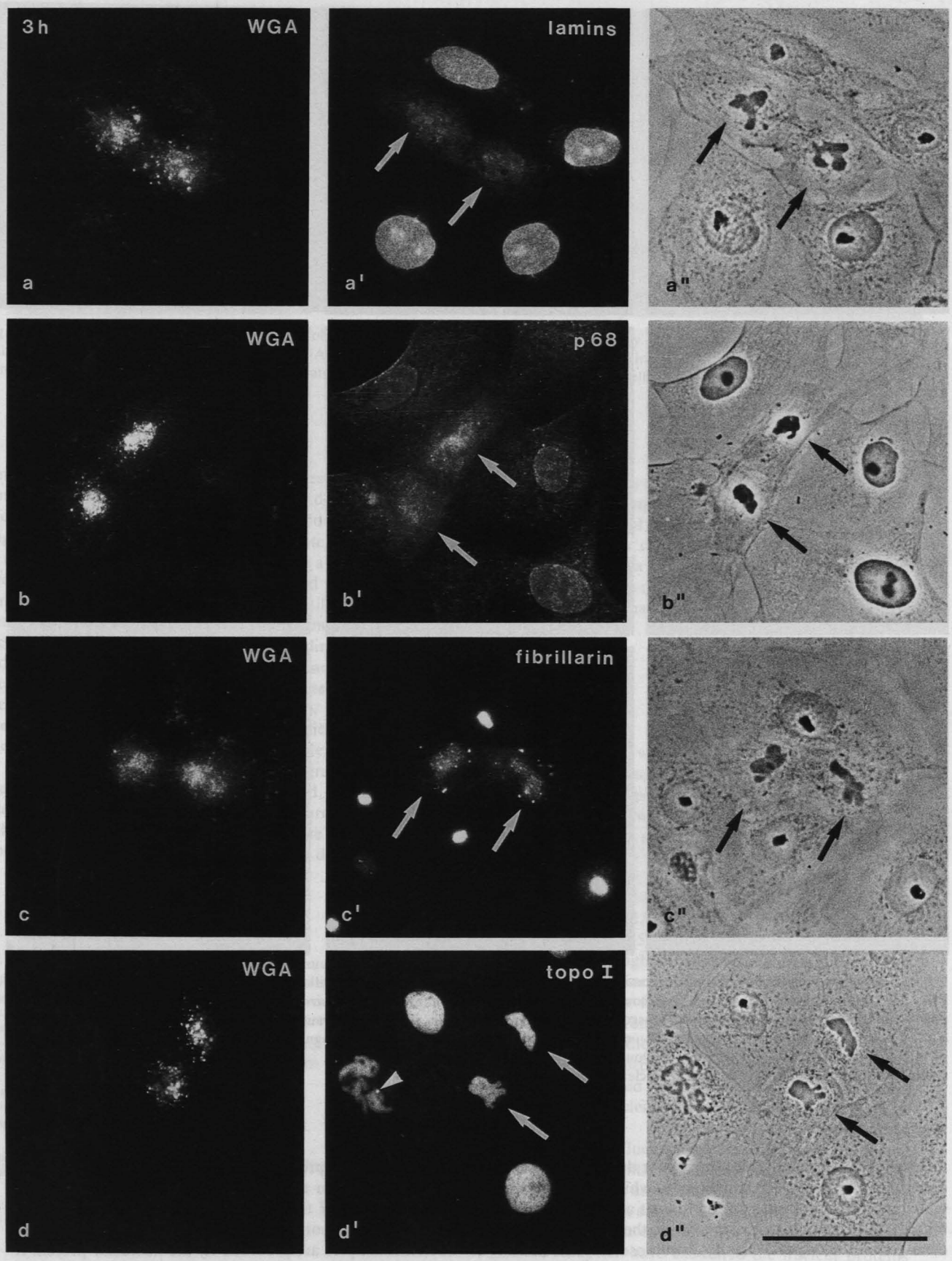


Fig. 3. Daughter cells (*arrows*) 3 h after microinjection of mitotic PtK_2 cells with antibody PI1 against pore complex glycoprotein p68 as shown by immunofluorescence microscopy (**a, a'; b, b'; c, c'**) and phase-contrast microscopy (**a"-c"**). — **a to c.** Distribution of microinjected antibody PI1. — **a' to c'.** Double-label immunofluorescence showing the distribution of nuclear lamins (**a'**), nucleolar protein fibrillarin (**b'**) and snRNAs (**c'**) in the antibody containing cells (*arrows*) as well as in adjacent non-injected cells. — **a" to c".** Daughter cells derived from the injected mother cell are clearly recognized by their telophase-like appearance (*arrows*). — Bar 50 μm.

and snRNAs listed in Table I in greater detail. Our results show that those nuclear constituents which remain associated with chromosomes during mitosis are directly transmitted to the telophase-like nuclei in the absence of nuclear protein transport. Clearly, chromosomal association during mitosis represents a mechanism that prevents mixing of a particular nuclear constituent with the cytoplasm in the absence of the nuclear envelope and thus ensures immediate incorporation into the newly formed telophase nuclei. Examples are provided by the nucleolar protein fi-


3h
WGA
a
lamins
a'
a"
WGA
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p68
b'
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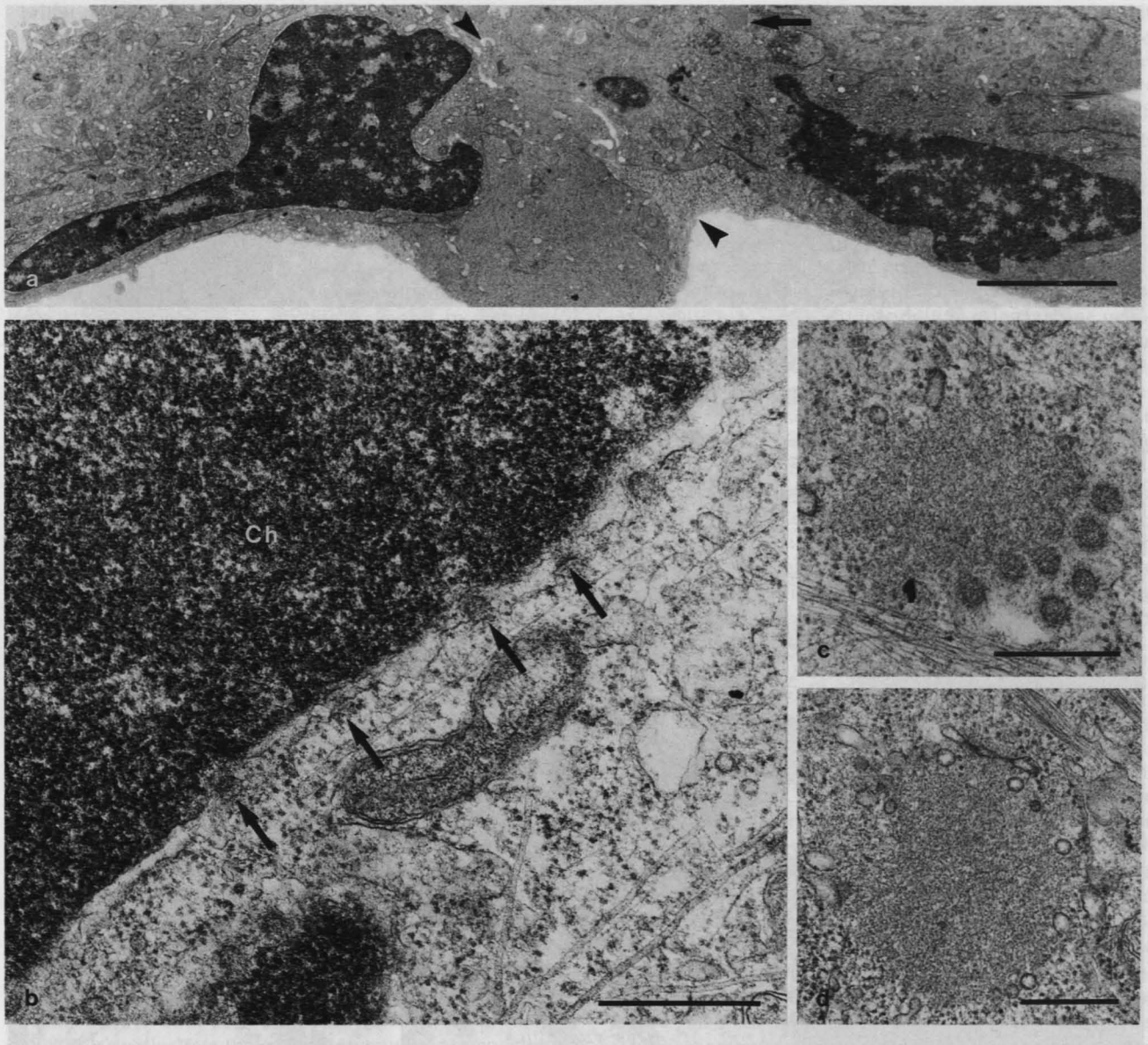

Fig. 5. Electron micrographs of daughter cells 3 h after microinjection of a mitotic PtK_2 cell with antibody PI1. Cytokinesis is essentially completed (*arrowheads* in **a**). The nuclei containing extremely condensed chromatin (Ch) are surrounded by nuclear envelopes with pore complexes (some of them are denoted by *arrows* in **b**). Large granular aggregates occur in the cytoplasm (*arrow* in **a**) and are covered by pore complex-containing membrane cisternae (**c**, tangential section; **d**, cross section of the pore complexes). — Bars 5 μm (**a**), 0.5 μm (**b**, **c**), 0.2 μm (**d**).

Fig. 4. Daughter cells (*arrows*) 3 h after microinjection of FITC-conjugated WGA. The distribution of WGA is shown in **a to d.** The distribution of lamins (**a'**), pore complex glycoprotein p68 (**b'**), fibrillarin (**c'**) and DNA topoisomerase I (**d'**) in the WGA-containing cells (*arrows*) and adjacent non-injected cells is shown by double-label immunofluorescence microscopy. — **a" to d".** Phase-contrast microscopy of the same cells. Note the telophase-like appearance of the WGA-containing cells (*arrows*). *Arrowhead* in (**d'**) denotes the NOR of a non-injected prometaphase cell which is positive for DNA topoisomerase I. — Bar 50 μm.

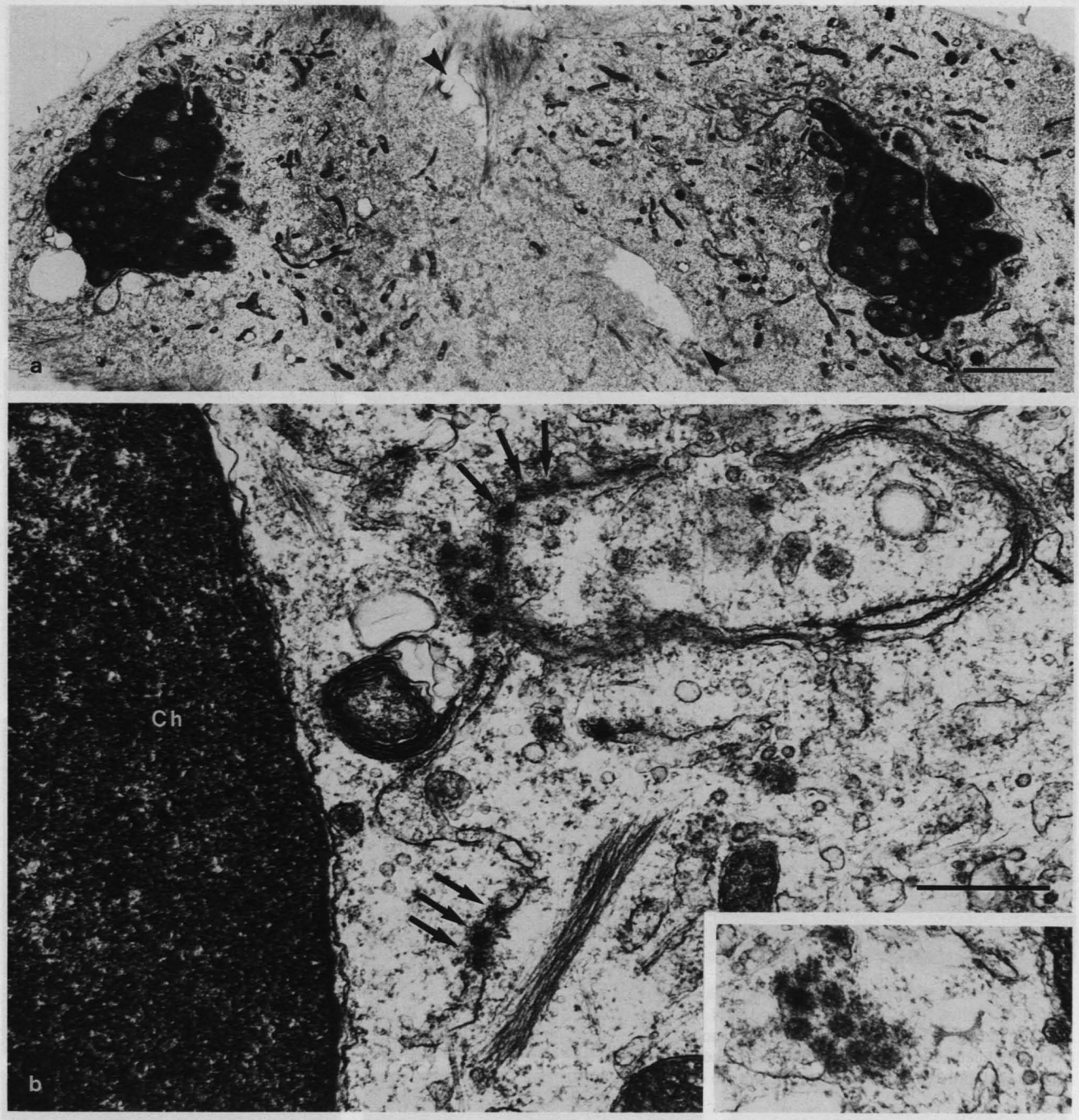

Fig. 6. Electron micrographs of daughter cells 3 h after microinjection of a mitotic PtK_2 cell with WGA. Nuclei contain highly compacted chromatin (Ch) and are surrounded by a nuclear envelope (**a, b**). Cytokinesis is essentially completed (*arrowheads* in **a**). Cytoplasmic membranes, often in the form of paired cisternae, contain numerous pore complexes (some of them are denoted by *arrows* in **b**). The *insert* of (**b**) shows a tangential view of such cytoplasmically located pore complexes. — Bars 5 μm (**a**), 0.5 μm (**b** and *insert*).

brillarin (Figs. 3b′, 4c′) and DNA topoisomerase I (Fig. 4d′). In addition, RNA polymerase I is also, at least in part, directly transmitted to the daughter nuclei by virtue of its association with the chromosomal nucleolar organizing region (NOR) (data not shown; see also [44]). On the other hand, our results indicate that some nuclear constituents which become diffusely distributed during mitosis are reacquired by the nuclear compartment via pore complexes. An example is provided by the snRNAs which remain essentially cytoplasmic when pore-mediated transport is blocked (Fig. 3c′).

The behavior of nuclear envelope components deserves special consideration. As previously shown by immunofluorescence microscopy, the lamins disperse throughout the cytoplasm during mitosis (e.g., [19, 28]). According to current concepts, the first step in nuclear envelope reformation is the binding of lamina proteins to chromosome surfaces which thus mediate the assembly of the double-layered nuclear membrane [18]. Our results demonstrate that a significant portion of the soluble lamin pool remains in the cytoplasm of daughter cells in the presence of antibody PI1 or WGA (Figs. 3a′, 4a′). Since the nuclei of the arrested cells are surrounded by a continuous double-layered nuclear membrane including pore complexes, our data indicate that the majority of the lamin proteins is not directly required for nuclear envelope formation but rather reenters telophase nuclei via pore complexes. Karyophilic signals required for such a pore complex-mediated uptake have been identified in type A and B lamins [8, 15, 29, 30]. Only a minority of lamins appears to be associated with chromosomes of the telophase-like cells (Figs. 3a′, 4a′). It is conceivable that a small population of early attaching lamins is sufficient to trigger nuclear envelope reassembly. The remaining lamina proteins would then be transported into the daughter nuclei and incorporated into the growing lamina layer during nuclear expansion accompanying the transition from telophase to early G1. That lamins are required for the structural reorganizations leading to interphase nuclei has been previously shown by experiments in which the mitotic soluble lamins were immobilized by microinjection of antibodies [2]. Taken together, while our results clearly illustrate that postmitotic nuclear remodelling processes are dependent on pore complex-mediated uptake of soluble lamin proteins, our present approach is not sensitive enough to clarify the important question of whether lamins are also critically involved in nuclear membrane assembly at the surface of the telophase chromosomes [6, 27].

During mitosis, pore complex glycoproteins have been shown to disperse throughout the cytoplasm (e.g., [5, 11, 38, 49]). From studies based on cell-free nuclear reassembly two alternative models for telophase pore complex reformation have been proposed (for review see [31]). The so-called vesicle precursor model implies that pore complexes are formed in association with membrane cisternae bound to the chromosome surface [33]. The pre-pore model, on the other hand, proposes that components of the pore complex bind first to the chromosomes in form of prepores, followed by fusion of membrane vesicles into the nuclear membrane [47]. As shown in the present study, microinjection of antibody PI1 and WGA does not inhibit pore complex formation but appears to interfere, at least to some extent, with their correct targeting. Thus, pore complexes were found not only in the nuclear envelopes surrounding the arrested nuclei but also in association with cytoplasmic membrane cisternae. Although these observations do not allow to distinguish between both models of nuclear pore complex formation, they do show, however, that pore complex assembly might occur in the absence of chromatin.

In the absence of nuclear protein uptake telophase nuclei do not swell but remain in a compact configuration. As a consequence, no further addition of membrane material to the nuclear envelope is required. Excess membranes apparently form flat cisternae in the cytoplasm of the arrested cells and are able to support pore complex formation there. This observation suggests the presence in these membranes of a kind of receptor [31] that allows binding of pore complex components and subsequent pore formation, irrespective of their position in the cell. A similar mechanism might also be involved in formation of annulate lamellae (for a recent review see [25]). Obviously, in the normal telophase situation excess nuclear membrane precursor material is rapidly integrated into the reforming nuclear envelope. As a result, the process of pore complex assembly and insertion takes place preferentially at the nuclear surface on correctly targeted cisternae (for postmitotic nuclear membrane targeting see also [51, 55]).

The results of the present and a previous study [5] suggest the following sequence of events in the reestablishment of cellular compartmentalization at the end of mitosis. As a first step, the condensed chromosomes become enclosed by a tightly associated double-layered membrane with some inserted pore complexes. This process necessarily leads to the exclusion of all of those macromolecules from the newly formed nuclei which are not associated with the chromosomal surface at this stage. The initial separation of nucleus and cytoplasm by the formation of the nuclear envelope appears to involve a general and non-discriminative exclusion of cellular material from the nucleus. This notion receives support from our experiments in which we injected non-specific immunoglobulins into mitotic PtK_2 cells. The results clearly showed that the IgG-molecules were excluded from the daughter nuclei as a consequence of nuclear envelope formation (see also [2]). Similarly, exogenous macromolecules such as large dextrans are also excluded from the postmitotic nuclei [53]. In the second step then, the characteristic nuclear environment is progressively restored by the selective uptake of nuclear proteins from the cytoplasm via pore complexes.

Our in vivo studies illustrate that although postmitotic nuclear reformation is a highly complex integrated temporal-spatial process it can be experimentally subdivided into defined steps. We hope that this approach will eventually lead, in conjunction with the use of cell-free systems aimed to analyze the components involved in nuclear reassembly processes (e.g., [6, 32, 35, 36]; for review see [37]), to a better understanding of the molecular mechanisms and regulative processes involved in nuclear reorganization during mitosis.

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