Formation of the Nuclear Envelope Permeability Barrier Studied by Sequential Photoswitching and Flux Analysis

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ABSTRACT In higher eukaryotes, the nuclear envelope breaks down during mitosis. It reforms during telophase, and nuclear import is reestablished within <10 min after anaphase onset. It is widely assumed that import functionality simultaneously leads to the exclusion of bulk cytoplasmic proteins. However, nuclear pore complex assembly is not fully completed when import capacity is regained, which raises the question of whether the transport and permeability barrier functions of the nuclear envelope are indeed coupled. In this study, we therefore analyzed the reestablishment of the permeability barrier of the nuclear envelope after mitosis in living cells by monitoring the flux of the reversibly photoswitchable fluorescent protein Dronpa from the cytoplasm into the nucleus after photoactivation. We performed many consecutive flux measurements in the same cell to directly monitor changes in nuclear envelope permeability. Our measurements at different time points after mitosis in individual cells show that contrary to the general view and despite the rapid reestablishment of facilitated nuclear import, the nuclear envelope remains relatively permeable for passive diffusion for the first 2 h after mitosis. Our data demonstrate that reformation of the permeability barrier of nuclear pore complexes occurs only gradually and is uncoupled from regaining active import functionality.

INTRODUCTION

In eukaryotic cells, the genome is separated from the rest of the cell by the nuclear envelope (NE). The exchange of macromolecules between the nucleus and the cytoplasm occurs via nuclear pore complexes (NPCs), large protein assemblies which span the nuclear envelope, forming aqueous channels. NPCs form selective gates: proteins that contain signals recognized by specific transport receptors can traverse the NPC channel rapidly by facilitated diffusion. Complexes transported by this pathway, for example, ribonucleoprotein particles and ribosomal subunits, can have a diameter as large as 40 nm (1). However, for proteins without specific nuclear transport signals, diffusion through the NPC is highly size-dependent and the size exclusion limit is at a diameter of ~9 nm (2).

Nuclear compartmentalization introduces an important regulatory level into the eukaryotic cell that is not present in prokaryotes. By separating the processes of transcription and translation spatially, the nucleus has allowed splicing to become a predominant process in eukaryotic gene expression. In addition, the access of, e.g., transcription factors to the genome can be regulated by the modulation of their transport across the nuclear envelope. Other proteins, like the mitotic regulators TPX2 and NuMA, are sequestered inside the nucleus during interphase, which prevents their action in the cytoplasm (3).

During mitosis in higher eukaryotes, the nuclear envelope breaks down and the separation of the nucleoplasm and the cytoplasm is transiently abolished. Rapid reformation of the nuclear envelope after mitosis and exclusion of cytoplasmic factors from the new nucleus is thought to be important for a normal progression of mitotic exit. Indeed, the nucleus regains its import capacity very rapidly, within ~8 min after anaphase onset (4). However, the process of nuclear pore assembly continues until ~20 min after anaphase onset (4–6), and studies in mammalian cells, as well as in some species of amoebae, have reported a higher permeability of the NE for colloidal gold particles within the first 2 h after mitosis (7–9). These studies suggested that the two main functions of the NE, nucleocytoplasmic transport and a permeability barrier for passive diffusion, may not be reestablished concomitantly. Contradictory to these findings, differences in NE permeability after mitosis were not observed in mammalian cells in which the equilibration of soluble enhanced green fluorescent protein between the nucleus and the cytoplasm was measured after bleaching of the nuclear signal (10).

To determine the precise time course of NE permeability barrier reestablishment after mitosis, we have developed an assay to repeatedly measure NE permeability in the same cell, which thus makes it possible to determine changes in NE permeability directly. We find that NE permeability only gradually decreases after mitosis and remains significantly above interphase levels for ~2 h after anaphase onset. Our results demonstrate that regaining nuclear transport function and establishing the full permeability barrier of the nucleus are separate processes. These findings have important implications for the cell cycle regulation of nucleocytoplasmic compartmentalization.

MATERIALS AND METHODS

Cell culture

Normal rat kidney (NRK) cells were cultured in standard Dulbecco's modified Eagle's medium. Transient transfection with pCS2-Dronpa (a generous gift from A. Miyawaki (11)) was performed by lipofection with FuGene 6 (Roche,

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Mannheim, Germany) 2 days before imaging according to the protocol of the manufacturer. A monoclonal NRK cell line stably expressing IBB-DiHcRed was established by selection of cells transfected with pIBB-DiHcRed (4) with 0.5 mg/ml Geneticin (Gibco BRL, Gaithersburg, MD). The cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 0.5 mg/ml Geneticin. For live-cell microscopy, NRK cells were grown in LabTek chambered coverglasses (Nalge Nunc International, Rochester, NY). At least 30 min before imaging, the medium was exchanged for prewarmed CO_2-independent medium without phenol red and without glutamine (Gibco BRL) supplemented with 20% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The chambers were sealed with silicone grease.

Measurements of nuclear permeability

Nuclear permeability measurements were performed at 37°C on a Zeiss LSM510 Meta microscope using the Zeiss LSM software (Carl Zeiss, Jena, Germany) with a Plan-Apochromat 40×/1.0 oil iris objective at numerical aperture 1.0, imaging with an open pinhole and low intensity of 488 nm laser light. Inactivation of the Dronpa fluorescence was achieved by brief (2–5 s) illumination with a mercury lamp via a yellow fluorescent protein filter (BP 500/20 FT 515 BP535/50). Activation was performed in an area of 70 μm^2 with a 405-nm diode laser for 200 ms. Image acquisition was at 1- to 5-s intervals with a scan speed of 1.28 μs/pixel. To exclude phototoxicity of the repeated 405-nm photoactivation illumination of Dronpa, we monitored cell cycle progression by measuring nuclear growth and, in some cases, the next cell division. Even after nine photoactivation cycles, cells showed normal nuclear growth and continued to divide, indicating no deleterious effects of the repeated photoactivation on cell cycle progression.

Intensity measurements were performed in the Zeiss LSM software or ImageJ. Mean intensities were measured in a manually segmented nuclear region selected on the transmitted light image. Mean whole-cell intensity was measured on a manually segmented area or on an automatic segmented area obtained by successive application of a Gaussian and an anisotropic diffusion filter and thresholding of the filtered image with an in-house-developed plugin (G. Rabut and J. Ellenberg, unpublished).

Evaluation of equilibration data

We describe equilibration of the fluorescent signal between the cytoplasm and the nucleus with a diffusion model between two compartments with fluorophore concentrations C_{Nuc} in the nucleus and C_{Cyt} in the cytoplasm. Flux between the two compartments via NPCs is governed by a permeability rate, k_{NE}, and is assumed to be the same in both directions, since passage of Dronpa through the NPC is expected to occur purely by passive diffusion. k_{NE} depends on the permeability coefficient of a single NPC as well as on the number of NPCs. The change of the number of molecules in the nucleus (N_{Nuc}) can then be described by

$$\frac{dN_{Nuc}}{dt} = k_{NE}\left(C_{Cyt} - C_{Nuc}\right) \tag{1}$$

The solution for photoactivation in the cytoplasm ($N_{Nuc}(0) = 0$) yields

$$N_{Nuc}(t) = \frac{N_{tot}\cdot V_{Nuc}}{V_{Nuc} + V_{Cyt}}\left(1 - \exp\left(-k_{NE}\left(\frac{V_{Nuc} + V_{Cyt}}{V_{Nuc}\cdot V_{Cyt}}\right)t\right)\right), \tag{2}$$

where N_{tot} is the total number of fluorescent Dronpa molecules in the cell and V_{Nuc} and V_{Cyt} represent the nuclear and cytoplasmic volumes, respectively. Formulated for the concentration of activated Dronpa molecules in the nucleus, this gives

$$C_{Nuc}(t) = \frac{N_{tot}}{V_{Nuc} + V_{Cyt}}\left(1 - \exp\left(-k_{NE}\left(\frac{V_{Nuc} + V_{Cyt}}{V_{Nuc}\cdot V_{Cyt}}\right)t\right)\right), \tag{3}$$

k_{NE} can be defined as the permeability of an individual NPC (P_{NPC}, in volume/time) multiplied by the number of NPCs (n) present on the nuclear surface (12):

$$k_{NE} = n\cdot P_{NPC} \tag{4}$$

To analyze the flux of activated Dronpa across the nuclear envelope, mean intensities of the nucleus (I_N) were background (I_{BG})-subtracted and corrected for acquisition photobleaching by whole-cell intensity (I_{WC}). According to Eq. 3, this ratio was fitted with a single exponential

$$\frac{I_N(t) - I_{BG}(t)}{I_{WC}(t) - I_{BG}(t)} = y_0 + A(1 - \exp(-st)), \tag{5}$$

where s will be called the equilibration rate, which depends on k_{NE} as well as on nuclear and cytoplasmic volumes. y_0 represents the initial fluorescence present in the nucleus. The first frame after activation was not considered for the fit due to the highly inhomogenous distribution of Dronpa at this time point. Fitting of the equation to the fluorescence equilibration data was performed with the Curve Fitting tool in MATLAB 7.5 (The MathWorks, Natick, MA) and Origin 7.5 (Northampton, MA).

RESULTS AND DISCUSSION

An assay for directly measuring changes in NE permeability in individual cells

NE permeability can be assayed in living cells by photobleaching of a diffusive probe in the nucleus and monitoring the equilibration of the signal from the cytoplasm (10, 13–15). However, the sensitivity of such measurements limited to single time points suffers from cell-to-cell variations in NE permeability. To directly determine changes in nuclear envelope permeability independent of cell-to-cell variations, we therefore developed an assay that allows repeated measurements in the same living cell. To this end, we used the photoswitchable green fluorescent protein Dronpa (11), which is green fluorescent in its native state but can be switched easily with blue light to a nonfluorescent state. Such deactivation is reversible, and fluorescence can be reactivated by a brief pulse of ultraviolet light. The deactivation/reactivation cycle can be repeated >100 times for single molecules (16).

Dronpa has a molecular mass of ~29 kD and distributes in both the cytoplasm and the nucleus when expressed in NRK cells (see Fig. 1, *lower right*). Reversible photoswitching of an entire field of view to the off state can easily be achieved by illumination of the sample with blue light from an arc lamp for a few seconds. After activation of a small region of the cytoplasm of an individual cell by a short pulse of 405 nm laser light, activated Dronpa quickly redistributes in the cytoplasm. In interphase, the nuclear envelope constitutes a barrier for passive diffusion, and equilibration with the nucleoplasm therefore requires several minutes (Fig. 1). To correct for acquisition photobleaching, the ratio of nucleoplasmic to whole-cell fluorescence intensity was used to monitor this flux. The data were fitted with a model for nuclear envelope permeability (see Materials and Methods) and yielded a mean equilibration rate constant of 0.9 ± 0.3 min^{-1}

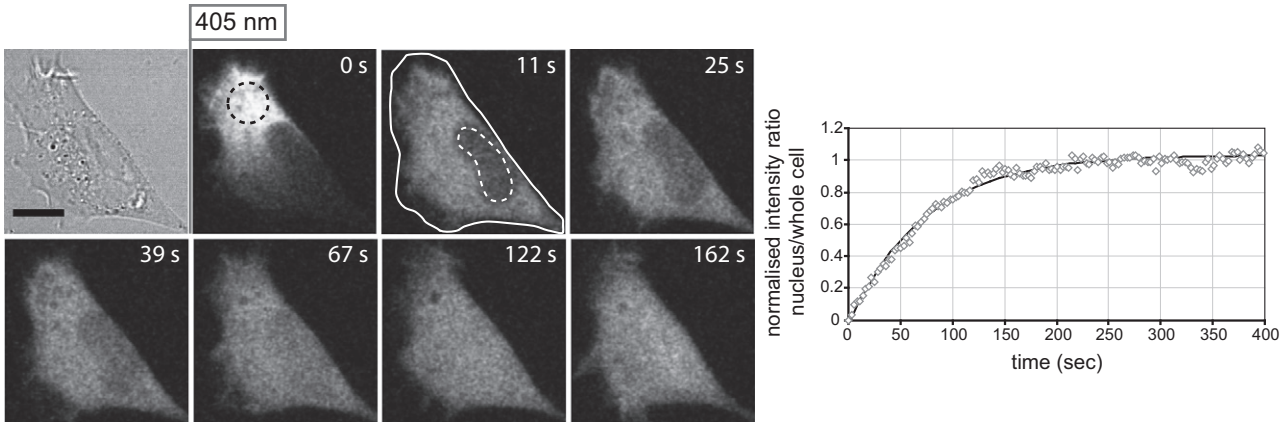

FIGURE 1 Assay for measuring nuclear envelope permeability in living cells. An interphase NRK cell expressing soluble Dronpa was reversibly photoswitched by 5 s of illumination with a mercury lamp via a YFP filter. Subsequently, the area indicated by a dashed circle was activated with a brief pulse of 405 nm laser light. Redistribution of the activated Dronpa was monitored. Nuclear fluorescence was quantified (*dashed white outline*), corrected for acquisition photobleaching with whole-cell fluorescence (*white outline*), and fitted with a single exponential (*black solid line*). Scale bar, 10 μm.

for interphase cells, which appeared to be in the S or G2 phase judging from the size of their nuclei ($n = 13$).

NE permeability increases after mitosis

Having established this quantitative assay for NE permeability, we then measured nuclear envelope permeability in individual NRK cells shortly after mitosis. Anaphase onset was defined as a reference time point to compare the behavior of different cells. To monitor the state of NPC assembly, we performed photoactivation in cells stably expressing the nuclear import marker IBB-DiHcRed (the importin β binding domain of importin α tagged with a tandem of the red fluorescent protein HcRed). Import of this reporter into the nucleus starts at ~6 min after anaphase onset and is completed within 2–3 min (Fig. 2). When Dronpa was activated in the cytoplasm of cells which were just starting to accumulate this reporter in the nucleus ~7 min after anaphase onset, no exclusion from the chromatin region could be observed, and redistribution throughout both cytoplasm and nucleoplasm could not be distinguished (data not shown). At 10–20 min after anaphase onset, diffusion of Dronpa into the chromosome region still occurred very rapidly, equilibrating with only a small delay compared to the cytoplasm, yielding high equilibration rates of >10 min^{-1} (Fig. 3 *A*, *top row*, and Fig. 4).

To determine when the full barrier function of the nuclear envelope is established, we performed photoactivation repeatedly in the same cell at different time points after mitosis (Fig. 3 *A*). As cells progressed into interphase, equilibration of the fluorescence between the nucleus and the cytoplasm occurred over an increasingly longer time frame. Equilibration rates declined rapidly during the first 30 min after anaphase onset but reached interphase levels only 1–2 h later (Fig. 4, *A* and *B*). The level reached 2 h after mitosis appears to represent the mature barrier of the interphase nucleus, as the

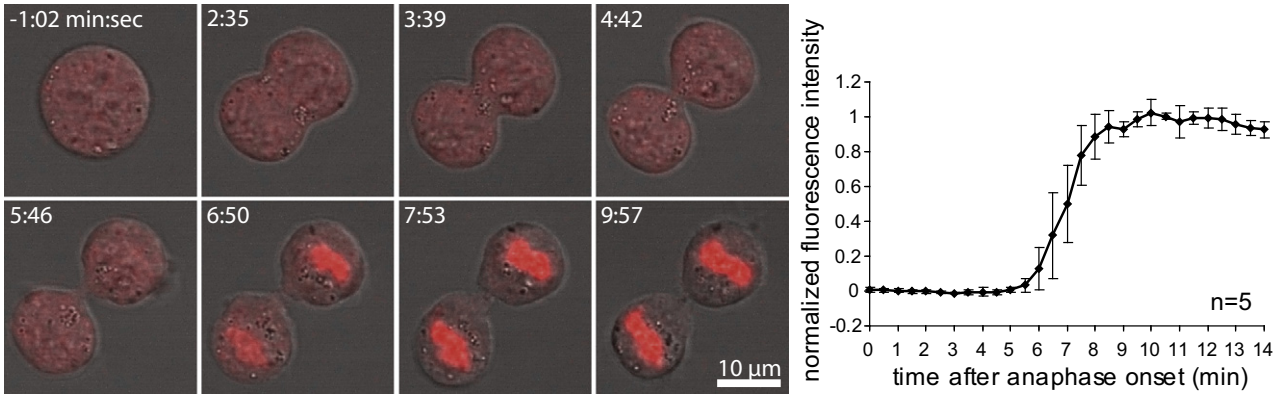

FIGURE 2 Kinetics of IBB import into the nucleus. NRK cells stably expressing IBB-DiHcRed were imaged at ~30-s time resolution through mitosis. IBB intensity in the nucleus was quantified in a manually segmented region. The quantification results of five different cells were normalized to a lower and upper plateau, aligned along the time point of anaphase onset, and averaged. Error bars show the standard deviation. Mean time from anaphase onset to half-maximal intensity in the nucleus was 6.8 $\pm$ 0.5 min. Images were filtered with a Gaussian blur filter (kernel size 4) for presentation purposes.

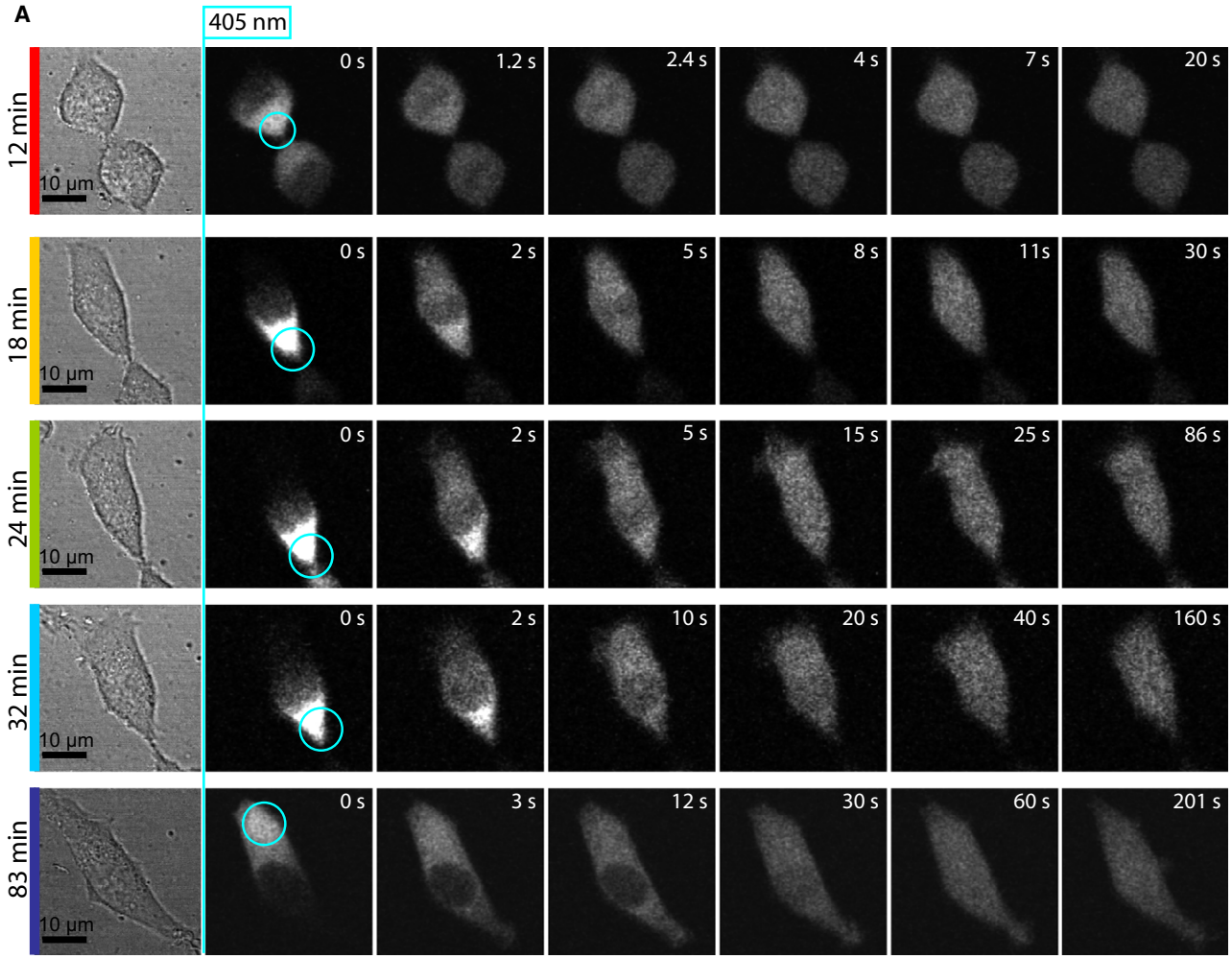

FIGURE 3 Nuclear envelope permeability is increased after mitosis. (*A*) Nuclear envelope permeability was probed repeatedly in the same cell during the first 1.5 h after mitosis. Dronpa was activated in the cytoplasm in the area indicated by a blue circle. (*B*) Mean nuclear fluorescence corrected for acquisition photobleaching is plotted. Curves were fitted with single exponentials. The fitted equilibration rate, *s*, and its standard error are given in C (see also Fig. 3). AO, anaphase onset.

equilibration rate remained constant in cells followed for up to 8 h after mitosis (Fig. 4 *A*). In summary, and in agreement with previous reports in amoebae and HeLa cells (7–9), we detect a long period of increased NE permeability for ~2 h after mitotic division in NRK cells. The establishment of the interphase permeability barrier thus lags considerably behind the

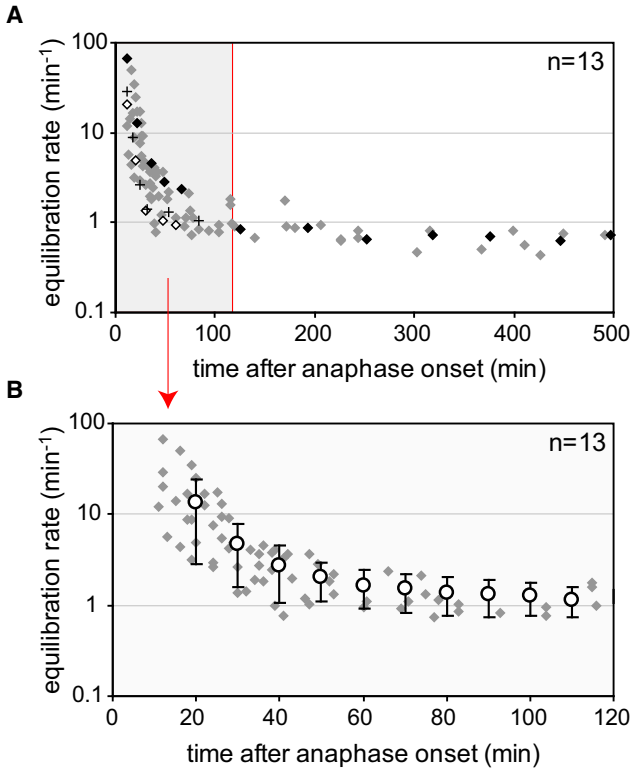

FIGURE 4 Equilibration rates decrease gradually during the first two hours after mitosis. (*A*) Equilibration rates for repeated permeability measurements after mitosis in 13 cells. The data points of three individual cells are highlighted (*crosses* for the cell shown in Fig. 2). (*B*) Close-up of the highlighted region in *A*. Data points displayed as empty circles show the average of all 13 cells. Error bars represent the standard deviation.

reformation of an import-competent nucleus (4), demonstrating that the two processes are kinetically uncoupled.

The increased leakiness of the nuclear envelope could be due either to incomplete sealing of the nuclear membranes or to a higher permeability of the newly formed NPCs early after mitosis. The accumulation of import substrates in the nucleus as early as 7–8 min after anaphase onset suggests that, at least for larger molecules of ~60 kD (e.g., NLS-BSA (17), NLS-2EGFP (18), and IBB-DiHcRed (4)), a certain diffusion barrier has already been established at this time point. However, it should be noted that in an elegant correlative light and electron microscopic study, gaps were observed in the NE by electron microscopy in cells that were already accumulating protein in the nucleus (17). This would suggest that the rate of active import could be sufficiently high to lead to nuclear accumulation despite an incompletely sealed NE. However, in this study also, NE sealing was estimated to occur by ~10 min after anaphase onset. In addition, the observation that large dextrans of >70 kD remain excluded from the nucleus in cells progressing through mitosis (19) suggests that no larger gaps remain in the NE when the chromatin is sufficiently decondensed to allow entry of larger molecules. Therefore, we can assume that the nuclear envelope is sealed at the time points analyzed here, and that the observed exchange of Dronpa molecules across the nuclear envelope occurs through NPCs.

By fitting our data and obtaining the equilibration rate, we do not directly assay the permeability of individual NPCs. As shown in Eqs. 3 and 4, the fitted equilibration rate, s, is related to the size of the cell and the nucleus and to the number of NPCs in the following way:

$$s = k_{NE} \cdot \frac{V_{Nuc} + V_{Cyt}}{V_{Nuc} \cdot V_{Cyt}} = n \cdot P_{NPC} \cdot \frac{V_{Nuc} + V_{Cyt}}{V_{Nuc} \cdot V_{Cyt}} \quad (6)$$

Since the geometry of cells undergoes dramatic changes immediately after mitosis, and since our measurements occur over a large fraction of the cell cycle during which the cell grows and new NPCs are formed, it is important to rule out the possibility that parameters other than the permeability of NPCs (P_{NPC}), such as the number of NPCs (n) or the changing volume of nucleus and cytoplasm (V_{Nuc} and V_{Cyt}), might be responsible for the dramatic decrease in the determined equilibration rate, s.

The number of NPCs doubles during interphase, and formation of new NPCs occurs throughout the cell cycle (20). However, an increase of NPC number would lead to an increase in NE permeability (k_{NE}) and, thus, a faster equilibration rather than a slower equilibration, as observed here. Since the NPC formation rate in early G1 is low (20), a constant NPC number during the first 2 h after mitosis is a valid and conservative assumption to interpret our equilibration data in terms of NPC permeability.

It remains to examine the effect of cell growth and the accompanying change in nuclear and cytoplasmic volume on the equilibration constant. Most of the measurements of postmitotic NE permeability were performed >15 min after anaphase onset. At this time point, postmitotic chromatin decondensation and the concomitant initially very rapid nuclear volume expansion are completed ((21) and E. Dultz and J. Ellenberg, unpublished results). In human cells, cell growth occurs continuously during interphase (22). Although it has been reported that nuclear volume growth occurs less homogenously (20,22), it is clear that both nuclear and cytoplasmic volumes only double throughout an entire cell cycle. However, the observed decrease in the equilibration rate in this study from 11 ± 9 min^{-1} 20 min after anaphase onset to 1.2 ± 0.5 min^{-1} 90 min after anaphase onset (i.e., by a factor of 9) (Fig. 4 *B*) would require a six- to sevenfold increase in the volumes of both nucleus and cytoplasm.

Therefore, we can conclude that the increased permeability of the nuclear envelope observed in cells during the first hours after mitosis is caused mainly by an increased permeability of nuclear pore complexes for passive diffusion.

Models for the molecular mechanism of increased NE permeability after mitosis

One possible explanation for the initial leakiness of NPCs after mitosis is that the NPCs formed during mitosis have

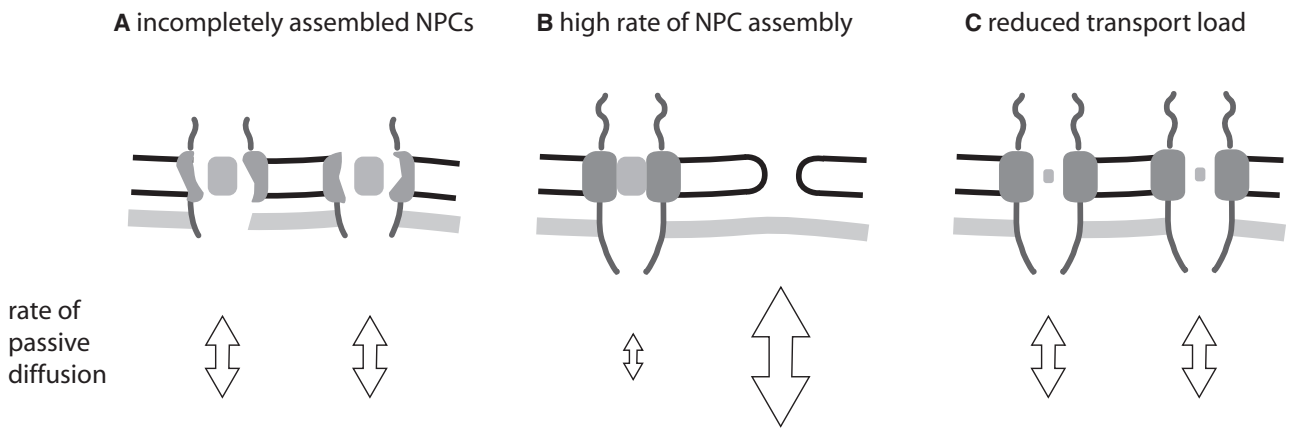

FIGURE 5 Models for increased NPC permeability after mitosis. (*A*) Although NPCs already contain most subunits shortly after mitosis, the addition of components continues into G1 and could be required to establish the full permeability barrier. (*B*) The assembly of new NPCs may occur at an increased rate early after mitosis. Early assembly intermediates probably include stages where the inner and outer nuclear membranes have already fused but the pore is not yet occupied by NPC proteins. (*C*) During mitosis, the transcription of RNAs is largely shut down, so that at early time points after mitosis, most of the large and abundant cargoes transported through the NPC in interphase are not present. This reduced transport load might lead to a higher permeability of the NPC for passive diffusion.

not yet completed their assembly although they are already import-competent (Fig. 5 *A*). Several peripheral nucleoporins associate with the NE only late in telophase and continue to assemble into G1 (4–6). The incorporation of these nucleoporins could thus be responsible for the observed decrease in NPC permeability. However, these late-assembling nucleoporins (Nup214, Tpr, and gp210) are considered to localize to the periphery of the NPC and for none of them has a role in establishing the permeability barrier of the NE been identified. An alternative explanation is that establishment of the permeability barrier could involve slow rearrangements of already bound components in a ''structural maturation process'' of the NPC.

A second possibility is that the assembly of new NPCs continues at an increased rate during the first hours after mitosis, which leads to the presence of ''open'' membrane fusion pores in the nuclear envelope and therefore to increased permeability of the nuclear envelope (Fig. 5 *B*). This is supported by the fact that many nucleoporins continue to assemble into the NE during late telophase and early G1 when nuclear import is already functional, since their density on the NE remains constant during the expansion of the nuclear envelope that occurs in telophase (4).

A third possible explanation is that the increased permeability after mitosis would be caused by differences in the metabolic state of the cell and in the types of cargo that are transported. For example, during mitosis, transcription is largely stopped, so that early after mitosis, the amount of mRNA and ribosomal particles that have to exit the nucleus can be expected to dramatically decrease. Since ribonucleoproteins and ribosomal subunits constitute two of the largest and most massively transported cargoes of NPCs, their absence in early G1 may lead to a larger volume of NPC channels available for passive diffusion (Fig. 5 *C*).

The increased NE permeability long after mitosis observed here has important implications for regulatory processes in the cell. Active transport begins to reestablish the specific protein composition of the nuclear compartment for molecules containing import (and possibly export) signals already in telophase. However, this process has to work against the initially relatively high accessibility of the nucleus for cytoplasmic proteins. For example, the exclusion of transcription factors from the nucleus, and potentially the sequestration of mitotic regulators, might be less efficient during early G1. It is tempting to speculate that cells might be able to regulate the NE diffusion barrier independently of active nucleocytoplasmic transport in circumstances other than mitosis, for example, after metabolic stimulation or during challenges by pathogens. Thus, regulation of the diffusion barrier could constitute a novel component among the regulatory functions of the nuclear envelope.

CONCLUSION

In summary, we found that the permeability of the nuclear envelope for passive diffusion is strongly increased early after mitosis compared to its permeability in interphase. Reestablishment of the permeability barrier occurs gradually during the first hours after mitosis and lags considerably behind the reestablishment of nuclear import. It will be very interesting to determine the molecular mechanism and potential biological function underlying this initial openness of the nucleus for passive diffusion in future work.

In addition, we have shown that the repeatedly photoswitchable protein Dronpa can be applied to repeatedly probe NE permeability in the same cell. We have thus established a versatile assay that can also be applied to analyze changes in NE permeability, e.g., in response to signaling

stimuli. For instance, increased permeability has been observed in response to Ca^{2+}, but conflicting data on this effect has been reported (23). Because of its independence from cell-to-cell variations, our assay provides the required sensitivity for a thorough analysis of the regulation of NPC permeability.

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REFERENCES

1. Ribbeck, K., and D. Gorlich. 2001. Kinetic analysis of translocation through nuclear pore complexes. *EMBO J.* 20:1320–1330.
2. Paine, P. L., L. C. Moore, and S. B. Horowitz. 1975. Nuclear envelope permeability. *Nature.* 254:109–114.
3. Harel, A., and D. J. Forbes. 2004. Importin β: conducting a much larger cellular symphony. *Mol. Cell.* 16:319–330.
4. Dultz, E., E. Zanin, C. Wurzenberger, M. Braun, G. Rabut, et al. 2008. Systematic kinetic analysis of mitotic dis- and reassembly of the nuclear pore in living cells. *J. Cell Biol.* 180:857–865.
5. Bodoor, K., S. Shaikh, D. Salina, W. H. Raharjo, R. Bastos, et al. 1999. Sequential recruitment of NPC proteins to the nuclear periphery at the end of mitosis. *J. Cell Sci.* 112:2253–2264.
6. Hase, M. E., and V. C. Cordes. 2003. Direct interaction with nup153 mediates binding of Tpr to the periphery of the nuclear pore complex. *Mol. Biol. Cell.* 14:1923–1940.
7. Feldherr, C. M. 1966. Nucleocytoplasmic exchanges during cell division. *J. Cell Biol.* 31:199–203.
8. Feldherr, C. M. 1968. Nucleocytoplasmic exchanges during early interphase. *J. Cell Biol.* 39:49–54.
9. Feldherr, C. M., and D. Akin. 1990. The permeability of the nuclear envelope in dividing and nondividing cell cultures. *J. Cell Biol.* 111:1–8.
10. Wei, X., V. G. Henke, C. Strubing, E. B. Brown, and D. E. Clapham. 2003. Real-time imaging of nuclear permeation by EGFP in single intact cells. *Biophys. J.* 84:1317–1327.
11. Ando, R., H. Mizuno, and A. Miyawaki. 2004. Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. *Science.* 306:1370–1373.
12. Keminer, O., and R. Peters. 1999. Permeability of single nuclear pores. *Biophys. J.* 77:217–228.
13. Lang, I., M. Scholz, and R. Peters. 1986. Molecular mobility and nucleocytoplasmic flux in hepatoma cells. *J. Cell Biol.* 102:1183–1190.
14. Peters, R. 1983. Nuclear envelope permeability measured by fluorescence microphotolysis of single liver cell nuclei. *J. Biol. Chem.* 258:11427–11429.
15. Peters, R. 1984. Nucleo-cytoplasmic flux and intracellular mobility in single hepatocytes measured by fluorescence microphotolysis. *EMBO J.* 3:1831–1836.
16. Habuchi, S., R. Ando, P. Dedecker, W. Verheijen, H. Mizuno, et al. 2005. Reversible single-molecule photoswitching in the GFP-like fluorescent protein Dronpa. *Proc. Natl. Acad. Sci. USA.* 102:9511–9516.
17. Haraguchi, T., T. Kojidani, T. Koujin, T. Shimi, H. Osakada, et al. 2008. Live cell imaging and electron microscopy reveal dynamic processes of BAF-directed nuclear envelope assembly. *J. Cell Sci.* 121:2540–2554.
18. Anderson, D. J., and M. W. Hetzer. 2008. Reshaping of the endoplasmic reticulum limits the rate for nuclear envelope formation. *J. Cell Biol.* 182:911–924.
19. Swanson, J. A., and P. L. McNeil. 1987. Nuclear reassembly excludes large macromolecules. *Science.* 238:548–550.
20. Maul, G. G., H. M. Maul, J. E. Scogna, M. W. Lieberman, G. S. Stein, et al. 1972. Time sequence of nuclear pore formation in phytohemagglutinin-stimulated lymphocytes and in HeLa cells during the cell cycle. *J. Cell Biol.* 55:433–447.
21. Mora-Bermudez, F., and J. Ellenberg. 2007. Measuring structural dynamics of chromosomes in living cells by fluorescence microscopy. *Methods.* 41:158–167.
22. Fidorra, J., T. Mielke, J. Booz, and L. E. Feinendegen. 1981. Cellular and nuclear volume of human cells during the cell cycle. *Radiat. Environ. Biophys.* 19:205–214.
23. Török, K. 2007. The regulation of nuclear membrane permeability by Ca^{2+} signaling: a tightly regulated pore or a floodgate? *Sci. STKE.* 2007:pe24.