

NUCLEAR PORE COMPLEXES

Elimination and Reconstruction during Mitosis

GERD G. MAUL

From The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

ABSTRACT

Nuclear structures similar to those of the nuclear pore complex were found on chromosomes. This finding indicates that part of the pore complex is retained by the chromosomes through mitosis in the absence of the nuclear membrane. The formation of approximately the same number of pore complexes in the presence and absence of protein synthesis during the first 4 h after mitosis proves the reassembly rather than new synthesis of the pore complex. The structure of pore complexes reconstructed in the absence of protein synthesis cannot be distinguished from the structure of those of control cells.

The nuclear pore complex has been shown to be a rather dynamic structure that can be rapidly formed and eliminated in the intact nuclear envelope (4, 21, 27). Very fast elimination and reconstruction of pore complexes also occur during mitosis. No structural information is available on the fate of the eliminated complex structure in prophase, nor of its reconstruction during telophase, though the presence of remnants of pore complexes has been shown in wholemount electron microscopy of chromosomes in some cell types (5, 6). In this communication, an attempt will be made to demonstrate that part of the pore complex structure may remain with the chromosome and is used in the formation of a new pore at telophase.

MATERIALS AND METHODS

Human melanoma cells (LeCa) were grown as previously described (19, 23). They were fixed for 1 h at room temperature 48 h after plating in 3-cm Falcon plastic petri dishes (Becton, Dickinson & Co., Rutherford, N. J.). The fixative contained 3% glutaraldehyde, 1 mM magnesium chloride in 0.1 M phosphate buffer (pH 7.4). Postfixation was carried out in 1% osmium tetroxide in the same buffer for 1 h, followed by 0.5% uranyl acetate in water for 1 h. The cells were then flat-embedded in

Epon. Appropriate stages of mitosis were preselected with a phase contrast microscope, sectioned, and double-stained with uranyl acetate and lead citrate. The sections were observed and photographed with a Hitachi HU-11E electron microscope at 75 kV. Freeze-etching of mouse L cells (clone 1D) was performed essentially as described previously (21). Nuclear size determination and calculation was made as described in detail elsewhere (20). Briefly, the nuclei of the cells growing as monolayers were measured by phase contrast microscopy. The height was determined from cross sections by electron microscopy, and the nuclear surface was determined by using a formula that describes the nuclei as a flat elliptical body with rounded margins.

Mitotic cells of mouse L cells were selectively removed from Blake bottles (Blake Industries, Inc., Springfield, N. J.) by gently shaking after 2 h of vinblastine treatment (0.05 μ g/ml). A hard shake before the addition of new medium with vinblastine removed debris and many of the still rounded doublets. Those doublets not removed during the hard shake appeared as binucleated cells if shaken off during the soft shake and observed 4 h after the removal of the drug. To half of the selectively detached cells cycloheximide was added, and vinblastine washed out in the presence of cycloheximide. About 20% of the cells were micronucleated after the short vinblastine treatment (13). They were not used for the nuclear size determinations presented in Results. An estimate of the combined nuclear surface of these multi-

nucleated cells indicates that it is somewhat larger than the surface of the single nuclei, but no definite size difference between the two samples (control and cycloheximide-treated) could be found within the limits of measuring these very small nuclei.

For the structural comparison of nuclear pore complexes of mouse L cells, 4 h after mitosis 20 large nuclear surfaces were photographed at low magnification, and selected areas at high magnification. After printing, the control and experimental groups were mixed and then analyzed. The following structures were evaluated: the number of central granules and the density of the traverse fibrils.

The effect of the protein inhibitor cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was tested 3.5 h after mitosis. Aliquots of the cells were incubated with 10 μ Ci of leucine (New England Nuclear, Boston, Mass., sp act 60 Ci/mmol) for 30 min. In preliminary experiments, it was found that 25 μ g/ml cycloheximide reduced protein synthesis in exponentially growing cells about 95%. The cells were washed three times in Hanks' balanced salt solution and then precipitated with TCA and collected on glass fiber filters.

RESULTS

The structure of the nuclear pore complex was analyzed in serial sections before components of it were looked for on chromosomes. Fig. 1*a-d* shows a series of micrographs illustrating the different appearances of the pore complex, depending on the plane of section. Pore complex 1 shows the projection of the traverse fibrils (16, 18) and a small central granule (Fig. 1*a*). In the next micrograph (Fig. 1*b*), radiating fibers and a central ring are apparent. In Fig. 1*c*, the central ring can still be seen (indicating a short tubule), though it is slightly larger; there is a larger ring structure visible in Fig. 1*b* which is larger than the pore perimeter. Fibrous material radiates laterally also in Fig. 1*d* where the central ring can be recognized, again somewhat larger than in the previous micrograph. Similar structures can be seen in other pore complexes, though the sequence may be different. In pore complex 4, a 50-nm central ring is attached to the traverse fibrils which converge at the cytoplasmic side (Fig. 1*b*). The central ring is also at the cytoplasmic side in pore complex 9 (Fig. 1*a*) and pore complex 6 (arrowhead), and is very obvious in pore complex 11 in Fig. 1*b*. It will be attempted to identify the arrangements of the non-membranous parts of the pore complex (1, 2, 3, 4, 7, 8, and 9) seen mainly in Fig. 1*c-d* at the chromosomes.

The images presented in Fig. 1 show that traverse fibrils pass through the pore proper and extend into the nuclear and cytoplasmic side at an

angle which makes them appear as short rods (pore complex 5, Fig. 1*d*; pore complex 6, Fig. 1*c*). The appearance of a ring structure larger than the pore membrane diameter does not come solely from tilted traverse fibrils, but also from some other material which seems to connect them. This material also appears as short cylinders without traverse fibrils attached (pore 9, Fig. 1*d*). According to the results obtained with preferential staining techniques, this material does not consist of chromatin (12); however, this material apparently connects with chromatin (14, 16, 17, 18).

During prophase, the chromosomes condense on the nuclear envelope, and pore complexes seem to be present less frequently on most areas of chromosome attachments to the membrane. In prometaphase, the nuclear envelope breaks at several points, but pore complexes can still be seen nearly intact at the areas where chromosomes and remnants of the nuclear envelope are closely attached. Areas where the nuclear envelope was removed from the chromosomes were analyzed for remnants of the nonmembranous pore complex structures. When found, these consisted mostly of the equivalent of the larger ring structure which was described as being in intimate connection with the traverse fibrils (Fig. 2, structure 1). Traverse fibrils are difficult to see in their projection since, if they are present at all, their recognition depends on the plane of the section. The membranous pores seem to expand before losing their recognizable identity. A rather compact and dense structure (Fig. 2, arrow pointing up) has been seen several times in the face-on views of chromosomes. It consists of a central density, radiating fibers, and a ring about 40 nm in diameter. Other ring and radiating fibrous structures suggestive of pore complex remnants are presented in Figs. 2-9. No complete pore complexes were seen during the brief anaphase. Four anaphase cells were analyzed. The rather dense annular component of the chromosome in Fig. 5 is particularly similar to structures seen in whole-mount preparations (5, 6, 7).

At early telophase, the first short membrane pieces are flatly apposed to the chromatin at various areas. In cross section, fine fibrils seem to emanate from the chromatin attached to the membrane pore (Fig. 8). In slightly more oblique sections (Fig. 9), one can discern some more lighter staining material with a central unstained spot at what does not yet seem to be a completed pore complex.

Some structures could be correlated with the

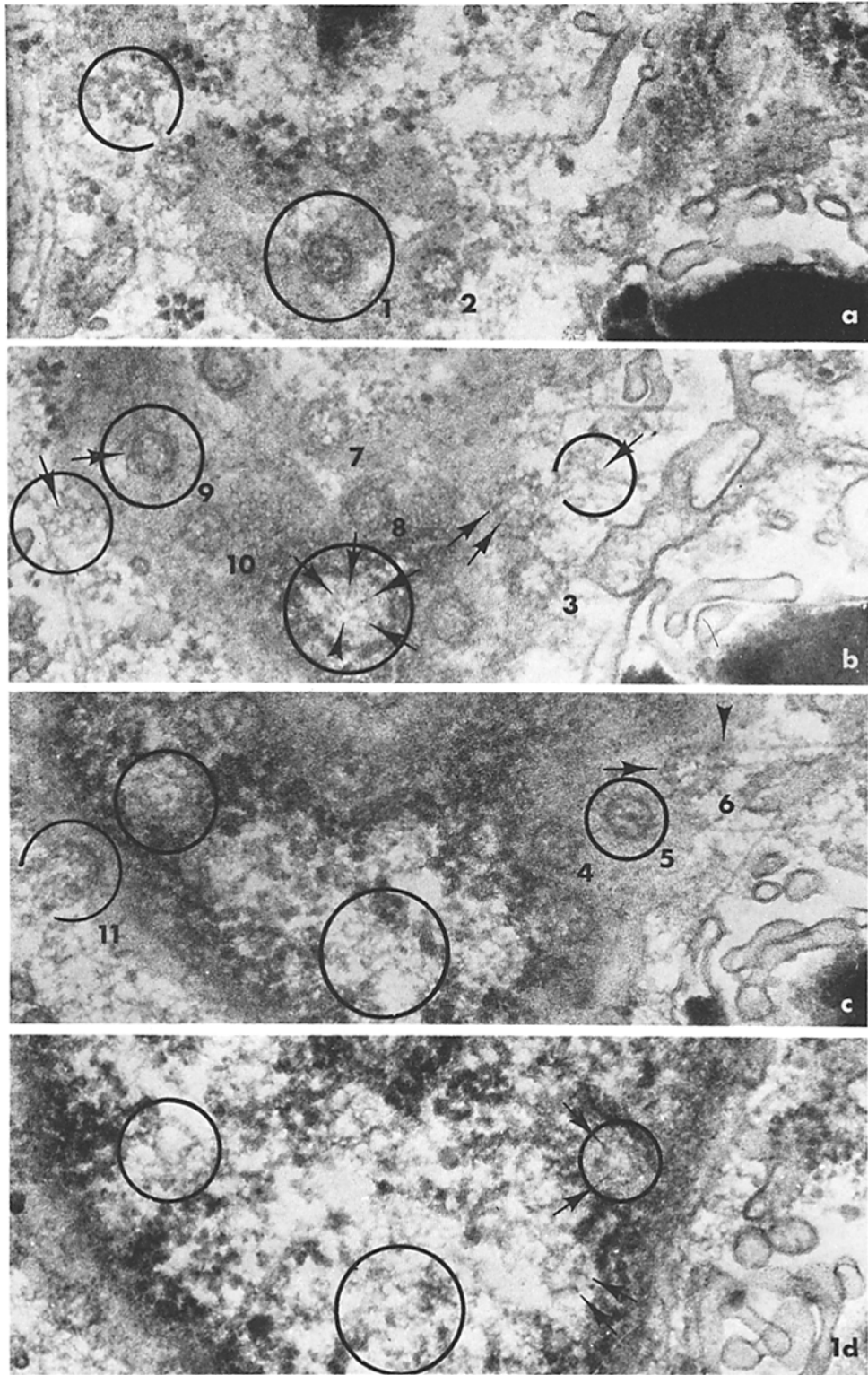


FIGURE 1a-d Consecutive sections of the nuclear envelope. Pore complexes are numbered and explained in the text. $\times 40,000$.

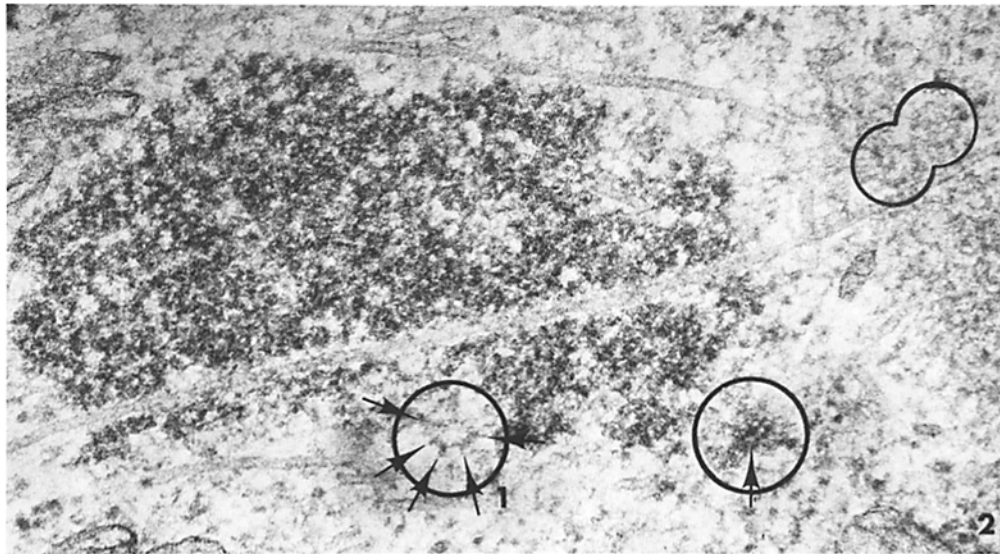


FIGURE 2 Tangential section of a chromosome in late prometaphase with different structures all resembling the pore complex arrangements, except for a compact structure apparently embedded in the peripheral part of the chromatin (arrow pointing up). $\times 75,000$.

underlying chromatin or membrane only by the use of serial sections. In Fig. 10a, there is a ring structure of the type described in Fig. 1 which seems to be only partly surrounded by membranes. It is situated over one of the smaller dense ring structures in the chromatin (Fig. 10b) described in Fig. 2. Since this arrangement has been observed several times, it does not seem coincidental.

If parts of the pore complexes are preserved through mitosis on the chromosomes, then they may be assembled during the time of nuclear envelope reformation without additional protein synthesis. From our results with HeLa cells, we knew that the total pore number doubles from a G_1 -phase plateau until the end of the cell cycle. Expressed differently, the daughter cells at G_1 have the same combined pore number as the cell before division (21). The hypothesis then that pore complexes are reassembled but not resynthesized was tested by comparing the total pore number of mouse L cells 4 h after removal of the mitotic block in an aliquot treated with 25 $\mu\text{g}/\text{ml}$ cycloheximide with the pore number of untreated cells. Mouse L cells were chosen since HeLa cells used in early synchronization experiments (21, 24) reacted rather strangely to even very low concentrations of colcemid or vinblastine. The G_2 phase was prolonged 2 or 3 h (few mitotic figures accumulate during this time) and many cells did not go

through the cell cycle after colcemid treatment because they did not enter S-phase (as tested autoradiographically). A large number of the cells became necrotic ~ 10 –12 h after the treatment with mitotic inhibitors. Since we wanted to add the protein synthesis inhibitor at the time of complete absence of the nuclear envelope, mouse L cells were used. The only disadvantage recognized was the presence of a sizeable number of micronucleated cells (21%). Binucleated cells were not used for nuclear size determination since they probably reflect an incomplete cell division. The estimated nuclear surface from three determinations was 290 μm^2 for the cycloheximide-treated sample and 302 μm^2 for the control if the average length and width of three synchronizations are used (Table I). The pore frequency for the cycloheximide-treated cells was less (7.38 ± 0.6 pores/ μm^2) than the control (8.49 ± 1.5 pores/ μm^2). There is a remarkably low standard deviation for the cycloheximide-treated sample. The difference is statistically significant ($P = 0.01$); the total pore number is slightly less than the control (G_1 control 2,564 pores/nucleus, G_1 cycloheximide-treated 2,140 pores/nucleus, or 16.5% less than control).

This clearly demonstrated that essentially all pores present in the nuclear envelope at G_1 can be reconstructed from proteins synthesized in the previous cell cycle or from preserved structures at the chromosomes. Protein synthesis was inhibited

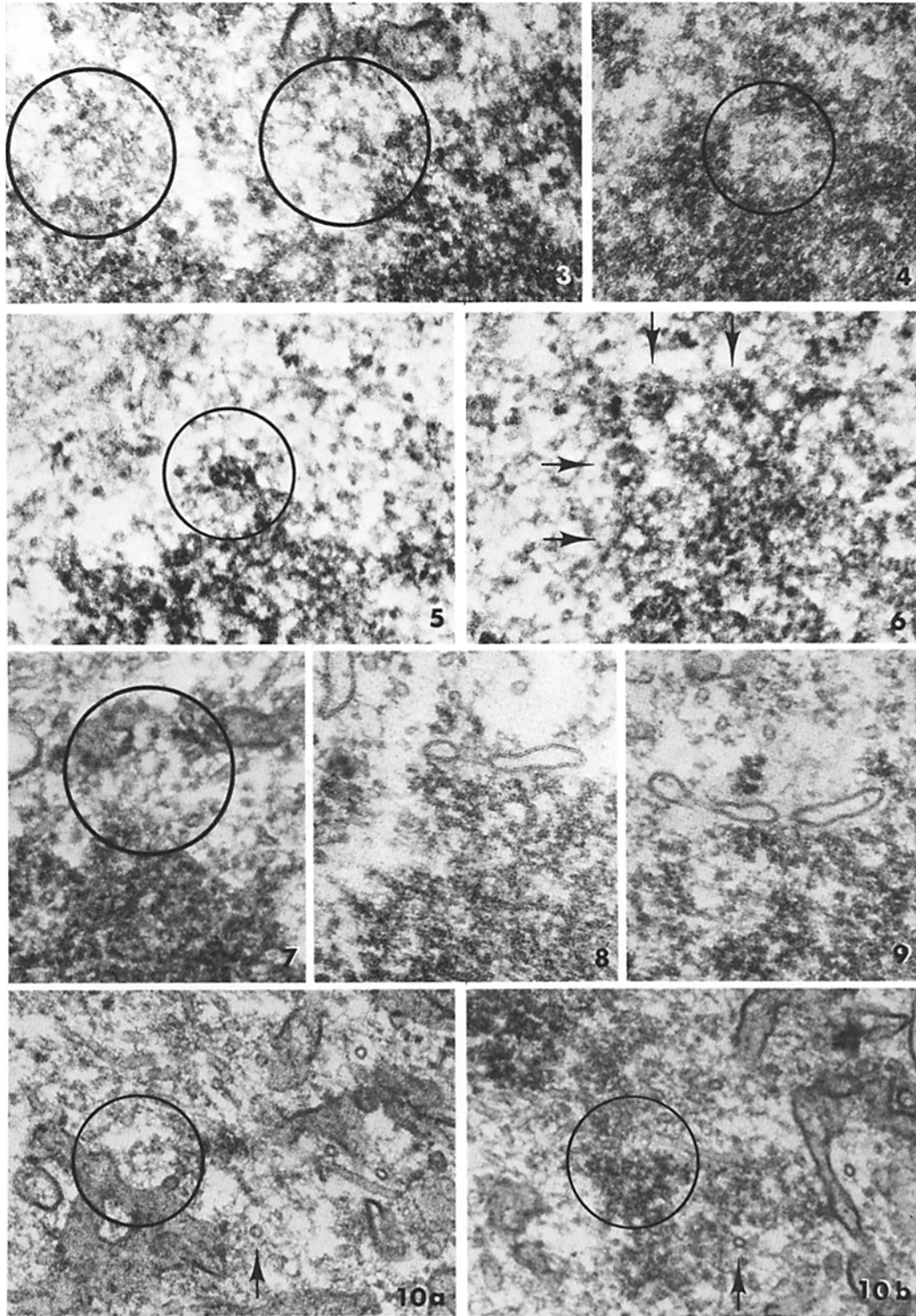


TABLE I
Quantitative Determination of the Nuclear Pore Number 4 h after Mitosis

Exp	Control			Cycloheximide-treated cells		
	Length \pm SD	Width \pm SD	Height \pm SD \ddagger	Length \pm SD	Width \pm SD	Height \pm SD
1	13.1 \pm 2.3	8.7 \pm 1.8	3.9 \pm 0.8	14.2 \pm 2.2	8.5 \pm 1.5	3.7 \pm 0.5
2	15.1 \pm 2.2	8.9 \pm 1.7		14.0 \pm 2.0	8.2 \pm 1.7	
3	14.4 \pm 2.0	8.3 \pm 1.5		15.6 \pm 2.2	7.7 \pm 1.7	
Mean	14.2	8.6	3.9	14.6	8.1	3.7
Nuclear surface		302			290	
Pores/ μm^2 *		8.49 \pm 1.5			7.38 \pm 0.61	
Pores/nucleus		2,564			2,140	

* The pores/ μm^2 were determined in the first experiment.

\ddagger The height was determined only in one experiment.

96% by using 25 $\mu\text{g}/\text{ml}$ cycloheximide. The test for the incorporation of [^3H]leucine was performed for 30 min from 3.5 h after mitosis by adding 10 μCi leucine to aliquots of the cycloheximide-treated and the control groups.

The structure of pore complexes was compared in the control vs. cycloheximide-treated cells in order to determine whether all structural elements are reconstructed. Mostly face-on views of large nuclear membrane areas were used for estimating the number of central granules and the size of the traverse fibrils. All nuclear pore complex structures were present in the cycloheximide-treated cells. In an attempt to quantitate, no significant difference was found measuring the diameters of the traverse fibrils but the central granules were seen less often ($P = 0.1$). The central granule frequency was determined for each envelope fragment and the means compared. A comparison of

central granules is afflicted with a number of variables which become obvious if different persons evaluate the same micrographs. One variable is the size and density differences within a population of central granules. The other variable is the location since it has been shown in serial sections that a central granule can be located at different levels of the pore complex (18). Other structural arrangements such as the ring at the nuclear side, the so-called radiating fibrils could also be seen in the cycloheximide-treated cells, but any smaller differences between the two samples may go undetected.

DISCUSSION

There are two pore complex systems in some cells—the nuclear and the annulate lamellae pore complex—which have a similar ultrastructure. The basic difference seems to be that one is formed

FIGURES 3–6 Metaphase chromosomes exhibiting structural arrangements of the pore complex. $\times 100,000$.

FIGURE 7 Early telophase attachment of membranes at the chromosomes. The pore complex is not complete but the ring structure with laterally radiating fibers seems to be attached to the membrane. $\times 100,000$.

FIGURE 8 Double membrane sheet attached to telophase chromosomes. The pore complex seems incomplete but fibers are attached to the rim of the membranous pore. $\times 100,000$.

FIGURE 9 Incomplete pore complex at telophase. The membrane pore in this slightly oblique section is situated at a circular arrangement of some material that stains lighter than chromatin. $\times 100,000$.

FIGURE 10a and b Two consecutive sections of a telophase chromosome near the kinetochore. Fig. 10a shows a larger ring structure partially surrounded by membranes, with some tenuous fibrils apparently touching it. At the same position in the next section, there is a densely staining structure like the one indicated by an arrow in Fig. 2. The cross-sectioned mitotubules can be used for orientation (arrows). $\times 75,000$.

without the physical proximity of chromatin, which suggests that pore complex formation is not necessarily dependent on induction by chromatin.

The nonmembranous pore complex structures are thought to be part of the fibrous lamina or the nuclear matrix, since they stay intact after DNA digestion and Triton treatment (1, 2, 6, 8, 26, 28). It is not known what happens to the fibrous lamina during mitosis. In most cells, it is so tenuous that it can be observed only after treatment of isolated nuclei (for instance in WI38 nuclei; reference 18). It is, therefore, not conspicuous even if it is present at the surface of the chromosomes. Under the assumption that the organized structures of the pore complex will be more easily identified, chromosomes were analyzed for parts of the pore complex at their periphery which has lately been shown to consist of an RNA-containing coat by the preferential EDTA staining technique and digestion with RNase (25).

Observations on the pore complex in intact nuclear envelopes preceded the analysis of micrographs with chromosomes in different stages of mitosis. The images of pore complexes corresponded best to those previously published by Abelson and Smith (3), who also used tissue culture cells, which have the advantage of extremely fast fixation. In their studies, the annular parts of the pore complex are wider on both sides of the nuclear envelope, resulting in an hourglass shape for this structure. Also, no annular granules were found at the nucleoplasmic side of the pore complex, but rather a ring connecting the traverse fibrils.

Preservation of the annular substructure at the nuclear side in some continuity with the condensed chromatin during the mitotic cycle seems probable from the images obtained. The danger of interpreting a randomly occurring ring arrangement for part of the remnants of the annular subunits is obvious, but few, if any, such arrangements were found in the cytoplasm. Also, there was a conspicuous accumulation of these images at the edges of chromosomes sectioned tangentially. However, owing to the variability of appearance of the larger ring structure in properly aligned nuclear envelope sections, i.e., face-on, it was essential to use serial sections to identify annular structures as being connected to chromosomes. These findings suggest a preservation of part of the pore complex, a preservation which may be instrumental in the rapid reformation of pores after mitosis (21), but it should not be used as

evidence that parts of all pore complexes are preserved at the chromosome.

During the reformation of the nuclear envelope, double-membrane sheets attach to the chromatin. No preferential attachment to the presumptive annular structures could be observed. Rather, the opposite seems to be the case. In incomplete pore complexes in cross-section, some lightly staining fibrous material is attached to the rim of the membrane pore. There is some evidence that the membrane pore can be developed either according to the scheme proposed earlier (22), i.e., by induction and fusion of the inner with the outer membrane, or by the wrapping of membranes around an annular structure (Figs. 7 and 10). This would also require a fusion and fission event. Underneath a few presumptive annular structures at the level of the condensed chromatin, a peculiar densely staining arrangement of fibers was found. Since this has been seen several times, perhaps certain sites exist on the chromatin where annular structures are retained or assembled.

The structural comparison of pore complexes reassembled in the absence of protein synthesis showed no obvious structural deviation from control cells. The apparently smaller number of central granules may be due to a lesser density in the cycloheximide-treated cells. Under the assumption that it represents the structural expression of material in transport, it would not necessarily have to be regarded as a pore structure per se. Under such an assumption, it may represent a slightly diminished transport in or out of the nucleus.

From this investigation, it cannot be judged whether or not all pore complexes remain with the chromosomes and are to be reassembled after mitosis. The structural integrity of the pore complexes in the absence of a membrane can be assumed not only from their presence in Triton-treated nuclear membranes (6, 8, 26, 28) but also from their presence in mosquito oocytes as membrane-free sheets (10, 11). Whether single intact pore complexes or subunits can exist free in the cytoplasm can not be determined at present by ultrastructural means.

Essential to the explanation of annulate lamellae pore complex formation which proceeds at the same time (15) is the assumption that some annular structures or their subunits can be released from the chromatin in mitosis, or from the nuclear envelope during pore elimination (see also reference 9), or that they are newly synthesized and can be assembled without the presence of chroma-

tin-inducing sites. The ability of the two daughter cells to form approximately the same total pore number 4 h after mitosis (see also reference 24) with or without any protein synthesis proves that pore complexes up to a certain number are re-assembled after mitosis and not resynthesized. The increased pore frequency in the control cells may be an expression of the continuous increase in frequency during the cell cycle as determined in HeLa cells (21). The preservation of parts of the pore complexes at the chromosomes may aid in the fast formation of pores during the reconstruction of the nuclear envelope.

The expert technical assistance of Ms. Patricia Connelly, Mr. Howard Nathan, and Mr. Joseph Weibel is gratefully acknowledged.

This research was supported, in part, by U. S. Public Health Service research grants CA 10815 from the National Cancer Institute, GM 21615 from the National Institute of General Medical Sciences, and RR 05540 from the Division of Research Resources. G. G. Maul is a recipient of a Faculty Research Award (FRA-118) from the American Cancer Society.

Received for publication 3 November 1976, and in revised form 31 March 1977.

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