observations on interphase nuclei

in relation to DNA replication

J.H.N. Schel

OBSERVATIONS ON INTERPHASE NUCLEI
in relation to DNA replication

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. A. J. H. VENDRIK
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN
OP DONDERDAG 20 OKTOBER 1977
DES NAMIDDAGS TE 2 UUR PRECIES

DOOR

Johannes Hendrikus Nicolaas Schel

geboren te Nijmegen

1977
DRUK: KRIPS REPRO MEPPEL

Aan mijn ouders,
aan Hermien en Noud

DANKWOORD

Bij het verschijnen van dit proefschrift wil ik graag allen bedanken, die op enigerlei wijze aan de totstandkoming ervan hebben bijgedragen.

De medewerkers van de afdeling Chemische Cytologie ben ik dank verschuldigd voor de prettige samenwerking. Met name wil ik Marianne van Geffen-Koning bedanken voor haar enthousiaste assistentie en daarbij de verzorging van de planten, die anders ongetwijfeld een kommervol bestaan tegemoet hadden gezien, als ze er al ooit geweest waren.

Jan Hoeymakers en Leo Steenbergen ben ik zeer erkentelijk voor de bijdragen, die zij in het kader van hun studie biologie aan dit onderzoek hebben geleverd.

Mijn collega Leon Mullenders, die een groot gedeelte van de extracties, beschreven in hoofdstuk 5, uitvoerde, dank ik voor de goede verstandhouding en zijn toestemming nog niet gepubliceerd materiaal voor deze dissertatie te gebruiken. Dit geldt ook voor Theo de Greeff, met wie ik in de afgelopen jaren vele stimulerende discussies mocht hebben.

Een groot gedeelte van het onderzoek werd uitgevoerd op de afdeling Submicroscopische Morfologie. De onbaatzuchtige hulp van veel medewerkers heb ik zeer gewaardeerd. De nimmeraflatende bereidheid tot hulp van met name de heer Dicke heb ik bijzonder op prijs gesteld.

Ine Grotens-de Cock dank ik voor de efficiente wijze van literatuurvoorziening, Han Hoes voor zijn veelal noodzakelijke verbeteringen van het geschreven engels en Hans Keeris voor de punctuele verzorging van de illustraties en het ontwerp van de omslag.

Een speciaal woord van dank aan Agnes Wijntjes-Lemmens voor de snelle en accurate verwerking van de tekst.

Mijn ouders zal ik altijd dankbaar blijven voor de studiemogelijkheden die ze mij hebben gegeven.

Hermien, niet in de laatste plaats bedank ik jou, omdat je hebt willen aanvaarden dat een proefschrift de aandacht weleens van andere zaken wil afhouden.

Deze onderzoekingen werden gedeeltelijk gesteund door de Stichting Scheikundig Onderzoek in Nederland (SON) met een subsidie van de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek (ZWO).

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ABBREVIATIONS AND SYMBOLS

Ci	curie
D	dalton
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNP	deoxyribonucleoprotein
dpm	disintegration per minute
EDTA	ethylenediaminotetraacetic acid
EF	exo- or endoplasmic fracture half
ES	exo- or endoplasmic surface half
^{3}H-TdR	(methyl-^{3}H)-thymidine
min	minute
NPC	nuclear pore complex
PAA	polyacrylamide
PF	protoplasmic fracture half
PHA	phytohemagglutinin
PS	protoplasmic surface half
PTA	phosphotungstic acid
Pt/C	platinum/carbon
Pt/Pd	platinum/palladium
RNA	ribonucleic acid
RNase	ribonuclease
RSB	reticulocyte standard buffer
RSM	rapidly sedimenting material
SDS	sodium dodecylsulphate
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl) aminomethane

CHAPTER I

GENERAL INTRODUCTION

" When a thing ceases to be a subject of controversy, it ceases to be a subject of interest "

William Hazlitt,
English essayist, 19th century

1. GENERAL INTRODUCTION

1.1 Organization of DNA in bacteria.

Ten years after the elucidation by Watson and Crick (1953) of the structure of DNA - the macromolecule which contains the genetic information in all living organisms - Jacob et al. (1963) proposed the concept of the replicon as a basic unit of DNA replication in bacteria. In their view, replication of the circular bacterial genome would be initiated at a specific site, the replicator (later often named initiation site or origin). Once started, DNA synthesis within a replicon continues until completion; the origin would be attached to a location on the bacterial cell membrane.

Leibowitz and Schaechter (1975), in their review on membrane attachment of the bacterial genome, conclude that " .. it is not yet possible to give an accurate picture of the role of membrane attachment in different functions of the bacterial chromosome. " They cautiously consider the replicon model to be correct in postulating that genome segregation in bacteria takes place by the attachment of daughter chromosomes to different sites of the cell membrane. They also conclude the origin of DNA replication to be attached to the membrane, although they think that the evidence for a location of the replication point itself on the membrane is not very strong (for other reviews on prokaryotes, see Jacob et al., 1966; Pato, 1972; Smith, 1973).

1.2 Chromatin and the nuclear envelope in eukaryotes.

The genome of eukaryotes is surrounded by a nuclear envelope, by which the nuclear contents are separated from the cytoplasm. The amount of DNA in one single nucleus varies from species to species but is, compared to prokaryotes, much more than needed to code for all proteins. There are many repeated sequences and the excess of DNA reflects many repeated genes or possibly has regulatory functions (see Bostock, 1971). In contrast with the prokaryotes is also the association of this mass of DNA with basic proteins, the histones, and with non-histone proteins. This associated complex is named chromatin or DNP (deoxyribonucleoprotein). During interphase the chromatin is more or less condensed (hetero- and euchromatin); in this phase the so important processes of transcription and replication take place.

Comings (1968) has paid much attention to "the problem of an ordered arrangement of chromatin in the interphase nucleus." He argued that nature's ability to create order from apparent disorder - as happens in mitosis, where the chromatin arranges itself into distinct chromosomes, which separate to opposite poles very exactly - would be aided by a non-random arrangement of chromatin in the nucleus. Comings suggested that:

a. chromatin is attached to the nuclear membrane;
b. the chromatin itself has specific attachment sites which may correspond to points of initiation for DNA replication, and

c. there is probably some degree of order in the arrangement of chromatin in the interphase nucleus.

Evidence in support of the concept that the sites of chromatin attachment are situated at specific locations on the nuclear envelope was mainly obtained from ultrastructural studies. In 1965, DuPraw had been the first to observe that the chromatin fibers in honeybee embryonic cells were attached to the nuclear envelope, typically at the edge of a part of the nuclear pore complex, the annulus. Comings and Okada (1970a) presented electron micrographs of whole-mount spreads of cultured mammalian and avian cells showing several chromatin fibers attached to one annulus. Other reports describing similar complexes have since appeared (Maul, 1971; Engelhardt and Pusa, 1972).

Further evidence comes from observations that chromosomes have fragments of nuclear membrane or nuclear pores attached to them (Roth and Daniels, 1962; Davies and Toose, 1966; Bajer and Molè-Bajer, 1969; Comings and Okada, 1970b; Blecher, 1971).

Since then, it has been proposed by several authors that the specific attachment sites of the chromatin should be located in the nuclear pore complexes (Lampert, 1971; Engelhardt and Pusa, 1972; Maul, 1972; Maul et al., 1972).

1.3 DNA replication and the nuclear envelope.

The interphase is usually divided into a G_1, S and G_2 phase. In the S-phase the daughter DNA is synthesized. To double the large amount of DNA in this rather short

period, a high number of tandemly arranged replication units are present within each molecule (Huberman and Riggs, 1968).

It is tempting, on the analogy of bacteria, to suggest also a role of a membrane - in this case the inner nuclear membrane - in the replication process of the eukaryotic cell. In 1968, therefore, Mosolov proposed a model for the function and packing of the DNA in the chromosome, taking into account the association between the genome and the nuclear envelope. He proposed the bases of replicon loops to be fixed to the inner surface of the nuclear envelope. In his view, the places where the replicons are attached to the membrane are the points of beginning and ending of replication.

In 1972, Comings proposes three major alternatives in the interpretation of the many available data:

a. DNA replication in some or all replicons may be initiated at the nuclear membrane;
b. the replication fork may remain at the nuclear membrane;
c. the nuclear membrane may have nothing to do with DNA replication and the patterns observed may be due merely to the association of heterochromatic DNA with the nuclear membrane.

He concludes that there is evidence both for and against these alternatives and that the data are insufficient to allow an unambiguous choice as to which one is correct.

Huberman (1973), however, states that "... one structure whose attachment to the nuclear membrane has recently been definitely disproved is the replication fork." He cites, besides work of his own (Huberman et al., 1973), the careful study by Fakan et al. (1972) who showed that

after a very short pulse with ^{3}H-TdR the label was found throughout the whole nucleus. Moreover, they showed that much of the biochemical evidence for attachment of replication forks to the nuclear membrane may also be explained by artificial attachment of single-stranded DNA to membrane-like material through inappropriate de-proteinization procedures.

Kay and Johnston (1973) also conclude that there is scanty support for the view that all DNA replication or its initiation is brought about by a machinery specifically located at the nuclear envelope. They suggest that the functional significance of the attachment of chromatin to the nuclear envelope may be in ordering chromosomes for mitosis and in the ordering of the interphase chromatin, but they admit to not being able to evaluate, at that moment, the significance of observations made by Pearson and Hanawalt (1971) and Hanaoka and Yamada (1971). These authors, using the M-band technique of Tremblay et al. (1969), showed that newly replicated DNA was selectively enriched in the M-band. From these findings they concluded that DNA was being synthesized at the nuclear membrane. Further, Yamada and Hanaoka (1973) present evidence suggesting that the initiation point of DNA synthesis must be associated with the nuclear membrane before S-phase; they indicate, that this process is probably accomplished with the aid of newly synthesized protein.

Fansler (1974), in his review, states that it is very difficult to draw firm conclusions from the biochemical and cell fractionation studies covered, although he thinks that the EM autoradiographic evidence overwhelmingly

indicates that DNA is replicated throughout the nucleus. He cites Comings and Okada (1973), who conclude - reinvestigating the relationship between DNA replication and the nuclear membrane in Chinese hamster cells - that neither the initiation nor the continuation of DNA synthesis occurs on the nuclear membrane.

Franke and Scheer (1974) think the results reported on this problem highly contradictory; in a note, added in proof, however, they mention an increasing number of articles in which it is concluded that replication sites are neither in early S-phase nor at any other time of S-phase exclusively or preferentially located at the inner nuclear membrane.

The reviews, cited in this section (Comings, 1972; Huberman, 1973; Kay and Johnston, 1973; Fansler, 1974; Franke and Scheer, 1974), cover most of the literature till mid 1973. Some other papers dealing with this subject have appeared since then. Hanania and Harel (1973), working with animal cells, are not able to exclude the possibility of initiation sites, preferentially associated with nuclear membrane. Hildebrand and Tobey (1973) showed an increased amount of DNA, complexed to membrane specifically during S-phase in synchronized Chinese hamster cells. They suggest, that DNA-membrane complexes may play a role in both spatial and temporal organization of DNA during the cell cycle. This view is supported by the work of Binkerd et al. (1974), who draw the conclusion, from their results with HeLa cells, that a nascent DNA-nuclear membrane complex might indeed occur at the beginning of S-phase. These complexes, formed during the first 10 minutes of the S-phase, might remain as stable structures throughout the cell cycle (Cabradilla

and Toliver, 1975; Dye and Toliver, 1975).

Also for plant cells (Gossypium) it was concluded that newly replicated DNA is attached to the nuclear membrane during periods of active synthesis (Clay et al., 1975). Another plant cell system (Haplopappus) shows DNA replication near or on the nuclear membrane, but only in the later part of the S period (Sparvoli et al., 1976). In the opinion of these authors, this result might be explained by a DNA fraction - probably present in heterochromatin - which replicates in late S-phase.

Observations by Hobart et al. (1976), made by means of EM autoradiography, support earlier biochemical findings that DNA replication occurs at membrane sites in cleavage nuclei of sea urchin embryos (Infante et al., 1973).

Recent papers, not covered by the reviews mentioned and speaking against a function of nuclear membrane in nuclear DNA replication are those by Hyodo and Eberle (1973) who used mouse leukemia cells, by Kuroiwa (1973, 1974), using Physarum polycephalum and Crepis capillaris and by Fakan and Hancock (1974) who worked with mouse cells.

So, at the moment there still exists a considerable controversy regarding the possible involvement of nuclear membrane components in DNA replication. Maybe some rather new insights in nuclear structure will contribute to making these now still conflicting results more compatible. A short survey of these developments will be given in the next section (my own observations are described in chapter 5).

1.4 The "nuclear protein matrix".

A fibrous network structure, remaining after extraction of rat liver nuclei, was observed first by Zbarsky et al. (1962). After extraction with 1-2 M NaCl, followed by a treatment with aqueous butanol, a network structure was left over as seen in thin-sections. In later years, also Smetana et al. (1963) and Shankar Narayan et al. (1967) describe the presence of a network after extraction with 1.4 or 2 M NaCl.

A fibrous lamina, remaining after extraction of nuclei with non-ionic detergent, was observed by Barton et al. (1971). These workers showed that the outer and inner membranes, the pore-annulus complex, the fibrous lamina and the perinuclear chromatin layer (Davies, 1967) were all interconnected, together constituting an integrated cortex surrounding the nucleus. They state, that this compound cortex preserves the normal appearance in the light microscope despite the absence of nuclear membranes. As suggested by them, the perinuclear chromatin layer is intimately associated with the fibrous lamina and with the pore apparatus, and may be involved in organizing these structures.

The presence of fibrous structures, interconnecting annular subunits and persisting after various nuclear extraction procedures, has been confirmed in several other reports (Comings and Okada, 1970a; chapter 2, Schel and Wanka, 1973; Fabergé, 1973; see also Kirschner et al., 1975; Scheer et al., 1976).

In 1974, two papers appeared dealing with this subject. Aaronson and Blobel (1974) notice the complete removal of both inner and outer membrane by use of 2% Triton X-100 in isolating rat liver nuclei. They observe, that the nuclear pore complexes do not require the membranes either for attachment to the nucleus or for their own structural integrity. Their observations confirm those by Barton et al. (1971). In their opinion, the composition of buffer, temperature, length of incubation and type and concentration of detergent may be critical in the release of phospholipid, as was also suggested by Kartenbeck et al. (1973).

The second paper, written by Hancock (1974), describes the isolation of chromatin in medium of low ionic strength with use of the detergent Nonidet P 40. The chromosomal DNP remains in the lysate as a discrete, rapidly sedimenting structure which retains the form of the nucleus, although a nuclear envelope is not detectable. The structures are bound by a peripheral region of fibrillar material, continuous with the internal network; this layer appears to maintain the form of the structures, which are highly fragile; chromatin fibrils are sometimes seen to have spread outside it.

A three-dimensional structural framework, after extraction of phospholipids and chromatin, was also observed by Berezney and Coffey (1974) for rat liver nuclei. This nuclear framework ("matrix") consists mainly of three polypeptide fractions. Since then, these residual nuclear protein frameworks have been observed in a variety of organisms (Riley et al., 1975; Hildebrand et al., 1975; Shelton et al., 1975; see also Coetzee et al., 1975; Keller and Riley, 1976a, 1976b; Herlan and Wunderlich, 1976; Schatten et al., 1976; Comings and

Okada, 1976).

Aaronson and Blobel (1975), using a series of extractions closely resembling those of Berezney and Coffey (1974), still observe large empty sacs of nuclear proportions by phase-contrast microscopy; electron microscopic examination showed the presence of nuclear pore complexes together with a lamina, obviously interconnecting them. A modified procedure was described recently (Dwyer and Blobel, 1976). The report by Scheer et al. (1976) confirms and extends these observations.

Berezney and Coffey (1975) describe the association of newly synthesized DNA with the nuclear structural network. These authors state, that the nuclear matrix and the DNA associated with this structure may have an important role in the initiation and replication of DNA. The finding, that the nuclear matrix proteins are phosphorylated and reach a maximal level of phosphorylation just prior to the onset of DNA synthesis supports this view (Allen et al., 1977).

As pointed out by Wunderlich et al. (1976), such a model for the topographical localization of DNA replication sites would agree with the evidence demonstrating replication sites throughout the nuclear interior; it would, however, also be consistent with the hypothesis of Jacob et al. (1963) in the more general sense of replication being associated with a surface or intranuclear structural component (see also Wanka et al., 1977).

1.5 Scope of the study; introduction to the chapters.

To acquire a better insight into such fundamental processes as e.g. RNA synthesis, RNA processing and DNA synthesis, knowledge of structural organization has to be combined with these functions. This study aims to obtain some more insight into the relation between structure and function in interphase nuclei.

We have focused our attention on two distinct structural features of these organelles: on the one hand, the nuclear envelope with its substructures, the nuclear pore complexes; on the other hand, the nuclear chromatin and replicating DNA. We have tried to reveal some of the interrelationships that may exist between them.

As a main object in this study the slime mold Physarum polycephalum Schwein. has been used. This myxomycete is very suited to study various aspects of the cell cycle (Rusch, 1970; Schiebel, 1973; Mohberg, 1974; Jockusch, 1975). For research on nuclear division and DNA synthesis the organism is particularly suited because of the naturally synchronous mitoses in all nuclei of the large, multinucleated macroplasmodium (see e.g. Werry, 1973).

To have some comparison with higher eukaryotes, however, and when larger amounts of nuclei were needed, some experiments were also done with an unsynchronized in vitro culture of bovine liver cells (Pieck, 1969).

The first paper (chapter 2) describes the morphology of nuclei, isolated from macroplasmodia of Physarum and prepared

according to modifications of the whole-mount spreading technique. Much attention has been paid to the ultrastructure of the nuclear pore remnants, the annular rings, which proved to be clearly revealed by this technique. The association of these rings with fibrils is described.

In the next paper (chapter 3) these observations are extended to the situation existing in liver cell nuclei. Here also, whole-mount spreading was used, combined with a replica technique. Annular substructures are being described, confirming the presence of eight subunits. A model for the nuclear pore complex is proposed.

Chapter 4 shows the results obtained by using the replica technique for a determination of nuclear pore numbers during the nuclear cycle of Physarum. The results are discussed in relation to biochemical data known about DNA synthesis in this organism. The possible function of the nuclear pore complex in the initiation of DNA replication is examined, using published values about elongation rate, replicon number and replicon size. The observations on nuclear pore structure and formation are compared and extended by means of the freeze-etch technique. It was possible to visualize the pore exchange process in Physarum with this technique.

The following chapter (chapter 5) describes the presence of nuclear pore complex remnants of liver cell nuclei - obtained by mass isolation - after a series of extractions. Even after high salt incubations, combined with detergent treatments, these pore complexes remained present, together with a nuclear fibrillar substructure. These findings are also discussed in relation to DNA replication.

The previous chapters all deal with morphological features of interphase nuclei, discussed in relation to DNA replication. The last two chapters (chapters 6,7) describe efforts to visualize this replication process for Physarum more directly.

Some replicating nuclear DNA molecules could be made visible by use of the aqueous spreading technique (chapter 6). In this way, an estimation of the chain elongation rate in Physarum was possible.

The last chapter (chapter 7) provides some preliminary results, obtained by DNA fiber autoradiography.

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ANNULAR STRUCTURES IN ISOLATED NUCLEI OF *PHYSARUM POLYCEPHALUM*

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Exp. Cell Res. 82, 315-318 (1973)

Experimental Cell Research 82 (1973) 315–318

ANNULAR STRUCTURES IN ISOLATED NUCLEI OF *PHYSARUM POLYCEPHALUM*

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SUMMARY

Whole-mount preparations of isolated interphase nuclei of *Physarum polycephalum* show annular structures The outer and inner diameters are about 1 080 and 580 Å respectively Ultrastructural features of the annuli are described; an association of the rings with fibers of various diameters, probably of chromatin nature, is also reported.

Annular structures have been observed in electron micrographs of nuclei from various animal cells, prepared by the whole-mount spreading technique [4, 5, 7, 11, 19]. They appear to be associated with nuclear pores. Detailed models of nuclear pore complexes, based mainly on study of ultrathin sections and negatively stained nuclear membranes, have been proposed, among others, by Franke [9] and by Abelson & Smith [1]. In recent years, a possible role of pore complexes in nuclear activities (e.g DNA replication) has been suggested [3, 8, 11, 14].

We report here a successful attempt to demonstrate the presence of annular structures in *Physarum polycephalum* by use of a whole-mount technique. Macroplasmodia of this slime mould were chosen because they proved to be particularly suitable for studies of nuclear activities due to the highly synchronous growth [17].

MATERIALS AND METHODS

The myxomycete *Physarum polycephalum*, strain M_3c IV (kindly supplied by Dr R Braun, Bern) was grown as described by Daniel & Baldwin [6]. Macroplasmodia were prepared according to the method of Guttes & Guttes [10] Stages of the nuclear cycle were determined by phase contrast microscopy of small pieces of plasmodia, fixed in 96% ethanol and embedded in a glycerol–ethanol mixture (1·1)

Nuclei were isolated, using procedure B of Mohberg & Rusch [15], as modified by Polman [16] One plasmodium, grown on filter paper, was rinsed in ice-water to remove growth medium and collected. Such macroplasmodia were in various stages of interphase after M II The plasmodium was washed once with buffer at pH 7.1 (30 mM NaCl, 1 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, and 0 1% Triton X-100; Rohm & Haas). After centrifugation the pellet was suspended in 25 ml of the same buffer and homogenized with a loose-fitting Teflon homogenizer (Tri-R-Stir-R, speed 3 5, 10 strokes) The homogenate was then diluted eight-fold and centrifuged for 10 min at 70 g The supernatant was filtered by gravity through two pieces of milk filter (Milac vacuum, Brocades N V , Nijmegen) The filtrate was centrifuged for 10 min at 3 000 g and the sediment washed twice with buffer and once with distilled water. The final pellet of swollen nuclei was resuspended in 0 5 ml distilled water; 5 to 10 µl of the suspension were transferred with the aid of a syringe needle onto the surface of freshly distilled water droplets (50 µl), placed on a flat piece of Teflon.

The nuclei preparation was picked up from the surface with a copper grid, covered with carbon-coated Formvar. The preparations were then fixed in 10% formalin (unbuffered, pH 5 5) for 10 min and dehydrated in a graded series of ethanol washes. They were stained with 2% uranyl acetate in the 70% ethanol step for 5 min. After 100% ethanol the grids were transferred into isopentane and air-dried. They were examined using a Philips EM 300 electron microscope operating at 60 kV.

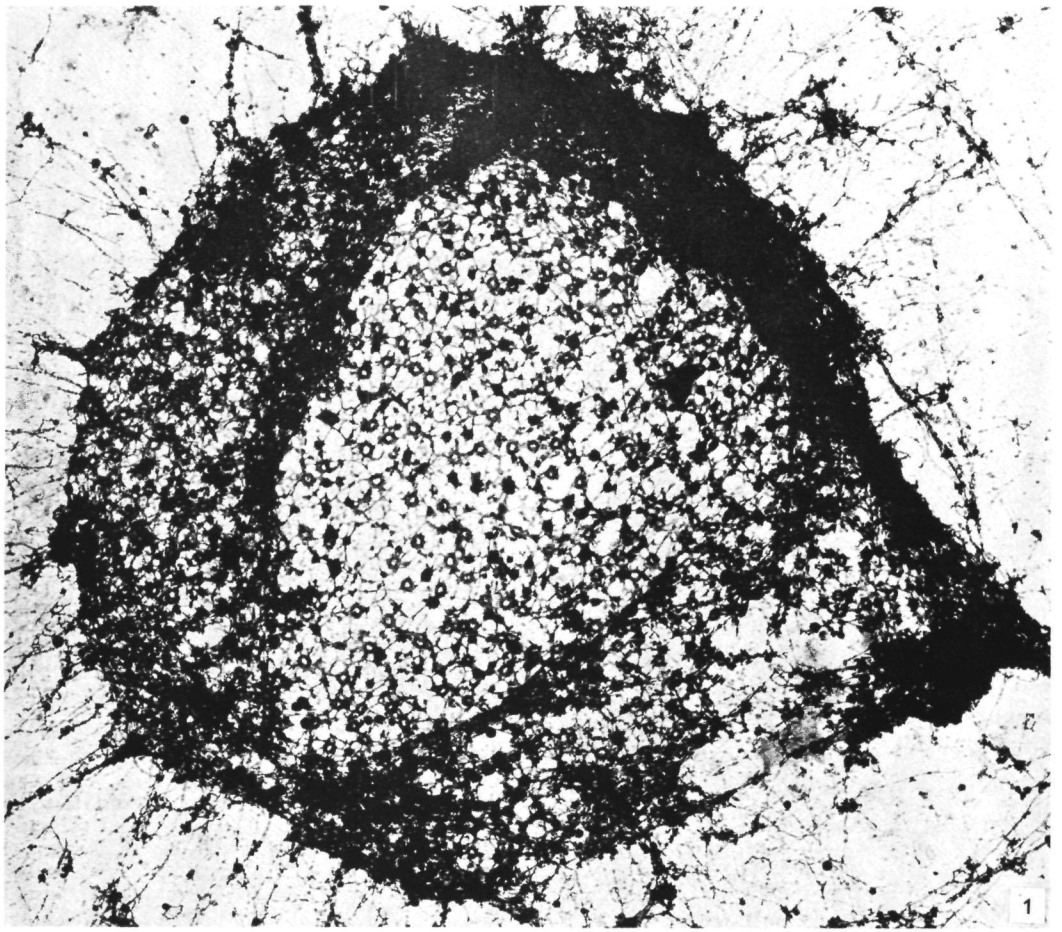


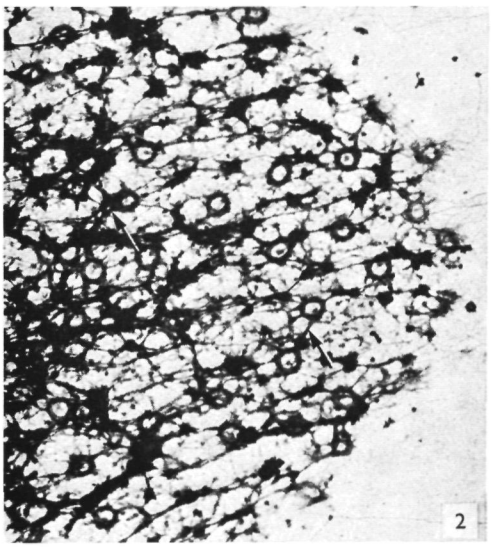
Fig. 1. View of a spread nucleus from the macroplasmodium of *Physarum polycephalum*. The central chromatin is largely displaced, exposing a layer of annuli. They are interconnected by a dense fibrous network, probably remnants of the nuclear envelope. Annuli also can be recognized on the left where the layer seems to be doubled by folding (× 18 000).

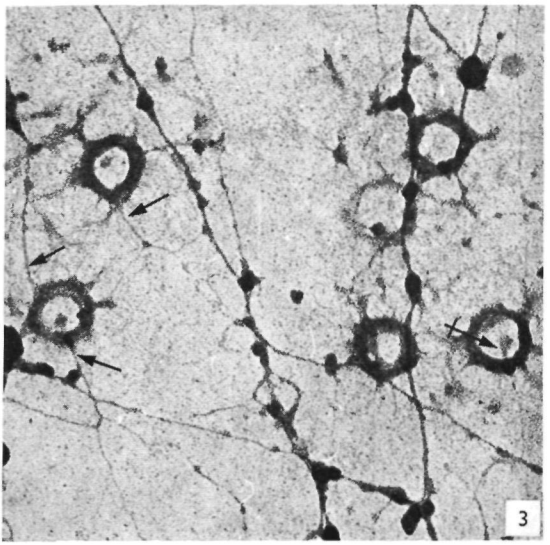
RESULTS AND DISCUSSION

Nuclei, displaying various degrees of spreading, were found in electron micrographs obtained from interphase plasmodia. Annular structures were sometimes present at the edges of fairly compact nuclei. In well-spread areas annuli were arranged in a way which suggests an association with the nuclear envelope (fig. 1).

Membranes were not recognizable, probably due to removal of lipid and protein components by detergent-distilled water treatment [5].

What we recognize as chromatin fibres are often attached to annuli (figs 2, 3) [4, 5, 7, 11]; for thin sections, see [8, 13]. They sometimes form a network—interconnecting the annuli—which is assumed to be a structural component of the nuclear envelope [2]. These observations are in close agreement with those recently described for *Physarum*





Figs 2, 3. Annuli with attached chromatin and central globules. Chromatin fibres of various thickness are attached to the annuli (→). Thinner fibres probably arise by stretching due to spreading forces. Central globules, sometimes with fibrillar connections (↔) to the annuli are often observed (fig. 2, × 29 000; fig. 3, × 80 000).

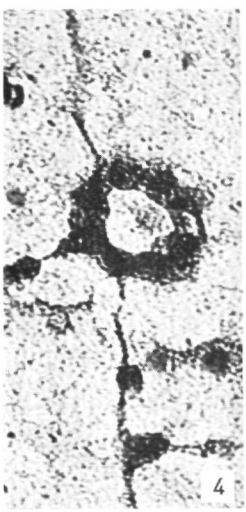
by Troncale et al. [18]. These authors report the presence of chromatin fibres, attached to annular rings; further, they mention the presence of a fibrillar network substructure in detergent- and trypsin-treated nuclear envelopes.

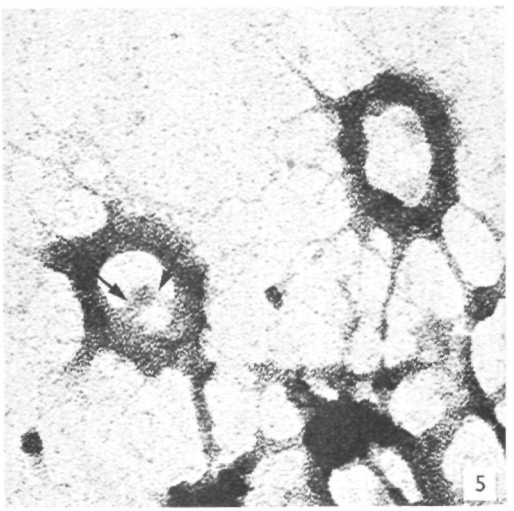
The annuli were slightly larger than those observed with this technique in other organisms [5, 11]. Outer and inner diameters were about 1 080 and 580 Å respectively (table 1). Their appearance was often bumpy and not fully concentric, suggesting a certain degree of stretching caused by the spreading forces (figs 4, 5). The bumpy appearance may also be due to the presence of remnants of detached chromatin fibers, as proposed by DuPraw [7], or indicate some fine structural elements of the annuli. Using the Markham analysis [12], we obtained predominantly an eight-fold symmetry, suggesting the presence of granular subunits as described by Franke [9] in nuclear pore complexes. However, reinforcements for $n=6$ and $n=9$ were obtained too frequently as to be certain about the significance of the finding. Central globules, sometimes with fibrous connections to the annuli, were found repeatedly (figs 2, 3, 5, 6). In all these cases the central granule was represented by a single dark spot. So far, we have not been able to detect differences in the annular shape at various stages of the interphase.

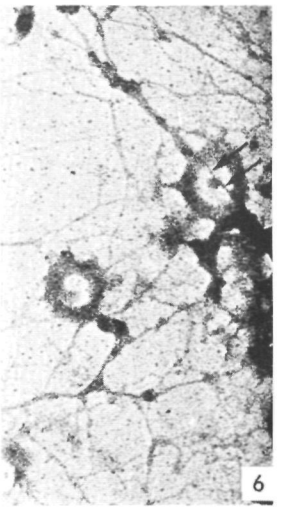
The results, mentioned above, strongly confirm the idea of the universality of nuclear pore complex structure [9]; their association

Table 1. *Dimensions (in Å) of the annular structures*

Diameter	No. of measurements	Mean	Range	S.D.
Outer	21	1 083	880–1 270	30
Inner	21	582	450–800	28
Of central granule	8	210	156–258	13







Figs 4–6. Higher magnifications of annuli, showing a bumpy appearance and deformation caused by the spreading process. Notice fibrillar connections between central globules and annuli (→). (Fig. 4, ×170 000; fig. 5, ×150 000; fig. 6, ×70 000).

with chromatin fibres (or their composition of chromatin, see [18]) supports the hypothesis that pore complexes may have an important function in nuclear activities [3, 8, 11, 14].

We thank Mr P. J. Camp for skilful technical assistance and Mr E. Kirby for kindly correcting the manuscript.

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Received March 13, 1973
Revised version received May 14, 1973

STRUCTURE OF THE NUCLEAR PORE COMPLEX IN MAMMALIAN CELLS. TWO ANNULAR COMPONENTS

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Exp. Cell Res. 87, 195–206 (1974)

Experimental Cell Research 87 (1974) 195–206

STRUCTURE OF THE NUCLEAR PORE COMPLEX IN MAMMALIAN CELLS

Two Annular Components

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SUMMARY

The ultrastructure of the nuclear pore complex has been investigated in isolated nuclei of an in vitro cultured bovine liver cell line. In shadow-cast replicas of the surface of nuclei isolated in Tris buffer containing low K^+ and Mg^{2+} concentrations (RSB) the rims of the pores appeared as annular projections with an outer diameter of 100 to 120 nm. When the nuclei were isolated in Tris buffer containing 0.1 % Triton the projections were essentially lost, together with the outer membrane of the nuclear envelope. In electron micrographs of whole-mount preparations the Triton-Tris nuclei—but not the RSB nuclei—were surrounded by numerous circular structures, which obviously had been detached from the nuclear surface during the preparation. They consisted of eight granules of about 20 nm diameter which were connected in a circular fashion by fibrous material. The circular structures had an inside diameter close to 65 nm. In broken nuclei many of these circular structures contained a second, smaller circular component and a central granule. From these observations it is concluded that the annulus of the nuclear pore consists of two components and that the outer component is located in the perinuclear space in intimate association with the membrane limiting the pore. A modified model of the nuclear pore complex which accounts for this location is proposed.

Evidence obtained from a great number of investigations has led to the assumption that nuclear envelopes of eucaryotic cells in general contain pore complexes with similar structures (for reviews see: [14, 15, 23, 36]). The diameters of the pore proper usually are close to 70 nm [20], but significantly smaller and larger pore diameters have been reported [5]. However, the effective opening is markedly reduced by an annular structure associated with the membrane around the pore perimeter [13, 28].

The annular material apparently consists of a number of subunits which can be observed in a variety of preparations, such as thin sections of glutaraldehyde–osmium fixed cells [8, 15] and whole-mount preparations of nuclei [6, 7, 9, 10, 33]. An eightfold symmetry was predominantly obtained with Markham image rotation analysis, while other symmetry numbers have been found occasionally [2, 15]. In negatively stained nuclear envelopes the eightfold symmetrical subunit structure was observed even more clearly [12, 16, 17, 20, 27]. It was generally located in a narrow zone around the pore perimeter and appeared sharply separated from the inner pore material by the unit membrane which limits the pore proper.

Basing themselves mainly on data of thin sections previous investigators have concluded that the annular subunits are located on

the cytoplasmic and the nucleoplasmic sides of the pore rim [15]; but from electron micrographs of negatively stained nuclear envelopes one gains the strong impression that this peripheral part is situated within the perinuclear space and is attached to the pore membrane. In the present study such an interpretation obtains support from submicroscopical data of specially treated mammalian cell nuclei.

MATERIAL AND METHODS

1. *Growth of cells and isolation of nuclei*

Monolayer cultures of bovine liver cells were grown in Carrel flasks as described by Pieck [29], except that the serum concentration was reduced to 10 %. The nuclei were isolated in two different ways

(A) *Isolation in RSB*: Cells were removed from the glass bottom by mild trypsinization and collected by centrifuging for 3 min at 200 g. All subsequent treatments were at 4 C. The cells were washed once with 25 % Hanks solution. They were then resuspended in 3 ml RSB (10 mM KCl and 1.5 mM $MgCl_2$ in 10 mM Tris-HCl buffer, pH 7.4 [30]) and passed 10 to 15 times through an injection needle of 0.7 mm diameter. The volume was increased to 25 ml by adding RSB and the nuclei were collected by centrifugation. The pellet was then passed through a 0.2 mm needle until approx. 90 % of the nuclei were found to be free of cytoplasmic contamination in the phase-contrast microscope. The sample was finally rewashed with 25 ml RSB.

(B) *Isolation in Triton-Tris*: Removal from the bottom of the Carrel flask and shearing of the cells occurred in a combined step at 37 C: The cells were syringed off the glass surface with the aid of 2.5 ml Triton-Tris (0.1 % Triton X-100 in 5 mM Tris-HCl buffer, pH 7.4) and simultaneously passed through the 0.7 mm needle 10 to 15 times. The subsequent steps were carried out at 4°C. After addition of 25 ml RSB the nuclei were collected by centrifugation. The pellet was passed through the 0.2 mm needle until the nuclei looked clean in the phase-contrast microscope, and the isolation was completed by a final wash with 25 ml RSB.

2. *Preparation of the replicas*

A droplet of the nuclear pellet was smeared out on a chloroform-cleaned microscopic slide. The slide was carefully rinsed in 70 % ethanol and allowed to dry in the air. Platinum shadowing was applied at an angle of about 45 and the shadow-cast was supported by a carbon coat. The replica was soaked from the glass slide in distilled water containing a few droplets of HF. The organic material was then removed by floating the replicas on, successively: concentrated H_2SO_4, eau de Javelle, and distilled water. The replicas were finally mounted on 150 mesh grids. Removal of the organic material was omitted in some cases.

3. *Whole-mount preparation and enzyme treatments*

A drop of distilled water or enzyme solution in distilled water was placed on a piece of Teflon. A small amount of the nuclear pellet was applied to the top of the drop and picked up by a Formvar carbon coated specimen grid. In the case of enzyme treatments the grid was allowed to float on the drop for the required time Enzyme treatments were at room temperature. Further details are given under Results. The preparations were then fixed for 10 min in 10 % formalin and dehydrated in a graded series of ethanol washes. They were stained with 2 % uranyl acetate in the 70 % ethanol step for 5 min. After the 100 % ethanol step the grids were transferred to amyl acetate and air-dried.

The enzymes used were: trypsin (bovine, Boehringer), ribonuclease A (pancreatic, Sigma), deoxyribonuclease I (pancreatic, Sigma).

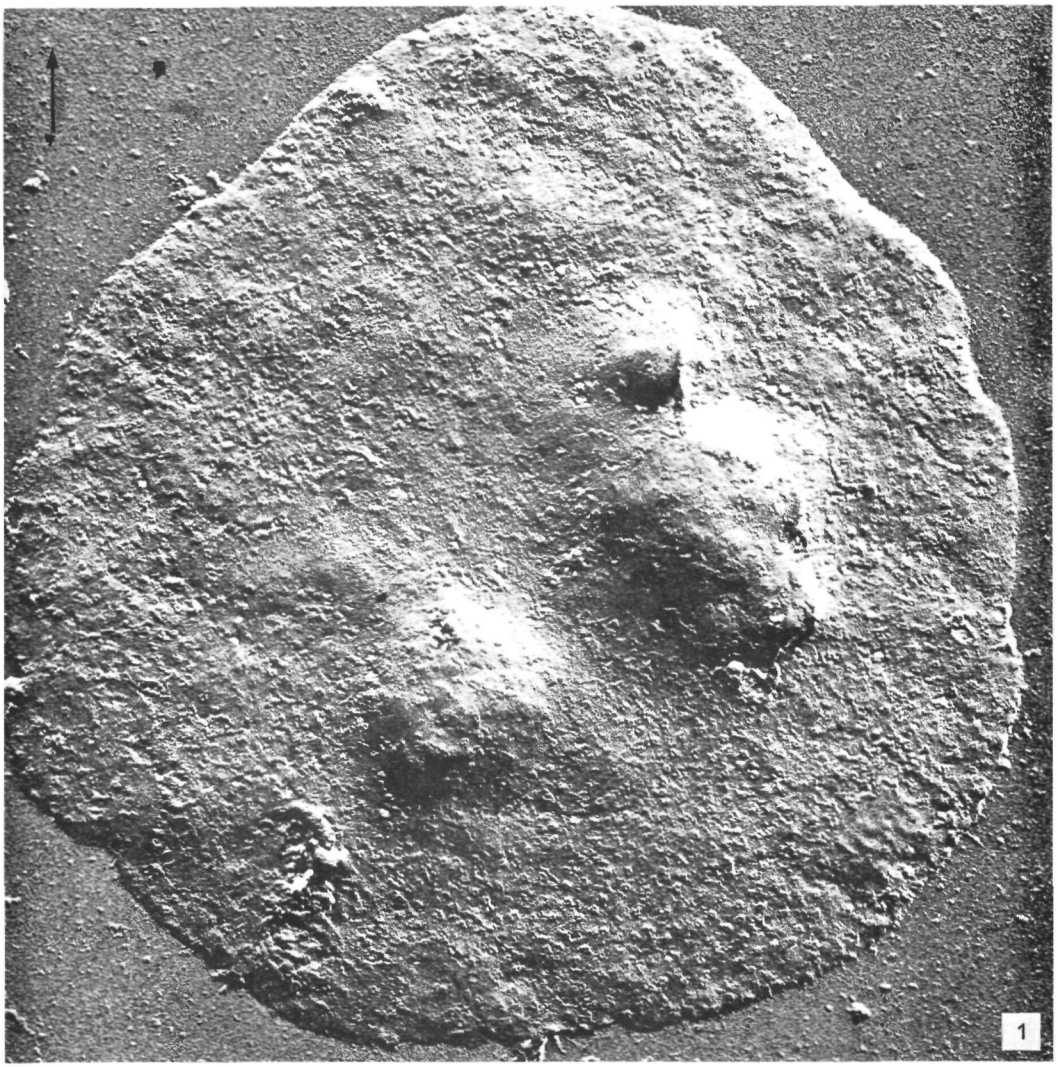
Electron micrographs were taken with a Zeiss EM 9S and a Philips EM 300 electron microscope.

RESULTS

1. *Replicas of the nuclear surface*

The replica technique proved to be highly suited for studying the envelope of unbroken nuclei. Up to 50 % of the surface area of a single nucleus could be examined in most cases. In many respects the surface structure of platinum-shadowed nuclei isolated with RSB resembled that of isolated nuclear envelopes of amphibian oocytes [18, 19]. Annuli with an outer diameter of 100 to 120 nm were the prominent structures projecting from the surface (figs 1, 2, 10*a*). Their more

Fig. 1. View of the surface of a nucleus isolated in RSB. Shadow-cast replica. × 9 500. Direction of shadowing in this and subsequent figures of replicas is indicated by the arrow.
Fig. 2. Surface area of a nucleus isolated in RSB. Shadow-cast replica showing annular projection (*open arrowheads*). 30 000.
Fig. 3. Similar preparation as in fig. 2 but no treatment of the replica with H_2SO_4 and eau de Javelle. Annular images appear reinforced (*open arrowheads*). 30 000.



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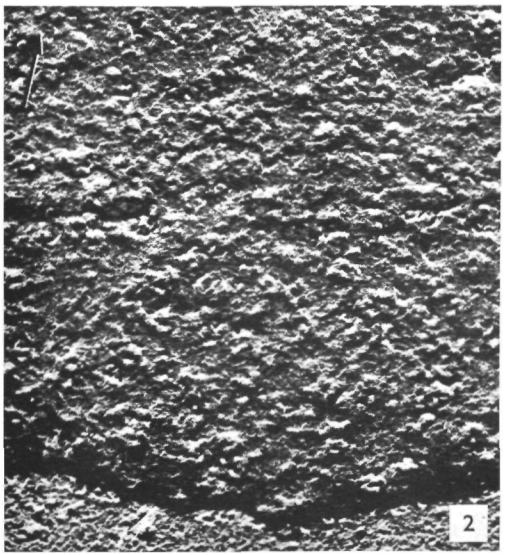

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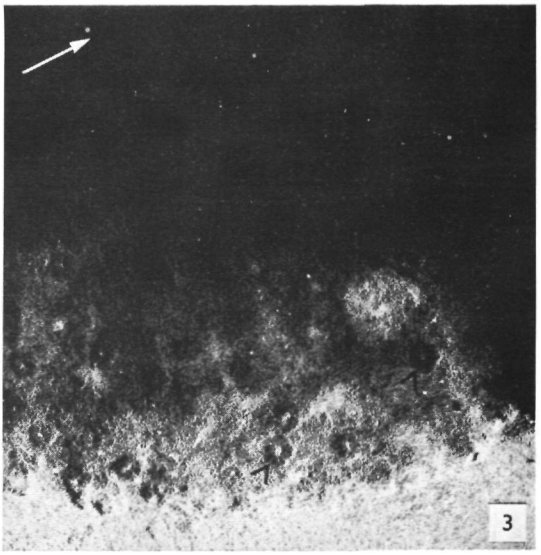

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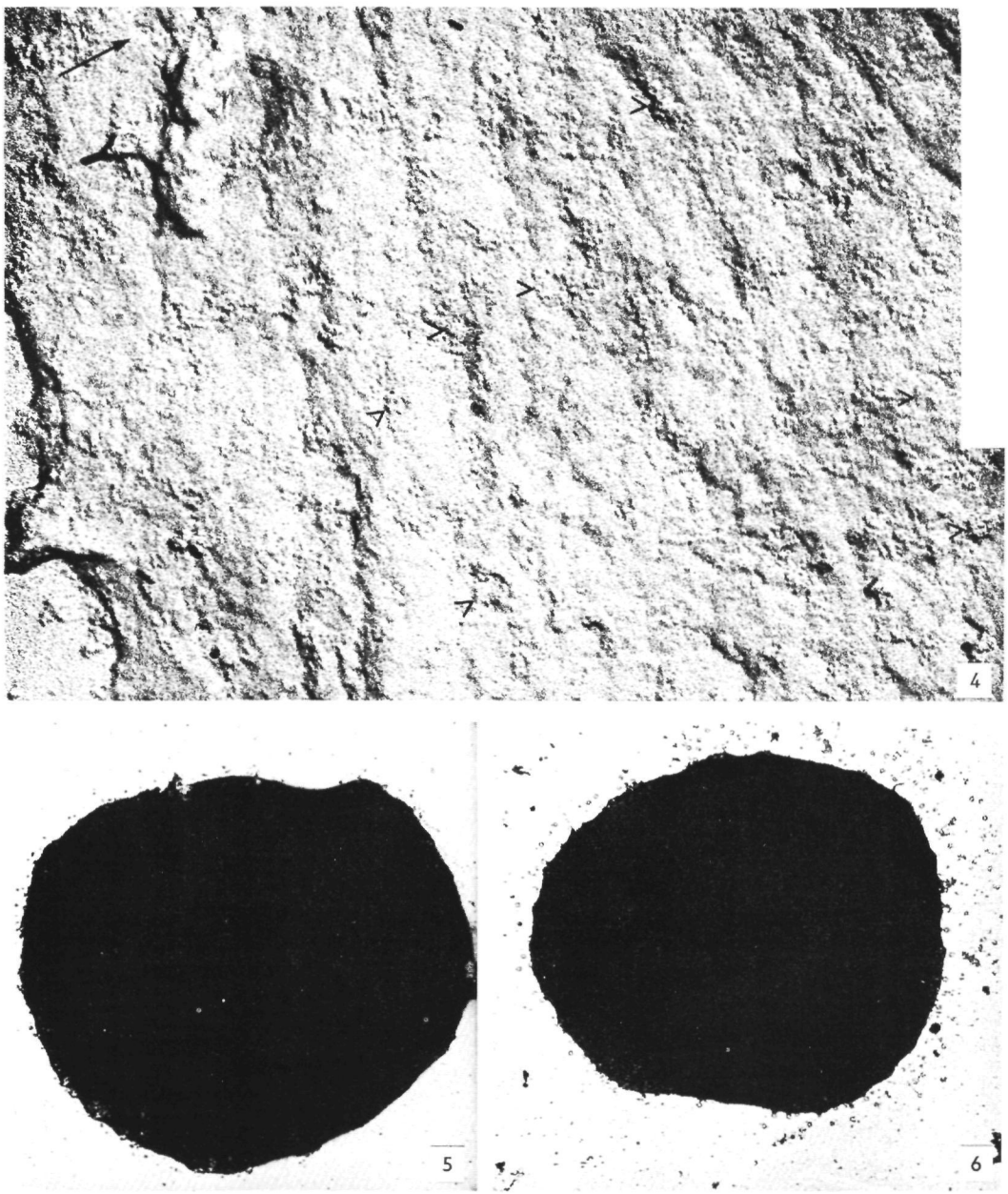


Fig. 4. Surface area of a nucleus isolated in Triton-Tris. Shadow-cast replicas showing patches of variable appearance and a diameter of 100 to 120 nm (*open arrowheads*). Frequently, small granules can be noticed at the margins of the patches. × 30 000.
Fig. 5. Whole-mount preparation of a nucleus isolated in RSB. × 6 800.
Fig. 6. Whole-mount preparation of a nucleus isolated in Triton-Tris. × 5 800.

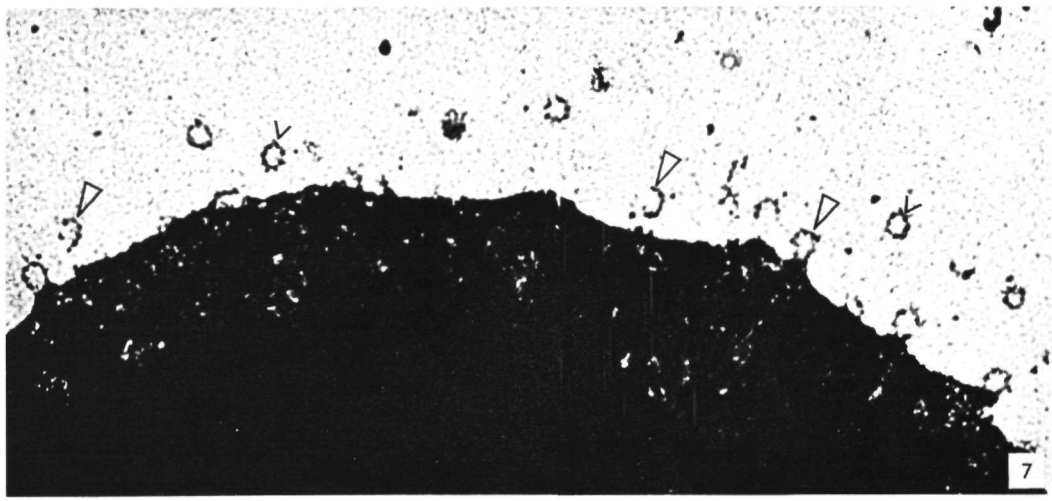


Fig. 7. Section of fig. 6 at higher magnification showing more or less deformed or disrupted circular structures (*closed arrowheads*). An eightfold subunit structure can be seen in some well preserved specimens (*open arrowheads*). ×30 000.

or less doughnut-like image was reinforced when the removal of the nuclear material from the replica was omitted (fig. 3). This suggests that the annular relief of the nuclear surface is caused by some electron-opaque material present in the unstained pore complex [10]. The number of annuli in an arbitrarily selected 1 μm^2 area could vary from zero up to slightly more than 15. This is in contrast to the dense and more regular distribution known, for example, from amphibian oocytes [16–19], but agrees well with the variable distribution patterns observed by freeze-etching studies of nuclei of different origins (e.g. [24, 26, 31]).

When the Triton-Tris medium was used for the isolation, the appearance of the nuclear surface varied according to the duration of the treatment. After a short exposure of less than 3 min the nuclear surface mostly appeared similar to that found without Triton, but when the time was increased to 5 min and beyond the annular reliefs were essentially lost. Instead, numerous patches of about the same 100 to 120 nm diameter (mentioned above) could be recognized (figs 4, 10*b*). They showed a variable appearance and sometimes a number of small granules at the margin. This change is primarily due to the removal of the outer membrane of the nuclear envelope [4, 35] and, presumably, of a more or less considerable portion of the annular material by the Triton treatment. The inner membrane which can be partially removed at higher Triton concentrations [3, 21], seems not to be significantly affected under the present conditions as indicated by the absence of any fibrous texture in such replicas.

2. *Whole-mount preparations*

With the whole-mount spreading technique only a few nuclei, probably those already disrupted during the isolation, were spread out on the support. They showed the same chromatin and annular components as described in detail by other authors [6, 7, 9, 10, 25, 33, 34]. Most nuclei retained their compact shape, revealing few structural details, but were frequently surrounded by a zone

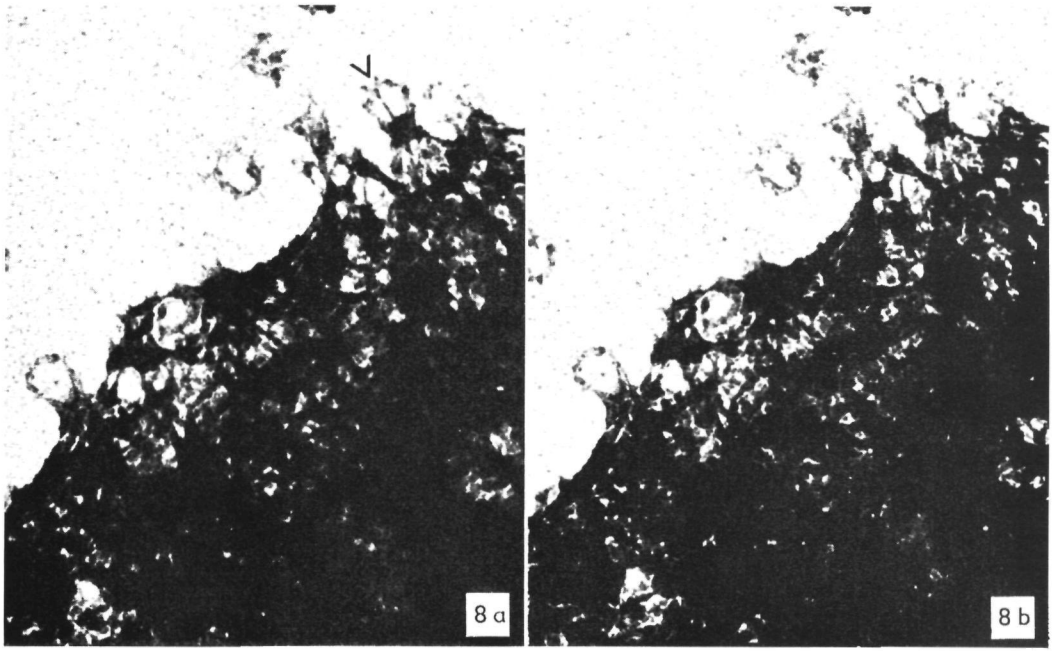


Fig. 8. Stereo electron micrographs of a section of fig. 6. Plug-like projection from the nuclear surface to a circular structure attached to the support. A connecting fibre is visible (*open arrowhead*). ×58 000. Tilting angles were +6° and −6° respectively.

which was covered by small pieces of electron-opaque material. As this zone sometimes broadened when the nuclei were caused to shrink by the electron beam, it can be concluded that it represents an area of the supporting film which had been in contact with the nucleus originally. This contact was lost when the nuclear volume decreased during the dehydration and drying process, but some material of the nuclear surface remained attached to the carbon coated support.

The material detached from nuclei isolated with RSB consisted of faint amorphous remnants (fig. 5). On the other hand, nuclei isolated with Triton-Tris revealed a great number of circular structures (fig. 6). At higher magnification each well preserved specimen showed 8 dense granules which were connected in a circular fashion by fibrous material (fig. 7). The outside diameter of this structure was about 105 nm which is in accordance with previously reported dimensions of spread annuli [7, 25], but the average inside diameter was significantly larger, namely 65 nm. The diameter of a granular subunit amounted to about 20 nm. Many of the circular structures had become deformed or even disrupted during the detachment. In certain cases nuclear projections were found to be still attached to the circular elements. They consisted of chromatin-like fibres merging into a distal plug. The spatial orientation of such structures can be observed in the stereo micrographs shown in fig. 8.

In disrupted nuclei the relationship of the above-mentioned circular structures to the previously reported annuli becomes obvious. In fig. 9 several of the circular structures contain a smaller ring-like structure and a central granule (see also figs 10*c*, *d*). The

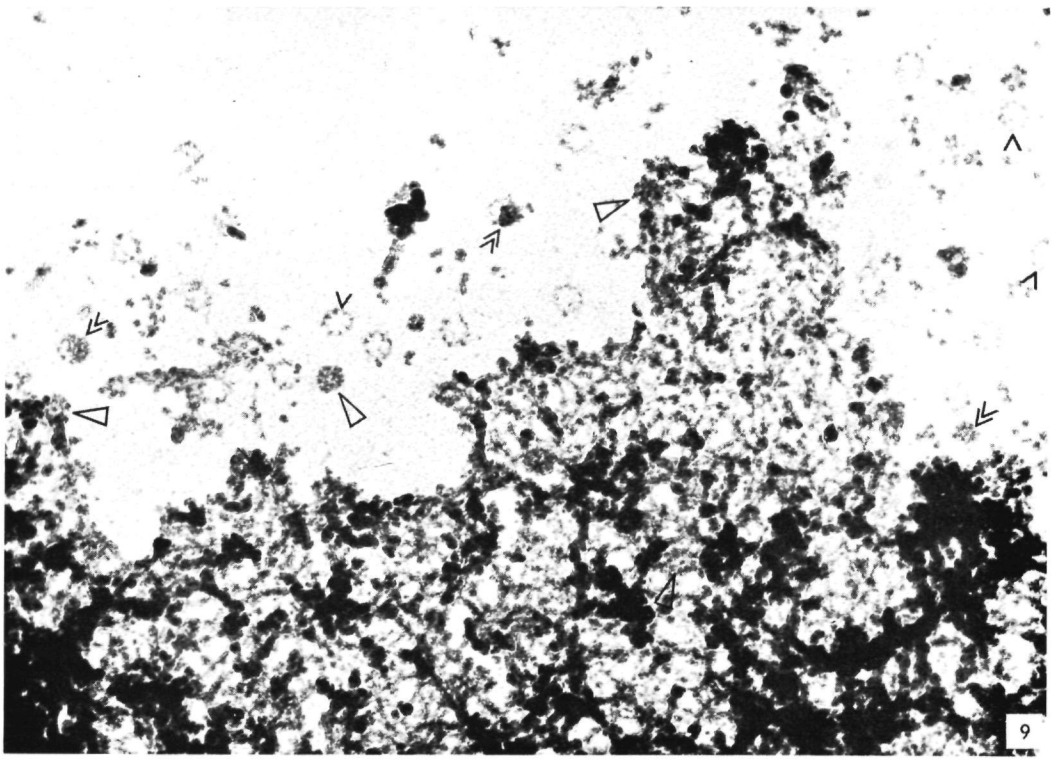


Fig. 9. Whole-mount preparation of a nucleus isolated in Triton-Tris. Only part of the disrupted nucleus remained attached to the grid showing chromatin fibres and fragments of the annular structures; plain circular structures (*open arrowheads*) and others containing a second, smaller ring and a central granule (*closed arrowheads*). The central part is sometimes displaced (*double arrowheads*). ×38 200.

inner ring was frequently somewhat displaced from its original site and looked less regular than the outer component. The most complete and least deformed structures were still associated with the chromatin of the nuclear fragment (figs 9, 10*c*, *d*). They strongly remind us of the electron micrographs of annuli which remain associated with the nuclear envelope when the chromosomes peel off during prophase [7].

3. *Enzyme treatments*

Enzyme treatments were performed with nuclei isolated in Triton-Tris. A brief deoxyribonuclease treatment (50 μg/ml; 8 min) did not visibly affect the annular structures. Longer treatments caused degradation of nuclei to amorphous masses which made further examination impossible.

A short exposure to trypsin weakened the nuclear structure to such an extent that the spreading was greatly improved and many annuli became released from the chromatin. Many annuli were deformed or showed traces of degradation, but practically all still contained most of the inner material (figs 10*e*, 11). This contrasts the situation found in the non-trypsin-treated nuclei, in which most annuli do not show the inner ring; probably both parts of the annulus are present but the central part is so firmly associated with the chromatin that it becomes detached from the

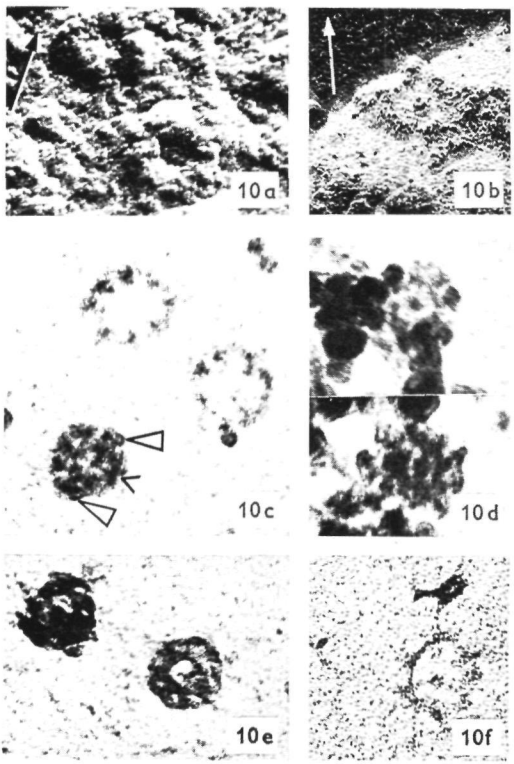


Fig. 10. Annular structure at high magnification. All micrographs are ×100 000. (*a*) Replica after isolation in RSB.; (*b*) replica after isolation in Triton-Tris; (*c*) whole-mount preparation showing outer components and a central (*open arrowhead*) part still associated with a fragment of the outer component (*closed arrowheads*), isolation in Triton-Tris; (*d*) same preparation as (*c*), showing two annuli with inner and outer components and attached chromatin; (*e*) whole-mount preparation treated for 4 min with 50 μg/ml trypsin. Slightly affected, complete annuli; (*f*) whole-mount preparation treated for 2 min with 200 μg/ml ribonuclease.

supporting film and thus separated from the outer part during the contraction of the nucleus. The trypsin treatment weakens the binding to the chromatin and therefore both parts remain on the support.

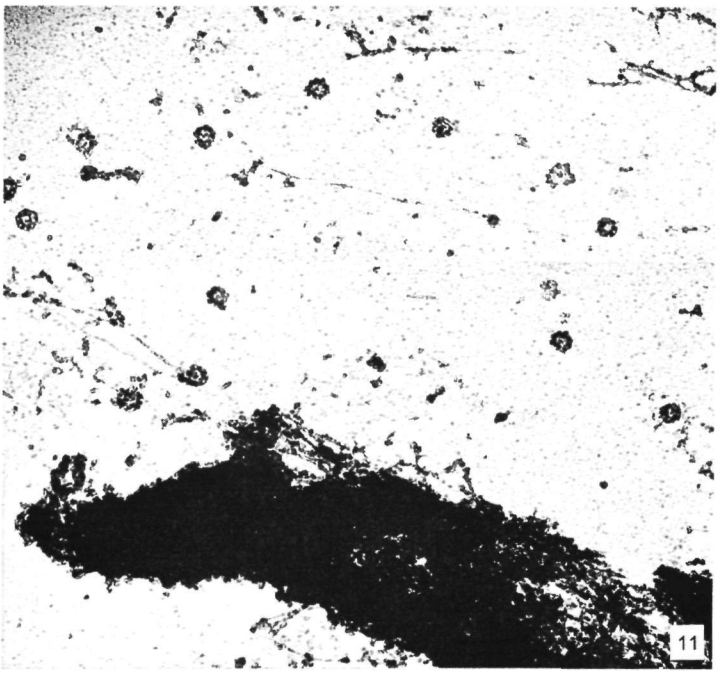
Ribonuclease caused a strong deformation and degradation of the annuli before a marked effect on the nuclei became apparent (figs 10*f*, 12). We were not able, however, to distinguish whether the deformed structures were derived from the complete annuli or from the outer component only. The observation supports the presence of RNA in the pore complex reported previously [32].

On prolonged treatments with trypsin or ribonuclease an aspecific association of degradation products of different nuclear constituents became obvious. Therefore, we found it difficult to further define the nature of the actions of the enzymes.

DISCUSSION

So far, efforts to elucidate the architecture of the nuclear pore complex have not been rewarded with a generally accepted model. The major difficulties rise from uncertainties encountered in the interpretation of electron micrographs (see recent reviews [23, 36]). It is generally agreed, however, that the nuclear pore proper is associated with annular material which frequently shows a symmetrical eightfold subunit structure. The evidence is primarily based on reinforcement patterns obtained by image rotation analysis and on direct examination of negatively stained envelopes and tangential sections [1, 5, 8, 12, 15, 16, 17, 20, 27]. The eightfold subunit structure is generally located in a zone immediately surrounding the pore perimeter. Interestingly, the few reported examples with other than the eightfold symmetry show an outer diameter which is not larger or only slightly larger than the diameter of the pore proper [2, 15].

Gall [20] has emphasized the point that in negatively stained nuclear envelopes the pore membrane serves as a barrier separating the stain in the pore from that in the perinuclear space where it appears in a pattern of 8 dark patches at the flat sides of the octagonally shaped pore. Subsequently, the lighter areas at the eight corners have been shown to occur as electron-transparent dots or granules and have been considered to represent an-



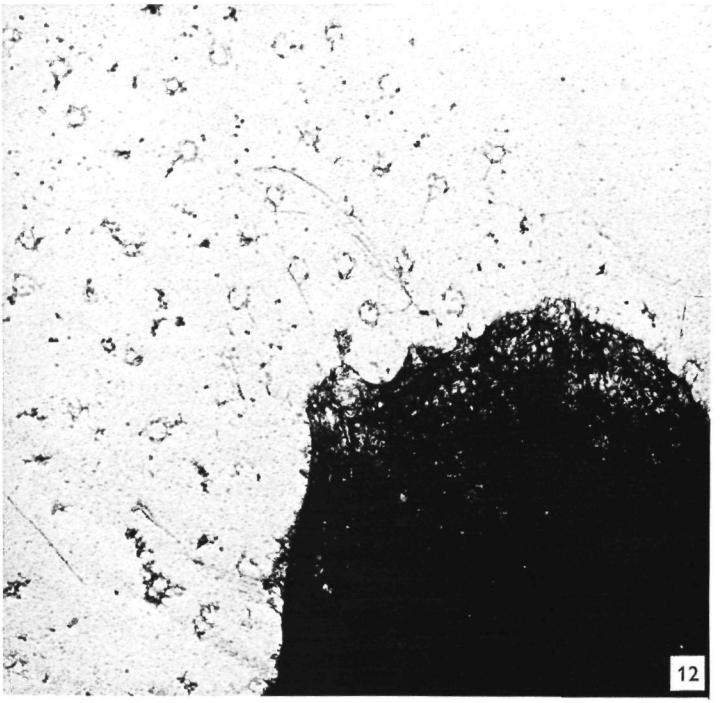


Fig. 11. Whole-mount preparation treated for 4 min with 50 μg/ml trypsin. $\times$ 30 000.
Fig. 12. Whole-mount preparation treated for 2 min with 200 μg/ml ribonuclease. $\times$ 30 000. Remnants of strongly degraded annular structures.

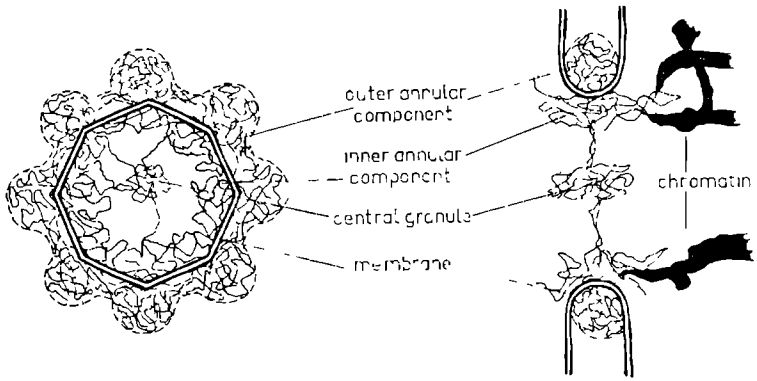


Fig. 13. Diagrammatic representation of the nuclear pore complex. Adapted from previously proposed models [15, 20, 22].

nular subunits [12, 16, 17]. These findings suggest that this part of the annulus is located in the perinuclear space in intimate association with the pore membrane as shown diagrammatically in fig. 13.

In this model the annulus is proposed to consist of two main components. The inner component which is located at the interior of the pore shows no regular substructure. This at least can be gathered from the many observations made upon nuclei prepared by several different techniques. The outer component consists of 8 symmetrically arranged globular subunits which are connected in a circular fashion by fibrous material. We identify this part of the annulus with the circular structures shown in figs 7, 8 and 9. This postulate is compatible with the finding that the inside diameter of the outer component is about 65 nm while pore diameters of some mammalian cell nuclei have been reported to vary between 64 and 72 nm [5]. The location of the outer component in the perinuclear space at the junction between the inner and outer membrane of the envelope provides for its more intimate contact with the membrane; this may promote the stability of the structure and especially its regular arrangement, and may also explain why some annular material can become removed under certain experimental conditions from the interior of the pore, while the eight subunits image becomes even more clearly visible [16].

The location of the outer annular component in the perinuclear space is further substantiated by the fact that it is only released from the surface of nuclei which have been deprived of the outer membrane by the Triton treatment. Another significant point is the clean dissociation of the two components during the detachment from the nuclear surface, because it might indicate a pre-existing separation of them by the membrane. This does not necessarily exclude the existence of connections penetrating the membrane. In fact thin fibres extending from one component to the other become sometimes visible during the separation process (fig. 8). But they seem to exist only temporarily and generally break in such a way that no noticeable fibre fragments remain associated with the outer annular component.

As the model presented here differs from the one put forward by Franke [15] we would like to draw attention to the following points. In the latter model eight subunits are proposed to be present on both the cytoplasmic and the nuclear side of the pore margin. No such subunits were detected in replicas of RSB nuclei and only some amorphous material but no regularly arranged subunits were found to

become detached in whole-mount preparations of such nuclei. If such subunits exist, they either must have been washed off during the isolation or have collapsed on drying. In any case they should have been removed with the outer membrane and a marked portion of the annular crest during the isolation in Triton-Tris, and thus, can not be identical with the eight-partite circular structure found in the corresponding whole-mount preparations. Moreover, in the only report which clearly shows the eightfold subunit structure in tangentially sectioned nuclei, the presented micrographs strongly suggest that the central granule lies in the same plane as the eight subunits [8]. This also holds true for sections which have been used with success for rotation analyses [1]. Thus, once more the most likely position of the eight subunits is in the perinuclear space.

Electron-opaque material which is located in the interior and at the cytoplasmic and nucleoplasmic sides of the pore must be more loosely and/or less regularly organized, except for the central granule. Such a view results from the highly variable appearance of the inner component, in particular in thin sections and negatively stained envelopes. This variable appearance might be due, at least in part, to preparation artifacts. Alternatively, it could indicate a more dynamic structure with some function in the regulation of nucleocytoplasmic exchange of macromolecules and small particles [14, 23].

Of the many proposed functions of the nuclear pore complex only that of providing attachment sites for chromatin fibres may be mentioned in relation to the present findings. One possible advantage of this association would be to determine the pattern of chromosome folding at the transition to the mitotic stage [7]. It has been suggested that the pore complex might function as a "press-stud" in the reversible attachment process [11]. The dissociability of the inner and outer annular components found in the present study could provide a mechanical basis for such a hypothesis. Functionally the inner component would then be homologous to the cyclomere [11]. It has to be acknowledged, however, that from the available evidence it seems more likely that the whole annulus is detached from the chromatin fibres and left with the envelope when the chromosomes condense [7].

We would like to thank Mr J. Eygensteyn for technical assistance and Dr R. Lesseps S. J. for kindly checking the English text.

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Received March 15, 1974

CHAPTER IV

ANALYSIS OF NUCLEAR PORE NUMBERS IN MACROPLASMODIA OF *PHYSARUM POLYCEPHALUM*

4. ANALYSIS OF NUCLEAR PORE NUMBERS IN MACROPLASMODIA OF PHYSARUM POLYCEPHALUM

4.1 Introduction.

The investigations of, among others, Feldherr (1972) have shown that nuclear pores may have a function in the regulation of nucleo-cytoplasmic exchange of macromolecules and small particles. In view of the complicated architecture of the nuclear pore complex (NPC), together with its universality (Franke, 1970), one is tempted to assume some function in other nuclear processes too.

One such function - serving, namely, as an anchoring place for the underlying chromatin - has been proposed by several authors; an association of the pore annuli with chromatin fibers was observed in various organisms (Comings and Okada, 1970; Maul, 1971; chapter 2).

The possible involvement of the nuclear envelope in DNA synthesis (chapter 1; see also Sneider, 1974) has led some workers to suggest a function of the NPC in the initiation and/or replication of the nuclear DNA (Lampert, 1971; Maul, 1972; Engelhardt and Pusa, 1972). Evidence, obtained by freeze-etching and supporting this view was given by Maul et al. (1972) who observed - for PHA stimulated human lymphocytes and synchronized HeLa cells - an increase of the number of nuclear pores, correlated with the onset of DNA synthesis. They further showed that the number of pores present in both types of cells corresponds to the estimated number of replicons that have to be active

simultaneously in order to replicate the DNA during S-phase. Vogel and Schroeder (1974), also using the PHA-stimulated lymphocyte system, but basing themselves on different theoretical considerations, show the same relationship.

This prompted us to investigate the number of pores during the nuclear cycle of Physarum. Macroplasmodia of this slime mold are very suited for this kind of study because of the rather short duration of the S-phase and the natural synchrony of nuclear divisions.

4.2 Materials and Methods.

Macroplasmodia of Physarum polycephalum were cultured as described earlier (chapter 2). At different times after metaphase 2 a plasmodium was dipped into water and scraped into 20 ml buffer (30 mM NaCl, 1 mM KCl, 5 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.1). It was centrifuged at 50 g for about half a minute. The pellet was suspended into 30 ml buffer, supplemented by 0.1% Triton X-100, and homogenized by two strokes of a Tri-R-Stir-R homogenizer, setting 3. After filtration through milk filter the homogenate was centrifuged for 1 min at 1000 g; the nuclear pellet was resuspended into 0.5 to 1.0 ml buffer without Triton X-100. The whole procedure took no more than about 10 minutes. All steps were carried out at $4^{\circ}C$.

Photographs, using phase-contrast microscopy of ethanol-fixed smears (chapter 2) were obtained with a Zeiss Photomicroscope III. Preparation of Pt/C replicas of nuclear suspensions took place as described in chapter 3; the shadowing angle, however, was routinely 10° and the prepa-

ration was rotated during evaporation.

Electron micrographs were made using a Zeiss EM 9 S electron microscope. In most cases, setting 3 (x 8500) was used. All negatives were printed at a final magnification of 17000. Measurements were made on pictures with this enlargement.

For freeze-etching, small samples of macroplasmodia were prefixed for 30 min at 4°C with 3.5% glutaraldehyde in 0.02 M Na-cacodylate buffer, pH 7.2 (Ryser, 1970). After a graded series of glycerol in buffer (until 30%) the pieces of plasmodium were frozen in Freon 22 on copper plates and conventionally freeze-etched (see e.g. Mühlethaler, 1971) using a Balzers freeze-etching device (BA 360 M, Balzers, Liechtenstein). Electron micrographs were obtained with a Philips EM 300 electron microscope operating at 60 kV. Nomenclature is according to Branton et al. (1975).

4.3 Results.

4.3.1 Phase-contrast microscopy and Pt/C replicas.

Fig. 1 shows phase-contrast micrographs of macroplasmodial smears, made at various stages of the nuclear cycle. As can be seen (pictures a to c), determination of the metaphase stage using the characteristics of the preceding stages as a marker is very exactly possible.

An estimation of nuclear diameters from these micrographs is presented in table 1. In the same table these values can be compared with measurements on diameters of

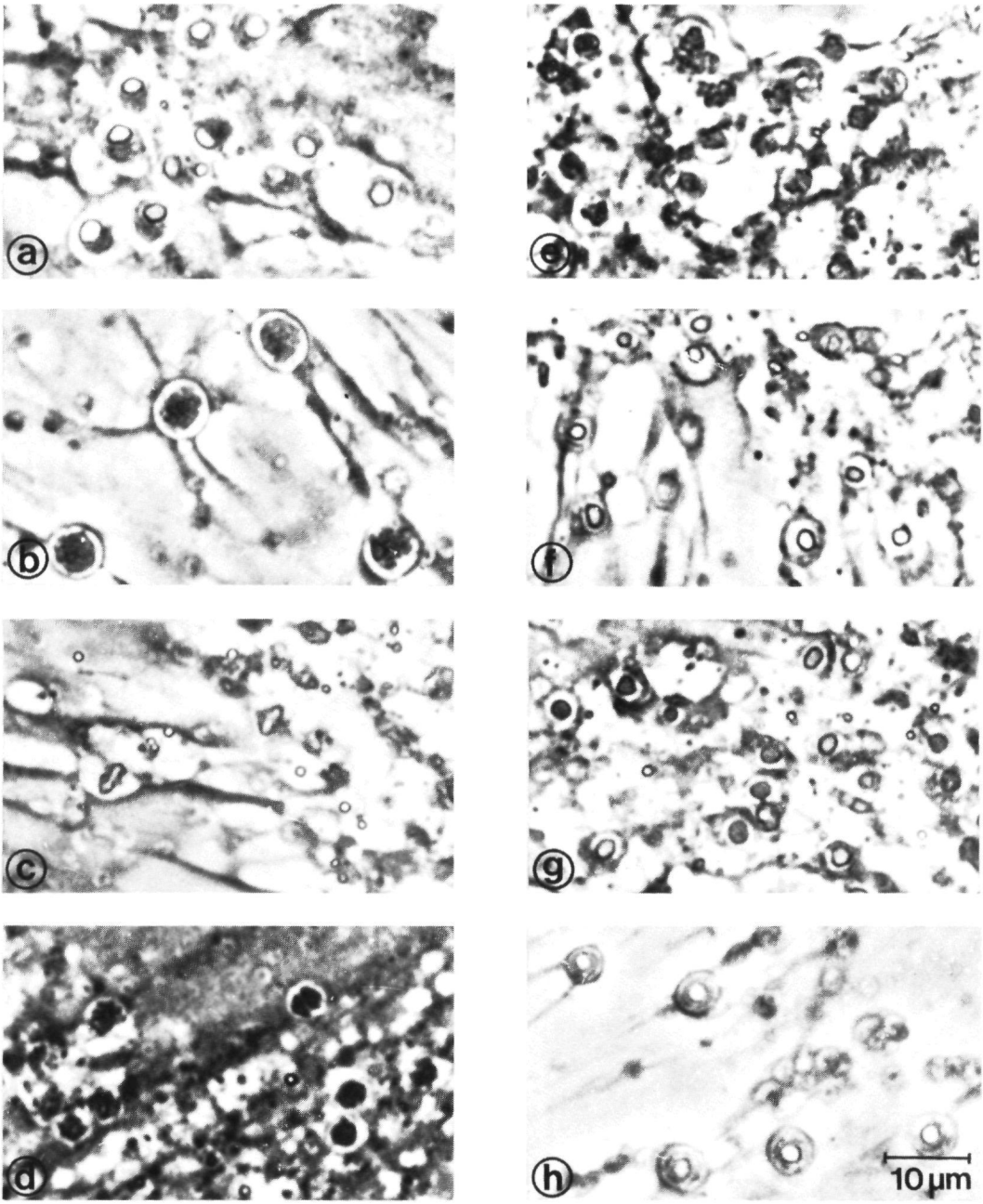


Fig. 1.: Phase-contrast photographs of macroplasmodial smears from Physarum polycephalum. a: prophase; b: prometaphase; c: metaphase; d: anaphase; e: metaphase + 30 min; f: metaphase + 60 min; g: metaphase + 135 min; h: metaphase + 240 min.

stage	nuclear (μm) diameter (a)	idem (b)	pores/ replica	pores/ nucleus (c)	pores/ μm^2 (d)	idem (e)
P	6.5					
PM	6.5					
M_2 + 30	4.8	3.7				
M_2 + 60	5.4	4.4	168 ± 18	336	11	(12)
M_2 + 135	6.0	5.8	284 ± 24	568	13	
M_2 + 240	5.8	5.7				(14)
M_3 - 150		5.8	370 ± 36	740	17	
M_3 - 50		5.8	385 ± 26	770	16	

Table 1. Dimensions of nuclei from macroplasmodia of Physarum polycephalum.

(a) as measured on phase-contrast micrographs of ethanol-fixed smears.
(b) as measured on electron micrographs of nuclear replicas.
(c) assuming that approx. 50% of the nuclear surface is exposed.
(d) measured on electron micrographs of nuclear replicas.
(e) Measured on electron micrographs of freeze-etching replicas.
(12): M_2 + 90 ; (14): M_2 + 300 min.

nuclear replicas. Nuclear diameters, obtained with both methods, agree rather well; the differences at smaller diameters may be due to the limitations of light microscopy in these measurements.

Some electron micrographs of nuclear replicas, prepared at various times after mitosis 2 are given in fig. 2. The nuclear pores appear as annular projections, having a diameter of about 100 nm. The protrusion of the replica, often in the center of the nuclei, probably reflects originally underlying and dehydrated nuclear contents or possibly marks nucleolar position. The excentric position as shown in stage d gives some support to this last assumption, because at the end of the G_2-phase the nucleolus moves towards the nuclear envelope (Guttes and Guttes, 1969; see also fig. 1a).

The main increase in nuclear size obviously takes place during the first 2.5 hrs after mitosis, i.e. during the S-phase (fig. 3). After that period the size of the nucleus remains fairly constant, except possibly in the prophase of mitosis. Fig. 3 also shows a diagram of the average number of pore counts/nuclear replica at different times in the nuclear cycle (see also table 1). There is a strong increase in nuclear pore number during S-phase. The pore number per replica increases from about 170 to 380 during the time investigated. But as the nuclear size also increases, there is only a minor change in pore number per μm^2.

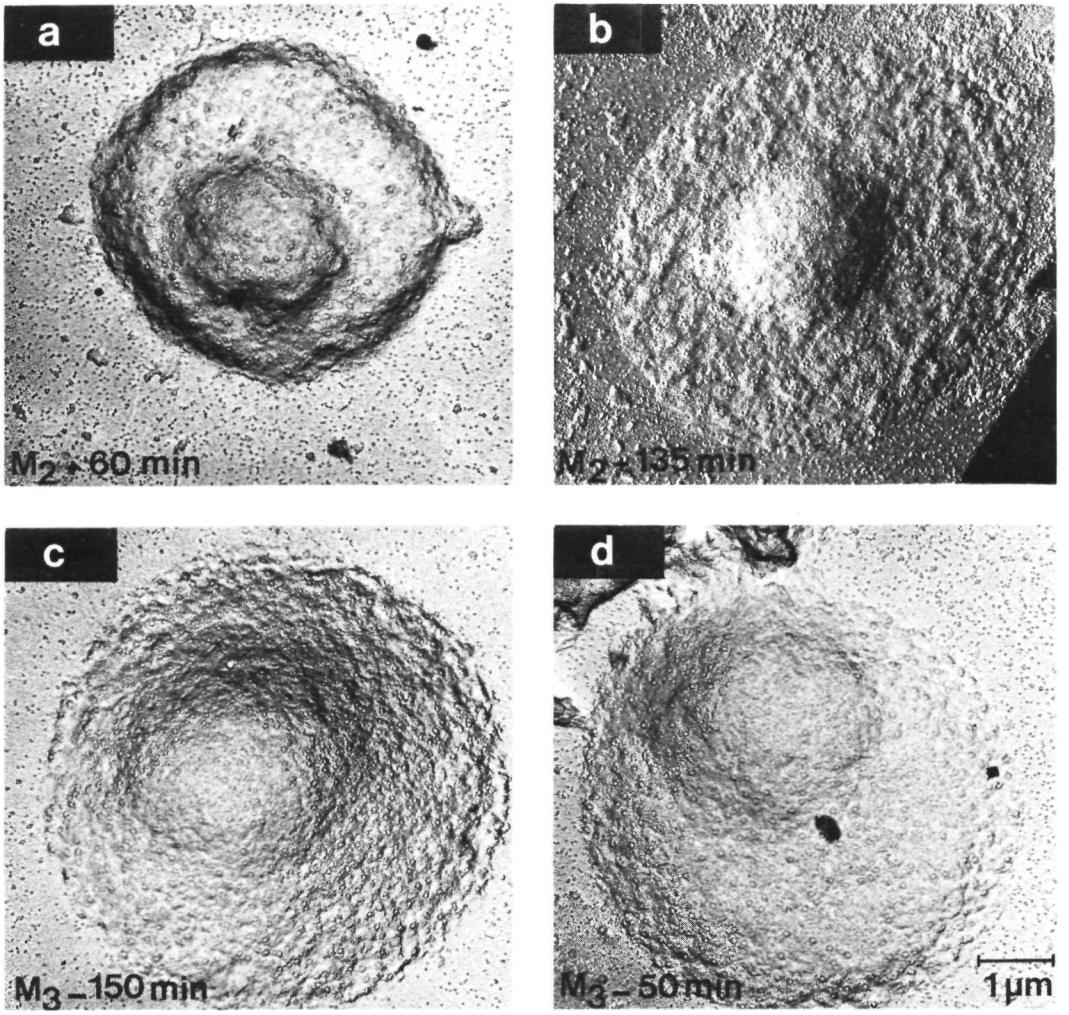


Fig. 2.: Micrographs of nuclear replicas, prepared at various times after mitosis 2. The protruding material possibly reflects underlying dehydrated nuclear contents or marks nucleolar position (note excentric position in d.).

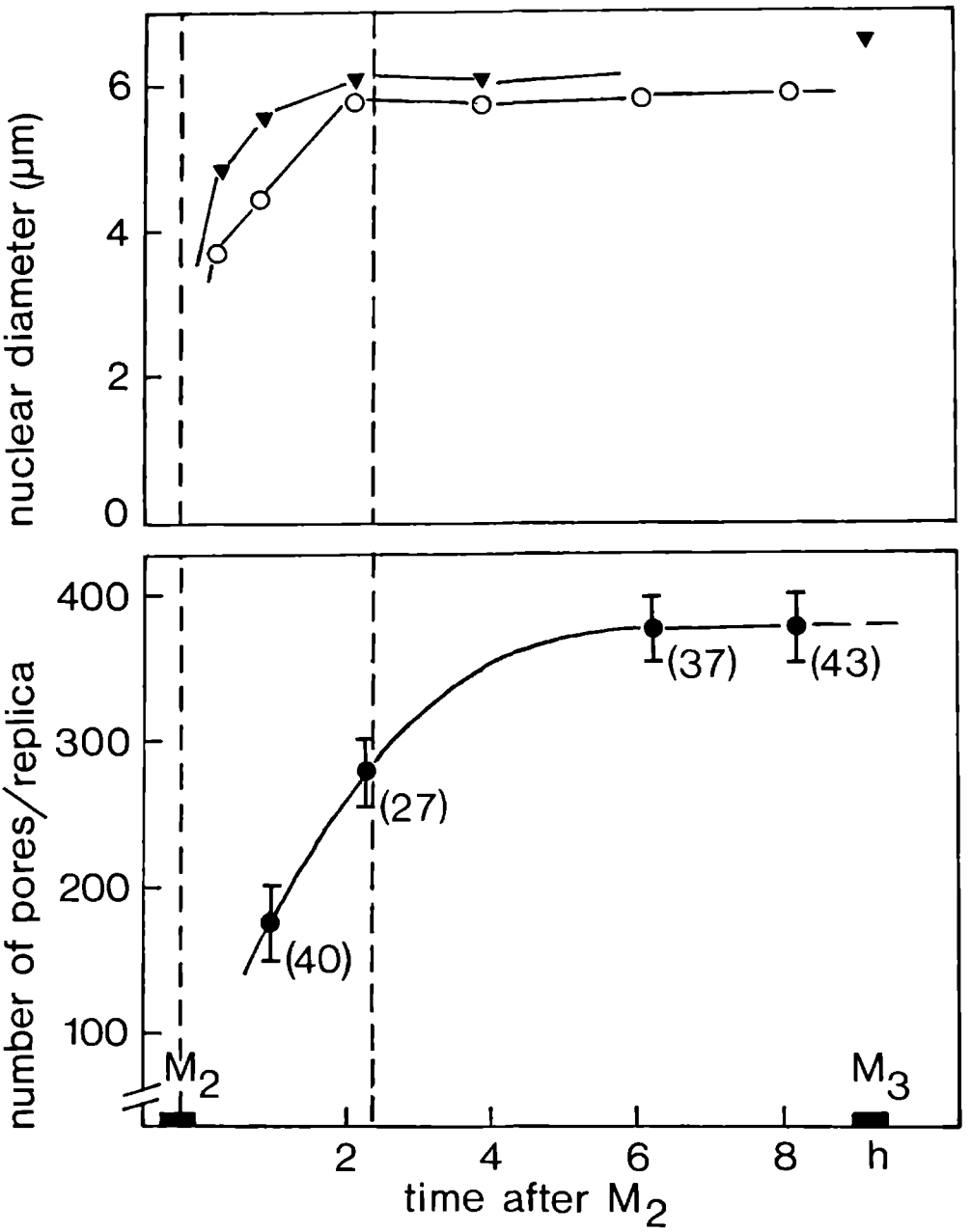


Fig. 3.: Schematic representation, showing the increase in nuclear diameter during the nuclear cycle in macroplasmodia of Physarum polycephalum. (upper drawing).
▼ : as measured on phase-contrast micrographs of ethanol-fixed smears.
○ : as measured on electron micrographs of nuclear replicas.
The number of pores/replica is given in the lower figure. The bars indicate the standard deviation; the number of nuclear replicas examined is given between parentheses. The S-phase is situated between the dashed lines. There is no G_1 in Physarum.

4.3.2 Freeze-etching.

In the photographs we have used the general nomenclature as proposed by Branton et al. (1975). For a better understanding, fig. 4 shows a schematic drawing indicating the most probable fracture faces for a frozen nucleus.

As is the case in all other organisms examined so far, PF is richly covered with intramembranous particles, as compared with EF (figs. 5,6,7). The nuclear pore complexes are clearly visible, having a diameter of about 100 nm. Annular subunits can also be observed, often together with a central plug (figs. 5,6); in an enlargement (fig. 6a) the subunits are evident, most probably eight, a number which has been reported for most organisms. The pore rim seems to be circular rather than octagonal (see e.g. Franke, 1974, for a review).

The occurrence of rings on the PF layer may support the model of the nuclear pore complex as proposed in chapter 3; the variable appearance of the pore complexes might also be interpreted according the classification of Severs and Jordan (1975), who discern domes, cylindrical holes, rimmed and cylindrical projection types of pores, depending on the fracture plane.

The number of pores/μm^2 is approx. 12 at M_2+135 min and 14 at M_2+300 min. This value agrees rather well with that obtained from replicas of isolated nuclei (table 1).

As was concluded under 4.3.1, nuclear pore formation in Physarum takes place during interphase, the main increase occurring during S-phase. Fig. 7 shows that the formation or

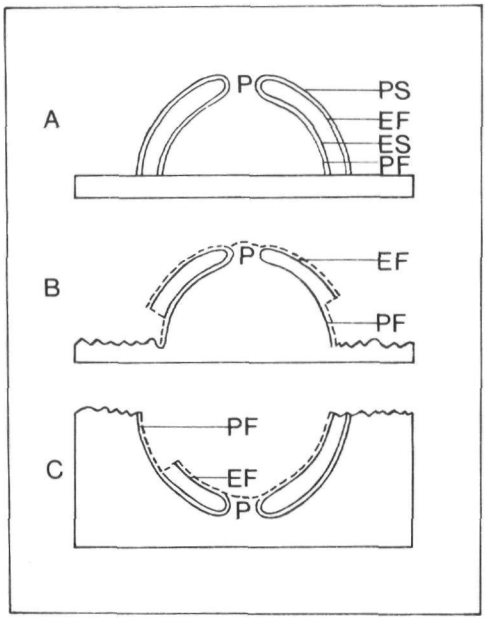
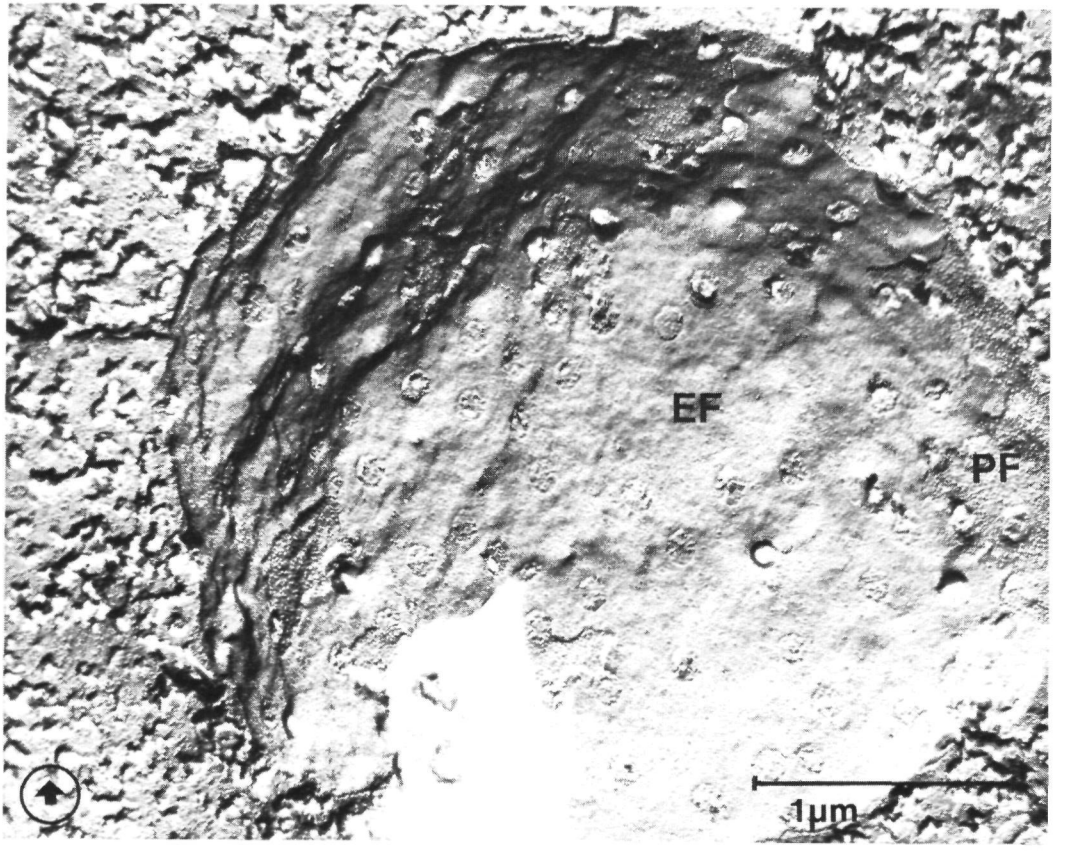


Fig. 4.: Schematic drawing showing the most probable fracture faces; nomenclature as proposed by Branton et al. (1975). Annular material is not shown in this diagram.
A: no fracturing; B: convex view; C: concave view; P: nuclear pore.

Fig. 5.: Freeze-etched nucleus of Physarum polycephalum. Stage: M + 90 min. Note the much higher particle frequency on the PF-face as compared with the EF-face. Subunits of the nuclear pore complexes are visible. The encircled arrow indicates the direction of the platinum shadowing.



disappearance of nuclear pore complexes can be demonstrated for Physarum. The regions of pore formation or breakdown are devoid of particles (arrows) and distributed in a regular way.

4.4 Discussion.

It has been pointed out by several authors (Speth and Wunderlich, 1970; Kartenbeck et al., 1971) that diameters of nuclei and other parameters, e.g. nuclear pore frequencies, may be altered artificially during the preparation procedure. This would explain the different values of pore counts in the same organism when using various techniques. Freeze-etching is considered to minimize these fixation artefacts due to the rapid freezing procedure. Therefore, the number of pores/μm^2 as obtained by this technique is probably most reliable, although it cannot be excluded that the prestabilization with glutaraldehyde and subsequent glycerination might have caused some alterations. Nevertheless, these prefixations were needed to obtain good replicas (Ryser, 1970; also our own observation).

The nice agreement of the number of pores/μm^2, as obtained by the Pt/C replicas, compared with the freeze-etching results, makes shrinkage artefacts, caused by the ethanol dehydration steps, not likely. For the determination of the pore number/nucleus we therefore used the Pt/C replica technique. It allowed a faster preparation of specimens together with the revelation of a larger nuclear surface area.

From our results we concluded that the main increase in nuclear size takes place during the first 2.5 hrs after

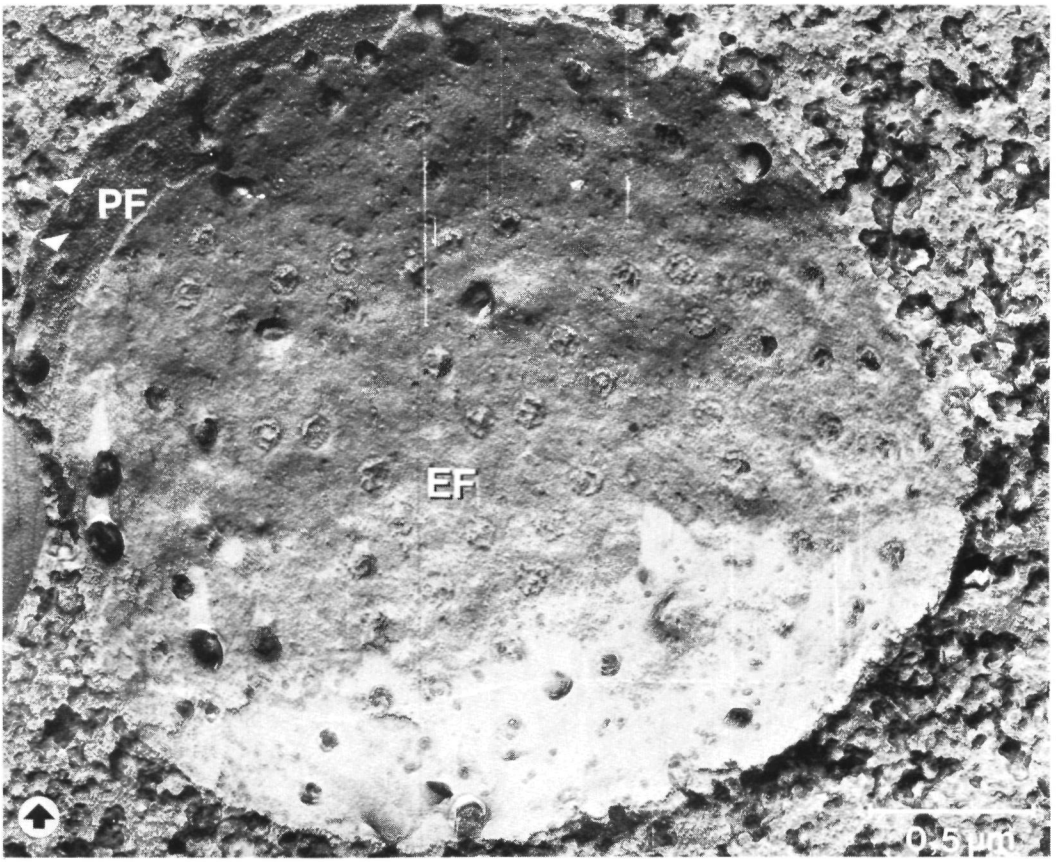


Fig. 6.: Freeze-etched nucleus of Physarum polycephalum. Stage: M + 300 min. Note the occurrence of ringlets on the PF layer (arrow-points).

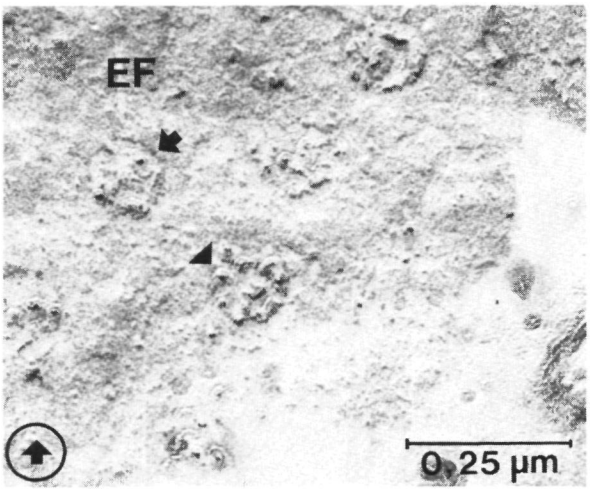


Fig. 6a.: Detail: nuclear pore complex with annular subunits (probably eight; arrow-point) and central granule. The pore rim seems to be circular rather than octagonal (arrow).

mitosis, i.e. during S-phase. This supports the ultrastructural and electrophoretic data of Kieffer (1966), which indicate a very rapid enlargement of the post-mitotic nuclei with a maximal rate in the first hour after mitosis. The results, on the other hand, do not agree with the observations by Bovey and Ruch (1972) who found an increase of nuclear volume during the whole intermitotic period.

We have interpreted fig. 7 as showing the nuclear pore formation or breakdown process during interphase. Regions, devoid of particles, are described by Wunderlich et al. (1974) as artefacts, caused by temperature changes. In that case, however, large and irregular regions are revealed; the pattern as observed by us seems to be too regular to be caused by this kind of processes. In our opinion, therefore, this picture shows nuclear pore dynamics in a very direct way. The picture closely resembles fig. 4 in the paper by Maul et al. (1971). This photograph was interpreted by them to demonstrate the nuclear pore formation process in rat kidney cells. Severs and Jordan (1975), on the contrary, using freeze-fracturing, did not succeed in revealing this formation process in nuclei of activated plant cells.

So, our data confirm the observations made by Maul et al. (1971), who showed for rat kidney cells and PHA-stimulated lymphocytes the formation of nuclear pores during interphase. The increase in nuclear pore number is related to nuclear size. The present results on Physarum, being a lower eukaryote, therefore seem to indicate that pore formation during interphase is a general mechanism, probably occurring in all eukaryotes.

The main increase in nuclear pore formation takes place during the S-phase, being the period of DNA synthesis. To

test the hypothesis that a NPC might be an initiation point for DNA synthesis (Lampert, 1971; Maul, 1972; Engelhardt and Pusa, 1972) we will compare the observed numbers with an estimated number of origins in Physarum.

The DNA content of a G_2 nucleus of Physarum polycephalum, strain m_3c IV, is 1.15 pg (Mohberg et al., 1973). A nucleus after mitosis, shortly before S-phase, contains half of this quantity, or approx. 0.5 pg. It corresponds to a molecular weight of 3.10^{11} D, representing a length of 1.7×10^5 µm. This total length has to be replicated during a S-phase of about 2.5 hrs (see e.g. Werry, 1973); this means 1130 µm/min.

It has been reported that in Physarum the average replication fork travels at a rate of at least 1.5 µm/min (Brewer, 1972; but see Brewer et al., 1974; see also chapter 6). Then, the elongation rate for one replicon, assuming a bidirectional replication, is at least 3 µm/min. The synthesis of 1130 µm DNA/min would, therefore, require approx. 375 replicons to be active throughout the S-phase.

As we have seen (table 1), the pore numbers/replica increase from about 170 to 380 during the time investigated. If we assume, that with this technique about 50% of the nuclear surface is exposed, this would give an increase from about 340 pores/nucleus at 1 hr after mitosis up to 760 in late G_2. Looking just at the increase during S-phase we might cautiously estimate, by extrapolation, a number of about 100 pores/nucleus at the start of the S-phase up to 600 pores/nucleus at the end of it. This yields an average number of 350 pores/S-phase nucleus.

As this value is in the same order of magnitude as compared with the calculated replicon number, it might be

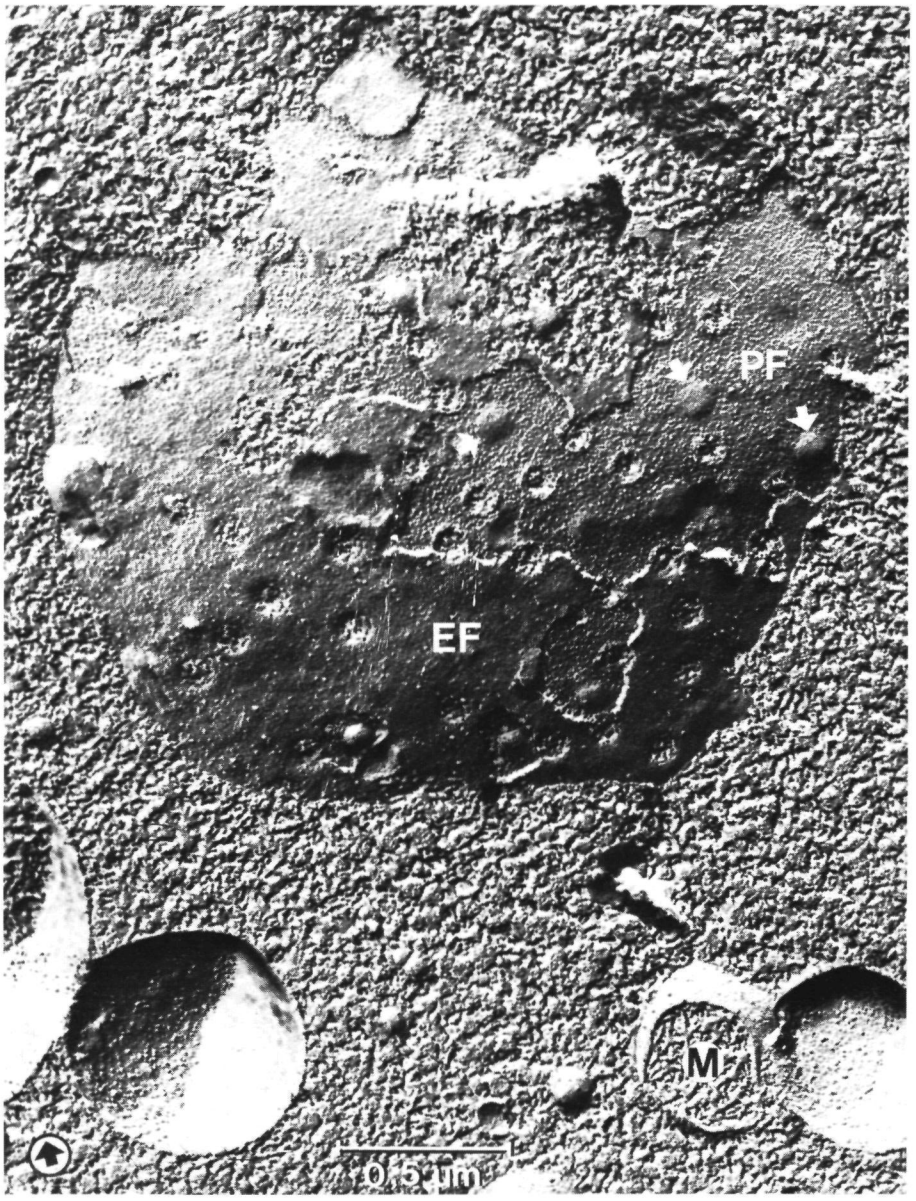


Fig. 7.: Freeze-etched nucleus of Physarum polycephalum. Stage: M + 240 min. The regions where the pores appear (or disappear) are devoid of particles (arrows). M: mitochondrion.

taken to indicate a causal relationship between pore and replicon number, as suggested by Maul et al. (1972). Some arguments, however, show that it is very difficult to draw firm conclusions from this result.

First, it has to be stressed that the many assumptions, necessarily made in the preceding calculations, together with the large number of parameters used, make this calculation just a rough estimation and no more than that. Secondly, the most straightforward consequence of the hypothesis of Maul and coworkers would, in fact, imply that the number of active replicons and, therefore, the over-all rate of DNA synthesis should increase during the S-phase. The highest rate of thymidine incorporation in Physarum, however, is found during the first part of the S-phase (Werry, 1973; see also chapter 6). Further, Jordan et al. (1977), using synchronized yeast cells, were not able to find a positive correlation between nuclear pore number and DNA synthesis, while, moreover, one of the calculations, used by Vogel and Schroeder (1974) to establish such a correlation, is erroneous.

On the other hand, Lott and Vollmer (1975) observed a very pronounced thymidine incorporation into the cotyledons of Cucurbita maxima at a time of maximum nuclear pore number. However, a positive correlation, as such, does not prove a causal relationship.

The increased pore number may also be related to the role of the NPC in nucleo-cytoplasmic exchange (see e.g. Scheer, 1973) or simply to the larger surface area of the nucleus. Also the anchoring function of the NPC (see 4.1) is possible.

Summarized, a function of the nuclear pore complex in the initiation of DNA synthesis is highly speculative and difficult to prove by direct evidence, while, in addition, much EM autoradiographic evidence speaks against it (see chapter 1). On the other hand, the presence of nuclear pore complex remnants in a nuclear structural framework, which seems to be involved in DNA synthesis (Berezney and Coffey, 1974; see also next chapter) might still indicate a function of these nuclear substructures in this process.

If, however, one is tempted to speculate about the whole problem, a hypothetical model might be proposed. In this proposal, the outer rings of nuclear pore complexes might serve as anchoring places for ring-like structures of chromosomes, the so-called "cyclomeres" (Engelhardt and Pusa, 1972). Formation of new outer rings (nuclear pore formation) would occur during S-phase; after this process the new pore would serve as a new attachment site for a cyclomere. This would give rise to the view of nuclear pore complexes, consisting of an outer ring and an inner ring, together with attached chromatin (chapters 2,3). After the formation of this pore complex it might serve as an initiation site for DNA synthesis; for the model itself, however, this is not necessary. During G_2, or just before mitosis, detachment of the cyclomeres takes place; the nuclear pores (outer rings) remain present, possibly interconnected by a fibrous network (chapter 5). Then the same process may start again in the two daughter nuclei.

This view, admittedly highly speculative, is based mainly on suggestions made by Engelhardt and Pusa (1972),

together with those of, among others, Comings and Okada (1970), Dingman (1974) and Vig (1975).

4.5 References.

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CHAPTER V

ULTRASTRUCTURAL OBSERVATIONS ON BOVINE LIVER NUCLEAR RESIDUES

5. ULTRASTRUCTURAL OBSERVATIONS ON BOVINE LIVER NUCLEAR RESIDUES

5.1 Introduction.

In recent years, some authors have described nuclear structures, persisting after extraction of nuclei with various methods (for a survey, see chapter 1.4). In all these cases, a combined procedure was followed, mostly a high salt/non-ionic detergent treatment, resulting in the extraction of DNP and membrane phospholipids. Even after DNase and RNase incubations nuclear structures, called "ghost" (Riley et al., 1975), "matrix" (Berezney and Coffey, 1974, 1976) or "lamina" (Aaronson and Blobel, 1974) were still recognizable. Electron microscopic examination revealed the presence of NPC-like structures in all these preparations. Moreover, Berezney and Coffey (1975) reported that newly synthesized DNA was preferentially associated with these isolated nuclear skeletons.

It was shown recently by Wanka et al. (1977) that after lysis in 1 M NaCl of nuclei, isolated from cultured bovine liver cells, a variable proportion of DNA remained associated with a structure, rapidly sedimenting in sucrose gradient centrifugation. Replicating DNA proved to be more closely associated with this structure than the bulk DNA, as was concluded from DNase experiments. Pronase digestions indicated that proteins were the main components that stabilized this complex. It was thought, that these rapidly sedimenting structures might be related to the residual nuclear frame-works described.

To test this assumption, ultrastructural studies, using whole-mount preparations, were carried out on the rapidly sedimenting material. The results were compared to those obtained after the use of the extraction procedures described by Berezney and Coffey (1974) and Aaronson and Blobel (1975).

5.2 Materials and Methods.

5.2.1 Culture of cells and isolation of nuclei.

Monolayer cultures of bovine liver cells were grown in Roux flasks containing 60 ml culture medium as described by Pieck (1971); the serum concentration was reduced to 10%. Cells from 1 Roux flask were washed off the glass surface with 20 ml Triton-Tris (0.1% Triton X-100 in 5 mM Tris/HCl, pH 8.0). Two portions of 10 ml were passed 5 times through a 0.7 mm needle and the nuclei were collected by centrifugation (1 min at 1000 *g*). This step was repeated. The resulting pellet was then resuspended into 5 ml Triton-Tris medium and sheared several times through a 0.2 mm needle, until the nuclei looked clean as judged by phase-contrast microscopy. They were collected by a final centrifugation step. Cells from 3 Roux flasks were used for each extraction procedure.

5.2.2 Preparation of rapidly sedimenting material.

This was done essentially according to Wanka et al. (1977), with some minor modifications. The final nuclear pellet from 1 Roux bottle was resuspended into 30 ml 50 mM Tris/HCl

buffer, pH 8.0, by means of the 0.7 mm syringe. Then an equal volume of buffer containing 2 M NaCl was added. The nuclear lysate was then sheared 5 times at 0.5 atm pressure through a 1 x 100 mm glass capillary. A short centrifugation (2 min, 100 g) followed to remove non-lysed nuclei; the supernatant was centrifuged for 20 min in a Sorvall RC 2-B centrifuge at 8000 g and 15°C. After a final wash with 1 M NaCl in 50 mM Tris/HCl, pH 8.0, the resulting pellet was used for further analysis. This material was called rapidly sedimenting material (RSM) because of its behaviour in neutral sucrose gradients.

5.2.3 Enzymic digestions of the RSM.

The final pellets were resuspended in 10 ml 50 mM Tris buffer, pH 8.0, supplemented with 7.5 mM $MgCl_2$, and incubated with DNase (100 µg/ml; beef pancreas, electrophoretically purified; Sigma) together with RNase (100 µg/ml; bovine pancreas, type II-A, Sigma; preincubated for 10 min at 80°C). Incubation occurred for 30 min at 37°C. Then, the suspension was centrifuged for 10 min at 1000 g and the pellet was washed once with 10 ml 50 mM Tris buffer, pH 8.0.

5.2.4 Extraction according to Berezney and Coffey (1974).

Starting with the final nuclear pellets, this extraction was carried out essentially in the same way as described by these authors. This, in short, includes two extractions with 0.2 mM $MgCl_2$ for 10 min, three with 2 M NaCl containing 0.2 mM $MgCl_2$ for 10 min, one with 1% Triton X-100 in 5 mM $MgCl_2$ and digestion with 200 µg/ml DNase combined with 200 µg/ml

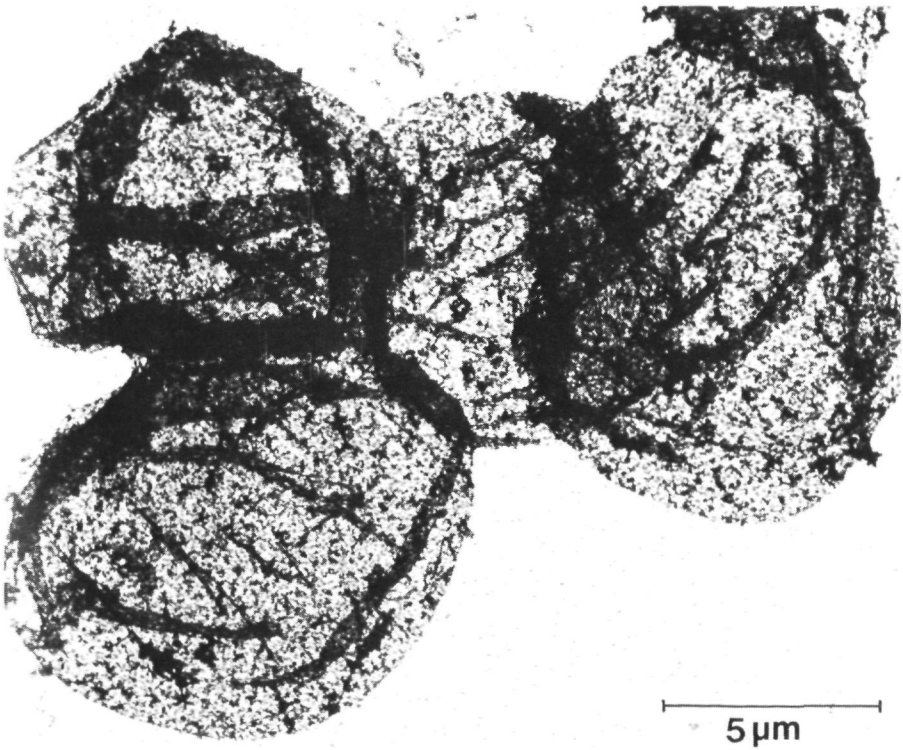


Fig. 1.: Low magnification electron micrograph of some nuclear ghosts after the extraction procedure according to Berezney and Coffey (1974). Nuclear shape remains present.

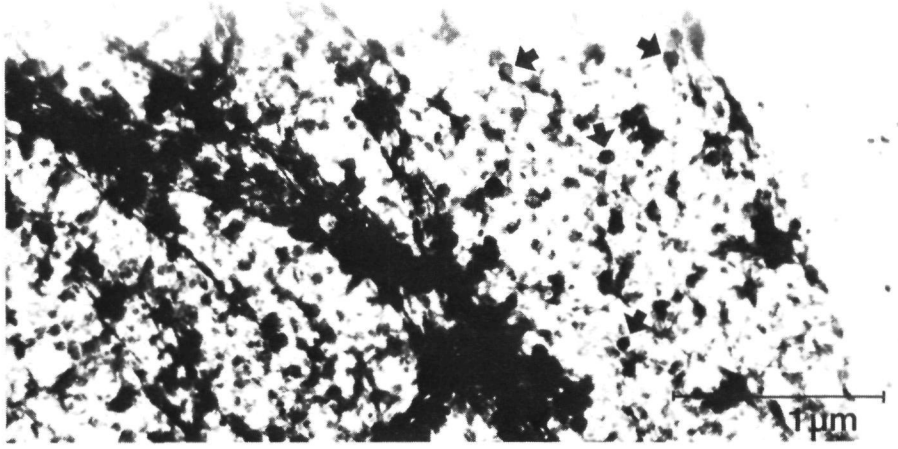


Fig. 2.: Higher magnification of a nuclear remnant after the extraction procedure of Berezney and Coffey (1974). Note the remainders of nuclear pore complexes (arrows).

RNase for 1 hr at 22°C (for enzyme specifications, see 5.2.3). All steps were carried out at 0°C in 10 mM Tris buffer, pH 7.4, and were followed by centrifugation (40 min, 780 g).

5.2.5 Extraction according to Aaronson and Blobel (1975).

The nuclear pellets were incubated for 15 min in 8 mM Tris/HCl, pH 8.5, 0.1 mM $MgCl_2$, 11 mM 2-mercaptoethanol, 0.25 M sucrose and 1 µg/ml pancreatic DNase. After the addition of an equal volume of cold double distilled water the preparation was centrifuged for 20 min at 20 000 g. The pellet was resuspended with 15 ml of 10 mM Tris/HCl, pH 7.5, 0.1 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 0.25 M sucrose, with DNase added to a concentration of 1 µg/ml. After 20 min at room temperature 30 ml cold water was added and centrifugation followed for 20 min at 1000 g.

The pellet was suspended into 12 ml of 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 25 mM KCl, 0.25 M sucrose, to which 3 ml 10% Triton X-100 solution was added. After incubation for 10 min at 0°C the suspension was centrifuged for 15 min at 1000 g. The pellet was then resuspended in the same buffer without Triton, but with $MgCl_2$ added at a final concentration of 0.3 M. A centrifugation step at 1000 g for 15 min followed.

5.2.6 Preparation of samples for electron microscopy.

The pellets, resulting after the procedures as described under 5.2.3, 5.2.4 and 5.2.5 were resuspended in 0.5 to 1.0 ml of the last used extraction medium, depending on the yield of the preparation. After carefully mixing, drops of 10 to

50 µl were put on a flat piece of Teflon. The drops were touched with 150 mesh copper grids, covered with a formvar-carbon supporting layer. The excess of fluid was removed by blotting the grids with filter paper.

The preparations were dehydrated in a graded ethanol series; they were stained for 10 min in the 50% ethanol wash with 1% PTA and air-dried after a final step in isopentane. The grids were examined using a Philips EM 201 or EM 300 electron microscope operating at 60 kV.

5.2.7 Electrophoresis.

The remainder of the pellets, as used for electron microscopy, was reserved for SDS polyacrylamide gel electrophoresis, according to Laemmli (1970); slab gels, containing a linear 6-18% gradient of polyacrylamide were used. The results of these experiments will be described in full detail elsewhere, together with some of the ultrastructural evidence presented here (Mullenders et al., submitted for publication).

5.3 Results.

Electron micrographs of whole mount preparations of material, obtained according to Berezney and Coffey (1974) and Aaronson and Blobel (1975) support the findings reported by these authors. Fig. 1 shows a low magnification electron micrograph of collapsed nuclear ghosts prepared after the extraction procedure of Berezney and Coffey. Nuclear shape is still present; fragments, however, were also observed frequently. Higher magnification of such a nuclear remnant reveals the

Fig. 3.: View of a nuclear ghost after the extraction procedure according to Aaronson and Blobel (1975). Nuclear shape remains present; the numerous nuclear pore complex remnants are indicated by arrows.

presence of several black dots, lying dispersed, with a diameter of about 72 nm (fig. 2, arrows). A fibrous network, more or less condensed, is visible too in this photograph. Possibly the more electron-dense material represents the extensions protruding from the nuclear margin into the interior of the nucleus. Residual nucleoli were not observed.

Fig. 3 shows a nuclear ghost after the extraction procedure of Aaronson and Blobel. Here too, nuclear shape has remained, while numerous black dots (arrows) or sometimes rings (arrowheads) are present, together with fibrous material. The diameter of the rings or dots is approx. 75 nm.

A whole-mount preparation of rapidly sedimenting material is shown in fig. 4. There is a striking resemblance when compared with the other two procedures. Often a rather spherical form is recognizable. A fibrous meshwork is present, together with black dots (arrows). In this case, however, the dots have a diameter of about 95 nm.

Mostly, the rapidly sedimenting material was observed as fragments. Fig. 5 shows such a fragment. The black dots obviously are interconnected, while thin fibrils, associated with the fragment, run away from it (arrow). After incubation with DNase/RNase these thin fibrils, which also occur between the dots (fig. 6a), have disappeared, while the dots themselves are still visible (fig. 6b). Some higher magnifications of another preparation confirm this observation (fig. 7).

5.4 Discussion.

As can be concluded from the photographs, there turned out to be an evidently similar morphology when using the three

different extraction procedures. In all cases, nuclear remnants were observed, either maintaining the rather spherical form of the nucleus ("ghost"), or occurring as fragments. The higher frequency of fragments in the rapidly sedimenting material might be caused by the lysis procedure or the absence of Mg^{2+} ions in the preparation (see Harris and Milne, 1975). The nuclear remnants showed a fibrous substructure, interconnecting a large amount of black dots. Often, also ring-like structures were seen, while in some cases an indication of subunits was present (e.g. fig. 7, open arrowhead).

In our opinion, these rings or dots are residual nuclear pore complexes - possibly often filled with stain caused by the positive staining procedure used - which remain present even after the high-salt extraction. The diameter varied from 72 and 75 nm to 95 nm, a value which accords with the dimensions reported by other workers for mammalian nuclear pores (see Comes and Franke, 1970). Using the same organism, we reported a value of 100 to 120 nm for annular structures, found in whole-mount preparations of isolated nuclei (chapter 3). We cannot explain, however, the lower dimensions - about 75 nm - obtained after the use of the extraction procedures of Berezney and Coffey or Aaronson and Blobel. On the other hand, we can rule out the possibility that the structures are RNP-particles, as described e.g. by Shankar Narayan et al. (1967). First, these particles are only approx. 20 nm wide and the largest RNP-particles observed so far are about 50 nm in diameter (Derksen, 1975). Secondly, the dots or rings were not digested by the combined DNase/RNase treatment, as was shown in figs. 6 and 7.

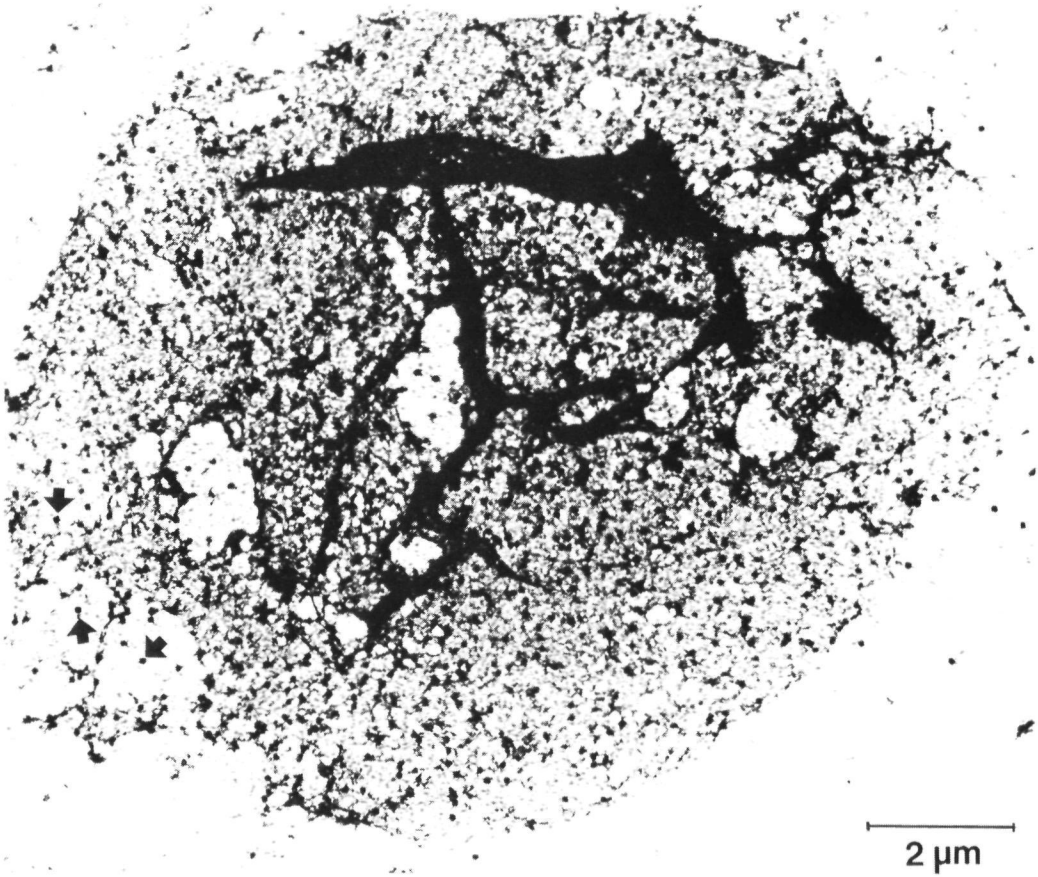


Fig. 4.: Rapidly sedimenting material. Note the rather circular form and also the presence of nuclear pore complex remnants (arrows).

The thin fibrils, which were observed in the rapidly sedimenting material, but not after DNase/RNase incubation, are supposed by us to be thin DNA fibrils, deprived of most proteins by the 1 M NaCl extraction. While the combined treatment with DNase/RNase does not supply full proof for this assumption, recent experiments using only DNase incubations have confirmed it (A.G.M. Bekers, pers. comm.). Moreover, recent results, obtained by use of similar methods, extend this observation to mouse liver nuclei (Comings and

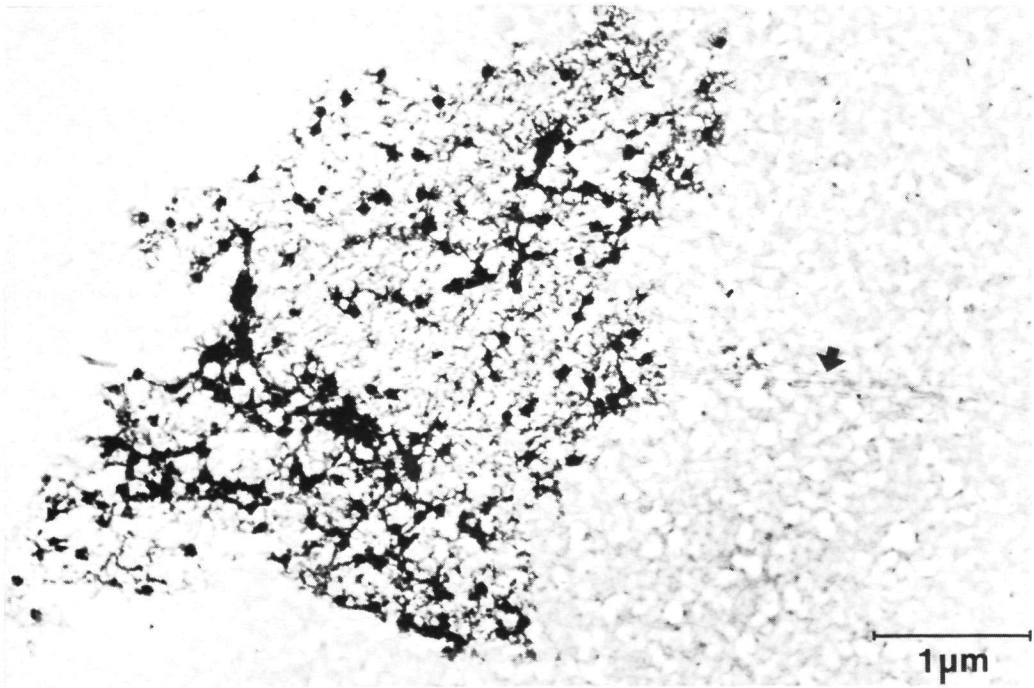


Fig. 5.: Higher magnification of a nuclear fragment, obtained from the rapidly sedimenting material. The nuclear pore complex remnants still seem to be interconnected; thin fibrils, probably DNA, associated with this fragment, run away from it (arrow).

Okada, 1976).

As noted in the first chapter, residual nuclear frameworks have been observed during the last few years in a variety of organisms with various ultrastructural methods (see pag. 19; see also Riley and Keller, 1976; Jackson, 1976; Hodge et al., 1977). In most cases, thin-sectioning techniques were used, which might explain the incidental failure to establish the presence of nuclear pore remnants in the preparations (e.g. Berezney and Coffey, 1974; Riley et al., 1975). As we have seen, they can be made visible with whole-mount technique in a very direct way (figs. 1 and 2; see also, however, Berezney and Coffey, 1976, using thin-sectioning; Keller and Riley, 1976; Scheer et al., 1976; Comings and

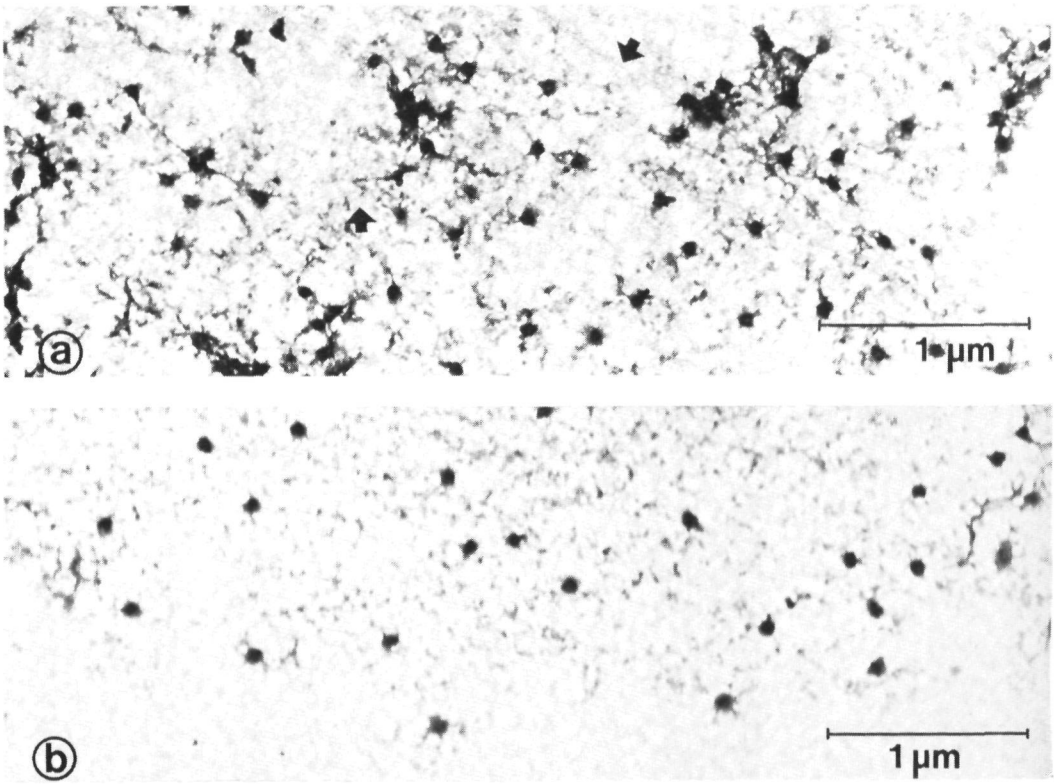
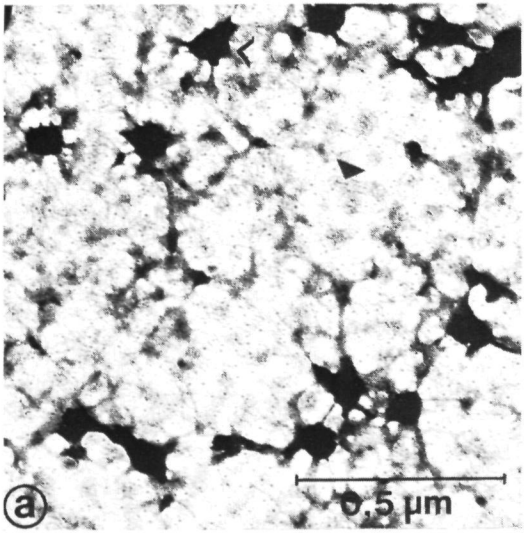


Fig. 6.: Rapidly sedimenting material.
a. without incubation with DNase/RNase. Note the presence of fibrils (arrows).
b. after incubation with DNase/RNase. The nuclear pore complex remnants are still visible, lying in a regular array. No fibrils, however, are visible.

Okada, 1976). The preparations of isolated nuclei from Physarum, as shown in chapter 2, in our opinion also represent a nuclear structural framework with residual nuclear pore complexes and attached deproteinized chromatin. It remains difficult, however, to discriminate with certainty between protein fibrils and DNA or chromatin fibers. Also, a three-dimensional impression of the residual framework is difficult to obtain because of the two-dimensional character of the whole-mount preparations.



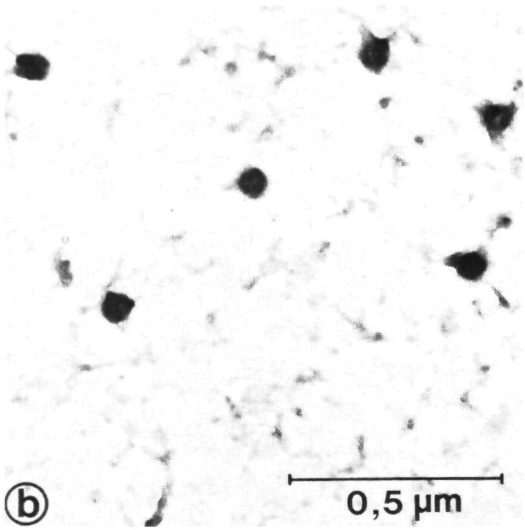


Fig. 7.: Rapidly sedimenting material. Some higher magnification photographs.
a. Annular subunits are sometimes visible (open arrow-head). Note again the presence of fibrils (closed arrow-head).
b. After incubation with DNase/RNase. Nuclear pore complex structure seems unaffected, but no fibrils are present.

Many authors have combined ultrastructural evidence with electrophoretic analysis (e.g. Berezney and Coffey, 1974; Aaronson and Blobel, 1975; Hildebrand et al., 1975; Shelton et al., 1975; Herlan and Wunderlich, 1976; Riley and Keller, 1976; Comings and Okada, 1976; Jackson, 1976). In most cases, three or four major bands of polypeptides were observed, with molecular weights in the range of 50 000 - 80 000 daltons, depending on the organism investigated. As can be seen in fig. 8, this also holds for the rapidly sedimenting material, which supports the nuclear framework function of this material (for a detailed account, see Mullenders et al., submitted for publication). Because of the presence of nuclear pore complex remnants in the material analysed, one is allowed to propose that at least one of the observed proteins will be involved in the composition of the nuclear pore complex.

Evidence about a role of these nuclear residual structures in nuclear function is scarce. A first attempt was made by Hodge et al. (1977) who showed an altered polypeptide pattern during the cell cycle of HeLa S_3 cells and after infection of cells with adenovirus. A function of these nuclear residues in DNA synthesis was suggested by Berezney and Coffey (1975), who reported that in rat liver nuclei the matrix DNA was newly replicated after a pulse-label with tritiated thymidine. It was also shown, that nuclear matrix proteins phosphorylate at a maximal level just before the onset of DNA synthesis (Allen et al., 1977).

Wanka et al. (1977) showed that replicating DNA was more closely associated with the rapidly sedimenting material than the bulk DNA. Therefore, also this observation might be taken to indicate that this material in some way may be involved in

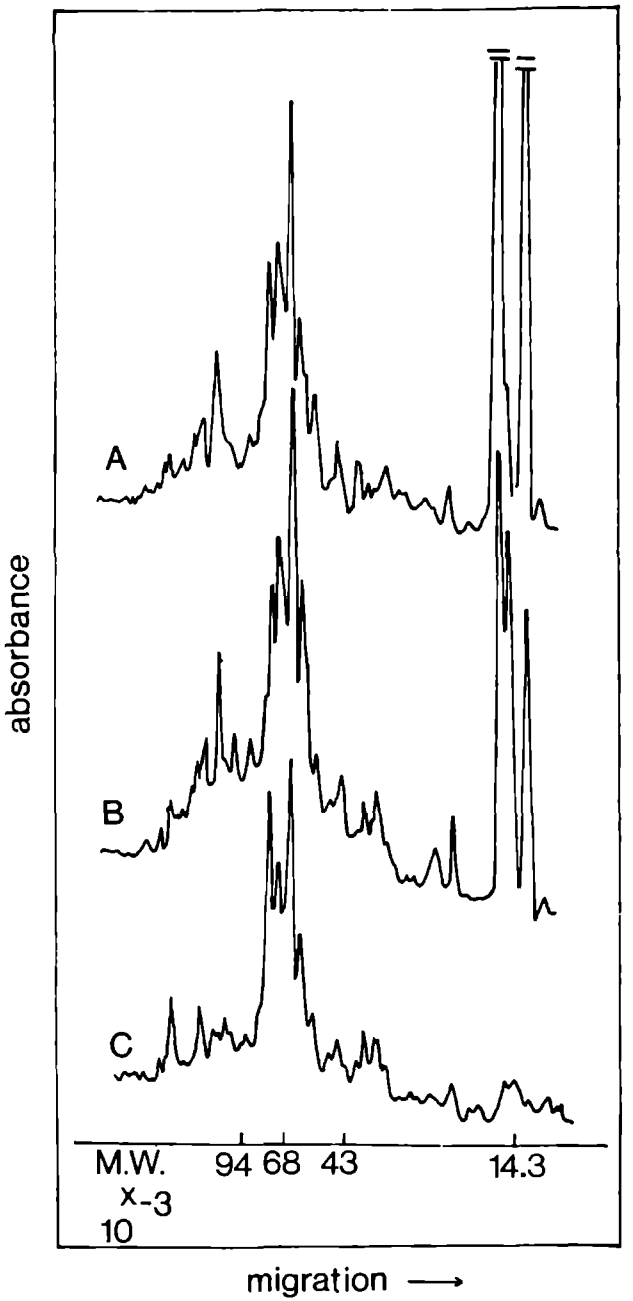


Fig. 8.: Densitometer tracings of PAA slab gels.
A: rapidly sedimenting material; B: according to Berezney and Coffey (1974); C: according to Aaronson and Blobel (1975). Molecular weight markers: phosphorylase a, 94 000; serum albumin, 68 000; ovalbumin, 43 000; lysozyme, 14 300. Courtesy of L.H.F. Mullenders.

the process of DNA synthesis. It is conceivable, that the nuclear residue (the RSM) provides initiation sites for replication of the attached DNA, possibly throughout the whole nucleus. Alternatively, this DNA might represent a series of sequences which interact with proteins to form fixing points for intranuclear organization (see also chapter 4, fig. 8).

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CHAPTER VI

VISUALIZATION OF REPLICATING DNA MOLECULES IN *PHYSARUM POLYCEPHALUM*

6. VISUALIZATION OF REPLICATING DNA MOLECULES IN PHYSARUM POLYCEPHALUM

6.1 Introduction.

Electron microscopy has proved to be a powerful tool in visualizing the replication process in small DNA molecules (e.g. bacteriophage DNA, Inman and Schnös, 1971; viral DNA, Meinke and Goldstein, 1971; mitochondrial DNA, Arnberg et al., 1974). It has made possible observations on the fine structure of replication forks, single-strandedness of regions (formamide technique) and also, by the use of recently developed techniques, on the attachment of other molecules, e.g. polymerases, involved in the replication process (Dubochet et al., 1971; Koller et al., 1974; Vollenweider et al., 1975; for reviews, see Griffith, 1973; Younghusband and Inman, 1974).

The high resolution of electron microscopy, on the other hand, is directly related to a rather small DNA length which can be analysed all at once. This implies, that preparations of larger DNA molecules have to be scanned thoroughly to observe changes in molecules as e.g. replication bubbles, and that often laborious overlapping micrographs have to be made (Bode and Morowitz, 1967).

These observations will be facilitated when the frequency of these changes/μm DNA increases. Wolstenholme (1973), therefore - to visualize replicating nuclear DNA molecules - made use of fertilized eggs of Drosophila melanogaster, an organism also used by Kriegstein and Hogness (1974). Fertilized eggs of Cochliomyia hominivorax were used by Lee and Pavan (1974).

organism	total DNA length/nucleus (µm)	chromosome number (haploid)	average chromosome length (µm)	duration of S-phase (min)	required replication rate/ chromosome (µm)	minimal number of replicons (origins)/ chromosome 1)	minimal number of replicons (origins)/nucleus	average minimal number of active replicons/µm DNA	references
Mycoplasma hominis	262	1	262	-	-	-	(1)	(1:262) (0.38%)	Bode and Morowitz, (1967)
Escherichia coli	1350	1	1350	41	33	-	(1)	(1:1350) (0.07%)	Cooper and Helmstetter, (1968)
Saccharomyces cerevisiae	7800	17	460	30	15	7	128	1:65 (1.5%)	Strauss, (1974); Petes et al., (1973a, 1973b)
Drosophila melanogaster (fertilized eggs)	38000	4	9600	3.4	2824	1412	5648	1:7 (14.2%)	Kriegstein and Hogness, (1974)
Physarum polycephalum	170000	25	6800	150	45	22	550	1:310 (0.32%)	Mohberg et al., (1973); Brewer, (1972); see also chapter 4
HeLa cells	2600000	40	65000	540	120	60	2400	1:1083 (0.09%)	Painter et al., (1966); Maul et al., (1972
1) assuming for all organisms a replication rate of maximal 1 µm/min/replication fork.									

Table 1. Comparison of Physarum polycephalum with some other organisms concerning DNA content, chromosome number and calculated replicon number.

adapted from Strauss, 1974.

The cleavage nuclei of these eggs have a very short S-phase, in which the whole DNA content has to be replicated, giving rise to a very high origin frequency.

Another organism, suited for this kind of studies, is the yeast Saccharomyces cerevisiae, mainly because of the very small DNA content of the chromosomes (Petes et al., 1973a, 1973b; Newlon et al., 1974). To augment the amount of replicating intermediates, temperature-sensitive mutants were used to synchronize the cultures. It could be shown, that most yeast DNA molecules initiate DNA synthesis internally and at several sites. Because of the varying sizes of eye-loops within one molecule it was thought to be likely that - assuming a constant replication rate at all sites - different units might initiate at different times.

Table 1 shows a comparison of Physarum polycephalum with some other organisms. The rather short S-phase, together with the low DNA content/nucleus (approx. 1 pg) gives rise to an average minimal number of active replicons/μm DNA which might be high enough to allow the visualization of replicating intermediates in this slime mold by electron microscopy. We thought it possible - knowing the stage of the samples - to draw some conclusions about replication rate and initiation starting points in a very direct way.

6.2 Materials and Methods.

6.2.1 Culture conditions.

The culture of Physarum polycephalum and preparation of macroplasmodia occurred as described earlier (chapter 2).

For determination of the mitotic stages, ethanol-fixed smears were made (Guttes et al., 1961) and observed by phase-contrast microscopy (chapter 4). As a marker stage (t=0) metaphase was used.

6.2.2 Preparation of DNA samples.

6.2.2.1 From crude lysates.

At various times after metaphase small pieces of the macroplasmodium (1 to 2 mm^2) were removed with a pair of forceps and immediately transferred into a welled microscopic slide, containing 0.1 to 0.3 ml of lysis medium, pre-incubated at 37°C. This lysis medium consisted of 15 mM NaCl, 10 mM EDTA, 5 mM Tris/HCl (pH 8.0) and 0.25% SDS; 1 mg/ml pronase (Calbiochem, nuclease-free) was added. In some cases, instead of pronase, proteinase K (Merck) was used at the same concentration.

A cover slip was put over the well and the preparations were left at 37°C for at least 3 hrs. Mechanical shearing, for example by pipetting, stirring or shaking, was avoided to minimize breakage of the DNA molecules.

These crude lysates were made because of the quick sampling of many various stages with minimum shear and, therefore, probably also minimal production of free DNase activities.

6.2.2.2 From isolated nuclei.

With this method the whole plasmodium was scraped from the

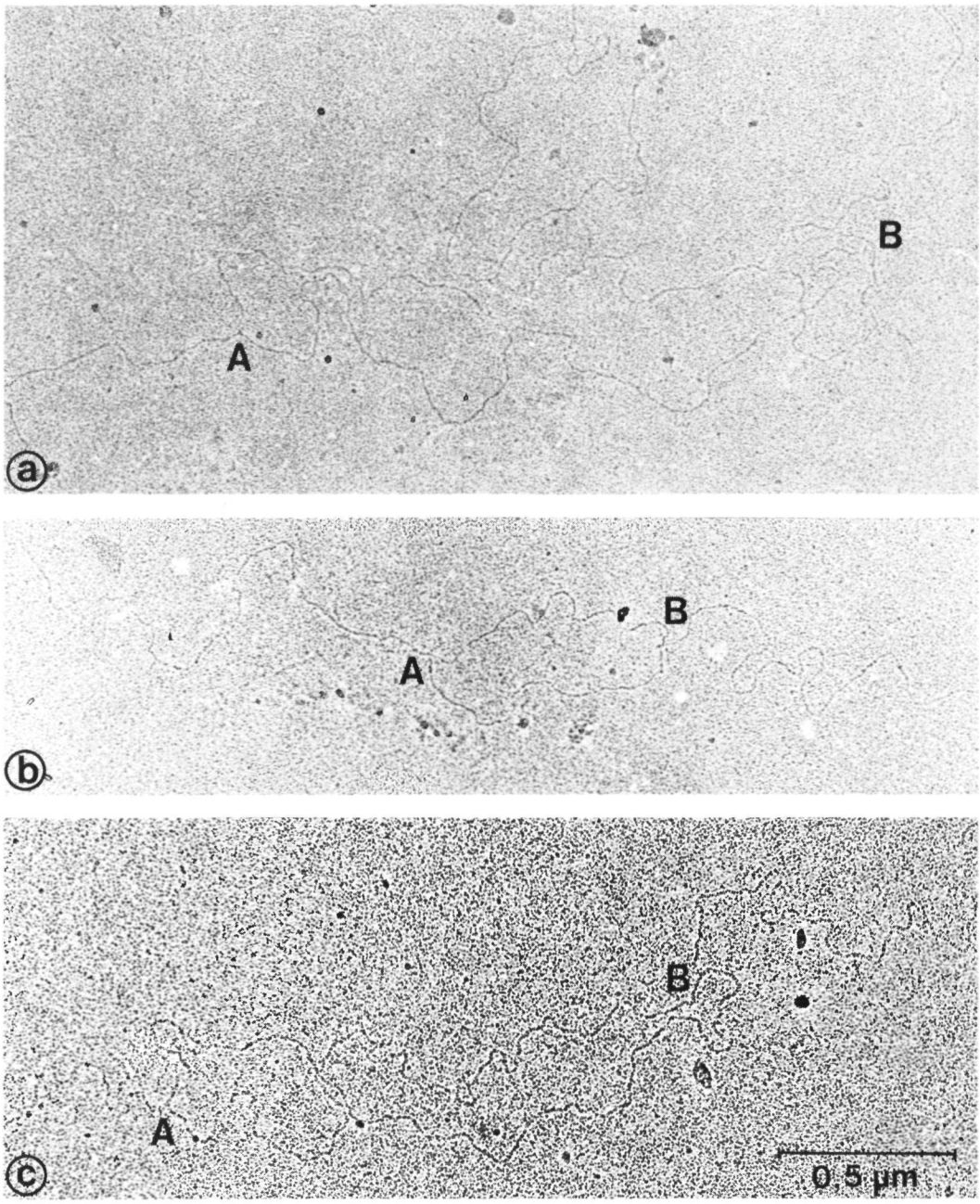

Fig. 1.: Assumed replicating intermediates from Physarum polycephalum.
a: from isolated nuclei; daughter strand length 3.2 µm.
b: crude lysate; daughter strand length 0.93 µm.
c: isolated nuclei; daughter strand length 2.8 µm.

filter paper at a definite time after mitosis and rinsed in ice-cold water. After transfer into buffer, nuclei were isolated as described in chapter 4. To the final pellet the same lysis medium as that mentioned under 6.2.2.1 was added in various amounts, mostly 1 to 2 ml, depending on the yield of nuclei. The solution was carefully vortexed and incubated for at least 3 hrs at 37°C.

In this way, one special stage could be analysed without the disadvantage of contaminating mitochondrial DNA being present in the samples.

6.2.3 Preparation of the lysates for electron microscopy.

Preparation for electron microscopy was done using a modification of the aqueous spreading technique of Freifelder and Kleinschmidt (1965), as used by Wolstenholme (1973; see also Davis et al., 1971). After incubation, small amounts of the lysates (approx. 1, 5 or 10 µl) were transferred with Eppendorf pipettes onto a piece of Teflon and carefully mixed with a freshly-made solution, consisting of 1 M ammonium acetate containing 0.05% cytochrome c (Merck) and 0.5% formaldehyde. From these mixtures samples were brought - also with Eppendorf pipettes - onto a hypophase of 0.3 M ammonium acetate in 0.5% formaldehyde. This hypophase consisted of drops of 1 ml, brought onto a piece of Teflon with small shallow cavities (see Inman and Schnös, 1970). All pipette tips were cut off with a razor blade to enlarge the inside diameter and thus minimize mechanical shear; to avoid DNase activity all glassware, pipette tips and Teflon sheet were sterilized before use.

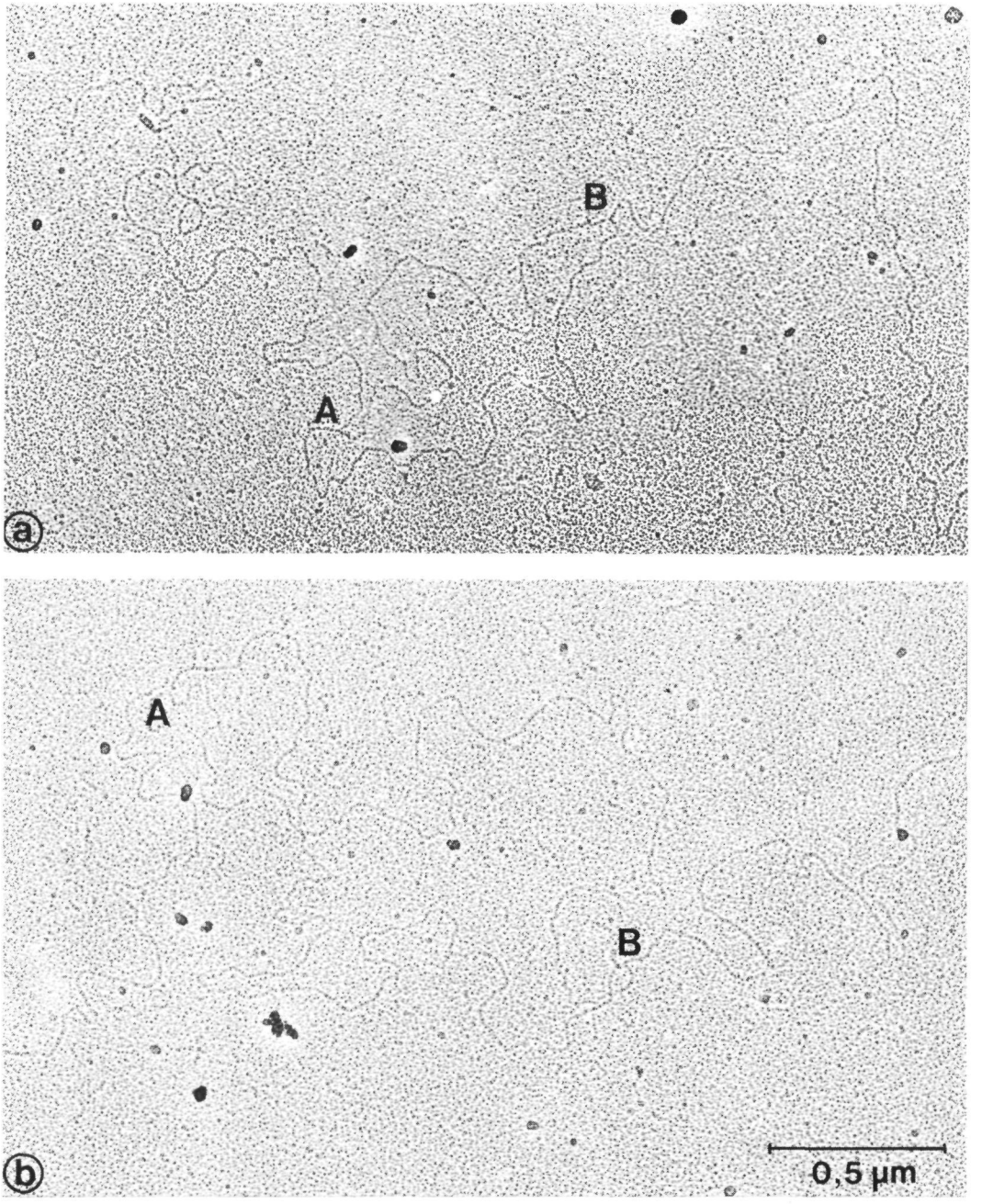

Fig. 2.: Idem as fig. 1.
a: isolated nuclei; daughter strands 2.4 µm.
b: idem; daughter strands 4.5 µm.

The surface film was picked up onto carbon-coated formvar films, supported on 150 mesh copper grids. Shadowing occurred at an angle of 6 to 10° with a Pt/Pd alloy (80 : 20, Drijfhout, Amsterdam). Preparations were rotated during evaporation.

6.2.4 Analysis of the preparations.

All preparations were scanned using a Philips EM 201 or EM 300 electron microscope operating at 60 kV. Magnifications were calibrated with a diffraction grating replica (Balzers, Liechtenstein; 54.864 lines/inch). After enlargement of the negatives measurements were made on projections of positive prints, using a Leitz episcope. Molecules were measured with a map ruler after tracing on a blackboard.

6.3 Results and discussion.

Figs. 1, 2 and 3 show the electron micrographs which are considered by us to visualize good candidates for replicating DNA-intermediates. The molecules observed were lying free, well dispersed and of even width. The lengths of the daugther strands, which were equal within each molecule, are given in table 2. As can be seen, all samples with the assumed replicating intermediates proved to be from stages shortly after mitosis (M_2 + 10 to M_2 + 15 min). In the many thousands of preparations examined, also at other times during S-phase and during G_2, these eye-loops were not observed.

It might be argued, that the molecules are of mitochondrial origin, which could have been the case especially in the crude lysates. Their total length, however, greatly exceeded

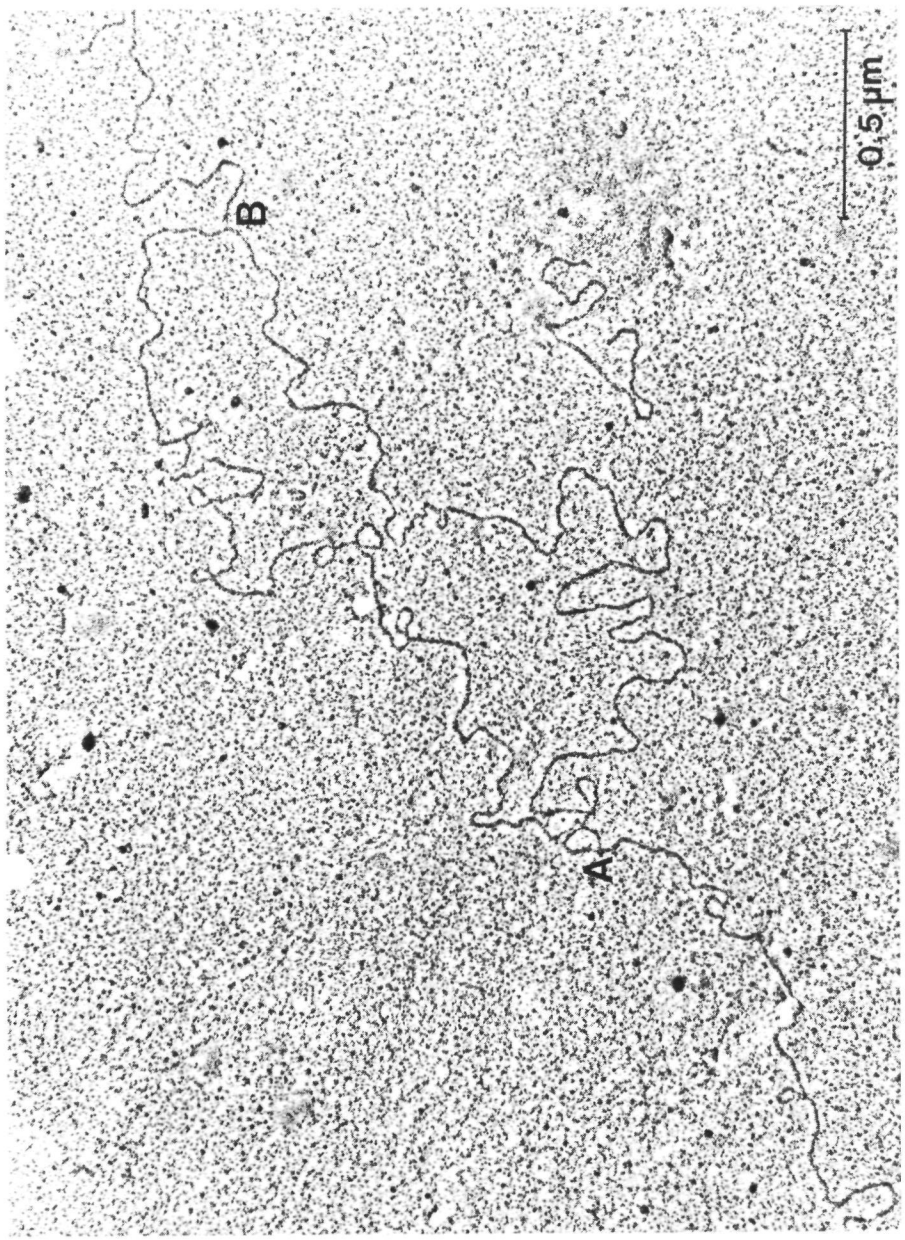

Fig. 3.: Replicating eye-form from a crude lysate; daughter strand length 4.8 µm.

20 μm, which is the largest value for mitochondrial DNA as reported for Physarum (Bohnert, 1977). We think it unlikely, therefore, that the pictures shown represent mitochondrial DNA.

Another possibility is the DNA being nucleolar DNA. Replicating nucleolar DNA of Physarum was made visible recently by Bohnert et al. (1975). About 15% of DNA from isolated nucleoli proved to be circular, having a contour length of 3.9 μm or multiples of it; but the major portion of nucleolar DNA was reported to consist of linear molecules, 2 to 50 μm in length. On the other hand, Vogt and Braun (1976) reported, that the nucleolar DNA in Physarum consists of independently replicating linear molecules with a discrete size, 37.10^6 D. However, Zellweger et al. (1972) showed, that nucleoli disaggregate early in mitosis, reappearing 30 to 40 min after metaphase; in the first hour after mitosis no isotope incorporation into nuclear satellite DNA was measured by these authors. Because our pictures are all from samples collected within the first 15 min after metaphase we think a nucleolar origin also unlikely.

We might state, therefore, that the observed structures represent chromosomal DNA molecules with replicating eye-loops. If so, some conclusions can be made from these photographs.

First - because no Y-forks were scored at all - in Physarum replication probably starts only internally in the DNA molecules. This was also concluded by Newlon et al. (1974) for the lower eukaryote Saccharomyces and, most likely, holds true for all eukaryotic organisms (see e.g. Edenberg and Huberman, 1975).

Secondly, we might make an estimation of the replication rate. It was proposed by Brewer (1972) that in Physarum polycephalum the average replication fork travels at the rate of at least 1.5 μm/min. This figure was obtained by

preparation	stage (min)	daughter strand length (μm)	initiation time (min) (a)	(b)
crude lysate	M_2 + 14	4.8	M_2 + 12.4	M_2 − 2.0
crude lysate	M_2 + 14	0.93	M_2 + 13.7	M_2 + 10.9
nuclei	M_2 + 14	3.2	M_2 + 12.9	M_2 + 3.3
nuclei	M_2 + 15	2.8	M_2 + 14.0	M_2 + 5.7
nuclei	M_2 + 15	0.4	M_2 + 14.9	M_2 + 13.7
nuclei	M_2 + 10	2.4	M_2 + 9.2	M_2 + 2.0
nuclei	M_2 + 10	4.5	M_2 + 8.5	M_2 − 5.0

Table 2. Calculated initiation times, based on the presented electron micrographs.

a) assuming a replication rate of 1.5 μm/min/fork (Brewer, 1972)
b) assuming a replication rate of 0.15 μm/min/fork (Brewer et al., 1974)

analysis of replicating DNA in neutral and alkaline sucrose gradients. However, in a later article, Brewer et al. (1974), using essentially the same techniques, estimate the average rate of DNA chain elongation to be 0.15 x 10^6 D/min/replication fork, or 0.15 µm/min/fork. In table 2, these two rather different values are used to calculate the initiation time of the observed replication bubbles, assuming a constant replication rate. As can be seen, the value of 0.15 µm/min/fork is very unlikely. In that case, four of the seven observed eyes would have started replication already during mitosis. It is known, however, that ^{3}H-thymidine incorporation just begins at 5 to 10 minutes after metaphase (fig. 4; see also Werry, 1973). The value of at least 1.5 µm/min/fork, as given by Brewer (1972), is considered by us to be a more reliable one. Assuming this value, none of the observed molecules starts before the onset of ^{3}H-thymidine incorporation. The observations of Funderud and Haugli (1975) are in agreement with this view.

The low frequency of observed eye-loops in this study has to be mentioned. Although artefacts during the preparation cannot be fully excluded - growing points, probably because of the presence of single-stranded regions, are more sensitive to shear; Hanawalt and Ray, 1964 - the low number of observed eye-loops makes it unlikely that all replicons are initiated at the start of the S-phase. This is also indicated by the varying length of the replicating eyes from the same stage. On the other hand - because all replicating molecules were from samples of the first part of the S-phase - a great deal of initiations might occur at the start of this period. To establish this with certainty, a more quantitative analysis

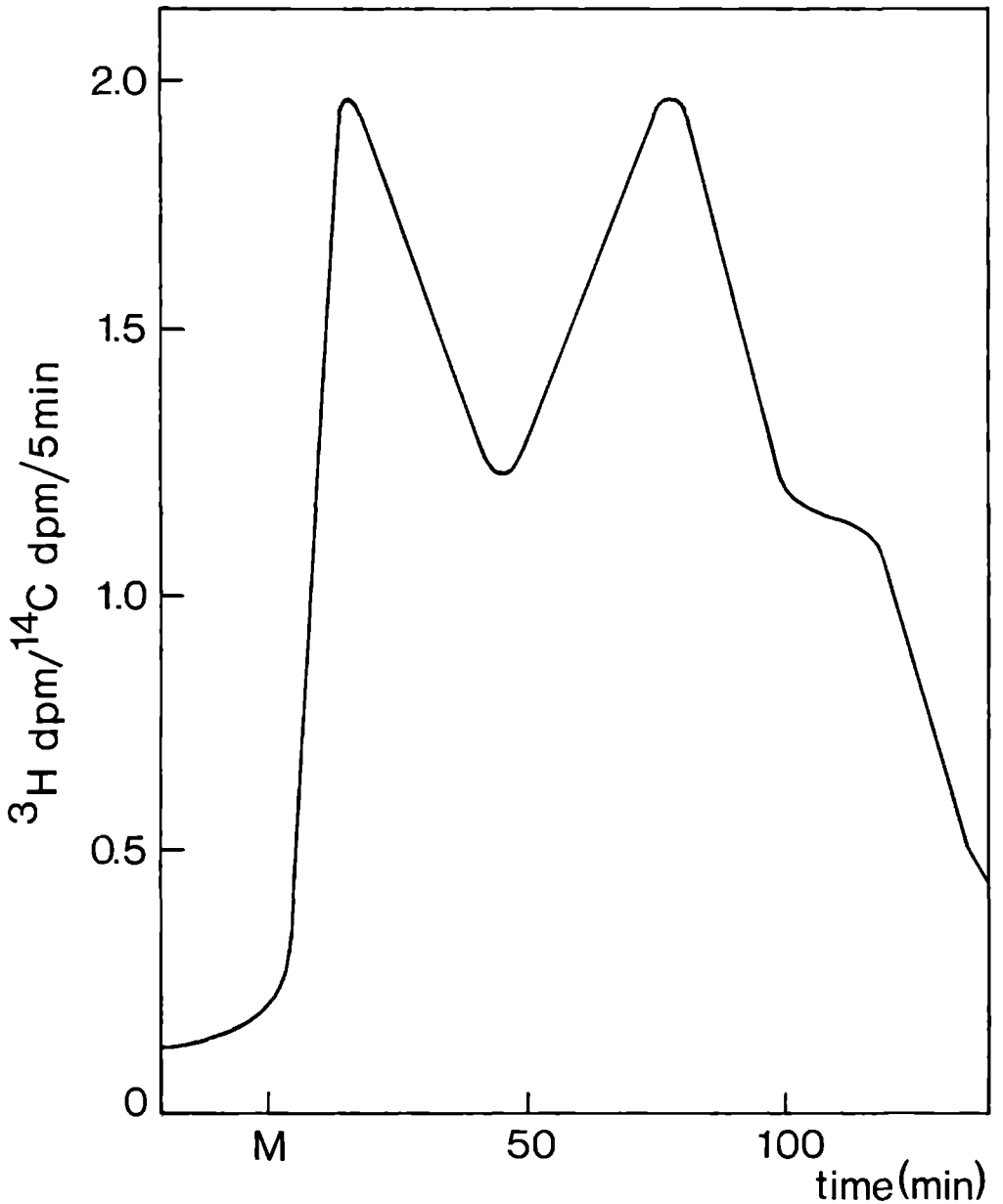

Fig. 4.: Incorporation of ^{3}H-thymidine into DNA of a macroplasmodium of Physarum polycephalum. DNA of one plasmodium was prelabeled from coalescence until 1 hr before mitosis with [2-^{14}C]-thymidine (0.2 µCi/ml; 52.8 mCi/mmol, obtained from New England Nuclear). After transfer of the macroplasmodium into fresh medium incubation was continued. Sectors of the plasmodium were subsequentially pulse-labeled for 5 min, starting at M_2, with [methyl-^{3}H]-thymidine (10 µCi/ml; 20 Ci/mmol, New England Nuclear). Courtesy of T.F.J. de Greeff.

is necessary.

In conclusion, basing ourselves on our photographs, we estimate for the first part of the S-phase a replication rate of 1.5 μm/min/fork, as proposed by Brewer (1972) more likely than a value of 0.15 μm/min/fork (Brewer et al., 1974). It has to be kept in mind, nevertheless, that these are average values, and the elongation rate might have varied between 0.15 and 1.5 μm/min (see e.g. also Housman and Huberman, 1975). For analysis of the origin-to-origin distances and maximal replicon sizes, however, the origin frequency in Physarum is apparently too low to make electron microscopic analysis attractive. For these kind of studies, DNA fiber autoradiography seems to be the method of choice. We have made some observations, therefore, using this technique. Some preliminary results are presented in the next chapter.

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CHAPTER VII

DNA FIBER AUTORADIOGRAPHY OF MACROPLASMODIAL SAMPLES FROM *PHYSARUM POLYCEPHALUM*: SOME PRELIMINARY RESULTS

7. DNA FIBER AUTORADIOGRAPHY OF MACROPLASMODIAL SAMPLES FROM PHYSARUM POLYCEPHALUM: SOME PRELIMINARY RESULTS

7.1 Introduction.

After the classic demonstration by Cairns (1963) of a replicating Escherichia coli genome with DNA autoradiography, this method has been used also for studying eukaryotic chromosome replication (Cairns, 1966; Huberman and Riggs, 1968; Callan, 1972). The basic method of DNA fiber autoradiography has been greatly simplified by Lark et al. (1971). They lysed the labeled cells with SDS directly on microscope slides instead of collecting the extracted DNA on Millipore filters. The lysate was then smeared out on the slide which, after drying, was rinsed in TCA and ethanol and coated with autoradiographic emulsion. A detailed description of the technique is given by Prescott and Kuempel (1973).

We have tried to use this simplified method for preparing autoradiograms of Physarum polycephalum. Some first results are presented below.

7.2 Materials and Methods.

Culturing of Physarum polycephalum and preparation of macroplasmodia occurred essentially as mentioned earlier (chapter 2). Determination of metaphase is described in chapter 4.

Exactly at metaphase, 1/8 part of a plasmodium was cut off, together with the filter paper, and transferred into

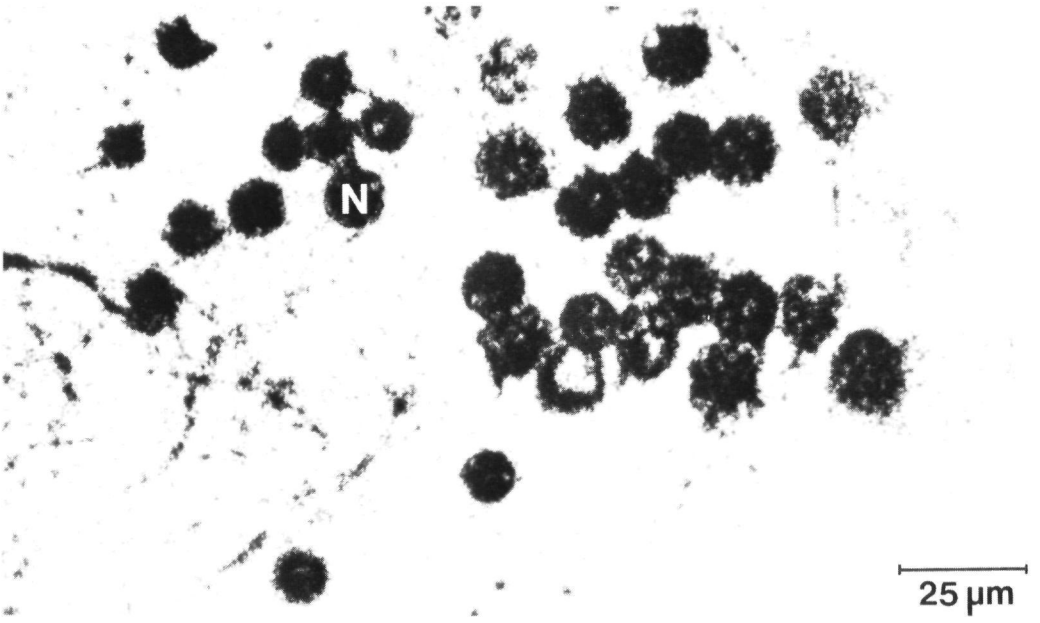

Fig. 1.: Autoradiogram of a macroplasmodial sample. Nuclei (N) were not lysed, resulting in an accumulation of label.

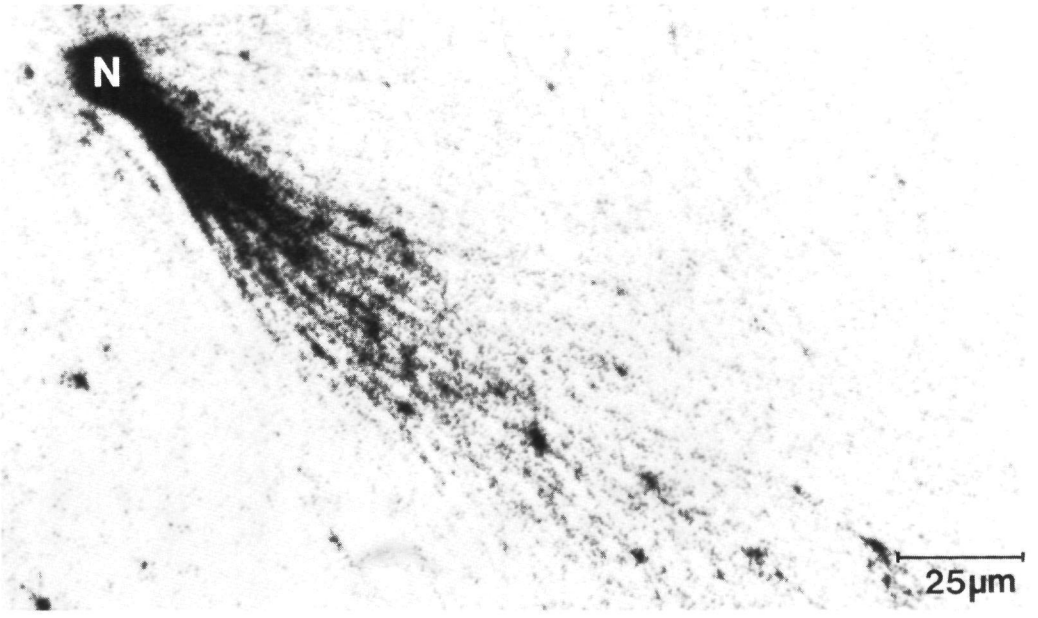

Fig. 2.: Autoradiogram of a macroplasmodial sample. Incomplete lysis, resulting in labeled DNA originating from a discrete nucleus (N).

a smaller Petri dish, containing approx. 5 ml pre-warmed culturing medium to which 100 µCi/ml methyl-^{3}H-thymidine was added (50 Ci/mmol; New England Nuclear). With intervals of a few minutes a small piece of plasmodium (about 1 mm^{2}) was transferred with a pair of forceps onto a clean microscopic slide coated with a thin layer of gelatin (see e.g. Caro, 1964). One or two drops of lysis medium, consisting of 2% SDS, 10 mM EDTA and 10 mM Tris/HCl, pH 8.0, were added with a Pasteur pipette and the lysate was allowed to stand for about 30 min at room temperature. In some cases, 0.25% SDS was used and pronase (nuclease-free, Calbiochem) or proteinase K (Merck) was added in a final concentration of 1 mg/ml to the lysis medium. In that case, incubation occurred at 37°C.

Then the lysate was allowed to run over the microscopic slide by slowly tilting the slide into a vertical position; in a modified procedure, the lysate was spread out over the slide with a second slide (see Prescott and Kuempel, 1973, for details). After drying, the slides were rinsed in 96% ethanol, allowed to dry, rinsed in two washes of ice-cold 5% TCA, then in several changes of 96% ethanol and air-dried. The slides were next covered with Kodak AR. 10 stripping film using conventional technique (see e.g. Fischer and Werner, 1971). The preparations were stored in light-tight slide boxes at room temperature for various lengths of time (3-6 months). Slides were developed in Kodak D-19 developer at 24°C for 5 min, rinsed in water, fixed in acid fixer for 10 min and finally washed in water for at least 60 min. After drying they were mounted with a large-size coverslip using Euparal as a mounting medium. For examination of the slides

a Zeiss Photomicroscope III was used with phase-contrast, bright-field or dark-field illumination.

7.3 Results and discussion.

With the various preparation methods used no significant differences in the final results were observed. In all cases, however, the degree of lysis was variable. Fig. 1 shows a preparation in which nuclei could still be located. In this case, the nuclei were not lysed and the label was only present above these nuclei. When lysis was incomplete, labeled DNA originating from a discrete nucleus was often observed (fig. 2). These kind of pictures were helpful, because they show that the patterns of grains observed were not artificial but indeed reflected the incorporation of ^{3}H-TdR into nuclei. Fan-shaped spreads (fig. 3) were also often observed.

The pictures shown closely resemble some of Hand and Tamm (1974), obtained with mouse fibroblasts (their figs. 3a and 3b) and of Weintraub (1974, plate 1) with chick erythroblast nuclei. The interpretation of the fan-shaped spreads - as given by the former authors - was, that in vivo neighbouring DNA fibres were closely associated. This seems to be also the case for Physarum. Weintraub (1974) suggests, that the many newly replicating DNA strands, radiating from a single point, indicate that several replication forks share the same replication complex. He refers to the association of pulse-labeled DNA with a lipo-polysaccharide containing matrix (Weintraub, 1972) and mentions the association of cyclomeres with DNA (Engelhardt and Pusa, 1972; see also

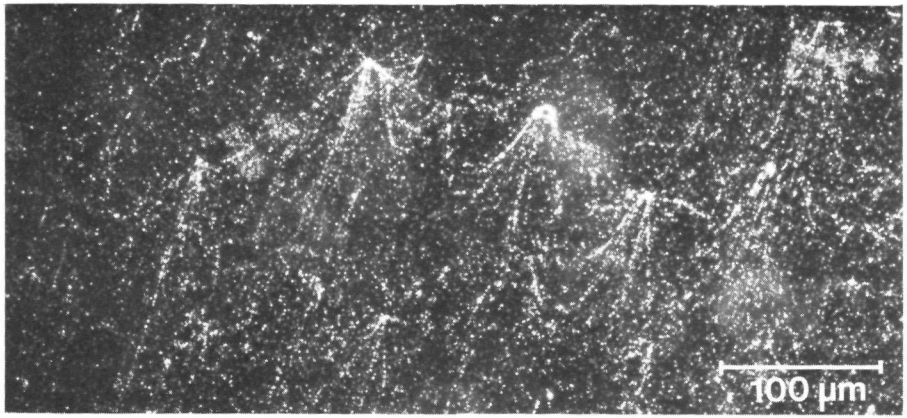

Fig. 3.: Autoradiogram, showing fan-shaped spreads, suggesting that neighbouring DNA fibres in vivo were closely associated.

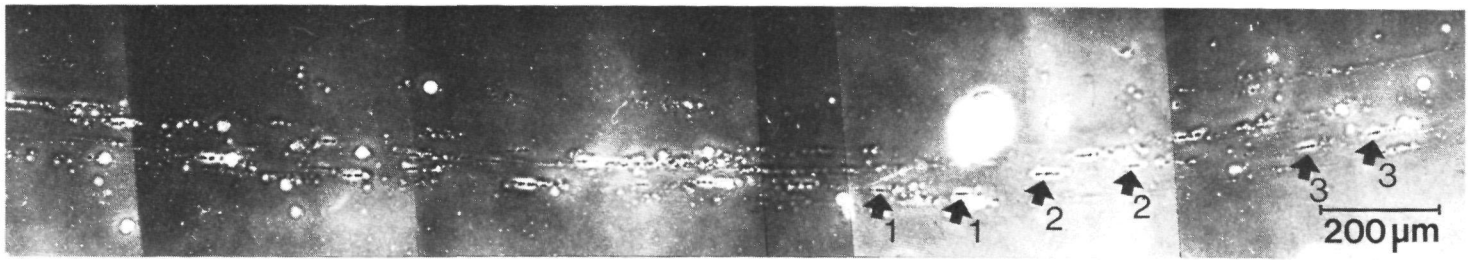

Fig. 4.: Autoradiogram, showing an array of post-pulse initiation figures lying closely together (arrows). Origin-to-origin distances seem to be about 100–150 µm, if one assumes that replicons with the same number are present in one and the same DNA fiber. Stage of the preparation unknown.

chapter 4). However, he admits, that much work remains to be done before an unequivocal interpretation of these structures can be made.

With the labeling protocol used by us, starting with the pulse at metaphase before the onset of DNA synthesis (chapter 6), only post-pulse initiation figures (Hand and Tamm, 1974) may be expected. This means that all tracks represent replicating replicons, which were initiated after the addition of label. The absence of pre-pulse initiation figures makes interpretation of the results easier. Fig. 4 shows such an array of post-pulse initiation figures lying closely together (arrows). As can be seen, most replicons are of the same size. This is in agreement with observations made by Hand and Tamm (1974) and Hori and Lark (1974). These authors proposed that replication occurs in clusters, in which initiations would start synchronously. The origin-to-origin distances in this picture seem to be in the order of 100-150 μm, a value which agrees well with observations made on other eukaryotes (see e.g. Edenberg and Huberman, 1975).

The last picture (fig. 5) comes from a sample, taken at M + 25 min. Here also, some post-pulse initiation figures are visible (e.g. between dashes). Their size is about 50-100 μm. If we assume that incorporation of ^{3}H-TdR starts at M + 7.5 min (see chapter 6) this would imply a length of 50-100 μm replicated in 17.5 min. This results into an estimated elongation rate of 3-6 μm/min/replicon, or 1.5-3 μm/min/fork assuming a bidirectional replication. Although this observation has a preliminary character and many of

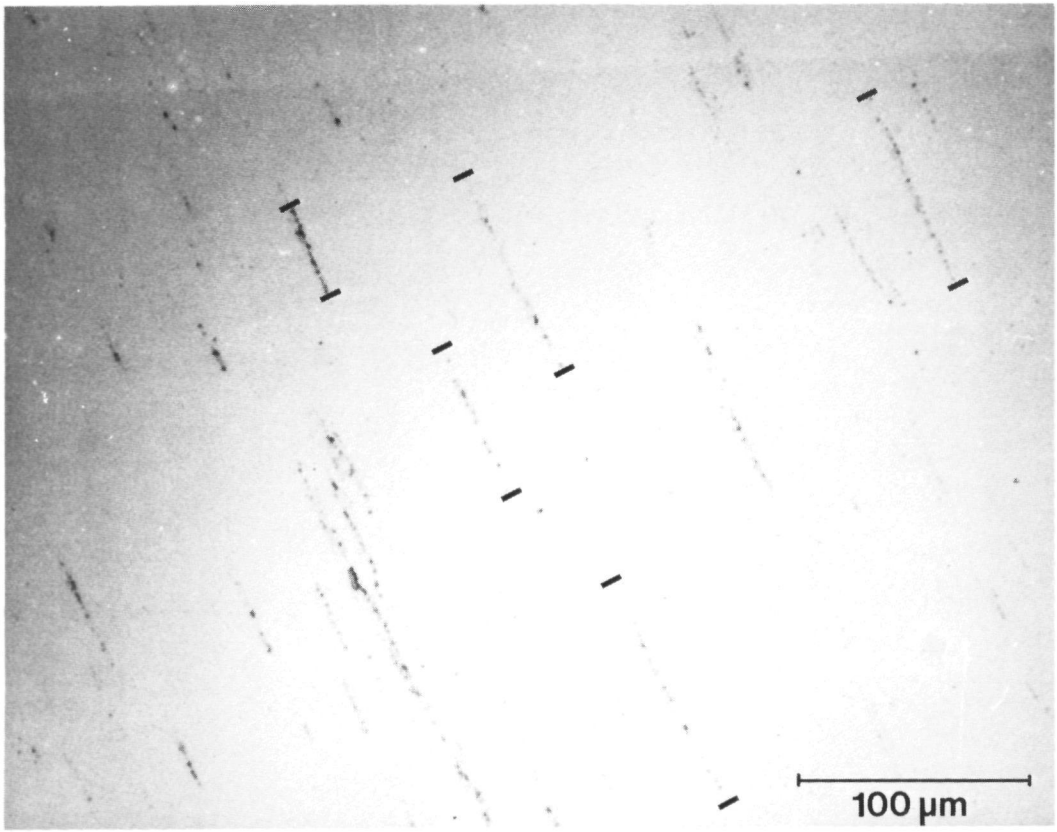

Fig. 5.: Autoradiogram from a macroplasmodial sample taken at M + 25 min. Post-pulse initiation figures are visible (between dashes).

such photographs are needed to make a statistical evaluation, we tentatively might conclude that this value of elongation rate agrees rather well with the one estimated for Physarum by an other technique (chapter 6).

These observations, though preliminary, show the possibility of making DNA fiber autoradiograms using Physarum samples. Therefore, also more sophisticated procedures (e.g. high-low labeling) might be combined with the method described, which might provide many data about direction of replication, origin-to-origin distances, replicon sizes,

clusters of initiation and, possibly, synchrony of the various events. Because of the natural synchrony of the macroplasmodium of Physarum, the results may be of great value, especially when compared to those obtained from other asynchronous or artificially synchronized systems.

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CHAPTER VIII

SUMMARY / SAMENVATTING

8. SUMMARY / SAMENVATTING

8.1 Summary.

This thesis mainly deals with fine structural observations made on interphase nuclei of two various eukaryotic organisms, the lower eukaryote Physarum polycephalum and the higher eukaryotic bovine liver cells, both cultured in vitro. The slime mold Physarum was used because of the synchrony in nuclear divisions, naturally occurring in the macroplasmodia. This allows the determination of the stage of a sample in the nuclear cycle and makes a comparison with other - e.g. biochemical - data possible. We have made use of this property in the chapters 4, 6 and 7. The bovine liver cells could be cultured in larger quantities and were therefore more suited for experiments in which extraction procedures with much loss of material were carried out (chapter 5).

Because of the possible involvement of nuclear membrane or related structures in eukaryotic DNA replication (chapter 1), much attention was paid to nuclear fine structure in relation to an ordered arrangement of chromatin during interphase. In this context, the occurrence and ultrastructure of nuclear pore complexes were emphasized (chapters 2 and 3).

In the first chapter a survey is given of literature about nuclear fine structure in relation to DNA replication, the literature cited being covered until May, 1977. The second chapter shows annular structures being present in whole-mount

preparations of isolated interphase nuclei of Physarum. Ultrastructural features of these annuli were described, while often an association of the rings with fibrils was observed.

The ultrastructure of the nuclear pore complex in bovine liver cells was investigated in chapter 3. By use of whole-mount technique the presence of eight annular subunits could be demonstrated unequivocally. The use of Triton X-100 obviously caused a loosening of the annular structures from the nuclear surface. This was also indicated by the fact that carbon replicas of the surface of nuclei, isolated in buffer without Triton X-100, showed annular projections which were not present after isolation with Triton. Often, a second smaller circular component was observed. A proposal for the arrangement of these components was given in a model.

The replica technique was also used to analyze the number of pore/nucleus during the nuclear cycle in Physarum (chapter 4). It was shown that the main increase in nuclear size took place during the S-phase, a period in which also a strong increase in nuclear pore number was observed. The results were compared with others obtained by freeze-etching technique. By use of this technique also the pore formation or breakdown process could be made visible. Based on the experimental data, a possible function of the nuclear pore complex in eukaryotic DNA synthesis was discussed.

Large-scale isolation of bovine liver cell nuclei allowed the electron microscopic visualization of nuclear ghosts remaining after a series of extractions (chapter 5). In these preparations annular structures could still be

recognized, often associated with thin fibrils which were not observed after combined DNase/RNase treatment. It was suggested that an association of deproteinized chromatin with these nuclear ghosts exists, and the function of such a complex was discussed in relation to DNA replication.

A direct demonstration of replicating nuclear DNA in Physarum was given in the next chapter (chapter 6). By use of a modified Kleinschmidt spreading technique some replicating DNA molecules, all from the first part of the S-phase, could be observed. Using these pictures, an estimation of the elongation rate in Physarum was made. This was also done in the last chapter (chapter 7), which describes some results obtained from Physarum by use of DNA fiber autoradiography. Visualization of replication clusters proved to be possible with this technique. The first preliminary results seem to indicate an elongation rate of 1.5-3 µm/min/fork.

8.2 Samenvatting.

Dit proefschrift beschrijft hoofdzakelijk electronenmicroscopische waarnemingen aan interfase kernen van twee verschillende eukaryotische organismen, nl. de lagere eukaryoot Physarum polycephalum en hogere eukaryotische runderlevercellen, beide in vitro gekweekt. De slijmzwam Physarum werd gebruikt vanwege de synchroniteit in kerndelingen, die van nature in de macroplasmodia aanwezig is. Dit maakt de plaatsing van een monster in de kerncyclus mogelijk alsmede een vergelijking met andere - bijv. biochemische - gegevens. We maakten gebruik van deze eigen-

schap in de hoofdstukken 4, 6 en 7. De runderlevercellen konden in grotere hoeveelheden worden gekweekt en waren derhalve meer geschikt voor experimenten waarin extractieprocedures met veel verlies aan materiaal werden toegepast (hoofdstuk 5).

Vanwege de mogelijkheid, dat de kernmembraan of verwante structuren betrokken zouden kunnen zijn bij de eukaryotische DNA replicatie (hoofdstuk 1), werd er veel aandacht geschonken aan de fijnstructuur van de kern m.b.t. een geordende rangschikking van chromatine tijdens de interfase. In verband hiermee werd de aanwezigheid en de ultrastructuur van kernporie complexen benadrukt (hoofdstukken 2 en 3).

In het eerste hoofdstuk wordt een literatuuroverzicht gegeven omtrent de submicroscopische structuur van de kern m.b.t. de DNA replicatie. Het overzicht strekt zich uit tot mei 1977. Het tweede hoofdstuk toont annulaire structuren, aanwezig in zgn. "whole-mount" preparaten van geïsoleerde interfase kernen uit Physarum. Ultrastructurele kenmerken van deze annuli werden beschreven, terwijl veelal een associatie van de ringen met fibrillen werd waargenomen.

De ultrastructuur van het kernporie complex in runderlevercellen werd onderzocht in hoofdstuk 3. Door gebruik te maken van "whole-mount" techniek kon de aanwezigheid van acht annulaire sub-eenheden ondubbelzinnig worden aangetoond. Het gebruik van Triton X-100 veroorzaakte klaarblijkelijk het loskomen van de annulaire structuren vanaf het kernoppervlak. Een aanwijzing hiervoor was eveneens het feit dat koolstof replica's van het oppervlak van kernen, geïsoleerd in buffer zonder Triton X-100, ringvormige verhogingen ver-

toonden die niet aanwezig waren na isolatie met Triton. Vaak werd een tweede, kleinere ringvormige component waargenomen. Een voorstel voor de rangschikking van deze componenten werd weergegeven in een model.

De replica techniek werd eveneens gebruikt om het aantal poriën/kern te onderzoeken gedurende de kerncyclus in Physarum (hoofdstuk 4). Het bleek dat de grootste toename in kerngrootte plaatsvond gedurende de S-fase, in welke periode tevens een sterke toename in de kernporie aantal werd waargenomen. De resultaten werden vergeleken met andere, verkregen m.b.v. de vries-ets techniek. Met deze techniek kon ook het proces van porie-vorming of -afbraak zichtbaar gemaakt worden. Aan de hand van de experimentele gegevens werd een mogelijke functie van het kernporie complex in de eukaryotische DNA-synthese besproken.

Isolatie op grote schaal van kernen uit runderlevercellen maakte de electronenmicroscopische aantoning mogelijk van kernoverblijfselen na een serie extracties (hoofdstuk 5). In deze preparaten konden nog steeds annulaire structuren herkend worden, veelal verbonden met dunne fibrillen die niet waargenomen werden na een gecombineerde DNase/RNase behandeling. De mogelijkheid werd geopperd dat er een verbinding bestaat tussen gedeproteiniseerd chromatine en deze kernrestanten, en de functie van zo'n complex werd besproken m.b.t. de DNA replicatie.

In het volgende hoofdstuk (hoofdstuk 6) werd replicerend kern-DNA in Physarum rechtstreeks aangetoond. M.b.v. een gewijzigde Kleinschmidt spreidingstechniek konden enkele replicerende DNA moleculen, alle uit het eerste gedeelte van

de S-fase, worden waargenomen. Gebruik makend van deze foto's kon een schatting van de elongatiesnelheid in Physarum gemaakt worden. Dit werd eveneens gedaan in het laatste hoofdstuk (hoofdstuk 7), dat enkele resultaten beschrijft, voor Physarum verkregen m.b.v. DNA vezel autoradiografie. Met deze techniek bleken replicatiegroepen zichtbaar gemaakt te kunnen worden. De eerste voorlopige resultaten wijzen op een elongatiesnelheid van 1.5-3 µm/min/vork.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren te Nijmegen op 4 augustus 1947. Vanaf 1959 bezocht hij het Canisius College te Nijmegen, waar in 1965 het diploma gymnasium-β behaald werd.

In hetzelfde jaar werd hij ingeschreven als student biologie aan de Katholieke Universiteit te Nijmegen, alwaar het kandidaatsexamen in 1968 werd afgelegd. Gedurende de doctoraalstudie werd een bijvak submicroscopische morfologie o.l.v. Dr. M.M.A. Sassen gedaan (de ultrastructuur van de celwandvorming bij het eencellige groenwier Chlorococcum ellipsoideum) en vervolgens het bijvak exobiologie (adaptatie in microörganismen) o.l.v. Dr. W. Heinen (onderwerp: de opname van fosfiet en fosfaat in Escherichia coli).

Het hoofdvak-onderwerp werd bewerkt op de afdeling Zoölogie II, later Genetica, o.l.v. Prof. dr. H.D. Berendes, waarbij de ultrastructuur van actief en inactief chromatine in Drosophila hydei werd onderzocht.

Op 11 januari 1972 werd het doctoraalexamen biologie (cum laude) afgelegd. Vanaf die datum was de auteur als wetenschappelijk medewerker verbonden aan het Laboratorium voor Chemische Cytologie (hoofd: Prof. dr. Ch.M.A. Kuyper) te Nijmegen, alwaar het proefschrift o.l.v. Dr. F. Wanka bewerkt werd.

Sinds 1 september 1976 is hij als wetenschappelijk medewerker werkzaam bij de afdeling Plantencytologie en -morfologie van de Landbouwhogeschool te Wageningen.

STELLINGEN

1

Aan het drukknoopjesmodel van Engelhardt en Pusa zitten - om in confectietermen te blijven spreken - nogal wat haken en ogen.

Engelhardt, P. en Pusa, K. (1972) Nature New Biol. 240, 163–166.

2

De waarde van 1.5 μm/min als snelheid voor de replicatievork in Physarum polycephalum, zoals door Brewer in 1972 is geschat, is waarschijnlijker dan de later genoemde waarde.

Brewer, E.N. (1972) J. Mol. Biol. 68, 401–412.

Brewer, E.N., Evans, T.E. en Evans, H.H. (1974) J. Mol. Biol. 90, 335–342.

3

Een der berekeningen van Vogel en Schreuder betreffende het aantal kernporiën in door phytohemagglutinine gestimuleerde lymfocyten is onjuist.

Vogel, F. en Schreuder, T.M. (1974) Humangenetik 25, 265–297.

4

Het is te betreuren, dat zulk een schitterend proza als "A fever of reason" in de vorm van een voorwoord in een review-bundel slechts door weinigen gelezen zal worden.

Chargaff, E. (1975) Annu. Rev. Biochem. 44, 1–18.

5

De opvatting van Rogers: "Onderwijzen is een sterk overschatte functie" verdient in de herprogrammering van het wetenschappelijk onderwijs meer aandacht.

Rogers, C.R. (1973), Leren in vrijheid, p. 91
(de Toorts, Haarlem).

6

In het woord "huisarts" betekent het voorvoegsel huis- in toenemende mate het huis van de arts.

7

Zolang "geëmancipeerde" vrouwen zich afzonderen in vrouwencafés zal hun emancipatie een vicieuze cirkel blijken.

8

De zinsnede: "de kinderen mogen wat later naar bed" in de zaterdagavond-tune van de NCRV-televisie verlaagt waarschijnlijk de kijkdichtheidscijfers van de erop volgende programma's.

J.H.N. Schel
Nijmegen, 20 oktober 1977