Z. Zellforsch. 127, 230–239 (1972)
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Actinomycin D and the Central Granules in the Nuclear Pore Complex:

Thin Sectioning Versus Negative Staining

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Received November 17, 1971

* The authors are indebted to Miss Marianne Winter and Miss Sigrid Krien for skilful technical assistance. We thank Dr. R. Berezney for reading and correcting the manuscript and helpful discussions. The work has been partially suppported by a grant of the Deutsche Forschungsgemeinschaft.

Summary. Thin section electron microscopy of Actinomycin D treated *Tetrahymena* cells and amphibian oocytes (*Xenopus laevis*, *Triturus alpestris*) reveal no reduction in the central granules in the nuclear pore complexes. Possible reasons for the diversity between these results and earlier observations using negatively stained isolated nuclear envelopes from the same objects are discussed. The results clearly show that the presence of central granules within the nuclear pores does neither depend on nuclear RNA synthesis nor does indicate nucleocytoplasmic RNA transport. This conclusion leads to a reconsideration of the nature of the central granule. The functioning of the central granule of the nuclear pore complexes is further discussed in connection with recent studies on the ultrastructure of various types of cisternal pores.

Key words: Nuclear pores — Nucleocytoplasmic exchange — Actinomycin D — *Tetrahymena* — Amphibian oocytes.

Introduction

The "central granule" has been shown to be a frequent constituent of the pore complex of nuclear membranes and annulate lamellae in ultrathin sections of whole cells as well as in negative staining preparations of isolated membranes (for review see, e.g., Kessel, 1968a; Scheer and Franke, 1969; Franke, 1970a). There are some cytochemical indications that the central granule contains ribonucleoprotein (RNP; for references see Franke and Falk, 1970; for some contrasting references, however, see the discussion of Franke and Falk, 1970, and Franke *et al.*, 1971). Furthermore a correlation between central granule frequency and nuclear RNA synthetic activity appears to exist in some cell systems (Merriam, 1962; Wunderlich, 1969; Franke and Scheer, 1970b). This, together with observations that electron dense clumps of obvious nucleolar or chromosomal origin often can exhibit a structural continuity with the center of nuclear pore complexes (e.g. Beermann, 1964; Stevens and Swift, 1966; Stevens, 1967; Kessel, 1966, 1968b; Takamoto, 1966; Lane, 1967; Clerot, 1968; Massover, 1968; Franke and Scheer, 1970b) has led to the hypothesis that the appearance of the central granule is generally caused by RNP material, centrally located in the pore complex, which is moving through the nuclear pore. In this context the central granule

was visualized by many authors as representing a transitory or dynamic structure rather than a static one. The observation of an actinomycin D (AMD)-induced decrease in central granule frequency in negatively stained, isolated nuclear envelopes (Wunderlich, 1969; Scheer, 1970b) was consistent with such an interpretation. However, as the present study shows, a decrease in central granule frequency after AMD treatment can not be found in ultrathin sections. Consequently, current views of the nature and functioning of the central granule have to be reconsidered.

Materials and Methods

Tetrahymena. Cells of *Tetrahymena pyriformis* GL (amicronucleate) from early exponential growth phase (for culture conditions see Franke *et al.*, 1971) were incubated with actinomycin D (50 μg/ml, Serva Feinbiochemica, Heidelberg) in culture medium for 90, 180, or 340 min at 28° C. Collection, simultaneous or sequential glutaraldehyde/osmiumtetroxide fixation of the cells was carried out as described previously (Franke *et al.*, 1971).

For ^{3}H-uridine incorporation a 200 ml log-phase culture was divided: to one portion AMD and ^{3}H-uridine (30 Ci/mmol, Radiochemical Centre, Amersham, England) was added simultaneously to give a final concentration of 50 μg/ml and 0.5 μCi/ml, respectively, and to the other half only the ^{3}H-uridine was added. At fixed time intervals samples of 5 ml were removed and precipitated with 1 ml of an ice-cold 50% trichloracetic acid (TCA) solution. After centrifugation the pellet was washed three times with 5% TCA, resuspended in 1 ml distilled water with mild sonication, and two aliquots (0.2 ml) were immediately taken. One aliquot was dissolved in NCS-solubilizer (Nuclear Chicago), mixed with 5 ml of scintillation fluid (5 g PPO, 0.5 g POPOP in toluene) and ^{3}H-uridine radioactivity was counted in a Packard Tri-Carb scintillation counter. The other aliquot was dissolved in 1 M NaOH and protein was determined according to the method of Lowry *et al.* (1951).

Autoradiographs of pulse labeled and AMD-treated cells were made of semithin sections (1 μm) using the Kodak AR-10 stripping film according to the standard methods (for details see Eckert and Franke, 1972).

Amphibian oocytes. Lampbrush oocytes of *Triturus alpestris* Laur. and *Xenopus laevis* Daudin were incubated at room temperature in the sterile commercial tissue culture medium TC 199 supplemented with 100 μg/ml penicillin and containing 20 μg/ml AMD. (The oocytes were fully intact, with their epithelial layer still attached.) Controls were kept in the TC 199 without AMD. After 1, 2, and 10 hrs incubation time AMD-treated and control oocytes were fixed in 4% glutaraldehyde (buffered with 0.05 M Na-cacodylate to pH 7.2) for 2 hrs at 0° C, washed thoroughly in the same buffer and then were postfixed in 2% OsO_4 (pH 7.2) for 3 hrs at 0° C. Dehydration was carried out through graded ethanol steps.

All specimes were embedded in Epon 812 and sectioned on a Reichert ultramicrotome OmU2. Micrographs were made with a Siemens Elmiskop IA.

Results

Exponentially growing cultures of *Tetrahymena pyriformis* and amphibian lampbrush stage oocytes were used in the present study for two reasons. First, they represent cellular systems which are known for their especially high efficiency in RNA synthesis (within one second the RNA is synthesized for about 6000 ribosomes in a *Tetrahymena* macronucleus, Leick and Anderson, 1970, and for about 300000 ribosomes in the nucleus of a rapidly growing *Xenopus* oocyte, Scheer, 1972) and nucleocytoplasmic transport (Franke, 1970b; Scheer, 1972), and secondly, the former positive results of an AMD effect on central granule frequency were from these objects (Wunderlich, 1969; Scheer, 1970b).

Tetrahymena. Fig. 1 illustrates the effects of the AMD concentration used (50 μg/ml) on RNA synthesis as monitored by the incorporation of ^{3}H-uridine. RNA synthesis ceases within 10 min after application of the drug and does not recover either in the presence of AMD or after thorough washing with growth

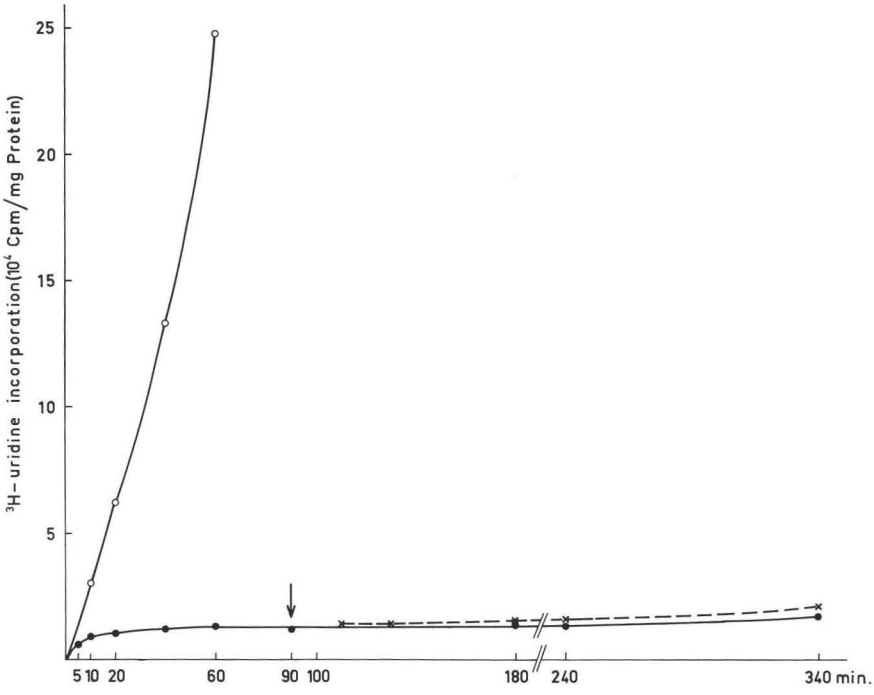
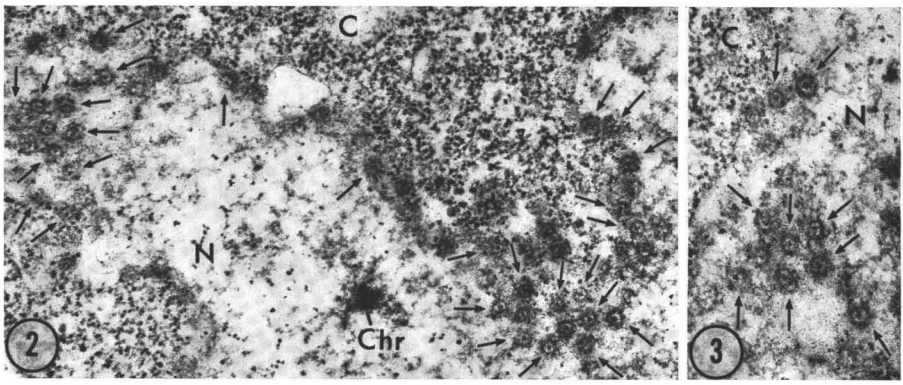


Fig. 1. Incorporation of ^{3}H-uridine into acid-precipitable material of an exponentially growing culture of *Tetrahymena pyriformis* GL in the control (open circles, solid line), and in the presence of 50 μg/ml Actinomycin D (filled circles, solid line). In another sample the Actinomycin has been washed out with culture medium at the time point indicated by the arrow (crosses, dashed line). The concentration of Actinomycin used is effective in the inhibition of uridine incorporation, and the incorporating activity is not significantly restored after removal of the drug

medium for up to 340 min after application and up to 250 min after the omission of AMD, respectively. The effectiveness of the washing procedure, especially against the retention of AMD within the cells, has been controlled in this type of experiments with the use of tritiated AMD, and it was evident that the ^{3}H-AMD was released from the animals during three subsequent washes and further incubation in AMD-free medium within 20–30 min (for details see Eckert and Franke, 1972). Furthermore, quantitative evaluation of the autoradiographs demonstrates that the transport of pulse labeled RNA from the nucleus to the cytoplasm rapidly diminishes and is totally stopped after about 60 min AMD incubation time (Eckert and Franke, 1972).

The viability of the cells in the course of the experiments was evident from their normal swimming behaviour, therefore the energy metabolism can not be seriously influenced at the AMD concentration employed (c.f. also Nachtwey and Dickinson, 1967). Ultrastructural studies of cells treated in such a way indicate, however, some drastic morphological changes which are most marked with the nucleoli and the cytomembranes (Franke and Eckert, 1972; c.f. also Satir and Dirksen, 1971). Whether such ultrastructural alterations are a more or less direct



Figs. 2 and 3. Tangential sections to the macronuclear envelope of *Tetrahymena pyriformis* GL which had been exposed to the Actinomycin D for 180 min (Fig. 2) and 340 min (Fig. 3). Central granules are recognized within the pores (arrows). *N* nucleus; *C* cytoplasm, *Chr.* chromatin. Fig. 2 $\times$ 33000; Fig. 3 $\times$ 45000

consequence of the primary inhibitory effect of the drug, or are to be ascribed, at least in part, to independent secondary effects can not be decided at the moment. Alternatively some kind of a general cytotoxity (c.f. Lorch and Jeon, 1969; Sawicki and Godman, 1971), perhaps due to an AMD induced degradation of RNA as assumed from biochemical studies with mammalian cells (Schwartz and Garofalo, 1967; Schwartz *et al.*, 1968; Stewart and Farber, 1968; Rovera *et al.*, 1970; c.f. also Sawicki and Godman, 1971) may be involved.

For the electron microscopic evaluation only such tangentially sectioned nuclear pore complexes were considered which revealed an electron dense margin, i.e. those in which a considerable portion of the pore wall was included in the section. Such sections fulfil the requirement of being roughly equatorial and thus should reveal the presence of a central particle within the pore widely independent from its shape and size. Evaluation of several hundred pore complexes from more than 50 different cells per sample showed consistently central granule frequencies between 90 and 100%, with no differences between the AMD-treated cells and the controls. Figs. 2 and 3 show examples of such tangentially sectioned macronuclear pore complexes of *Tetrahymena* treated for 180 and 340 min with the drug: almost every pore possesses a central granule.

Amphibian oocytes. The incubation of growing oocytes in AMD concentrations above 10 µg/ml inhibits the chromosomal as well as the nucleolar RNA synthesis (see also Izawa *et al.*, 1963; Lane, 1969; Snow and Callan, 1969). Ultrathin sections grazing the nuclear envelope of oocytes fully blocked in transcriptional activity revealed no differences in the percentage of central granule containing pore complexes even after 10 hrs incubation time in AMD as compared to the controls (Fig. 4). Other pore parameters such as the pore diameter and the pore frequency were likewise unaltered, and no striking differences could be detected in the pore complex subarchitecture of AMD-treated and control oocytes.

An AMD-induced segregation of the nucleolar regions, as has been described for various other cell types (reviewed in Busch and Smetana, 1970), was recognized

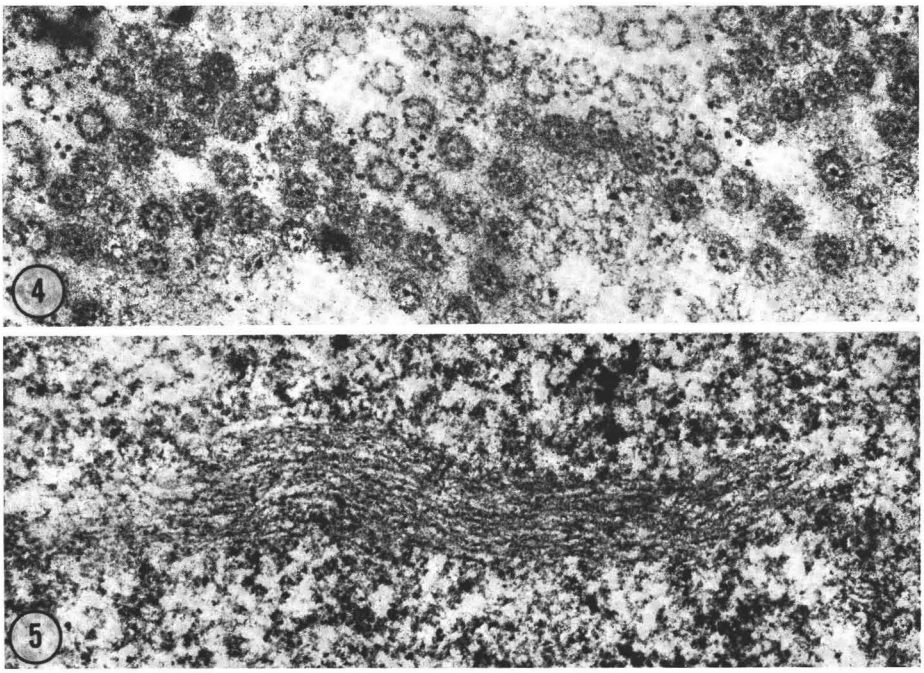


Fig. 4. Tangentially sectioned nuclear envelope of a lampbrush stage *Xenopus laevis* oocyte after the Actinomycin D treatment. No differences in central granule frequencies are recognizable in the Actinomycin-treated and control oocytes. Pores which do not reveal the pore wall are out of equatorial section plane and were not taken into account for the present study. Several ribosomes attached to the outer nuclear membrane lie in between the pores. × 50000

Fig. 5. Fibrillar body in the nucleoplasm of a lampbrush stage *Xenopus laevis* oocyte, incubated for 2 hrs in 20 μg/ml Actinomycin D. × 70000

with these oocytes as well (for *Triturus* see Lane, 1969). Furthermore, bundles of fibrillar material appeared in the nucleoplasm of AMD-treated oocytes, but were never observed in control nuclei (Fig. 5). Such fibrillar aggregates have been earlier described by Lane (1969) as being typical for nuclei of *Triturus viridescens* oocytes incubated in AMD.

In the view of the divergent results of AMD effects on central granule frequency due to the special electron microscopic method employed (thin sections vs. negative staining), one of the authors (U. Sch.) has repeated in the course of the present investigation the negative staining preparations described earlier (Scheer, 1970a, b) and found them reproducible with oocytes from both species, *Triturus alpestris* and *Xenopus laevis*.

Discussion

The nuclear pore complex is thought to be the main route of RNA translocation from the nucleus to cytoplasm (e.g. Anderson and Beams, 1956; Beermann, 1964; Kessel, 1966, 1968b; Stevens and Swift, 1966; Stevens, 1967; Verhey and Moyer, 1967; Allen and Cave, 1968; Kessel and Beams, 1968; Clerot, 1968; Massover, 1968; Franke and Scheer, 1970b; Franke, 1970a, b). In particular, the electron

microscopic appearance of the "central granule" within the pores is considered by the majority of present days' investigators to represent a "snap shot image" of RNP material being in transit in nucleocytoplasmic direction (see Introduction, p. 230). The data from the present thin section study, however, show that the central granule frequency is unaltered even after prolonged treatment with AMD. This makes clear that the occurrence of this structure in the central part of the pore is independent of concomitant RNA synthesis and nucleocytoplasmic transport of RNA molecules. Therefore the interpretative views of central granule dynamics, which are based on earlier results reporting a decrease of central granule frequency in negatively stained isolated nuclear envelopes after a treatment with AMD (Wunderlich, 1969; Scheer, 1970b), have to be reconsidered.

A possible explanation of the diversity of the two methods in such experiments may be derived from their different efficiencies in the demonstration of nucleoprotein structures. The non-membranous constituents of the nuclear pore complex are likely to be composed of more or less coiled fibrillar material, presumably RNP (e.g. Gall, 1956; Mentre, 1969; Abelson and Smith, 1970; Franke, 1970a; Franke and Falk, 1970; Franke and Scheer, 1970a, b; Scheer, 1970a, 1971). The degree of local coiling of such strands can vary, particularly in the case of the central granule and the annular subunits (e.g. Franke, 1970a, Franke and Scheer, 1970a). Thus, it is to be expected that only a very compact fibrillar accumulations in the central part of the pore can prevent the negative staining molecules (e.g. phosphotungstic acid) from complete penetration, and thus give rise to the appearance of a well defined relatively electron-transparent granulum with a diameter larger than 40 Å. What is actually seen in the electron micrographs of negatively stained preparations is a rather wide range from a more compact granule to looser fibrillar aggregates, and the decision as to what is a distinct granule is necessarily somewhat arbitrary. Moreover, the fibrillar aspect of such nucleoprotein coils should be especially emphasized in the high salt conditions of the phosphotungstic acid solution during the final stages of the drying process which favour an unravelling and solubilization of the RNP material. Fibrillar arrangements, however, were not included in the central granule counts of the above mentioned reports. On the other hand, the dehydration steps in the course of thin section preparations precipitate any RNA material present within the pore and thus in every case, independent of the particular state of the material in vivo or after fixation. should lead to the appearance of a condensed mass in this region.

The great differences in central granule frequency of the *Tetrahymena* macronucleus in thin sections, where almost every pore shows this structure, as compared to negatively stained isolated nuclear envelopes, where far less central granules have been reported (Wunderlich, 1969), probably are at least partially due to losses of pore complex material during the isolation procedures. With the amphibian oocytes, though, where the manual isolation of the nuclei is a matter of a few minutes, this effect should be negligible. This then would explain the coincidence of central granule frequency determination in this material after both methods. The two divergent results in central granule frequency after AMD treatment could then be brought into some accordance only by assuming that AMD alters the structural state of the central granule material but does not lead to its disappearance.

It is obvious from the present study that what is visualized as a "central granule" in electron micrographs of the nuclear pores does not disappear when nuclear RNA synthesis and nucleocytoplasmic translocation of RNA has totally ceased. Thus it is either (1) a nonmigrating component, or (2) a complex structure composed of a static moiety fixed to the pore complex and another superimposed dynamic moiety, or (3) migrating material which, however, can be arrested for long times whithin the pores under certain conditions. In fact, the presence of "central granules" within the pore complexes of intranuclear as well as of cytoplasmic annulate lamellae (for review see, e.g., Kessel, 1968a; Scheer and Franke,

1969; Wischnitzer, 1970) and also in the "pores" of Golgi cisternae and endoplasmic reticulum cisternae (e.g. Franke *et al.*, 1971; Franke and Scheer, 1972) and in the fenestrae of the capillary endothelium (Friederici, 1969; Maul, 1971) clearly speaks against the concept that such a central density in a nuclear pore is only the visualization of material in a state of nucleocytoplasmic transition. Thus, the central granule cannot be used as a morphological marker for nucleocytoplasmic transport of RNA through a given pore complex. Moreover, some structural homologies of the arrangement of granular and filamentous components in pore complexes (such as in the nuclear envelope and the annulate lamellae) and in other types of pores (e.g. in the dictyosomal cisternae and the extracellular fenestrae of the endothelium) make it highly questionable that a central granule contains only RNP material (see also Franke and Falk, 1970; Franke *et al.*, 1971).

There are various indications, on the other hand, especially from studies on annulate lamellae, that the pore complexes have a RNA storage function (Verhey and Moyer, 1967; Bal *et al.*, 1968; Babbage and King, 1970; Franke and Scheer, 1971). In this respect, biochemical results of an accumulation of uridine-labeled RNA in high salt treated nuclear membrane fractions from *Tetrahymena* macronuclei during an in vivo AMD chase are interesting (see Eckert and Franke, 1972, for details). Such an accumulation of pulse labeled RNA at the level of the nuclear envelope, presumed to be caused by a local pile up at the pore complexes as a consequence of the general AMD effect on RNA transfer to the cytoplasm, would be fairly compatible with a non-decreased central granule frequency in the presence of the AMD.

The conclusion that central granules do not disappear from nuclear pores after cessation of RNA synthesis is further supported by the observation that nuclei of special differentiated cells which are known to have very low rates of RNA synthesis also frequently show central granules in their pores such as, e.g., in the mature oocyte (Franke and Scheer, 1970a) and the later stages of spermiogenesis in amphibia (about 80% central granule frequency in maturing spermatids of *Triturus alpestris*, Scheer, unpublished; see also Folliot and Picheral, 1971). It is also strengthened by the finding that pore complexes can reveal a central granule even after break-down in prometaphase of mitosis (Bajer and Molè-Bajer, 1969, Fig. 9a) as well as in the mitotic telophase (Ryser, 1970). So with respect to cessation of RNA synthesis, the central granule basically behaves in a similar fashion as the other pore complex components such as the annulus which also do not reveal remarkable structural alterations. In this connection it is worth mentioning the view that such annulus material could, for instance, represent a transitory stage of nucleocytoplasmically migrating RNP as well (c.f. Mepham and Lane, 1969; Franke and Scheer, 1970b).

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