

Nuclear envelope assembly around sperm chromatin in cell-free preparations from *Drosophila* embryos

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Chicken sperm chromatin initiated an assembly of interphase-like nuclei in a cell-free cytoplasmic preparation from 1–6 h old *Drosophila melanogaster* embryos. The formation of these interphase-like nuclei from the condensed sperm chromatin happened in a series of distinct steps. Anti-*Drosophila* lamin monoclonal antibody stained the assembled nuclei in a pattern indistinguishable from normal *Drosophila* nuclei. This assembly process required an ATP regenerating system and could be blocked by the addition of novobiocin into the cell-free extract.

Chromatin; Nuclear envelope; Lamin; Chicken sperm; (*Drosophila melanogaster*)

1. INTRODUCTION

The ability to use cell-free extracts to assemble nuclei from either naked DNA or condensed chromatin, has facilitated the in vitro study of some of the biological activities of the nucleus. Such cell-free systems were established using extracts derived from *Xenopus* eggs [1–3], and mitotic mammalian cells [4]. Cell-free extracts were used to analyze different stages of chromatin assembly [2]; transport between the nucleus and the cytoplasm [3]; interaction between the lamins and the chromatin [4]; possible role of inner-membrane proteins in nuclear assembly [5]; DNA replication and cell cycle regulation [2,6].

Each of the cell-free extracts were prepared from cells whose cytoplasm contained sufficient nuclear precursors for thousands of nuclei that form during the rapid cell divisions of the early embryo [7].

As in *Xenopus* eggs, *Drosophila* embryos contain large pools of precursors to support many nuclear divisions [8]. Previous work in *Drosophila* revealed that protein-free DNA injected into *Drosophila* embryos can induce nuclei formation in vivo in a hierarchical fashion [8]. Extracts of *Drosophila* embryos were shown to mediate the assembly of a chromatin-like structure from histones and DNA under physiological conditions [9]. The chromatin-like product assembled by the *Drosophila* embryo extract was similar to the one produced in soluble in vivo extracts from *Xenopus* eggs [10].

This paper describes a *Drosophila* cell-free cytoplasmic extract derived from 1–6 h old embryos that

reconstitutes nuclear structure around added chicken sperm chromatin. Nuclear assembly from sperm chromatin appeared to involve distinct intermediate steps of chromatin condensation and decondensation and nuclear envelope formation. An ATP regenerating system was essential for nuclear assembly and topoisomerase II activity was found to be essential for this process.

2. MATERIALS AND METHODS

2.1. Materials

Monoclonal anti-*Drosophila* lamin antibody 611A3A6 [11,12] was a kind gift from Dr Bruce Alberts. Affinity-purified Texas Red-conjugated rabbit anti-mouse IgG was purchased from Jackson Laboratories (West Grove, PA). Novobiocin, ATP, phosphocreatine and creatine kinase were purchased from Sigma. Lysolecithin was purchased from ICN Biomedicals.

2.2. Preparation of *Drosophila* cytoplasmic extracts

Cell-free extracts were prepared essentially as described [2]. 1–6 h old *Drosophila melanogaster* (Canton S) embryos were collected on feeding plates, washed with ST solution (0.7% NaCl/0.1% Triton X-100), dechorionated for 90 s in 1.25% sodium hypochlorite and washed again in ST and phosphate-buffered saline (PBS). The dechorionated embryos were rinsed 3 times with 3 vols of a solution containing 250 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, 100 mg/ml cycloheximide, 5 µg/ml cytochalasin B and 1 mM dithiothreitol. The embryos were then packed by a 30 s centrifugation at 500 × g, transferred into an Eppendorf tube and squashed. The homogenized embryos were centrifuged (4°C/5 min/14 000 × g), and the supernatant was transferred into 0.6 ml tubes and recentrifuged under the same conditions. The slightly cloudy yellowish cytoplasm was removed with a syringe and used directly for the reconstitution experiments. Rapid freezing of the extracts in liquid nitrogen resulted in no substantial loss of activity.

2.3. Preparation of demembrated sperm

Demembrated sperm nuclei were prepared essentially as described [6]. Briefly, rooster testes were milked and the sperm were

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homogenized in 2 ml SuNaSp (250 mM sucrose, 75 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine). The sperm was concentrated by centrifugation at $1000 \times g$ for 5 min and resuspended in 0.5 ml SuNaSp. Demembration was performed by the addition of 20 μ l of 1 mg/ml lysolecithin. After 20 min, the reaction was stopped by the addition of 1 ml SuNaSp containing 30% bovine serum albumin at 0°C. The sperm were washed three times in SuNaSp and finally resuspended in SuNaSp containing 30% glycerol. For long-term storage, aliquots were stored at -70°C .

2.4. Nuclear reconstitution

Sperm chromatin (1×10^3 sperm/ μ l extract) was incubated at 22°C in 15–20 μ l of reconstitution extract (~ 2000 embryos) containing an ATP regenerating system which consisted of 2 mM ATP, 20 mM phosphocreatine and 50 μ g/ml creatine kinase. To monitor the progress of nuclear reconstitution, 3- μ l aliquots of the reaction mixture were removed at different time intervals, mixed with 3 μ l of a solution containing 7 mM MgCl_2 , 200 mM sucrose, 80 mM KCl, 15 mM PIPES, 15 mM NaCl (pH 7.2), 10 μ g/ml of the DNA specific fluorescent stain bisbenzimidide, and viewed under fluorescence microscope.

2.5. Immunolabeling

The lamin content during various stages of nuclear reconstitution was determined by indirect immunofluorescence using 611A3A6 anti-*Drosophila* lamin monoclonal antibody. Following 2 h incubation of the sperm in the extract, the nuclei were placed on gelatin-coated glass coverslips, fixed for 10 min at room temperature in PBS containing 3.7% paraformaldehyde and 0.1% Triton X-100, washed in PBS, and incubated for 1 h with a monoclonal anti-lamin antibody. Following washes in PBS, the nuclei were incubated for 1 h with Texas Red conjugated goat-anti-mouse IgG (Fab'), washed as above and stained for 10 min in PBS containing 10 μ g/ml bisbenzimidide. Following washes in PBS and mounting in PBS containing 90% glycerol, the nuclei were viewed under a Leitz microscope equipped with epifluorescence.

3. RESULTS

When demembrated sperm were incubated in a cell-free extract prepared from *Drosophila* embryos, morphological changes occurred comparable to those described in *Xenopus* extracts [1,2,6]. During the first 5 min of incubation in the *Drosophila* reconstitution extract, the sperm chromatin was found in a long thin form characteristic of sperm nuclei (stage I, fig.1A). Within 5–10 min, all nuclei decondensed and a remarkable enlargement of the chromatin was observed (stage II, fig.1B). In the next 30–40 min, condensation of the chromatin was observed in 40–60% of the cases (stage III, fig.1C). During the following 30–60 min, these condensed areas enlarged and their peripheral chromatin decondensed to form interphase-like nuclei (stage IV, fig.1D). When the reconstituted nuclei were viewed with phase optics, a defined sharp border enveloping them was observed.

To determine whether nuclei reconstituted in vitro contained *Drosophila* nuclear envelopes, an immunofluorescence study was performed using 611A3A6 antibody. This antibody is highly specific to the *Drosophila* lamin and does not cross-react with the vertebrate lamins (Gruenbaum, Y., unpublished results). Only decondensed nuclei at stage IV displayed an intense peripheral staining pattern when stained with the antibody (fig.2). DNA at either stage II or III of nuclear assembly

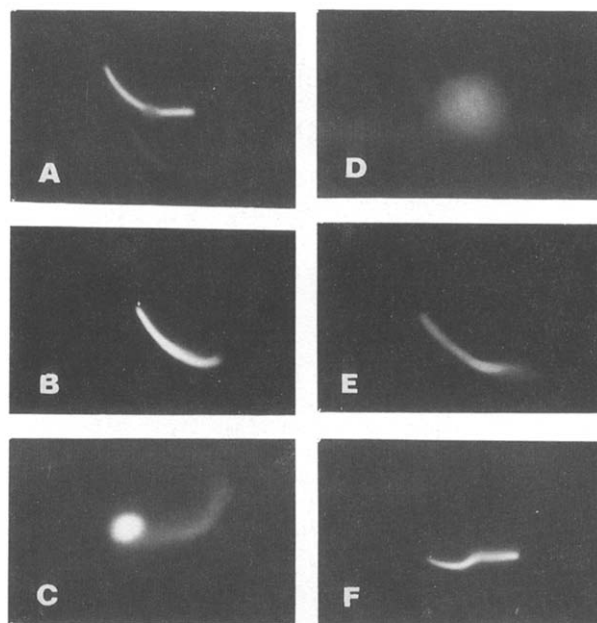


Fig.1. Intermediate steps in nuclear reconstitution. *Drosophila* embryonic extract containing 2 mM ATP, 20 mM creatinephosphate, 50 μ g/ml creatine kinase and 1×10^3 demembrated sperm/ μ l extract was incubated at 22°C . The progress of nuclear assembly is shown following 0 min (A, stage I), 5 min (B, stage II), 20 min (C, stage III), 90 min (D, stage IV). Aliquots from the reaction mixture were removed, stained with the DNA specific fluorescent dye bisbenzimidide, and observed with a fluorescence microscope. Control of *Drosophila* cytoplasmic extract without an ATP regenerating system is shown at 90 min (E), and sperm incubated with an ATP regenerating system but without embryonic extract at 90 min (F). Magnification $\times 1600$.

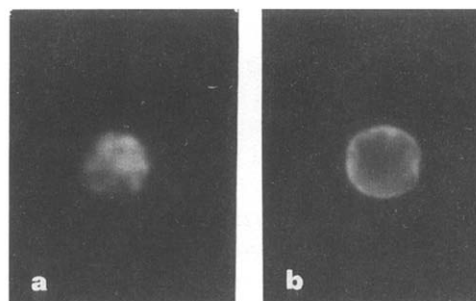


Fig.2. Lamin staining of assembled nuclei. Following a 2-h incubation of the demembrated sperm in the *Drosophila* extract, the nuclei were stained with 611A3A6 anti-*Drosophila* lamin antibody. (a) A selected nucleus stained for DNA with bisbenzimidide. (b) Immunolabeling of the same nucleus. Staining these nuclei with secondary antibody alone did not give a positive signal (data not shown). Magnification $\times 1600$.

did not reveal detectable anti-lamin staining (data not shown).

ATP is probably required for nuclear reconstitution since cell-free extracts without an ATP regenerating system were essentially not reactive; no change was observed in the sperm chromatin morphology and as a result nuclei were not formed (fig.1E). In order to assess whether topoisomerase II activity is required for the nu-

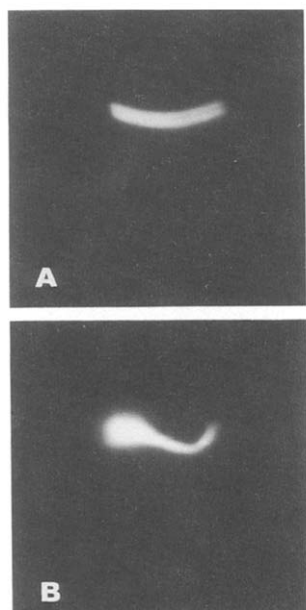


Fig.3. The effect of novobiocin on nuclear assembly. Demembrated sperm were incubated in the assembly extract for 2 h. Novobiocin (500 μ g/ml) was added to the incubated sperm at different time points. (A) 0 min, (B) 30 min. Magnification $\times 1600$.

clear assembly, novobiocin was added to the extract at various stages of assembly [13]. When added at the onset of the reaction, the chromatin failed to decondense and remained in a slightly longer condensed form (fig.3A). Nuclear assembly was completely blocked. Addition of novobiocin at stages II and III prevented the chromatin from decondensing and hence from assembling into a nucleus (fig.3B). Beyond stage III, novobiocin did not prevent nuclear envelope formation, as judged by phase microscopy (data not shown).

4. DISCUSSION

The present study shows that a cytoplasmic preparation of *Drosophila* embryos can induce a series of changes in sperm nuclear morphology which culminates in interphase-like nuclei. These nuclei contained chicken sperm DNA and *Drosophila* nuclear envelopes. The observed hierarchical fashion of nuclear reconstitution was generally similar to that described in *Xenopus* extracts [2,6]. In the *Drosophila* cell-free extracts, the first stage of nuclear reconstitution is characterized by a remarkable enlargement and dispersion of the sperm chromatin, followed by recondensation mostly at the chromatin's ends. In some cases, several nuclei formed from one sperm, apparently containing few chromosomes only. Unlike *Xenopus* cell-free extracts, an ATP regenerating system is required not only to sustain nuclear reformation [2,6], but also for the onset of the nuclear assembly process itself. One possible explanation for the different ATP requirement might be that the endogenous ATP pool in *Drosophila*

extracts is lower than that in *Xenopus* extracts, and thus cannot sustain even the first stage in nuclear reformation. Alternatively, the *Drosophila* embryo may consist of an as yet unidentified ATP-dependent chromatin associated factor(s) which are not necessary in *Xenopus* chromatin decondensation and nuclear reformation. These possibilities are currently under investigation.

Topoisomerase II is known to have an important role in the spatial organization of the DNA within metaphase chromosomes and interphase nuclei [13-16]. The *Drosophila* ATP-dependent topoisomerase appears to be closely related to *Escherichia coli* DNA gyrase in that both use a similar mechanism to change the topology of DNA, require ATP and are inhibited by novobiocin [13]. The presence of an enzyme that allows one DNA helix to pass freely through another could not only be useful in relaxation of topological constraints, but may also be involved in the folding and unfolding of eukaryotic chromosomes. Thus, it was not surprising to find that topoisomerase II activity was required for several stages of nuclear assembly, which is in agreement with the observations in *Xenopus* [2]. The failure of sperm chromatin to decondense in the absence of ATP or by inhibiting topoisomerase II in the cell-free system might be explained by the ATP dependence of the enzyme.

Antibodies to most characterized nuclear envelope proteins in *Drosophila* are available. These antibodies will enable examining the role of different nuclear proteins in the different stages of chromatin organization and nuclear envelope assembly. Two of the genes that encode major proteins of *Drosophila* nuclear envelope, the lamin [17] and the otefin [12, Gruenbaum, Y., unpublished results] have been cloned. Site-directed mutagenesis may allow the future use of modified proteins in this in vitro system and might further advance our understanding of nuclear envelope assembly.

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