Nuclear assembly of purified *Crythecodinium cohnii* chromosomes in cell-free extracts of *Xenopus laevis* eggs

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ABSTRACT

Incubation of dinoflagellate *Crythecodinium cohnii* chromosomes in cytoplasmic extracts of unfertilized *Xenopus laevis* eggs resulted in chromosomes decondensation and recondensation, nuclear envelope assembly, and nuclear reconstitution. Dinoflagellate *Crythecodinium cohnii* is a kind of primitive eukaryote which possesses numerous permanently condensed chromosomes and discontinuous double-layered nuclear membrane throughout the cell cycle. The assembled nuclei, being surrounded by a continuous double membrane containing nuclear pores and the uniformly dispersed chromatin fibers are morphologically distinguishable from that of Dinoflagellate *Crythecodinium cohnii*. However, incubation of dinoflagellate *Crythecodinium cohnii* chromosomes in the extracts from dinoflagellate *Crythecodinium cohnii* cells does not induce nuclear reconstitution.

Key words: *Dinoflagellate Crythecodinium cohnii, chromosome, nuclear reconstitution, cell-free system, Xenopus laevis egg extracts.*

INTRODUCTION

Dinoflagellates are a group of diverse eukaryotic protists, possessing a number of peculiar traits of nuclear organization, suggestive for the transition between the prokary-

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otic and eukaryotic state. Among these are: (a) permanently condensed chromosomes during the whole cell cycle[1], (b) chromatin devoid of histone and nucleosomes[2], (c) very complex cell cycle[3]. These prokaryotic affinities of the dinoflagellates could indicate their closeness to the ancestral state of all present-day eukaryotes, at least regarding nuclear organization[4]. Molecular data suggest that *Crythecodinium cohnii* is one of the most primitive of the dinoflagellate Peridinales[5]. How can dinoflagellate chromosome keep highly condensed when replication, transcription and translation are proceeding during the whole cell cycle? What are the important components, DNA, histone or nonhistone in *C. cohnii*? However, very few studies about nuclear organization of dinoflagellate have been done.

Eggs of *Xenopus laevis* have proved to be exceptionally favorable sources of cell-free systems for studying the assembly of the cell nucleus. *Xenopus laevis* eggs store abundant protein and membrane components required to generate a functional nuclear envelope around exogenously added DNA or chromatin[6],[7]. Egg extracts can be stably prepared in either interphase or metaphase. Interphase extracts can support nuclear assembly and transport, whereas mitotic extracts can disassemble nuclei. Since 1989, in our laboratory, we have successfully got the nuclei assembled in vitro around exogenous naked DNA from animals[8], plants[9], rDNA[10] and demembranated sperm chromatin [11]. Meanwhile, we have done some research concerning the mechanism of nuclear envelope reconstitution[12]. We wonder whether the intact dinoflagellate chromosomes can be reconstituted or not in a cell-free system derived from *Xenopus laevis*. Here we reported the details of *Crythecodinium cohnii* chromosomes being reconstituted in a cell-free system of *Xenopus laevis* eggs, followed by nuclear reassembly. Differences of the nuclei and nuclear envelope between that of dinoflagellate and assembled nuclei were also reported.

MATERIALS AND METHODS

Cell culture

Crythecodinium cohnii was kindly provided by Dr. Joseph T.Y. Wong (Hong Kong University of Science and Technology). Strains were maintained on 1.5 % MLH agar medium according to the method of Tuttle and Loeblech[13]. For intensive cultures, clones were subcultured in MLH liquid medium and put in the dark at 27°C.

Isolation of Dinoflagellate C. cohnii chromosomes

Dinoflagellate *C. cohnii* cells were harvested by centrifugation for 5 min at 3,000 g and suspended in isolation medium (0.75 M sucrose, 2.5 % w/v Ficoll, 5 mM CaCl_2 , 0.5 mM DTT, 0.01 M Tris-HCl, pH7.5, and 0.1 mM PMSF). Cells were disrupted briefly in a W-220 ultrasonicator. Nuclei, the brown pellet, sedimented from the homogenate by centrifugation at 2,000 g for 5 min, and homogenized in isolation medium containing 0.05 % (v/v) Triton X-100 using a Potter-Elvehjem homogenizer. The homogenate was layered over a 2-step discontinuous gradient consisting of the following: 2.4 M sucrose, 5 mM CaCl_2 and 0.01 M Tris-HCl (pH 7.5), overlaid with 2.2 M sucrose, 5 mM CaCl_2 and 0.01 M Tris-HCl (pH 7.5). The homogenate were centrifuged at 48,000 g for 1 h. The pellet was suspended with isolation buffer containing lysolecithin at 330 μg /

ml for 10 min, and centrifuged at 3,000 g for 10 min. The pellet was chromosome extracts. The extracts were washed with ice-cold isolation buffer containing 3 % bovine serum albumin (BSA), and then with isolation buffer containing 0.4 % BSA for three times, finally resuspended in isolation buffer with 30 % glycerol and stored at -70°C.

Preparation of Xenopus egg extracts

Healthy *Xenopus laevis* females were injected with 600 units of human chorionic gonado-tropin (Sigma) the night before use and were placed in 5 liters of 100 mM NaCl. Eggs were dejellied with 2 % cysteine (pH 7.8), rinsed twice in MMR (0.1 M NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 5 mM Hepes, 0.1 mM EDTA), and activated by incubation in a solution containing MMR, 5 % Ficoll, and 0.2 mg/ml of the Ca²⁺ ionophore A23187. The eggs were rinsed three times with 1/4 (MMR plus 5 % Ficoll) and were allowed to incubate another 20 min. Following this the eggs were rinsed three times with three volumes 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. Then the eggs were crushed at 10,000 g, 4°C for 15 min. The material between the top lipid layer and yolk pellet was removed and centrifuged again at 10,000 g, 4°C for 15 min. The supernatant was used as egg extracts.

Preparation of dinoflagellate C. cohnii cell extracts

Dinoflagellate *C. cohnii* cells were harvested by centrifugation for 5 min at 3,000 g and suspended in 1/2 volume 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 cells were crushed in a W-220 ultrasonicator. The homogenate was centrifuged at 10,000 g, 4°C for 15 min. The supernatant was centrifuged at 100,000 g, 4°C for 1 h. The supernatant was used as dinoflagellate *C. cohnii* cell extracts.

Nuclear assembly in vitro

8 µl dinoflagellate *C. cohnii* chromosomes extracts were mixed with 100 µl freshly prepared *X. laevis* egg extracts or 100 µl dinoflagellate *C. cohnii* cell extracts respectively. Then the ATP regenerating system (2 mM ATP, 50 µg/ml creatine kinase, 2 mM phosphocreatine) was added. The mixture was incubated at 22°C.

Fluorescent microscopy

After incubation, the samples were fixed in 2.5 % glutaraldehyde, stained with DAPI (DNA dye), and examined by fluorescent microscopy.

Electron microscopy

Aliquots of the incubation mixture were fixed in ice-cold 2 % glutaraldehyde and osmium tetroxide successively, dehydrated through a graded concentration series of acetone and then embedded in Epon 812. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and finally examined under JEM-100CX transmission electron microscope.

RESULTS

Successful isolation of the dinoflagellate *C. cohnii* chromosomes has been scarcely reported because dinoflagellate *C. cohnii* possesses a thick theca and numerous “starch” granules which make it very difficult to isolate its chromosomes. Thus, we first isolate the nuclei as described by Rizzo[14]. Then, lysolecithin was used to remove nuclear enve-

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loped effectively and to get the chromosome extracts.

*The unique structure of the chromosomes of dinoflagellate *Cryptercodinium cohnii**

Dinoflagellate *C. cohnii* possesses 99-100 rod-like acentric chromosomes per cell, which keep condensed during the whole cell cycle (Fig 1). When they were incubated with *Xenopus laevis* egg extracts, some interesting changes were happened: chromosomes decondensation, recondensation, followed by nuclear reconstitution in steps. The highly condensed dinoflagellate *C. cohnii* chromosomes could be stained with DAPI (Fig 2A). At the ultrastructural level, there were numerous arched whirls of chromosomal fibrils in a single *C. cohnii* chromosome (Fig 3A), which is very different from those of higher eukaryotic metaphase chromosomes, but similar to those of bacterial nucleoids. The diameter of dinoflagellate chromosomes is about 30-50 nm.

Fig 1.

The ultrastructure of dinoflagellate *C. cohnii*.

(A) Dinoflagellate *C. cohnii* cells were fixed, embedded, and sectioned for electron microscopic observation. N: nucleus. Bar=5 μ m

(B) Inset: the nucleus of dinoflagellate *C. cohnii* was stained with DAPI (DNA dye). Note the dot-like permanently condensed chromosomes. Bar=2 μ m

*Nuclear assembly of *C. cohnii* chromosomes in cell-free system from *Xenopus laevis* eggs*

Stage I. This stage covered in the first 20 min of incubation. The chromosomes began to elongate and swell, showing discernible decondensation but still keeping rod-like in shape (Fig 2B, 3B).

Fig 2.

Stages of Nuclear reconstitution of *C. cohnii* chromosomes in *X. Laevis* egg extracts. A mixture containing 100 μ l of *Xenopus* egg extracts, 2 m M ATP, 20 m M creatine phosphate, 50 μ g/ml creatine kinase, and 8 μ l of Dinoflagellate *C. cohnii* DNA was incubated at 22 °C. At the end of incubation (A) 0 min, (B) 20 min, (C) 60 min, (D) 90 min, (E) 150 min, and (F) 180 min, aliquots of the mixture were removed, stained with the DNA-specific fluorescent dye DAPI, and observed with a fluorescence microscope at a magnification of 1,000 $\times$. During the 180 min incubation, the initial Dinoflagellate *C. cohnii* chromosomes (A) became elongated and swelled (B), then decondensed (C), recondensed (D), reorganized as a packed sphere (E), and (F) assembled into a nucleus. Bar=1 μ m

Stage II. This stage covered the period between 20-60 min of incubation. The chromosomes decondensed continuously so that they no longer maintained their rod-like shape, but displayed some chromatin-like fibrils (Fig 2C, 3C). Meanwhile, there were many membrane vesicles appeared around the chromatin-like structures (Fig 3C, arrow).

Stage III. This stage covered the period between 60-90 min of incubation. The chromatin-like structures became recondensed, and many of them reorganized into tightly

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Fig 3.

Electron micrograph of Dinoflagellate *C. cohnii* chromosomes at different stages of nuclear reconstitution. Nuclei were reconstituted around Dinoflagellate *C. cohnii* chromosome for 180 min, as described in Material and Methods, and then fixed, embedded, and sectioned for electron microscopic observation. (A) At the beginning of incubation, Dinoflagellate *C. cohnii* chromosomes keep highly condensed. One chromosome was showed. Note the peculiar arrangement of chromosomal fibers. (B) During the first 20 min of incubation, chromosomes were elongated and swelled. (C) At 60 min, rod-like elongated chromosomes were decondensed to assume chromatin-like structures. There were many membrane vesicles (arrow indicated) around it. (D) Following the recondensation of chromatin-like structures, the vesicles fused into double-layered membrane fragments which can be seen in the upper part of the figure. (E) The chromatin-like structure was reorganized into packed spheres.

(F) At 180 min, the packed spheres were encapsulated within a well-defined membrane.

(A) and (B): Bar=0.2mm (C), (D), (E) and (F): Bar=2 μ m

packed spheres (Fig 2D). This was followed by the gradual fusion of the membrane vesicles into double-layered membrane segments around the chromatin-like structure and packed spheres (Fig 3D).

Stage IV. This stage covered the period between 90-150 min of incubation. The chromatin-like structures condensed continuously until they were contained within a membrane structure (not fully complete) which was larger in volume than the initial highly packed sphere (Fig 2E, 3E compared with Fig 2D, 3D).

Stage V. This stage covered the period between 150-180 min, of incubation The packed spheres acquired a continuous nuclear envelope with pores and became assembled nuclei (Fig 2F, 3F). The chromatin in assembled nuclei was dispersed uniformly at this stage.

Fig 4.

The difference of the nuclear envelope between Dinoflagellate *C. cohnii* and that of assembled nuclei from *C. cohnii* chromosomes in cell-free extracts of *Xenopus* eggs.

(A) The discontinuous nuclear envelope of dinoflagellate *C. cohnii*. NE: nuclear envelope. CH: chromosome. Bar=10 μ m

(B) The continuous nuclear envelope with nuclear pores (arrow indicated) of assembled nuclei. Bar=10mm

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The newly assembled nuclei were morphologically different from those of in dinoflagellate cells (compare Fig 1B with Fig 2F). Electron micrograph showed that the nuclear envelope of dinoflagellate *C. cohnii* is discontinuous (Fig 4A). However, the reconstituted nuclei possess a well-defined membrane with nuclear pores (Fig 4B) which is morphologically indistinguishable from that of normal higher eukaryotes interphase nuclei. Contrary to the highly condensed chromosomes being attached to the dinoflagellate *C. cohnii* nuclear envelope, the chromatin in the newly assembled nuclei disperses uniformly, similar to that of typical higher eucaryotic interphase nuclei, in which chromatin is not easily observable directly by TEM (transmission electron microscope), and is much thinner than that of dinoflagellate.

There is no nuclear assembly in the cell-free system from dinoflagellate

In contrast, nuclear reconstitution did not occur when dinoflagellate *C. cohnii* chromosomes were incubated with cell-free system from *C. cohnii* cells. The chromosomes (Fig 5A) were decondensed (Fig 5B) and could not be recondensed to become packed spheres and intact nuclei.

Fig 5.

Dinoflagellate *C. cohnii* chromosomes can not induce nuclear assembly in cell-free extracts from dinoflagellate cells. Being incubated with cell-free extracts from dinoflagellate *C. cohnii*, the rod-like or dot-like dinoflagellate chromosomes (A) can be decondensed (B), without being recondensed into nucleus-like structure. Samples were stained with the DNA-specific fluorescent dye DAPI, and observed with a fluorescence microscope at a magnification of 1000 $\times$.

DISCUSSION

Since Lokha and Maisui first demonstrated that demembrated sperm chromatin can be induced to perform nuclear reassembly in a cell-free system from amphibian eggs components in 1983[6], a great deal of works followed later suggested that nuclear assembly *in vitro* can be successfully induced around exogenous DNA or chromatin derived from animals[11], plants[9] and bacteria[7]. Thus, nuclear assembly is independent of the

species from which DNA was purified. Nuclear assembly is also independent of the length of DNA[9], or any specific DNA sequence like genomic DNA or rDNA[7],[10]. Here we showed that exogenously added purified intact chromosomes can be induced to carry out nuclear reconstitution successfully. Our results reinforced the fact that *Xenopus* egg extracts is a good cell-free system which is extremely stable at room temperature and can be indefinitely stored at -70 °C for studying the assembly of cell nucleus.

The decondensation, recondensation, encapsulation and dispersion of dinoflagellate chromosomes in *Xenopus* egg extracts suggested that the dinoflagellate chromosome must have been reassembled. It is well known that nucleosome is the basic repeating subunit of typical eukaryotes, which contains a variable length of DNA ($\approx$ 165-220 bp) and all of the histones. A histone octamer (H2A, H2B, H3, H4) is wrapped by 146bp of DNA in 1.75 super-helical turns in the nucleosome core. In evolutionary terms, histones are among the most highly conserved proteins known. It is surprising, however, that these highly conserved proteins are totally lacking in dinoflagellates and the absence of nucleosomes in dinoflagellate chromatin is due to the absence of histones. This group of unicellular algae must have evolved a different means of packing the nuclear DNA into chromosomes. On the other hand, more than two decades ago, a single histone-like protein has already been reported in dinoflagellate chromatin using one-dimensional gel electrophoresis by Rizzo's group[15]. Later, an electrophoretic study on *C. cohnii* using two-dimensional gels showed that a single band in urea gels and SDS gels called HCc[16] was actually composed of three similar proteins. These three proteins possess similar biochemical traits just like H4 in higher eukaryotes[17]. The *Xenopus* egg extract is a specialized system which possesses two histone variants: histone H2A. X, and histone B4 which correspond to H2A and H1 histones found in eukaryotic somatic cells. This system is also enriched in the nonhistone protein HMG2[18]. It has been demonstrated that in *Xenopus* egg extracts naked DNA actually acts as a template for nucleosome assembly[19], followed by higher-order chromatin assembly[20]. Now, in the reconstitution of dinoflagellate chromosomes in *Xenopus* egg extracts, the detailed molecular changes involved in the different stages of nuclear assembly, especially the changes of dinoflagellate chromosome DNA and their associated proteins and their interaction with histones and other components in the egg extract, are all not-known. These will be the subjects of our future studies.

Self-assembly is the basic characteristic of many macromolecular structures in cells. Nuclear reconstitution is also present in the other kinds of cell-free extracts including CHO cells[21] and *Drosophila melanogaster* embryo[22]. The *Xenopus* egg is arrested in metaphase of the second meiotic division, which means that stockpiled components (including lamins, pore proteins, and nuclear membrane vesicles) are stored in their disassembled mitotic form until the egg is fertilized or activated. Therefore, the egg extract system contains full components or factors necessary for chromatin decondensation and recondensation to take place in nuclear assembly. In contrast, the dinoflagellate cells extract does not support nuclear reconstitution. We think that this is most probably due

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to the lack of components, e.g. histones and other necessary factors, in cell-free extracts from dinoflagellate cells. The nuclear envelope of dinoflagellate can be maintained intact during the whole cell cycle[3], and chromosomes can be kept condensed permanently[1]. So there is no necessity to keep a biochemical machine of nuclear assembly and disassembly in dinoflagellate.

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