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Assembly of Envelope Structure with Vesicles Associated with Ku-Homologous Protein in Xenopus Egg Extract in the Absence of Chromatin

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ABSTRACT. **To study the process of nuclear envelope assembly at the end of mitosis, we developed a chromatin-free in vitro system for assembly of envelope structures in *Xenopus* interphase egg extract, and examined the participation of Ku-homologous protein in the assembly. The envelope structure assembled spontaneously in the absence of chromatin or DNA between glass plates under a condition that minimized generation of flow of the extract. Morphological study using an electron microscopy has revealed that the membrane surrounding the envelope structure is a double membrane that contains gaps resembling nuclear pore complex. Their assembly was dependent on ATP and was inhibited by the addition of GTP-γ-S or N-ethylmaleimide. Depletion of a prenuclear vesicle by preincubating the interphase egg extracts with large excess of sperm head chromatins impaired the assembly. The membrane vesicle, which was associated with Ku-homologous protein of *Xenopus*, participated in the assembly as proven by reaction with monoclonal antibody made specific for Ku p70 protein. However, the assembly process of the envelope structure was inhibited only slightly by the antibody, suggesting that the Ku-homologous protein does not participate in the fusion process of vesicles to form the envelope structure.**

At the beginning of mitosis, the nuclear envelope breaks down and its components are disassembled. Upon reconstitution of nuclei at the end of mitosis, compact chromosomes undergo decondensation along with targeting prenuclear membrane vesicles onto their surface to reform the nuclear membrane. Studies using cell-free system from CHO (1, 2) and *Drosophila* (3) describe the direct role of lamins in this process of membrane vesicle binding to decondensing chromosomes. However, in *Xenopus* egg extract system it has been found that the lamin protein cannot by itself mediate the binding and fusion of membrane vesicles, but rather is essential for extending the nuclear membrane and organizing a nuclear structure capable of DNA replication (4, 5).

We have studied the Ku-homologous protein of *Xenopus* which has close homology with the human Ku protein known as an autoantigen in a patient with systemic lupus erythematosus (6, 7). We found that pronuclei reconstituted *in vitro* from sperm chromatins under inhibition by Ku-homologous protein specific antibody possessed an anomalous nuclear membrane but lacked the lamin layer. Furthermore, the pronuclei did not sustain DNA replication (7). These results indicated that the Ku-homologous protein of *Xenopus* has a role either directly in a second step of nuclear membrane growth or indirectly in the reconstitution of an unknown nuclear structure that may be involved in lamin assembly beneath the nuclear envelope. To investigate both these possibilities for the role of Ku-homologous protein we searched for a simple system of nuclear envelope assembly *in vitro*. After many trials, we found that nuclear envelope-like structures were formed in large number on a glass slide with coverslip in unfractionated *Xenopus* interphase egg extract without adding the chromatin or DNA. In this study we examined the process of the assembly of the envelope structure and showed that it shared several characteristics of the nuclear envelope. Using this system we examined whether the Ku-homologous protein of *Xenopus* participated in the assembly of the envelope structure.

MATERIALS AND METHODS

Reagent and buffers. GTP-γ-S was purchased from Boehringer; N-ethylmaleimide and apyrase (type VIII) were from Sigma; dithiothreitol was from Seikagaku Kogyo LTD. Buffer A was 250 mM sucrose, 15 mM PIPES, pH 7.4.

Preparations of sperm chromatin and interphase egg extract. The demembranated *Xenopus* sperm head chromatins and the extracts from interphase *Xenopus* eggs were prepared as described previously (7).

Envelope assembly on glass slide. 3 μl of the extract from eggs of *Xenopus* were diluted twofold with buffer A and put on a microscope glass slide. The extract was then covered with a coverslip (18 mm × 18 mm), sealed with silicon grease, and

incubated at 23°C for the time indicated in the figure captions. In an alternate experiment, the coverglass was not sealed with grease, but the preparation was placed in a moist chamber to avoid drying of the sample during incubation.

Monoclonal antibodies. Hybridoma cell lines that secreted monoclonal antibodies against nuclear antigens of human origin were prepared by the methods described previously (6). The monoclonal antibodies in ascitic fluid were purified according to the method described by Cambell (8). Antibodies mAbH6 and mAbD2 used in this study are IgM type.

Immunochemical staining and microscopy. The envelope structures were assembled on a glass slide coated with poly-L-lysine (Sigma) for 90 min incubation at 23°C. The slide was washed briefly with buffer A and then the envelope structures were fixed by immersing them to methanol-acetate (3 : 1 in volume) at 4°C for 10 min. The fixed preparation was treated with monoclonal antibody mAbH6 followed by FITC-conjugated secondary antibody, and examined by Olympus fluorescence microscope or an Olympus high-speed beam scanning laser confocal microscope as previously described (6).

Electron microscopy. 30 μl of egg extract were incubated for 90 min between the glasses, and then 1 ml of 0.05 M sodium phosphate (pH 7.4) containing 2% glutaraldehyde was added. After incubation for 3 h at 4°C, the sample was centrifuged at 1,500×g for 5 min. The resultant pellet was rinsed three time with 50 mM sodium phosphate buffer (pH 7.4), stained for 2 h at 4°C with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) then rinsed three times with 0.1 M barbitol-acetic acid buffer (pH 7.4). The pellet was then dehydrated in a graded series of ethanol and propylene oxide and finally embedded in Epon. Sections were counterstained with 1% aqueous uranyl acetate followed by 0.3% lead citrate and viewed using JEM-100S transmission electron microscope.

RESULTS

Envelope structures are assembled in egg extract enclosed between glass plates in the absence of chromatin. In order to establish the chromatin-independent nuclear envelope assembly system, we examined several incubation procedures. When the crude extract of *Xenopus* interphase eggs was enclosed between glass slide and coverslip and seaed with silicon grease, large envelope structures were assembled in a time dependent process as shown in Fig. 1A to D. Their morphology closely resembled nuclei formed from demembranated sperm chromatins in interphase egg extract. However, the average diameter of envelope structures was about half the diameter of the nucleus. Envelope structures also assembled on the glass slide in a moist chamber when it was covered with coverslip but without sealing with grease. However the average size was smaller than those shown in Fig. 1. On the other hand, such envelope structures did not assemble when the extract was placed in an Eppendorf microcentrifuge tube; instead, very small (<2 μm in diameter) envelopes were occasionally observed. The addition of 0.4 mM $CaCl_2$ or 1 to 10 mM $MgCl_2$ to the extract in Eppendorf tubes did not induce envelope assembly, indicating that the divalent cations present in the glass system were not relevant to the process.

Assembly of envelope structure is dependent on ATP, and is inhibited by GTP-γ-S and N-ethylmaleimide. Fusion of vesicles in the nuclear envelope assembly is dependent on GTP and ATP (9, 10) and is inhibited by N-ethylmaleimide (10, 11). Thus, we first examined the requirement of ATP in the assembly of the envelope structure in our system. As shown in Fig. 2B, the ATP-depleted interphase egg extract by treatment with ATPase, apyrase, did not sustain envelope structure assembly. Next, to determine whether GTP was required for the envelope assembly, the effect of GTP-γ-S, a hydrolysis-resistant GTP analogue was examined. As shown in Fig. 2C, the envelope assembly was inhibited in the interphase extract containing 1 mM GTP-γ-S, whereas the control incubation containing 1 mM GTP was not (Fig. 2D). In the GTP-γ-S inhibited culture, the envelope smaller than 2 μm was occasionally observed (indicated by arrowhead in Fig. 2C), but the envelope did not grow anymore after prolonged incubation up to

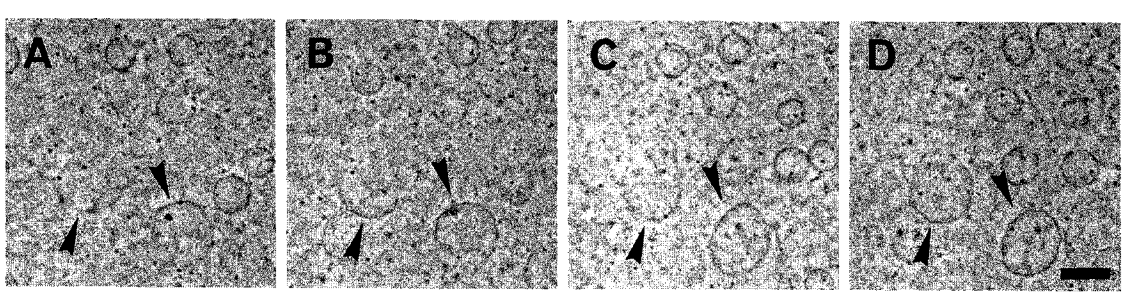

Fig. 1. Time course of spontaneous assembly of the nuclear envelope-like structure in Xenopus egg extract.
The interphase egg extract was diluted with an equal volume of buffer A, and then incubated after being confined between slide and coverslip at 23°C. Photographs of the same field were taken under phase contrast at appropriate time intervals in order from A to D. Arrows show the growing envelope structures. Bar, 10 μm.

3 h. These results suggest that envelope assembly is dependent on GTP and ATP, which is in good agreement with the case for the fusion process of nuclear envelope assembly on chromatin. Dependency on GTP also suggests that a GTP-binding protein such as those that participated in vesicle fusion process (12) are at work in the envelope assembly. Next, we examined the effect of AlF^{-4}, which affects the action of G proteins by blocking the G_α subunit in its active configuration (13), on the envelope assembly. Addition of 5 mM KF and 1 mM $AlCl_3$ inhibited the envelope assembly as well as the nuclear envelope assembly on the sperm chromatin (data not shown).

The addition of 5 mM N-ethylmaleimide inhibited the envelope assembly (Fig. 2E) while this effect of N-ethylmaleimide was negated by the addition of 10 mM DTT (Fig. 2F). The result demonstrated that a protein inactivated by N-ethylmaleimide was involved in the vesicle formation.

Assembly of envelope structure is inhibited by the addition of large excess of sperm chromatins. Heterogeneous population of membrane vesicles has been found in *Xenopus* egg extract (11). However, only a small portion of the membrane vesicles has nuclear envelope-forming activity (14). The membrane vesicle obligatory for nuclear vesicle formation can be titered out by pre-incubating with a large number of sperm chromatins. Thus, we could determine whether the envelope structure assembled in the absence of chromatin are fusion-products of a subpopulation of membrane vesicles with the nuclear envelope-forming activity. To examine this, the interphase extract was incubated for 30 min at 22°C with sperm chromatins (9,000/μl extract) in an Eppendorf tube, before pipetting it between the glasses. The sperm chromatins were supersaturated with the nuclear envelope assembly as evidenced by the fact that most of the chromatin remained in a partially swollen, snake-like configuration over the 1 h incubation period. As shown in Fig. 3, the ability to assemble envelope structures was not impaired by pre-incubation alone for 30 min at 23°C (A) whereas pre-incubation with an excess amount of the chromatins completely inhibited the as-

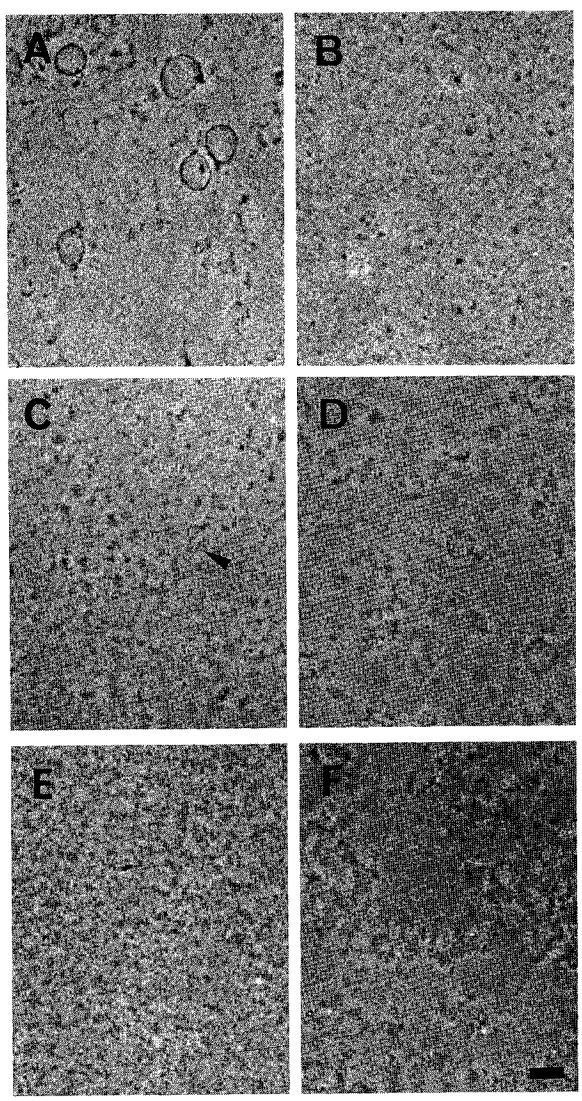

Fig. 2. Assembly of envelope structure dependent on ATP and inhibited by GTP-γ-S and N-ethylmaleimide.
The interphase egg extracts were incubated as for Fig. 1 under the following conditions: the extract was incubated between glass plates (control) (A); ATP-depleted by preincubating with apyrase (10 units /ml) for 20 min at 30°C (B); 1 mM GTP-γ-S (C) or GTP (D) was added to the extract before incubation; 5 mM N-ethylmaleimide (E) or 5 mM N-ethylmaleimide plus 10 mM dithiothreitol (F) were added to the extract before incubation. Photographs were taken 90 min after starting the incubation. Arrow in C shows small vesicle. Bar, 10 μm.

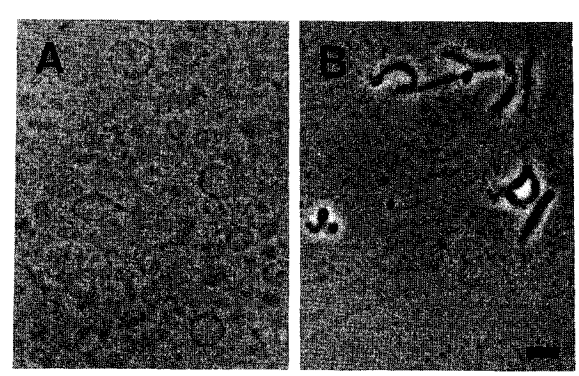

Fig. 3. Effect of addition of large excess of demembranated sperm chromatin to the egg extracts.
Interphase egg extracts were incubated for 30 min in the absence (A) or presence (B) of 9,000 demembranated sperm chromatins per microliter extract followed by incubation for 90 min as Fig. 1. The sperm chromatins are seen as dark, rod-like images in (B). Bar, 10 μm.

sembly of envelope structures (B).

Envelope structure is constituted from double-membrane morphologically resembling typical nuclear envelope. To determine if the envelope structure is constituted from a nuclear double-membrane or a single membrane such as the endoplasmic reticulum, the ultrastructure of the envelope was examined. As shown in Fig. 4, the envelope consisted of a double-membrane with several structures resembling nuclear pore complex (Fig. 4B). Occasionally, another type of vacuoles was observed under the electron microscope. This type of vacuoles was centered around an electron dense body of unknown origin and was constituted from onion skin-like membrane lamella, in which no nuclear pore complex was seen. This membrane lamella is thought to be a dark vacuole which was occasionally noticed by phase contrast microscopic observation during the experiment. At present the origin of this structure is unknown.

Vesicles associated with Ku-homologous protein assemble to form envelope structure. We showed previously that the Ku 70 kDa protein was distributed to the nucleus of HeLa cells (6), and the *Xenopus* protein homologous to the human Ku p70 protein localized to the membrane fraction of interphase *Xenopus* egg extract and accumulated to nuclei reconstituted from sperm

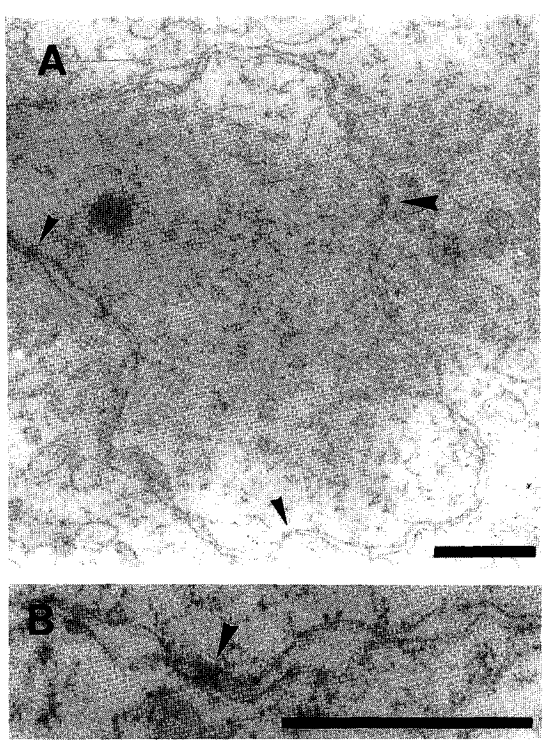

Fig. 4. Electron micrograph of the envelope structure.
The sample was prepared as described in Materials and Methods. Photograph (A) shows a single structure. Arrows show the positions of a structure morphologically resembling nuclear pore complex in membrane. (B) High magnification photograph of one of the structures (arrow). Larger arrow in (A) shows the location of the structure enlarged in (B). Bar, 1 μm.

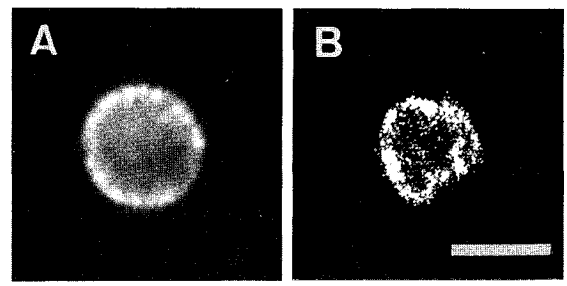

Fig. 5. Immunofluorescence staining of envelope structure with mAbH6 antibody.
The envelope structures assembled on glass were fixed and stained as described in Materials and Methods using mAbH6 antibody. Two different envelope structures stained with mAbH6 and FITC-conjugated second antibody were photographed by fluorescence microscope (A) or laser confocal microscope (B). Bar, 10 μm.

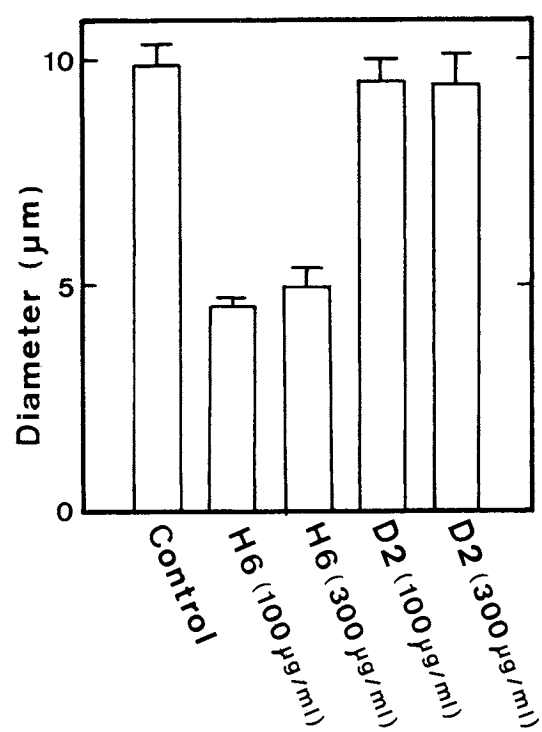

Fig. 6. Effect of mAbH6 antibody on diameter of envelope structure.
The envelope structures were assembled in the absence (Control) or presence of different concentration of monoclonal antibodies as indicated for 90 min at 23°C. The diameter was measured blind in the micrographs taken under phase contrast microscopy. To ascertain the specificity of mAbH6, monoclonal antibody mAbD2 was used as a control. The mAbD2 stained immunochemically the nuclei of HeLa cells as fine dots, but did not react with the *Xenopus* source.

head chromatin in the cell-free system for nuclear reconstruction (7). To determine if the membrane fraction associated with the Ku-homologous protein was involved in the assembly of the envelope structure as well as the nuclear envelope, we examined the envelope structure for existence of Ku-homologous protein by using the mAbH6 monoclonal antibody. The mAbH6 antibody was made against the human Ku protein and decorated the nuclei of HeLa cells (6). As shown in Fig. 5A, the envelope structure was peripherally immunostained with the mAbH6. The peripheral localization of Ku-homologous protein was also determined using laser scanning confocal microscopy (Fig. 5B). This indicates the involvement of the membrane vesicle associated with the Ku-homologous protein in envelope assembly. However, the assembly itself was not inhibited by the addition of mAbH6, although the diameter of the envelope structure assembled in the presence of mAbH6 was reduced by half relative to the control containing irrelevant antibody as measured after 90 min incubation (Fig. 6).

DISCUSSION

In order to study the nuclear envelope assembly we developed an *in vitro* system for assembly of envelope structure using *Xenopus* egg extract. We have found that the envelope structure assembled in the absence of chromatin shares some characteristics with the nuclear envelope of nuclei reconstituted from sperm chromatins in *Xenopus* interphase egg extract. Our results indicate that the enclosure of interphase egg extract in a narrow space between two glasses is necessary for effective assembly of a large envelope structure. This setup might serve to minimize generation of extract flow during incubation which was found to destroy the fragile growing structure.

Xenopus egg extract contains a large variety of vesicles destined to form membrane other than nuclear envelope such as the endoplasmic reticulum membrane, Golgi membrane, and plasma membrane (15, 16). Although efforts have been made to separate membrane vesicle for nuclear envelope assembly (11, 14), it remains to be elucidated what properties characterize the prenuclear envelope vesicle. Several lines of evidence suggest that at least a part of the envelope structure observed in this study was constituted from membrane identical to the nuclear membrane. First, the membrane of the envelope structure was constituted from a double membrane with a structure resembling nuclear pore complex. Second, the addition of large excess of demembranated sperm chromatin inhibited the assembly of the envelope in interphase egg extract. Third, the Ku-homologous protein, which localized to the nucleus of *Xenopus* liver (7), was distributed on the membrane of the envelope structure. Further, the experimental results thus far obtained by others suggest the possibility of nuclear envelope assembly in the absence of chromatin. In the egg extract, a large amount of materials for nuclear envelope assembly is stored to enable the egg to divide rapidly following fertilization. Dabauvalle et al. (17) have shown that these materials spontaneously assemble *in vitro* to form pore complex containing membranes (annulate lamellae) in the absence of chromatin. The annulate lamellae have been observed in several cell types with no association to chromatin, DNA or lamina structure (reviewed by Kessel) (18). Thus, we infer these observations to imply that spontaneous assembly of the nuclear envelope-like structure might be possible in the egg extract if an appropriate physical structure is provided in place of the chromatin.

On the other hand, it has been known that the outer nuclear membrane is frequently contiguous (19) and shares many biochemical properties with endoplasmic reticulum (20, 21), and that the annulate lamellae may be the precursor to the endoplasmic reticulum in *Xenopus* oocyte (22) and other organisms (reviewed by Kessel) (18). Moreover, Newport and Dunphy (10) observed the formation of endoplasmic reticulum with isolated membrane vesicles from *Xenopus* mitotic egg extract. Thus, we also realize there remains the possibility that the envelope structure may be assembled from both the precursor vesicles of nuclear and endoplasmic reticulum membranes.

The specific antibody mAbH6 proved that the Ku-homologous protein localized to the membrane region of the envelope structure. In our previous study (7), we showed that the antibody did not inhibit either the binding or fusion of vesicles of nuclear envelope on the sperm chromatin, but did disturb the completion of nuclear envelope formation. The low level of inhibition of the antibody on the assembly of the envelope structure shown in this study further suggests no participation of the Ku-homologous protein in the initial fusion process of vesicles in the assembly of nuclear envelope. Thus, it has been suggested that the Ku-homologous protein reconstitutes some unknown structure on the chromatin for either lamin assembly or further growth of the nuclear envelope.

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