The structure and interactions of components of nuclear envelopes from Xenopus oocyte germinal vesicles observed by heavy metal shadowing

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Summary
We have examined the structure of the nuclear envelope of oocytes of Xenopus laevis by electron microscopy of metal-shadowed specimens. Material was prepared by either freeze-drying or a rapid protocol using air-drying after dehydration in ethanol followed by amyl acetate. These methods emphasized different aspects of the structure and enabled an integrated view of the arrangement of nuclear pore complexes, nuclear lamina and pore-connecting fibrils to be assembled. In specimens prepared by either air-drying or freeze-drying, the lamina meshwork beneath the nuclear face of the envelope was well preserved, but the fine structure of the nuclear pores was superior in freeze-dried preparations. Both methods also showed pore-connecting fibrils that were clearly not components of the lamina. By using stereo pairs, we established criteria for recognizing the cytoplasmic and nucleoplasmic faces of shadowed nuclear envelopes. These views also enabled us to identify the levels at which different fibrous components were attached to the pores. In particular, we were able to visualize the nuclear lamina fibres and pore-connecting fibrils simultaneously and show that they attach to the pore complexes at different levels. We supplemented this work by using a range of treatments to disrupt the nuclear envelope slightly and gained several insights into this structure as a result. Sometimes pore complexes and their connecting fibrils were stripped from the envelope. This enabled a clearer view of these connections to be obtained without the lamina present. Moreover, in some conditions, the nuclear pore complexes and fibrous lamina began to disintegrate, thereby showing some of the morphological components from which they were assembled.

Key words: nuclear pores, nuclear lamina, pore-connecting fibrils.

Introduction
The nuclear envelope of eukaryotic cells separates DNA from the cytoplasm and controls transport into and out of the nucleus. The envelope is constructed from a double membrane that is perforated by a large number of nuclear pore complexes, which are thought to be responsible for selective transport (see Gerace, 1986, or Newport & Forbes, 1987, for reviews). The nuclear pore complexes project above the cytoplasmic face of the nuclear membrane (Kirschner et al. 1977) and are roughly cylindrical structures composed of eight globular units linked within a circumferential ring (see Kartenbeck et al. 1971; Franke, 1974; Maul, 1977; Unwin & Milligan, 1982, and references therein). A fibrous lamina lies under the nucleoplasmic face of the envelope, below the level of the pores (reviewed by Gerace, 1986). The lamina is composed of proteins called lamins, that appear to resemble closely intermediate filaments in sequence and structure (McKeon et al. 1986; Aebi et al. 1986; Fisher et al. 1986; Parry et al. 1986; Krohn et al. 1987; Wolin et al. 1987). The lamina is composed of beaded fibres about 10 nm in diameter that sometimes form a remarkably regular basketweave pattern (Aebi et al. 1986) and which are thought to have an important function in organizing the nuclear pore complexes and possibly also chromosomes (Benevento et al. 1984; Gerace, 1986; Gerace & Blobel, 1981; Gerace et al. 1984; Newport & Forbes, 1987). In addition to the lamina, there have also been reports of fibres linking one pore complex directly to another (Kartenbeck et al. 1971; Keller & Riley, 1976; Riley et al. 1975; Scheer et al. 1976; Kirschner et al. 1977; Maul, 1977). During cell division the nuclear envelope breaks down and then re-forms (Gerace et al. 1986).
The Xenopus oocyte nuclear envelope is an attractive system in which to investigate the structure and also the interactions between its components. The specialized nucleus (or germinal vesicle) of these cells is so large that it can be easily isolated manually. Moreover, it contains a high density of nuclear pore complexes (Scheer et al. 1976; Stiek & Krohne, 1982) and its overall composition has been studied in considerable detail (Krohne et al. 1978, 1981). Its lamina contains a single lamin isomer (Scheer et al. 1976) and both its pore complexes and lamina have been characterized by a number of biochemical and structural methods (Krohne et al. 1981; Unwin & Milligan, 1982; Aebi et al. 1986).

Most studies of nuclear envelope structure have used sectioned material or freeze-fracture replicas (see Kartenbeck et al. 1971; Franke, 1974; Franke et al. 1981; Maul, 1977; Newport & Forbes, 1987 for reviews). However, Aebi et al. (1986) recently demonstrated that shadowing can produce remarkable images of the nuclear envelope, in which some aspects of the structure of the nuclear lamina are seen particularly clearly. We have extended the work of Aebi et al. (1986) to examine the structure of Xenopus oocyte nuclear envelopes and the interconnections between their constituents in greater detail. Using freeze-dried and air-dried specimens, coupled with stereo pairs, we have established criteria that permit the nucleoplasmic and cytoplasmic faces of the envelope to be distinguished. Moreover, in these preparations, it has been possible to visualize simultaneously pore complexes, pore-connecting fibrils and the nuclear lamina. Stereo pairs show that lamina fibres and pore-connecting fibrils attach to the pore complexes at different levels. Also, partial disruption of the envelope, by prolonged washing in low salt buffers or with detergents, produced some intriguing fragments that gave novel insight into its structure.

Materials and methods

Nuclear envelopes
Stage six oocytes of Xenopus laevis were broken open using fine forceps into low salt buffer (10 mM-Hepes, 1 mM-KCl, 0.5 mM-MgCl₂, pH 7.5, at room temperature, which was generally close to 20°C) and whole nuclei were extracted from the yolk (Feldherr & Richmond, 1978). The nuclei were washed several times by aspiration in a fine drawn-out Pasteur pipette and then deposited, in a drop of low salt buffer, on carbon-coated 400 mesh copper electron microscopy grids that had been coated with polylysine. The nuclei were then broken open using fine glass needles to release the nucleoplasm and to enable more of the nuclear envelope to become attached to the carbon film. Often a number (up to 4) of nuclear envelopes were attached to the same grid.

Grids with nuclear envelopes attached were then washed on drops of low salt buffer for between 1 and 20 min to remove the last traces of yolk and also to disrupt the structure slightly as required. Generally, little disruption of the envelope was found for washes of less than 5 min, but for longer washes progressively greater disruption occurred. Additional disruption of the structure could be produced, if required, by treating for 5–15 min with detergents, such as Triton X-100 and sodium deoxycholate, which were both applied at a concentration of 0.1% in 0.3 M-KCl, 10% sucrose, 2 mM-EDTA, 1 mM-dithiothreitol, 20 mM-Tris-HCl, pH 7.0, at room temperature. After the low salt wash (and detergent treatment, if employed), samples were washed on a drop of 10 mM-phosphate buffer (pH 7.0 at room temperature) to remove the Hepes or Tris buffer and then fixed on a drop of 1% glutaraldehyde in 10 mM-phosphate buffer (pH 7.0) for at least 1 min on ice. After fixation, specimens were washed briefly in 10 mM-phosphate buffer (pH 7.0 at room temperature).

Preparation of specimens for shadowing
Samples for shadowing were prepared either by freeze-drying or air-drying as described in detail by Stewart & Whytock (1988). For air drying, samples were dehydrated by 30-s washes through ethanol series, immersed for 1 min in amyl acetate and then dried at room temperature. Specimens for freeze-drying were first washed with at least four changes of HPLC grade water (BDH Chemicals, Poole, Dorset, UK) to remove all salts and other non-volatile material. After washing, specimens were processed essentially as recommended by Nermut (1977). Samples were plunged into liquid nitrogen and loaded into a grid holder, similar to that described by Smith (1980), which was cooled by liquid nitrogen. The charged specimen holder was then transferred to a Balsers BA300 freeze-etching apparatus. When a vacuum of better than 5 × 10⁻⁷ Torr (1 Torr = 133.3 Pa) had been achieved, residual water was removed at −80°C. Removal of water was generally complete after 40 min.

Shadowing and electron microscopy
Specimens were unidirectionally shadowed at a nominal angle of 40° or rotary shadowed at a nominal angle of 45° with platinum/carbon in a Balsers BA300 apparatus equipped with electron beam guns.

Shadowed samples were examined at 80 kV in Philips EM301, EM400, EM420 or CM12 electron microscopes using standard imaging conditions. Stereo pairs were recorded with nominal tilts of ±12° using a eucentric single-tilt goniometer. Micrographs were recorded on Kodak SO-163 cut film, usually at nominal magnifications near ×15000 and, as an aid to interpretation, were printed with contrast reversed, so that the heavy metal appeared white and shadow black.

Results

General appearance of the nuclear envelope
To obtain a comprehensive view of the architecture of
the Xenopus oocyte nuclear envelope, we prepared samples in several ways each of which tended to accentuate different aspects of the structure. Specimens were prepared by freeze-drying or air-drying, and then either unidirectionally or rotary shadowed. Unidirectional shadowing was particularly useful because, with this sort of object, interpretation of images was usually straightforward. This method also showed up the fibrous components of the envelope very clearly. Rotary shadowing, on the other hand, gave useful insights into pore structure and was helpful when assessing the dimensions of connections between components because, with unidirectionally shadowed material, fibrous components parallel to the shadowing direction were often not well contrasted.

In low-magnification micrographs, the overall appearance of shadowed nuclear envelopes from Xenopus oocytes (Fig. 1) was generally that seen previously using a number of different preparative methods (Kartenbeck et al. 1970; Franke, 1974; Aaronson & Blobel, 1975; Scheer et al. 1976; Krohne et al. 1978a; Unwin & Milligan, 1982). In freeze-dried specimens treated with Triton X-100, or in air-dried specimens that had been dehydrated through an ethanol series, the lipid components of the membrane appeared to have been substantially removed, so that the shadowed material showed principally the cylindrical nuclear pore complexes and the fibrous components of the envelope. Two patterns were clearly present (Fig. 2), corresponding to the cytoplasmic and nucleoplasmic faces of the envelope. Isolation of nuclei in low-salt buffer caused them to swell (probably due to osmotic effects, see, e.g., Scheer et al. 1976) and this seemed to manifest itself by the presence of areas in which mechanical stress had increased the spacing between the pores (see Fig. 2).

At higher magnifications (Figs 3–9), the differences between freeze-dried and air-dried material, and between unidirectional and rotary shadowing, became more apparent. The rings of granules associated with the cytoplasmic face of the pore complexes (see Unwin & Milligan, 1982) were most easily seen in freeze-dried preparations (Fig. 3). Pore-connecting fibrils could be easily seen in both freeze-dried and air-dried speci-
mens, particularly in areas in which the envelope appeared to have been slightly stretched, although usually the most striking views were obtained with air-dried specimens (Figs 5 and 8). Generally the fibrous lamina was easily seen on views of the nucleoplasmic face (Figs 4, 6 and 9) using either method.

Cytoplasmic and nucleoplasmic faces of the nuclear envelope
We used stereo pairs to distinguish between views of the nucleoplasmic and cytoplasmic faces of the nuclear envelope. Our initial criterion was the knowledge, from studies on sectioned embedded material (Dwyer & Blobel, 1976; Gerace et al. 1978), that the nuclear lamina is attached to the pore complexes near their nucleoplasmic end. Thus, views in which the lamina lay at the base of the pore complexes were clearly of the cytoplasmic side of the envelope, whereas views in which the lamina was attached to the top of the pore complexes were of the nucleoplasmic surface. In stereo pairs of well-preserved areas of either freeze-dried or air-dried material, it was usually possible to make an unequivocal identification on this basis. Subsequent study of areas characterized in this way enabled us to identify several additional criteria that could be used to classify views, often without the necessity of obtaining stereo pairs.

As shown in Figs 3 and 4, it was fairly easy to distinguish nucleoplasmic from cytoplasmic faces in material that had been freeze-dried before unidirectional shadowing. In views of the nucleoplasmic face (Fig. 4), the lamina clearly dominated the image and formed a dense fibrous canopy supported by the pore complexes. Where the underlying pore complexes were widely separated, the lamina often tended to droop slightly, but rarely by so much as to touch the carbon support film. By contrast, views of the cytoplasmic face (Fig. 3) were dominated by the pore complexes. In stereo pairs, the pore complexes were clearly standing out for a considerable distance from the lamina, which, because it was usually in fairly intimate contact with the carbon support film, could only be seen faintly in the background.

Differentiation between views of the cytoplasmic and nucleoplasmic faces was a little more difficult for air-dried material because, in these preparations, the lamina was usually in closer contact with the carbon film and, in views of the nucleoplasmic face, was not so
Figs 3, 4. Stereo pairs of unidirectionally shadowed freeze-dried preparations of *Xenopus* germinal vesicle nuclear envelopes that had been extracted with Triton X-100.

**Fig. 3.** Cytoplasmic face, in which the pattern is dominated by the roughly cylindrical pore complexes, which generally have distinct granules on their uppermost face. Pore-connecting fibrils (arrow) were often seen linking pore complexes. Bar, 200 nm.

**Fig. 4.** Nucleoplasmic face, in which the pattern is dominated by the fibrous lamina that appears as a canopy supported by the nuclear pore complexes. Bar, 200 nm.
obviously supported by the pore complexes. However, as shown in Figs 5–9, the sidedness of each area could be determined by noting the level at which the lamina fibres attached to the pore complexes. In views of the nucleoplasmic face (Figs 6 and 9) the fibres clearly attached at the top of the pore complexes, whereas in views of the cytoplasmic face (Figs 5 and 8) they were attached near the base. Only when the pore complexes

**Figs 5, 6.** Stereo pairs of a unidirectionally shadowed preparation of *Xenopus* germinal vesicle nuclear envelopes that had been dehydrated through an ethanol series and then amyl acetate before air-drying.

**Fig. 5.** Cytoplasmic face in which the outline of the pore complexes is more circular and in which pore-connecting fibrils (arrows) can also be seen, together with granules, on the uppermost surface of the pore complexes. Bar, 200 nm.

**Fig. 6.** Nucleoplasmic face, in which the lamina has collapsed somewhat except near the pore complexes, which have taken on a star-like pattern as a result of the attachments. In views of either face, areas were often seen in which the lamina fibres were arranged in a basket-weave-like pattern. Bar, 200 nm.
Figs 7–9. Rotary-shadowed preparations of Xenopus germinal vesicle nuclear envelopes after air-drying.

Fig. 7. A view analogous to that in Fig. 2 in which both cytoplasmic (left-hand side) and nucleoplasmic (right-hand side) faces can be seen. Bar, 300 nm.

Fig. 8. Cytoplasmic face dominated by images of the pore complexes topped with distinct granules (particularly clear in the circled pore complex), and which contained a deep central cavity. Pore-connecting fibrils were often seen linking pore complexes (arrow). Bar, 200 nm.

Fig. 9. Nucleoplasmic face showing pore complexes with a star-like outline, and whose uppermost surface was topped with a comparatively smooth annulus in which a central protuberance (arrow) was often seen. Bar, 200 nm.

in air-dried material were relatively close together did the lamina not droop so much as to contact the carbon film.

Once the sidedness of areas had been established from stereo pairs, it was possible to see if there were any other structural features that could be employed to identify cytoplasmic or nucleoplasmic faces. In both air-dried and freeze-dried material, the presence of up to eight globular units in the nuclear pore complexes was usually seen only in views of the cytoplasmic face.
Thus, the presence of this feature could be taken as virtually diagnostic for this view although, of course, its absence should not be taken to indicate the nucleoplasmic face (for example, the failure to see this feature could also be a result of inadequate preservation of the pore complexes). The difference between the cytoplasmic and nucleoplasmic sides of the nuclear pore complexes was particularly clear in material that had been rotary shadowed (Figs 7 – 9). In this material, the cytoplasmic face of the pore complexes contained a deep central cavity surrounded by a ring of granules, whereas the nucleoplasmic face had a comparatively smooth ring with a shallow depression that often contained a central protuberance. Pore-connecting fibrils (Figs 3, 5 and 8) were generally seen clearly only on views of the cytoplasmic face and so, like the granules in the pore complexes, these served as useful diagnostic features for this view, provided that (as was usually the case) they could be distinguished from the fibrous lamina. Generally, the pore-connecting fibrils were thicker and rather short and straight compared to the lamina fibres.

In unidirectionally shadowed material (Figs 3 and 5) in which the cytoplasmic face was uppermost, the pore-connecting fibrils were usually easily recognized on the basis of the shadow they cast, which indicated that they were located well above the support film and lamina, whereas, in rotary-shadowed material (Fig. 8), these fibrils accumulated more metal than the underlying lamina fibres and so appeared in higher contrast (white in Fig. 8). In air-dried preparations, the connections between the pore complexes and nuclear lamina were usually clearly seen in views of the nucleoplasmic side of unidirectionally shadowed envelopes (Fig. 6). In areas closely adjacent to the pores, these connections were a very striking feature of images, and usually gave the pores a distinctive star-like appearance (Fig. 6) that was easily differentiated from the more rounded appearance of pore complexes in air-dried views of the cytoplasmic face (Fig. 5). In rotary-shadowed preparations with the cytoplasmic face uppermost (Fig. 8), this star-like appearance was also present, but the lamina and its constituent fibres were perhaps not quite as clear as in unidirectionally shadowed material. In freeze-dried specimens, the sheer contrast of the lamina was usually sufficient to distinguish between the faces: views of the nucleoplasmic face (Fig. 4) were completely dominated by the lamina fibres and the pore complexes could be only faintly made out. In contrast, views of the cytoplasmic face (Fig. 3) were dominated by the pore complexes with only a very faint underlying lamina.

**Nuclear lamina**

Generally the most striking views of the substructure and arrangement of the lamina fibres were obtained with unidirectionally shadowed air-dried material (Fig. 6). Individual strands of the lamina often showed a fine granularity, analogous to that observed by Aebi et al. (1986) using freeze-dried material, and were continuous with the periphery of the pore complexes. Freeze-dried preparations gave the impression that the lamina was a partially ordered fibrous mat in which the pore complexes were embedded (Figs 3 and 4). Particularly in areas in which the envelope appeared to have been stretched (Fig. 6), the fibrous network appeared as a roughly tetragonal interlacing network of beaded fibrils, as suggested by the earlier studies of Aebi et al. (1986). Connections between pores and lamina could be seen in freeze-dried material, but were not as clear as in air-dried specimens. However, in both types of specimen it seemed that the number of connections between a pore complex and the lamina was of the order of eight. This could represent an underestimate of the number in vivo, since some connections may have been lost in those areas of the envelope where the pore complexes were spaced more widely, probably as a consequence of the envelope’s stretching in low ionic strength buffer. Moreover, some connections may have been obscured by overlapping fibres. The fibres of the lamina appeared to be about 10 nm in diameter in rotary-shadowed air-dried preparations (mean 10.5 nm, ±2.5 nm s.d., n = 127). It was often difficult to make out individual fibres in freeze-dried material but they seemed to have essentially the same size of diameter as those seen in air-dried specimens.

**Nuclear pore complexes**

The eightfold rotational symmetry of the granules on
the cytoplasmic face of the pore complexes was best seen on views of Triton-extracted freeze-dried envelopes (Fig. 3). As would be anticipated from the structure of the pores suggested by electron microscopy of negatively stained material (Unwin & Milligan, 1982), this feature was not seen on views of the nucleoplasmic face. Air-drying did not preserve this feature of the structure so well, particularly in material that had been shadowed unidirectionally, although it could sometimes be observed in specimens that had been so extensively washed in low-salt buffer that the lamina and pores became detached from each other (Fig. 10). Rotary-shadowed, air-dried preparations (Fig. 8) emphasized the rather deep cavity surrounded by granules at the cytoplasmic entrance to the pore complexes. This feature contrasted with the smoother ring and shallow depression on the corresponding nucleoplasmic face (Fig. 9). A central granule was often seen on the nucleoplasmic face of pore complexes, and was particularly clear in rotary-shadowed material (Fig. 9). Central granules generally seemed to be absent in views of the cytoplasmic face of the pore complexes. These observations were consistent with the indications from both sectioned embedded material (for reviews, see Franke, 1974; Maul, 1977) and three-dimensional reconstructions of negatively stained preparations (Unwin & Milligan, 1982), which indicated that central granules were located in the nucleoplasmic portion of the pore complex. In some instances (e.g. Fig. 10), small groups of nuclear pore complexes appeared to be arranged on a tetragonal lattice analogous to that seen by Unwin & Milligan (1982). However, these areas were usually of very limited extent and areas of extensive pore crystallinity were not seen. Moreover, this sort of tetragonal ordering was conspicuously absent from areas in which the pores were more widely separated and in which a tetragonal arrangement of the lamina could be seen.

Pore-connecting fibrils
Stereo views of the cytoplasmic face of shadowed nuclear envelopes showed pore-connecting fibrils.
Fig. 12. Unidirectionally shadowed preparation of rings derived from the disruption of nuclear pore complexes by washing in low salt buffer and Triton X-100. The rings appear to be constructed from eight subunits. Bar, 200 nm.

Fig. 13. Unidirectionally shadowed preparation of rings derived by treatment of Xenopus germinal vesicle nuclear envelopes with 1% sodium deoxycholate. In these preparations the eightfold rotational symmetry of the subunits in the rings was often particularly clear. Moreover, in some instances the rings began to dissociate into smaller subunits. Bar, 200 nm.

clearly (Figs 3, 5 and 8). These views also indicated that the pore-connecting fibrils were attached to the cytoplasmic half of the pore complexes, often seeming to be at or just below the level of the granules at the cytoplasmic face of the pore complexes. This was in contrast to the lamina fibres that were clearly attached near the nucleoplasmic end of the pore complex cylinders. The pore-connecting fibrils were usually seen only when the pores were close together. They were generally absent in areas in which the pores were more widely separated, such as those in which the lamina was most visible, and some may have been broken in areas in which the nuclear envelope appeared to have been stretched. The pore-connecting fibrils seemed to be wider than the fibres of the nuclear lamina and, in rotary-shadowed preparations, had an apparent diameter of 19 nm (mean = 18.6 nm, ±3.8 nm s.d., n = 106) which was almost double that measured for the lamina fibres in corresponding preparations (mean 10.5 nm, ±2.5 nm s.d., n = 127). Generally up to four pore-connecting fibrils seemed to be attached to each pore complex and sometimes two separate connecting fibrils could be seen between the same two pores, giving a characteristic doublet appearance (see Figs 10 and 11). Generally, the pore-connecting fibrils seemed to be rather more constant in length than the lamina fibres. This was particularly so in isolated islands of pore complexes, where the connecting fibrils seen were usually about the same length as the diameter of the pores (Figs 10 and 11).

Disruption of the nuclear envelope

Prolonged incubation of the nuclear envelope in low salt buffer resulted in a gradual disruption of the structure and, after prolonged treatments, isolated groups of pores, often with some pore-connecting fibrils still attached, could often be seen (Fig. 10). Moreover, in some areas, the pores themselves appeared to have been disrupted into smaller components. In some instances (Fig. 12) rings with approximately eightfold rotational symmetry were seen, often with smaller, roughly spherical particles near them. These probably corresponded to the rings and cytoplasmic granules seen in three-dimensional reconstructions of negatively stained nuclear pores by Unwin & Milligan (1982).

Treatment of the nuclear envelope with detergents such as 0.1% Triton X-100 or deoxycholate tended to accentuate disruption slightly. For example, treatment of envelopes with 0.1% deoxycholate for 5 min at room temperature caused sufficient disintegration to permit isolated clumps of pores with pore-connecting fibrils between them to be seen (Fig. 11). Often these images gave the impression that the positioning of the pores in these islands was maintained by the connecting fibrils (Figs 10 and 11). Sometimes the pore complexes themselves began to disintegrate as a result of these treatments, giving rise to small isolated spherical particles and rings (Figs 11 and 12). In Fig. 11, it appeared that the rings derived from the nucleoplasmic face of the pore complexes. The rings seemed to be
Fig. 14. Unidirectionally shadowed air-dried preparation in which the envelope appeared to have been substantially stretched, as suggested by the low density of pore complexes and widely separated lamina fibres. In areas such as this, the lamina fibres often appeared to be arranged in a basket-weave-like pattern and sometimes appeared to have a somewhat beaded outline as described by Aebi et al. (1986). Bar, 200 nm.

Fig. 15. Unidirectionally shadowed preparation of an air-dried sample of *Xenopus* germinal vesicle nuclear envelope that had been treated with deoxycholate. In this specimen, the lamina fibres are starting to dissociate further so that they appear almost like strings of beads. Bar, 200 nm.

made up of eight particles (Fig. 12) and sometimes themselves began to break down into smaller components (Fig. 13).

A beaded substructure was often seen in lamina fibres, particularly after extensive washing in low salt buffer or Triton X-100 (Fig. 14). This appearance was generally similar to that reported by Aebi et al. (1986). Some further dissolution of the lamina, so that its fibres appeared to be composed from strings of beads (Fig. 15), was also occasionally seen with deoxycholate treatment.

Discussion

We have used complementary methods to prepare *Xenopus* oocyte nuclear envelopes for shadowing and examination by electron microscopy. Superior preservation of nuclear pore complexes was obtained by freeze-drying, whereas air-drying gave particularly clear views of the lamina and pore-connecting fibrils and their attachment to the pores. Unidirectional shadowing was most useful for accentuating the fibrous components of the envelope and had the additional advantage that images could usually be interpreted correctly by simple inspection. Rotary-shadowed material, particularly when stereo pairs were employed, gave more informative views of the pore complexes. Fig. 16 illustrates schematically the main features of the *Xenopus* oocyte nuclear envelope that were deduced by integrating the information obtained from the range of shadowed preparations we investigated.

The ability to distinguish between the two faces of the nuclear envelope is an essential prerequisite to studying its fine structure, by using antibodies and other labels, or to studying the transport of material through the envelope. By using stereo pairs of shadowed preparations, we determined which face was being observed on the basis of the position of attachment of the lamina to the pore complexes. Views of the cytoplasmic face had the lamina at the base of the pore complex cylinders, whereas views of the nucleoplasmic face had the lamina attached near the top of the pore complexes. We then used micrographs in which orientation had been established in this way to develop criteria for recognizing the cytoplasmic and nucleoplasmic faces of the envelope, without the necessity of recording stereo pairs. The cytoplasmic face could be most easily recognized by the presence of cylindrical pore complexes topped with granules having approximate eightfold rotational symmetry, the presence of clear pore-connecting fibrils, and the relatively poor contrast of the lamina, whereas in views of the nucleoplasmic face the pores had lower contrast and a star-like outline, lacked granules and eightfold symmetry, and the pattern of the lamina was much more prominent.

One advantage of shadowed images of the nuclear...
Fig. 16. Schematic illustration of the architecture of the *Xenopus* oocyte nuclear envelope suggested by integrating the information obtained from different shadowed preparations. Note that the two membranes present in this structure *in vitro* have not been included in this figure because they were removed by the preparative methods we employed. The nucleoplasmic surface of the envelope is dominated by the nuclear lamina, which is composed of somewhat beaded fibres that are sometimes arranged on an approximately rectangular lattice. The lamina fibres attach to near the base of the roughly cylindrical nuclear pore complexes, perhaps by way of a disc-like component that forms the lower face of the pore complex as shown in the exploded view on the right. Eight granules are arranged on the cytoplasmic face of the pore complex so that there is a pronounced central cavity that contrasts with the smoother nucleoplasmic face of the pore, which often contains a central protuberance. Pore-connecting fibrils, that are roughly twice the diameter of the lamina fibres, attach to the cytoplasmic half of the pore complex cylinder, probably just below the level of the granules, and may serve to arrange the pores within the envelope on a roughly square lattice.

The precise location of the pore-connecting fibrils in relation to the inner and outer nuclear membranes was not clear in our shadowed preparations, since the membranous components of the envelope had been removed. However, their attachment to the cytoplasmic half of the pore complex cylinder would indicate that the pore-connecting fibrils could be located in the lumen between inner and outer membranes; within the outer membrane itself; or in the cytoplasm on top of the outer membrane. In their painstaking study of the fibrous components of amphibian nuclear envelopes using sectioned embedded material or negatively stained specimens, Scheer *et al.* (1976) detected fibrous components only at the level of the inner nuclear membrane. However, in their sections of material extracted with Triton X-100, on which this observation was based, the pore-connecting fibrils may not have been preserved or, because of their low abundance compared to the lamina fibres, may have been difficult to detect. Kartenbeck *et al.* (1971) illustrated a freeze-fracture replica in which a pore-connecting fibril seemed to be present within a membrane (presumably the outer nuclear membrane), whereas Maul (1977) suggested that these fibrils were located on the cytoplasmic side of the outer membrane. Clearly the precise location of the pore-connecting fibrils relative to the nuclear membranes and their protein composition needs to be resolved. In addition to the lamina and the envelope was that they permitted simultaneous observation of the fibrous lamina and pore-connecting fibrils. Only indications of the presence of these fibrils had been obtained previously (for reviews, see Kartenbeck *et al.*, 1971; Scheer *et al.*, 1976; Maul, 1977), because of the difficulty of detecting them in sectioned embedded material or in freeze-fracture replicas, and there was some uncertainty as to whether these fibrils were components of the lamina. Stereo views enabled the level of attachment of the lamina and pore-connecting fibrils to be observed. The pore-connecting fibrils were clearly attached nearer the cytoplasmic end of the pore complexes, whereas the lamina fibres were attached at the nucleoplasmic end. Moreover, the pore-connecting fibrils had a characteristically different appearance from that of the lamina fibres. The former were generally all of similar length and much shorter than the latter. The pore-connecting fibrils were also smoother, lacked the characteristic beaded appearance of the lamina fibres, and appeared to be thicker. These criteria would seem to establish clearly that the lamina fibres and pore-connecting fibrils were different structural components of the nuclear envelope, as illustrated schematically in Fig. 16. On this basis it seems likely that the pore-connecting fibrils would have a different protein composition from that of the lamina fibres, although it is conceivable that both structures could be composed of lamins arranged in different ways.

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pore-connecting fibrils there are other filaments attached to both the cytoplasmic and nucleoplasmic faces of the nuclear envelope (see, e.g., Franke & Scheer, 1970), but we did not observe them in our preparations, possibly because they were lost during the various washing steps we employed. We do not think it likely that these filaments could have been confused with pore-connecting fibrils, since the former are probably up to micrometres long and often appear to be beaded (Franke & Scheer, 1970), whereas the latter were always smooth and very short (generally of the order of 100 nm long). Moreover, the observation of pore-connecting fibrils in specimens prepared by a range of other techniques (see, e.g., Kartenbeck et al., 1971; Scheer et al., 1976; Kirschner et al., 1977; Maul, 1977) argues strongly against their being an artefact arising from the collapse of cytoplasmic or nucleoplasmic filaments. Furthermore, in their recent study of the assembly of nuclear envelopes in vitro, Sheehan et al. (1988) suggested the possibility of connections forming between pores that did not involve the lamina. It is tempting to speculate that these connections could have been pore-connecting fibrils linking the pore complexes into rafts, analogous to those seen in Figs 10 and 11, at an early stage of the assembly process.

The different fibrous components of the envelope that interconnected the pore complexes were often disrupted by the preparative methods. The pore-connecting fibrils often appeared to have been disrupted in those areas in which the envelope seemed to have stretched, either as a result of swelling of the isolated germinal vesicle in low salt buffer or of mechanical stress during mounting and drying of the specimens. In these stretched areas, the lamina was most clearly seen and its fibres most often appeared to lie on a regular tetragonal lattice, as first reported by Aebi et al. (1986). Conversely, the pore connecting fibrils were most easily seen in views of the cytoplasmic face of the envelope in which the pores were relatively closely packed. They were also seen in views of small fragments that had been removed from the envelope by various disruptive treatments. Given the rather constant length of the pore-connecting fibrils, it may be that these connections, rather than the underlying lamina, were primarily involved in maintaining small areas of pore complexes in either lines or, more rarely, two-dimensional tetragonal arrays. A possible role for the lamina, suggested by the general appearance of the areas in which the spacing between pore complexes had increased, may be to provide a general mechanical stability to the envelope, which would prevent its being stretched too much.

The images of isolated islands of pores (Figs 10 and 11) suggested that the relative positioning of pore complexes within the plane of the envelope was probably determined to some extent by the pore-connecting fibrils. The number of connections on a pore that involved these fibrils did not seem to correspond very precisely to the eightfold rotational symmetry that is such a striking feature of nuclear pore complexes (Franke, 1974; Maul, 1977; Franke et al., 1981; Unwin & Milligan, 1982; Newport & Forbes, 1987). Therefore, it would appear that the potential binding sites on the pore complexes for these fibrous connections may not be fully saturated in the nuclear envelope, but instead probably form only between adjacent pore complexes. The roughly tetragonal packing of pore complexes usually observed would be consistent with roughly four connections, which corresponds to the number of pore-connecting fibrils often observed. The lack of long-range crystalline ordering of the pores in the nuclear envelope could be a consequence of the promiscuity of attachment that could result from all binding sites on the pore complexes not being saturated. In these circumstances, one could imagine that a regular structure, resulting from every second unit around a pore complex binding to a connecting fibril, could be disrupted if two adjacent pore complex subunits were to bind fibrils instead. This sort of model would also be consistent with the double pore-connecting fibrils sometimes observed. The density of pore complexes in Xenopus oocyte germinal vesicle nuclear envelopes is higher than that found in many other cells (see Stick & Krohne, 1982). Thus, although pore-connecting fibrils form quite a striking feature of the Xenopus envelope, they may not be such a prominent feature of other envelopes, such as those from rat liver, in which the pore complex density is lower. Furthermore, when pore complexes are more widely separated, it may not be possible for the pore-connecting fibrils to form. Consequently, it is not certain at this stage how general the observation of pore-connecting fibrils is and clearly this point should be addressed by examining nuclear envelopes from a broad range of species.

The images obtained of partially disrupted pore complexes suggest that this approach, used in conjunction with the high image contrast obtainable with heavy metal shadowing, may give some insight into the structural components of the pore complexes, their internal arrangement, and their connections to fibrous components of the nuclear envelope. Thus, the partially disrupted material shown in Fig. 11 indicated that, near the nucleoplasmic surface of pore complexes, there was a ring-like structure, containing eight subunits, that can be detached from the rest of the pore complex cylinder. This ring-like structure probably corresponded to the comparatively smooth annulus seen most clearly on rotary-shadowed views of the nucleoplasmic face of the pore complexes (Fig. 9). Although the eightfold rotational symmetry was not clear in the whole mounts, it could be that this feature was more pronounced on the inner face of the annulus.
that was exposed only when the pore complexes were disrupted. This annulus may be the site of attachment of the lamina, but, as it was observed only in material from which the lamina had been detached, it may be that the lamina is instead attached to a component that binds to the ring and which was itself removed with the lamina. Unwin & Milligan (1982) also showed images of isolated rings. Their three-dimensional reconstruction of the nuclear pore complex also had a ring-like structure surrounding the cylinder. However, as this ring was located nearly mid-way between the cytoplasmic and nucleoplasmic surfaces of the pore complex cylinder, it probably does not correspond to the morphological unit we have seen, which instead appears to be located at the nucleoplasmic face of the pore complex. In their recent study of the assembly of Xenopus oocyte nuclear envelopes in vitro, Sheehan et al. (1988) observed 'prepore' structures that seemed to be thinner than mature pores. It is tempting to speculate that these prepores could correspond to the cylinders remaining after removal of the nucleoplasmic ring in our disrupted preparations (Fig. 11) and, therefore, that the different morphological components we have observed might correspond to pore complex assembly intermediates.

The central protuberance seen in views of the nucleoplasmic face of some nuclear pore complexes may correspond to the central granule detected by Unwin & Milligan (1982) and many other workers (see, e.g., Franke, 1974; Maul, 1977). However, this feature clearly varied between pore complexes and was present in only a proportion of pores in any one view. It may be that this feature was related to transport of material through the pores, and could represent either the material actually in transit or, alternatively, some conformational change in the pore complex associated with transport.

We are currently employing these shadowing methods to study further the architecture of the nuclear envelope and the interconnections between its components. We are also using this system to investigate the effect of various disruptive treatments and lamina fragments altered using recombinant DNA methods.

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References


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