

High Resolution Scanning Electron Microscopy of the Nuclear Envelope: Demonstration of a New, Regular, Fibrous Lattice Attached to the Baskets of the Nucleoplasmic Face of the Nuclear Pores

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Abstract. The nuclear envelope (NE) of amphibian oocytes can be readily isolated in relatively structurally intact and pure form and has been used extensively for structural studies. Using high resolution scanning electron microscopy (HRSEM), both surfaces of the NE can be visualized in detail. Here, we demonstrate the use of HRSEM to obtain high resolution information of NE structure, confirming previous data and providing some new information. NEs, manually isolated from *Triturus cristatus* oocytes, have been mounted on conductive silicon chips, fixed, critical point dried and coated with a thin, continuous film of chromium or tantalum and viewed at relatively high accelerating voltage in a field emission scanning electron microscope with the sample within the objective lens. Both nucleo-

plasmic and cytoplasmic surfaces of the nuclear pore complexes (NPC) have been visualized, revealing the cytoplasmic coaxial ring, associated particles, central plug/transporter and spokes. The nucleoplasmic face is dominated by the previously described basketlike structure attached to the nucleoplasmic coaxial ring. In *Triturus*, a novel, highly regular flat sheet of fibers, termed the NE lattice (NEL) has been observed attached to the distal ring of the NPC basket. The NEL appears to be distinct from the nuclear lamina. Evidence for the NEL is also presented in thin TEM sections from *Triturus* oocytes and GV's and in spread NEs from *Xenopus*. A model is presented for NEL structure and its interaction with the NPC's is discussed.

THE nuclear envelope (NE)¹ is the semi-permeable barrier that surrounds the interphase chromosomes of all eukaryotic cells (Franke et al., 1981; Gerace, 1986; Newport and Forbes, 1987; Gerace and Burke, 1988). It consists of two membranes. The outer nuclear membrane (ONM) is continuous with the rough ER, and is decorated with ribosomes. The inner nuclear membrane (INM) is joined to the ONM at the nuclear pore complexes (NPCs), and is parallel to the ONM and separated from it by the perinuclear cisterna which may be continuous with the cisterna of the rough ER. Between the INM and the nuclear contents is the nuclear lamina (Gerace, 1986). This consists of a network of lamin proteins (Aebi et al., 1986; Akey, 1989), which are a class of intermediate filament proteins (McKeon et al., 1986; Fisher et al., 1986; Franke, 1987), probably performing a structural role (Gerace, 1986). The lamins may be joined to the INM, the chromatin and the NPCs. The NPCs are attached to the lamina and penetrate both INM and ONM resulting in an aqueous channel connecting the nucleoplasm and the cytoplasm.

1. *Abbreviations used in this paper:* GV, germinal vesicle; HRSEM, high resolution scanning electron microscopy; INM, inner nuclear membrane; LSB, low salt buffer; NE, nuclear envelope; NEL, nuclear envelope lattice; NPC, nuclear pore complex; ONM, outer nuclear membrane.

Although the NE appears to be freely permeable to ions, other small molecules and even small macromolecules it is able to regulate the passage of larger macromolecules, such as nucleophilic proteins and mRNA, into and out of the nucleus (Gerace and Burke, 1988; Garcia-Bustos et al., 1991; Silver, 1991). Passive diffusion of molecules up to 40–60 kD occurs (Peters, 1986), although there is also active transport of small proteins such as histone H1 (21 kD) (Breeuwer and Goldfarb, 1990). Diffusion and transport of molecules across the NE occur primarily through the NPC's. Transport is temperature dependent and requires ATP (Dingwall et al., 1982; Newmeyer et al., 1986). Nuclear import is a two step process involving binding of nucleophilic proteins, which contain a nuclear localization signal (Garcia-Bustos et al., 1991; Silver, 1991), to a receptor on the cytoplasmic side of the NPC in an ATP-independent manner, followed by ATP-dependent translocation (Newmeyer and Forbes, 1988; Richardson et al., 1988). Transport is inhibited by wheat germ agglutinin (Yoneda et al., 1987; Dabauvalle et al., 1988a) which is thought to bind to a subset of O-linked N-acetylglucosamine containing NPC proteins (Hart et al., 1989). Transport is also inhibited by antibodies to such proteins (Dabauvalle et al., 1988b) which have been used to identify this class of eight glycoproteins and localize them

to the NPC (Snow et al., 1987). In addition, another glycoprotein, gp210, which binds the lectin Concanavalin-A, has been localized to the periphery of the NPC and may act as an anchor for NPCs in the NE membranes (Gerace et al., 1982).

An understanding of the fine structure of the NPC and the composition and function of each component of the structure is essential to our understanding of the transport mechanisms. It may also be important to understand how the NPC interacts with other structures such as the lamina, chromatin, membranes and cytoplasmic structures and how this interaction is related to function. In the present morphological models (Unwin and Milligan, 1982; Akey, 1989; Reichelt et al., 1990), the NPC consists of two coaxial rings, one at the ONM and one at the INM. Between the two rings is a third ring consisting of eight spoke regions which are each divided into two domains: the inner spoke ring which is towards the center of the central channel and the outer spoke domains which abut against the membrane border. Eight peripheral channels may also be apparent (Hinshaw et al., 1992). Vertical supports connect the spokes to each of the rings. A central plug or transporter may be present in the central channel. Radial arms may also be present at the NPC periphery and may be involved in anchoring the NPC in the membrane (Akey, 1989). It has recently been shown by high resolution scanning electron microscopy (HRSEM) and subsequently by transmission electron microscopy (TEM) that the nucleoplasmic face of each NPC is formed by a basketlike structure protruding into the nucleoplasm (Ris, 1989, 1991; Jarnik and Aebi, 1991). The complete NPC has an estimated molecular mass of ~ 125 MD (Reichelt et al., 1990) and may consist of several hundred proteins (Gerace and Burke, 1988).

The nuclear lamina appears to be an interwoven meshwork of intermediate-type filaments into which the NPCs are embedded at the level of the INM (Aebi et al., 1986; Stewart and Whytock, 1988). The lamins (Gerace and Blobel, 1980) can be assembled into filaments in vitro (Aebi et al., 1986; Heitlinger et al., 1991). There are two types of lamin proteins: A-type lamins may interact with chromatin (Glass and Gerace, 1990), whereas B-type lamins may interact more intimately with the INM, probably via the lamin B receptor (Worman et al., 1988) or other integral membrane proteins (Senior and Gerace, 1988; Powell and Burke, 1990; Bailer et al., 1991) and are found to be isoprenylated (Wolda and Glomset, 1988).

The NE of amphibian oocyte germinal vesicles can be easily and cleanly isolated to give large areas of both the cytoplasmic and nucleoplasmic faces of the NE and hence has been used in a large majority of electron microscopical studies of NPC and lamina structure (Unwin and Milligan, 1982; Aebi et al., 1986; Stewart and Whytock, 1988; Akey, 1989; Reichelt et al., 1990). We have used such material to investigate NPC and lamina structure and their interaction with one another using HRSEM. NPC baskets were first identified using HRSEM (Ris, 1989) which has the potential for direct visualization of molecular structures present on surfaces such as the NE.

Scanning electron microscopy is an ideal technique for the visualization of surface structure. Recently it has been possible to visualize molecular detail by the use of field emission sources and a specimen stage within the objective lens (Nagatani et al., 1987). HRSEMs can resolve to ~ 1 nm and

have been used to visualize macromolecular detail such as tail fibres of T-even type phages, Fab fragments bound to the tail fibers and 1 nm colloidal gold attached to the Fab fragment (Hermann et al., 1991), expelled DNA and capsomeres (Muller and Hermann, 1990), crystal structure of tooth enamel (Apkarian et al., 1990) and chromatin substructure of spread nuclei (Inaga et al., 1990) and of chromosomes (Allen et al., 1986), as well as details of NPC components (Ris, 1989, 1991). In this work, specimens have been coated with a thin film of chromium or tantalum (Apkarian, 1986; Peters, 1985; Hermann et al., 1988) and visualized with an accelerating voltage of 27–30 kV.

Materials and Methods

Isolation of Germinal Vesicle NE

Oocytes were obtained and germinal vesicles were isolated manually into 5:1 medium (83 mM KCl, 17 mM NaCl, 10 mM Hepes pH 7.4) as described (Macgregor and Varley, 1988) from mature female *Triturus cristatus* (kindly provided by Professor H. C. Macgregor, University of Leicester, UK). Briefly, oocytes were pierced with a sharpened needle and contents extruded. The GV was located and cleaned by gently sucking up and down a narrow bore pipette. Low salt buffer (LSB: 10 mM Hepes pH 7.4, 1 mM KCl, 0.5 mM MgCl₂) has also been used with similar results. Germinal vesicles were then transferred in a narrow bore pasteur pipette onto a silicon chip (Agar Scientific Ltd., Stansted, Essex, UK) immersed in 5:1 or LSB where they were allowed to settle and attach by natural adhesion. The NE was then broken open with a fine glass needle and spread on the chip. Nuclear contents were removed with a pasteur pipette. For clean preparations GVs were washed extensively to remove contaminating cytoplasm and yolk. After transfer to the chip they were allowed to settle, attach, and swell for 1–5 min before being broken open and washed with a stream of buffer and then incubated in buffer for 20–60 min on ice. For quick isolation, GVs were only briefly washed, transferred to the chip, and spread immediately. Nucleoplasm was removed and the chip transferred to fix.

Electron Microscopy

Silicon chips with attached NEs were transferred to a petri dish containing 2% glutaraldehyde, 0.2% tannic acid (Polysciences, Inc., Warrington, PA) in 100 mM Hepes pH 7.4 for 10 min. They were then briefly washed in double distilled water, postfixed for 10 min in 0.1% OsO₄ and 10 min in 1% uranyl acetate (Ris, 1991) followed by dehydration through ethanol and critical point drying via Arklone (ICI, Runcorn, Cheshire, UK) as a transitional fluid. 100% ethanol and Arklone were sometimes dried with molecular sieve (Merck Ltd., Poole, Dorset). High purity CO₂ with <5 ppm water was used with or without a Tousimis oil/water filter 8782 (Tousimis Research Corporation, Rockville, MD). Dried samples were mounted on aluminum stubs with Eccobond (Emerson and Cuming Inc., Canton, MA). They were then coated with a thin film of chromium or tantalum, measured to be 1.5 nm on the film thickness monitor in an Edwards Auto 306 (Edwards High Vacuum International, Crawley, West Sussex, UK) cryopumped vacuum system with advanced magnetron sputter head which had been pumped to at least 5.0×10^{-7} mBar before introducing an atmosphere of high purity argon at 8.0×10^{-3} mBar. Specimens were then introduced into the top stage of a Topcon (ABT) ISI DS 130F field emission scanning electron microscope (Topcon Corporation, Tokyo, Japan) and viewed at 30kV accelerating voltage.

For TEM, isolated GVs and whole oocytes were fixed in 2% glutaraldehyde, 0.2% tannic acid, 100 mM Hepes pH 7.4 for 1 h, post fixed in 1% OsO₄ for 1 h, dehydrated and embedded in Agar 100 resin. Thin sections were cut, stained with uranyl acetate and lead citrate and examined in a Philips EM 400 TEM (Philips, Eindhoven, Nederland) at 80 kV.

Results

Overall Appearance of Spread NEs from *Triturus cristatus*

NEs were manually stripped from isolated germinal vesicles

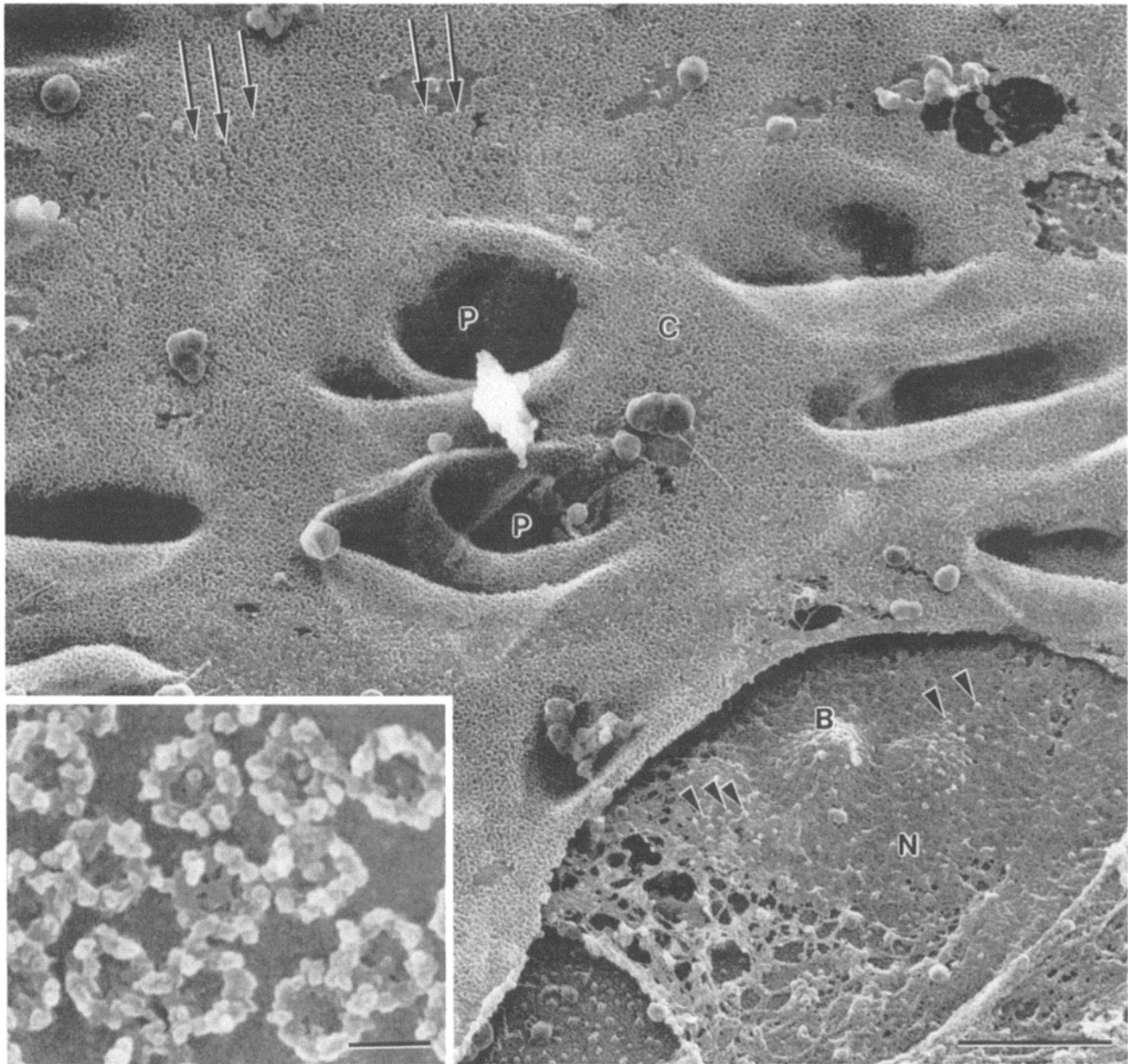


Figure 1. Low magnification of spread NE tilted to 30° showing cytoplasmic surface (C), nucleoplasmic surface (N), pockets (P) with corresponding nucleoplasmic bulges (B). Some NPCs are arranged in rows (arrows). Nucleoplasmic face of NPCs are just apparent (arrowheads). Inset shows cytoplasmic face of NPCs at higher magnification. Bar = 2.0 μm , bar (inset) = 100 nm.

from oocytes of the newt *Triturus cristatus* and spread on silicon chips, followed by fixation, critical point drying and coating with a thin film of chromium or tantalum (Peters, 1985). Fig. 1 is a low power micrograph of such an NE, tilted to 30°. The cytoplasmic face (C) is densely packed with NPCs (Akey, 1989) which are seen here as very small rings. In some regions, these rings are arranged in rows (arrowed) instead of being close packed, possibly due to stretching. The NE is highly invaginated with many deep pockets, which are more apparent in SEM images of whole isolated GVs and in thin TEM sections of both oocytes and isolated GVs (not shown). In Fig. 1 is an area of the nucleoplasmic face (N) showing a small bulge (B) which is a nucleoplasmic view of the pockets. The nucleoplasmic faces of the NPCs are only just apparent (arrowheads) at this magnification. The inset

in Fig. 1 shows a cytoplasmic view of the NPCs at higher magnification. The cytoplasmic ring is decorated with up to eight particles of 15–25 nm diam. Central granules or plugs and spokes are visualized in some NPCs.

Nucleoplasmic Face

The nucleoplasmic face (Fig. 2) is dominated by two structures: the NPC “baskets” or “fishtraps” which have previously been described (Ris, 1989, 1991; Jarnik and Aebi, 1991) and patches of fibers interwoven into a regular network apparently lying as a canopy over the NPC baskets and attached to them. Although fibers have previously been observed attached to the baskets (Jarnik and Aebi, 1991), this regular network is, to our knowledge, a novel structure.

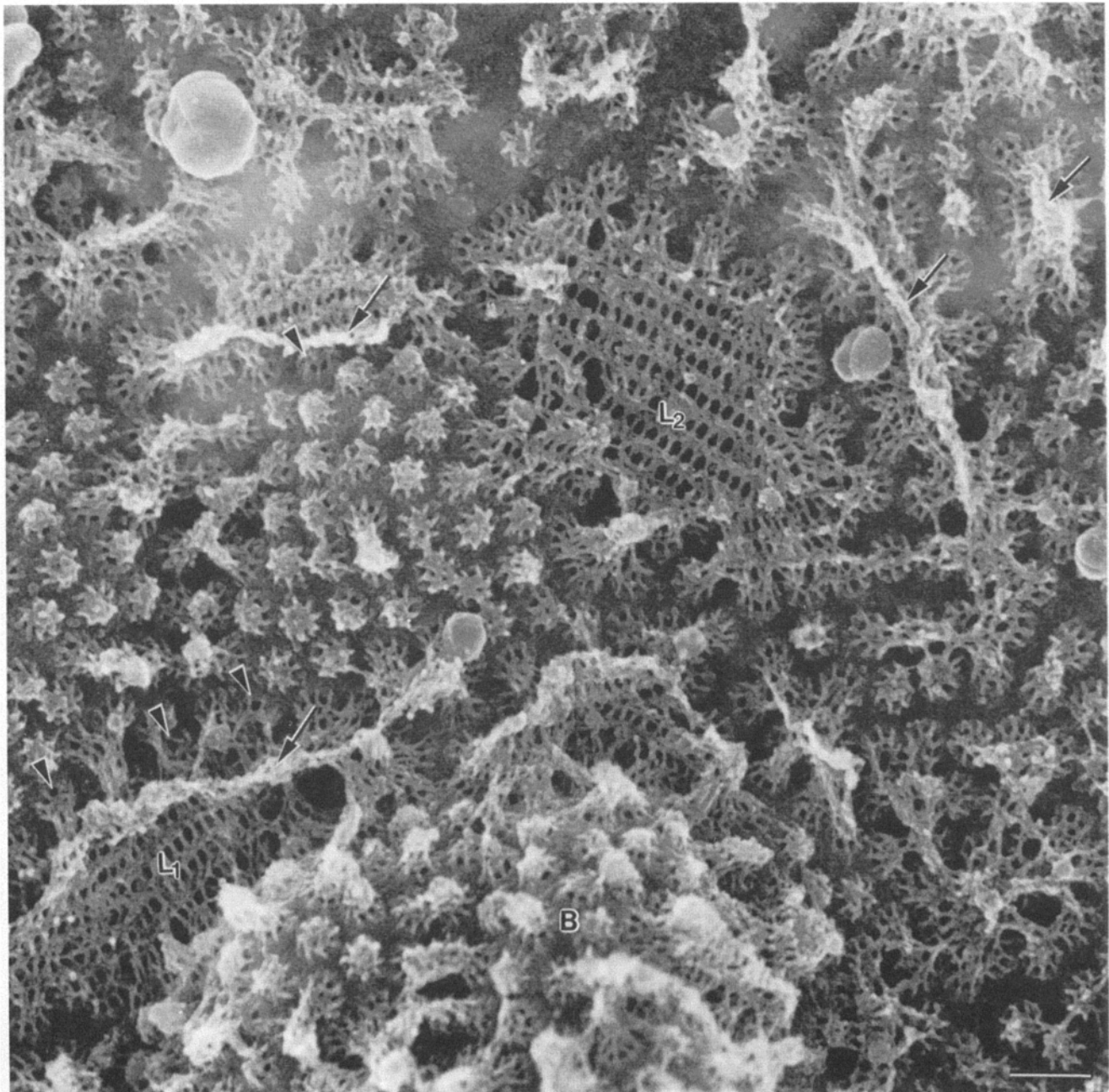


Figure 2. Nucleoplasmic face of "clean" NE showing NPC baskets, NEL (L_1) around base of bulge (B) and on flat area (L_2). Some NEL appears rolled up (*arrows*). NPC baskets are apparent beneath rolled up NEL (*arrowheads*). Bar = 200 nm.

NPC Baskets

The nucleoplasmic face of the NPCs (Figs. 2 and 3) consists of the nucleoplasmic coaxial ring, ~ 110 nm diam, which appears to be largely embedded in the INM. Eight fibers, each with an 8–10 nm diam are attached to the periphery of this ring. The fibers are ~ 40 nm long and extend into the nucleoplasm where they are attached to a smaller ring of ~ 60 nm diam, the "basket ring." The basket ring may have a central aperture, or be a solid disc, or may be decorated with fibers or other material (Fig. 3 *a*). This structure is in general agreement with previous results (Ris, 1989, 1991; Jarnik and Aebi, 1991).

Fibrous Network

The regular fibrous network has been termed the NE Lattice (NEL). Several questions regarding the NEL have been considered: (*a*) its occurrence over the INM surface; (*b*) its structure and the authenticity of the structure; (*c*) its relation to the NPCs; (*d*) its relation to the nuclear lamina; and (*e*) whether it occurs in species other than *Triturus* (e.g., *Xenopus*).

Occurrence of NEL

The amount of NEL observed is variable between NEs and

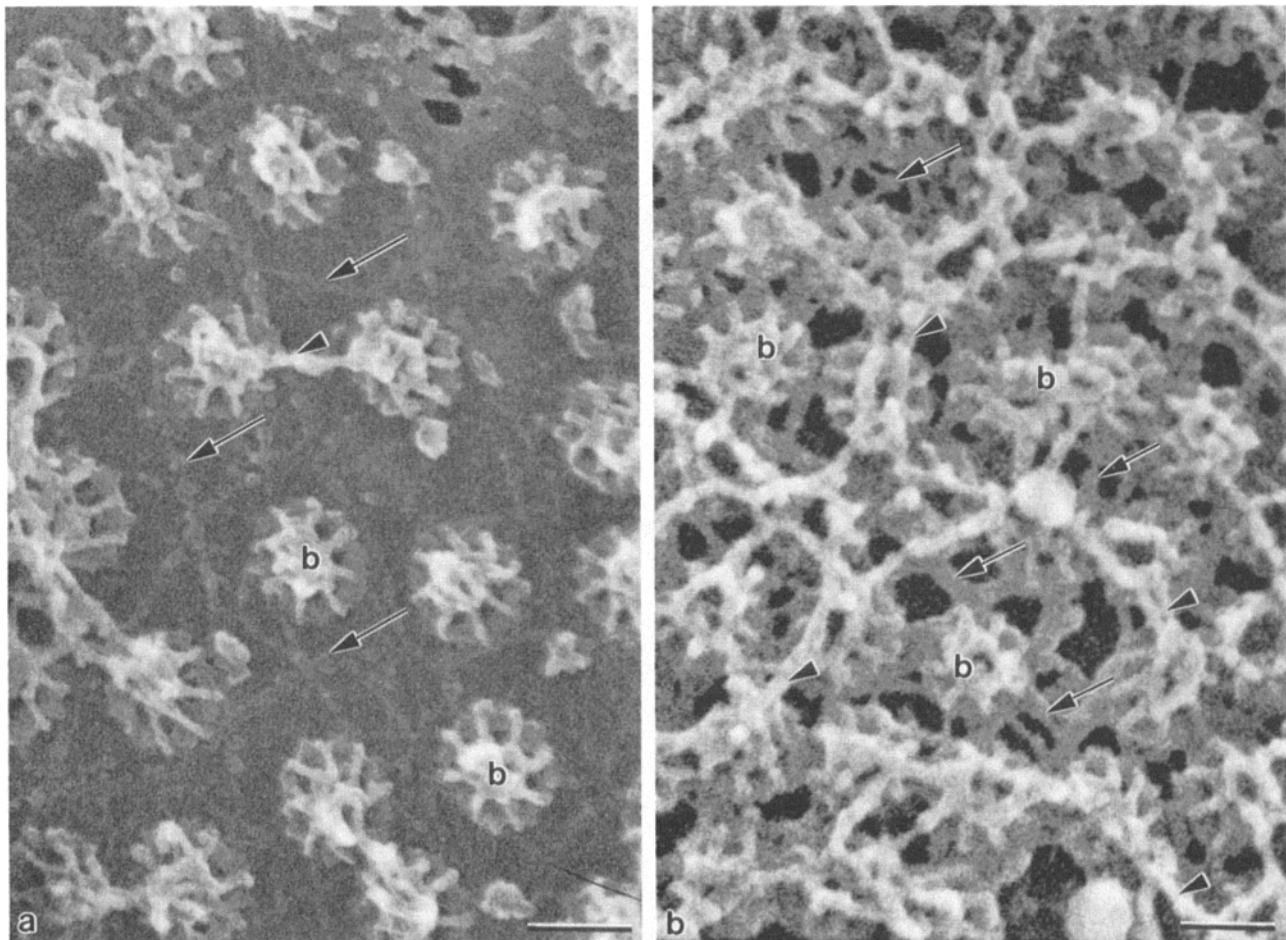


Figure 3. (a) Nucleoplasmic face of “clean” NE. NPCs are well spaced due to stretching of NE. Possible NEL remnants join some basket rings (*arrowheads*). Arrows indicate fibers attached to INM. (b) NE treated with 0.5% Triton X-100 for 5 min in 5:1 medium to remove membranes. NPC baskets (*b*) remain intact but NEL is only observed as remnants joined to basket rings (*arrowheads*). Other fibers, possibly lamina, join NPCs apparently at the level of the nucleoplasmic coaxial rings (*arrows*). Bars = 100 nm.

is dependent on conditions and speed of NE isolation. *Triturus* oocytes were stored dry on ice for 1 h to 2 d before NE isolation, the GVs were allowed to swell before spreading the NE and the isolated NEs were incubated in 5:1 buffer for up to 1 h on ice before fixation. After this procedure, clean looking preparations are obtained (Fig. 2). Baskets were clearly visible and patches of NEL are observed attached around their edges to the baskets. NEL is often found around the base of bulges (L_1) but also on flat areas (L_2), although intact sheets of NEL have not been observed on the bulges themselves.

However, if the NE is isolated rapidly after removal of oocytes from the animal, without chilling and with minimal swelling of the GV, the NEL appears to be much more widespread. In such preparations (Fig. 4), NPC baskets are rarely seen free of NEL over most of the INM, and the NEL is the dominant feature, largely obscuring baskets from a surface view. The exception to this are the bulges, which again only have NEL around the base (bottom right hand corner of Fig. 4) and are essentially free of intact NEL. However, baskets on bulges are decorated with less organized aggregates, possibly remnant NEL (see Discussion).

Although most baskets in rapidly-isolated preparations are

attached to NEL, the NEL does not appear as a continuous canopy over the NPCs but appears in small patches (Figs. 2 and 4). Patches are usually parallel to the surface of the INM but may appear orientated at an angle or perpendicular to it (Fig. 4), possibly due to mechanical disruption during isolation.

Structure of NEL

Low magnification micrographs (Fig. 2 and 4) show that the NEL is a fibrous sheet with regular repeating substructure. Fig. 5 shows aspects of the NEL at high magnification or as stereo pairs. The NEL consists of thin parallel fibers of 8–10 nm diam (Fig. 5 *a*, *small arrows*). These fibers appear branched at each end. The branches appear woven to form a perpendicular, more complex element (Fig. 5 *a*, *arrowheads*) which has a ~30-nm diam. The thin fibers separate the complex, woven element by 30–40 nm. The structure is repeated from one thin fiber to the next on average by 36 nm (Fig. 5 *a*) and from one complex, woven element to the next by 72 nm (Fig. 5 *a*).

In general the nature of the woven element is clearer in preparations that have been washed (Figs. 2, 5 *a* and *b*) com-

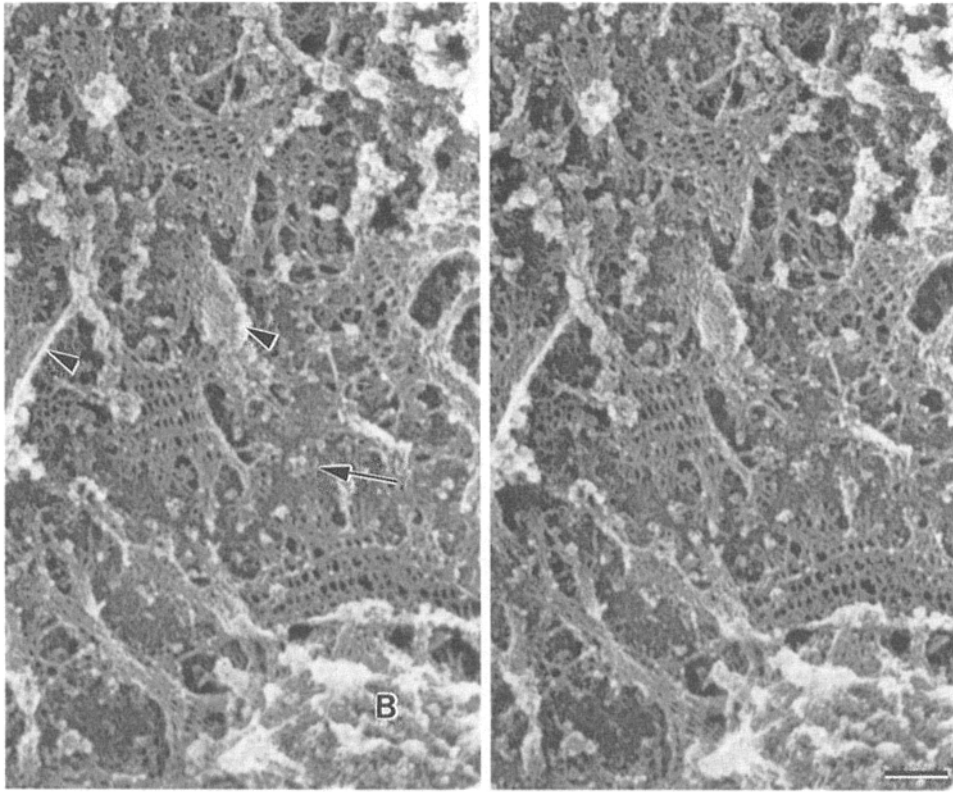


Figure 4. Stereo pair (10° tilt) of low magnification of nucleoplasmic face of "quick-isolated" NE showing wide distribution of NEL. All baskets are attached to NEL, except one (*arrow*). NEL is found on flat areas and around the base of the bulge (*B*) but not on the bulge itself. Some NEL is orientated at an angle or perpendicular to the INM (*arrowheads*). Bar = 200 nm.

pared to quick-isolated preparations (Figs. 4 and 5 *c*). The thin filaments appear to be branched at each end (Fig. 5). The complex elements appear to be composed of these branches. In addition to two branches emanating from each end of each fiber, another fiber is sometimes observed joining adjacent thin fibers at one end only (*large arrow*, Fig. 5 *a*).

The precise arrangement of fibers within the complex element is not particularly clear. However, there are areas where the path of the fibers can be followed, at least tentatively, possibly because they are particularly "clean" or because they have been stretched very slightly, resulting in a resolvable separation such as shown in Fig. 5 *b*. Each thin fiber appears branched at each end. Each branch possibly connects to two other thin fibers that are diagonally opposed across the thick element. The top (in Fig. 5 *b*) of each thin fiber, therefore, appears joined to the bottom of two diagonally opposite thin fibers on each side on the row of thin fibers above. The top (in Fig. 5 *b*) of each thin fiber also appears connected to each adjacent fiber on the same row (see also Fig. 5 *a*). The left hand branch from the top of a thin fiber (in Fig. 5 *b*) appears to be woven over the top of the right hand branch of the adjacent fiber. Although not very clear, this interpretation is suggested at in areas of both Fig. 5 *b* and *c*, as indicated. This is illustrated in the inset of Fig. 5 *b*. However, it is clear that further work on imaging and specimen preparation and manipulation will be required to test this model.

It is also possible that the NEL and/or its structure is an artifact of specimen preparation, particularly of critical point drying (Ris, 1985). To avoid the problems caused by incom-

plete dehydration, ethanol and Arklone used in these studies have been dehydrated with molecular sieve and the high purity CO₂ used, was itself passed through a molecular sieve filter in some instances (Materials and Methods) giving consistent results.

TEM of NEL

We have also attempted to demonstrate the NEL by conventional ultrathin section TEM (Fig. 6) of isolated GVs and of whole oocytes. The NEL is not striking in TEM sections. However, structures can be identified that are suggestive of it, particularly in isolated GVs (Fig. 6, *a* and *b*). Fig. 6 *a* shows a section that appears perpendicular to the surface of the NE. On the nucleoplasmic side of the NE is a filamentous network of the right size and shape with several features consistent with being a patch of NEL that appears orientated perpendicular to the NE surface such as that shown in stereo in Fig. 4. There are rows of fibers parallel to the NE that have a zig-zag appearance (*arrowheads*) similar to the complex element seen in the SEM (Fig. 5). These appear to be joined by perpendicular thin fibers (*arrows*) which are similar to the thin fibers observed in the SEM (Figs. 2, 4, and 5). Fig. 6 *b* is of a glancing section that is parallel to part of the NE and shows a structure similar to that in Fig. 6 *a* as indicated.

Fig. 6, *c* and *d*, shows sections through whole oocytes where the NEL is much less apparent than in isolated NEs. However, there are features which are consistent with it, as indicated, either as a single layer a short distance from the NE, presumably at the base of the baskets (Fig. 6 *c*), or as a network in glancing sections (Fig. 6 *d*).

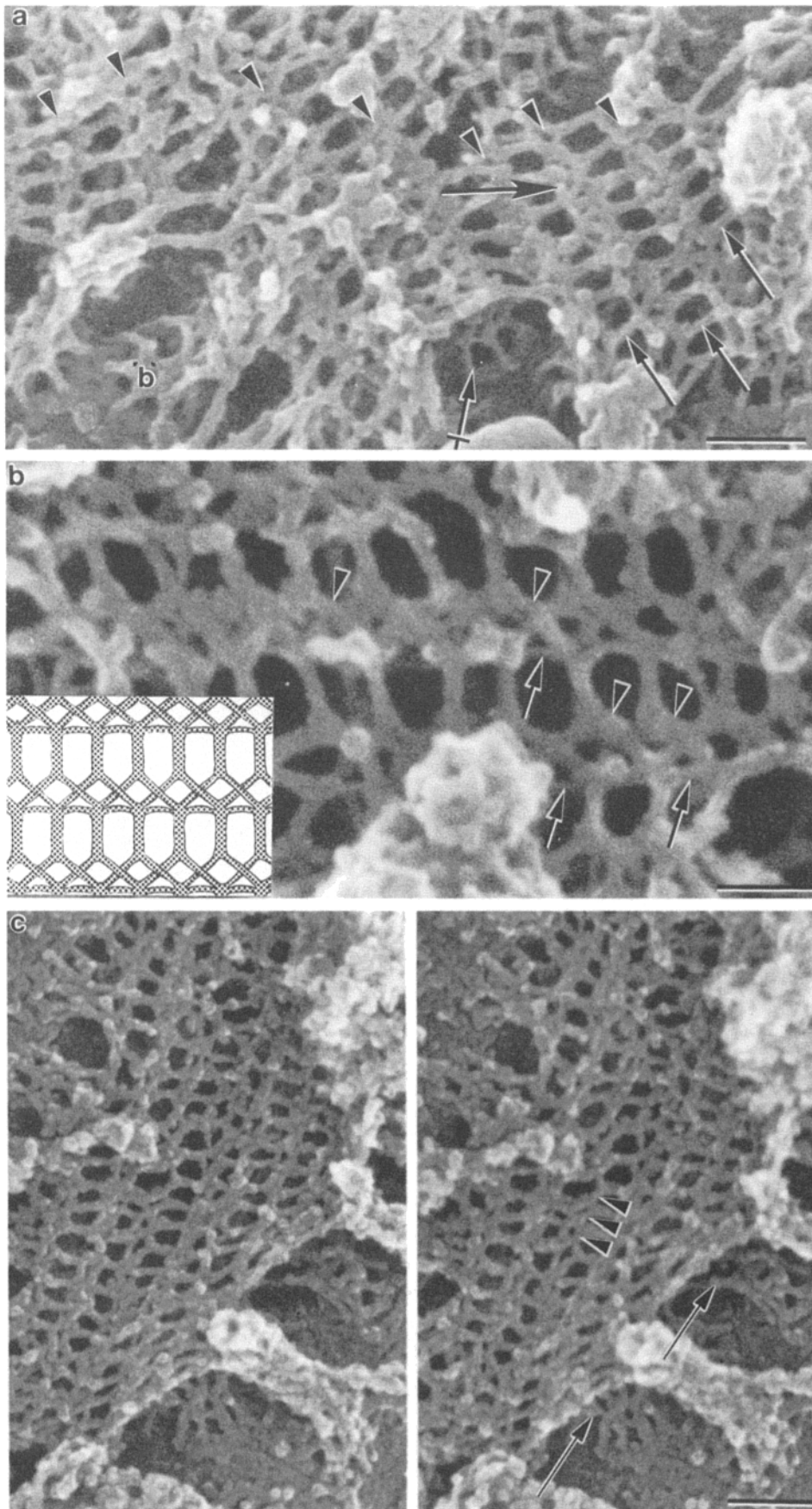


Figure 5. (a) Nucleoplasmic face of clean NE showing NEL structure including woven complex element (arrowheads), thin fibers (arrows), fibers joining adjacent thin fibers (large arrow), NPC located beneath NEL (crossed arrow) and NPC at edge of NEL (b). Bar = 100 nm. (b) NEL substructure for clean NE showing detail of complex woven element including fibers joining adjacent thin fibers (arrows) and fibers which appear to be branches of the thin fibers over the top (arrowheads) of other branches. A model for NEL structure is presented in the inset. Bar = 50 nm. (c) Stereo pair (10° tilt) of quick-isolated NEL showing fibers woven over top (arrowhead) of other fibers and fibers attaching NPCs beneath NEL to the NEL (arrows). Bar = 100 nm.

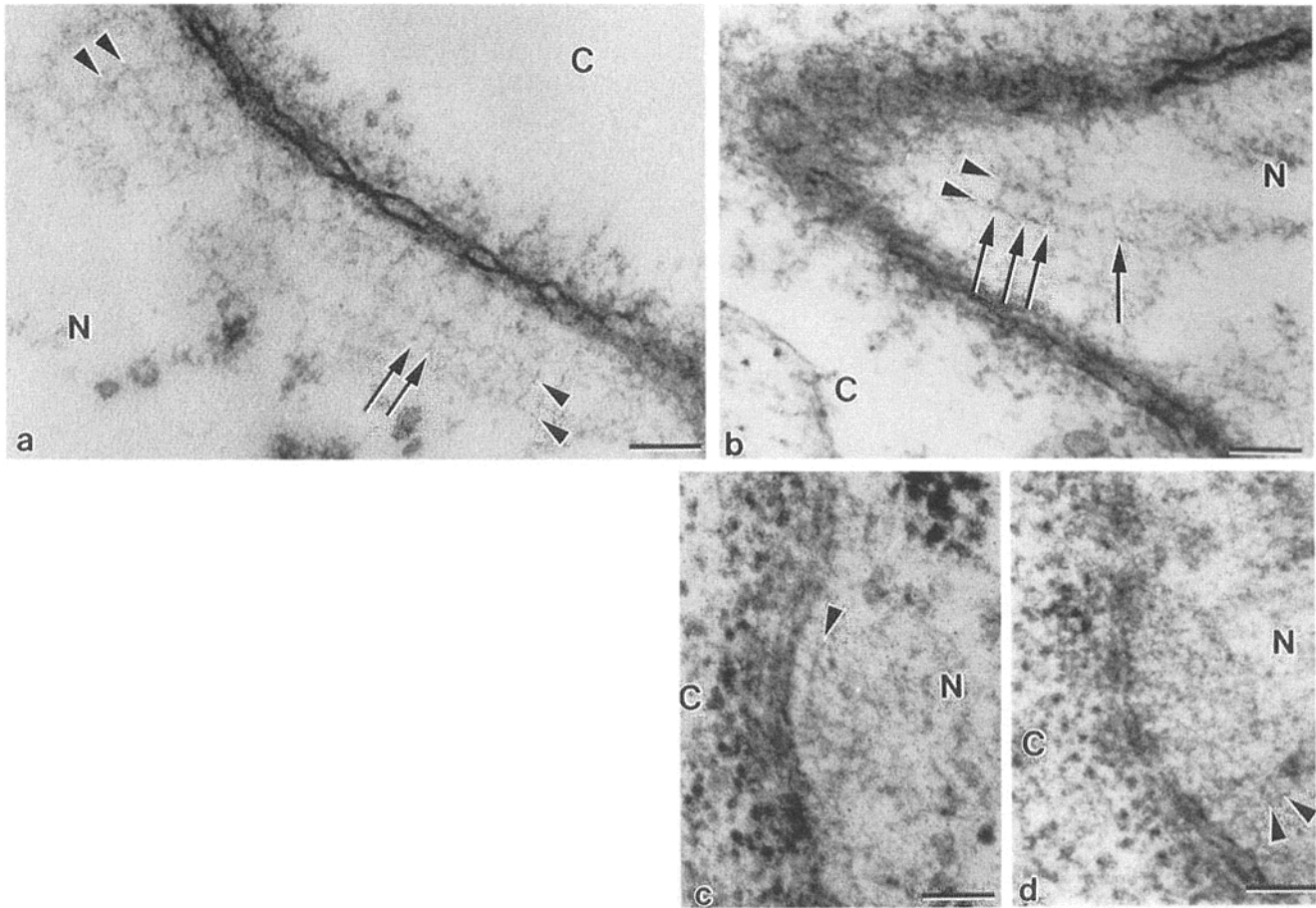


Figure 6. Ultrathin TEM cross-section (a) or tangential section (b) of isolated GVs and of whole, unswollen oocytes (c and d). Zig-zag complex element (arrowheads) and thin fibers (arrows) of NEL are indicated. C is cytoplasmic side of NE; N is nucleoplasmic side. Bars = 100 nm.

Relation of NEL to NPCs

The NEL patches are always attached around their edges to NPC baskets (Figs. 2, 4, and 5). NPC baskets also appear to be present beneath the NEL, as indicated, where the NEL appears to have rolled up in Fig. 2 and also in Figs. 5 a and c where NPCs are observed around the edge but beneath the NEL.

Where NPC baskets are attached to the edge of the NEL

patches there are fibers, which may be NEL thin fibers, that join to the basket ring of the NPC baskets (Fig. 7; see also Figs. 2, 4, and 5 c). These micrographs suggest that baskets are attached directly to the NEL, at least around the edges of the patches. However, in some instances where baskets are beneath the NEL (Figs. 2 and 5 c) there appear to be fibers spaced between the baskets and the NEL.

So far, the position of the baskets relative to the NEL suggests that they are attached to the complex element of the

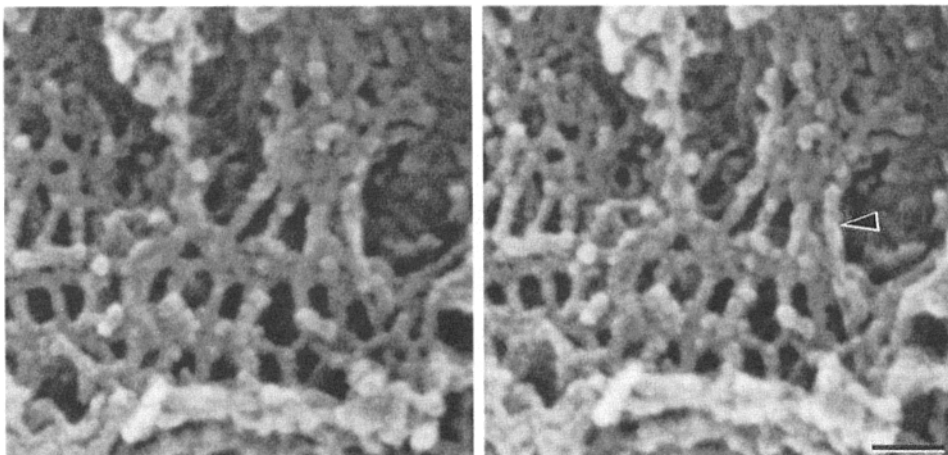


Figure 7. Stereo pair (10° tilt) showing connection of NPC basket ring to NEL, apparently via NEL thin fibers (arrowheads). Bar = 50 nm.

NEL (Figs. 5 *a* and 7). However, more evidence is required to show if this really reflects their relative *in vivo* positions.

Relation of NEL to the Nuclear Lamina

The NEL does not appear consistent with structures previously described as the lamina (Aebi et al., 1986; Stewart and Whytock, 1988; Akey, 1989; Reichelt et al., 1990) (see Discussion). In regions where NEL is not present, in untreated specimens (Fig. 3 *a*), fibers can be observed lying on the surface of the INM which sometimes coincide with the edge of the NPCs. These fibers may be the lamina (Akey, 1989) although they could represent NEL or actin filaments (Stewart and Whytock, 1988; Jarnik and Aebi, 1991) that have collapsed onto the INM.

Treatment of NEs from *Triturus* oocytes with 0.5% Triton X-100 for 5 min reveals structures consistent with previous descriptions of the lamina (arrows, Fig. 3 *b*). Absence of the NE removes the plane of reference, however, nucleoplasmic rings of the NPCs are apparent at the base of the baskets (*b*) and appear joined by fibers at this level (arrows) in contrast to remnants of the NEL (whose structure is not fully preserved after Triton treatment) which is seen as fibers joined at the level of the basket rings (arrowheads).

Occurrence of NEL in *Xenopus laevis* GV

Previous studies using surface imaging techniques (Aebi et al., 1986; Stewart and Whytock, 1988; Ris, 1989, 1991; Jarnik and Aebi, 1991) have not revealed NEL on *Xenopus* oocyte NEs and it has not been recognized in a number of species using other techniques. This might suggest that possibility that it is an unusual structure, possibly unique to *Triturus cristatus*. However, Jarnik and Aebi (1991) have demonstrated filaments frequently connecting basket rings in *Xenopus* which are similar to those seen in *Triturus* (Figs. 2 and 3) which could be remnants of NEL.

We have, therefore, attempted to demonstrate structures resembling NEL in *Xenopus*. Although our results are usually similar to those of Jarnik and Aebi (1991), if NEs are isolated rapidly, as described earlier, considerably more fibrous material may remain attached to the NPC baskets. In particular, where NPCs remain close packed, a network of fibers is observed joining several NPC baskets (Fig. 8, *a* and *b*), although they do not have the regular structure of the *Triturus* NEL. However, very occasionally small patches that are consistent with NEL are observed (Fig. 8, *c* and *d*). These consist of thin parallel fibers that appear branched (large arrow, Fig. 8 *c*) with a thicker element perpendicular to these. The dimensions are consistent with NEL.

Discussion

The structure of both nucleoplasmic and cytoplasmic surfaces of isolated NEs from amphibian oocytes have previously been studied using both TEM of metal shadowed specimens (Aebi et al., 1986; Stewart and Whytock, 1988; Jarnik and Aebi, 1991) and low voltage HRSEM (Ris, 1989, 1991). Here, similar methods have been used to those of Ris (1991) except specimens are coated with a refractory metal (tantalum or chromium) instead of platinum and are viewed at relatively high accelerating voltages (up to 30 kV instead of 1.5 kV). These conditions allow the microscope to perform op-

timally and in our experience produce higher resolution results with thin specimens such as isolated NEs than by using low accelerating voltages. Results presented here of each face of the NPC in *Triturus* are similar to low voltage HRSEM results of Ris (1989, 1991) who studied critical point dried oocyte NEs of *Notophthalmus* and *Xenopus*. Our results also show a striking similarity to quick-frozen, freeze dried, metal shadowed NEs of *Xenopus* oocytes studied by TEM (Jarnik and Aebi, 1991). In the same manner as Ris (1985), we attempted to remove as much water as possible from solvents and CO₂ to avoid previously described CPD artifacts (Ris, 1985), however, in this work omission of molecular sieves gave the same results (presumably our Arklone and CO₂ are sufficiently dry).

In addition to the NPC baskets and features of the cytoplasmic face of the NPCs, which have been shown by others (Ris, 1989, 1991; Jarnik and Aebi, 1991) we have also observed a previously undescribed structure, the NEL. The regular but apparently complex structure (as suggested in the inset in Fig. 5 *b*) of these patches of fibrous sheet that are attached as a canopy over the NPC baskets found in *Triturus* oocytes raises several questions: (*a*) How widespread is the NEL over the NE surface? Is it present as a few small patches (Fig. 2), many small patches (Fig. 4) or as a continuous sheet over much, or all, of the NE? (*b*) Is it a structure common to all NEs, just oocyte NEs, or just *Triturus* oocyte NEs? (*c*) Is the structure observed by SEM of critical point dried specimens authentic or could it be altered in some way by specimen preparation? (*d*) How might it interact with the NPCs and lamina? (*e*) What does it consist of? (*f*) What is its function and how does that relate to its structure? We have gone some way to answering the first four of these questions. We can only, as yet, speculate on the last two.

(*a*) **Occurrence of NEL over the NE surface.** We have observed considerably more NEL attached to NEs that have been isolated quickly from the animal compared to those that have not. During rapid isolation each step was kept to a minimum possible time, whereas in the latter, nuclei were allowed to swell during isolation. Quick isolation reduces the swelling and consequently may result in less disruption of the NEL. However, it does not eliminate swelling, which may be why the NEL appears fragmentary. Evidence of rolled up parts of the NEL (arrowed in Fig. 2) in *Triturus* suggest fragmentation. Fibers joining basket rings in *Triturus* (Figs. 2 and 3), also observed by ourselves (Fig. 8) and others (Jarnik and Aebi, 1991) in *Xenopus*, may be remnants of the NEL and are usually observed when NPCs remain close together in relatively unstretched areas. Intact NEL in *Triturus* is only observed when NPCs are close packed and the NE is unstretched. Regions with widely spaced NPCs (Fig. 3), which have presumably been stretched, have few fibers attaching the basket rings together, although most basket rings are not completely "clean" and still have some material attached. It should be noted that even a small increase in the diameter of the nucleus would lead to a relatively large increase in the surface area of the NE (the surface area of a sphere increases by the square of the radius). Unfortunately, NEL is difficult to see in thin sections of whole, unswollen oocytes which could either mean that it is only present in small patches or alternatively that it is just not usually visible amongst other nuclear material (it appears as a very fine structure in thin sections, Fig. 6 *a*). It is likely, therefore, but not certain, that

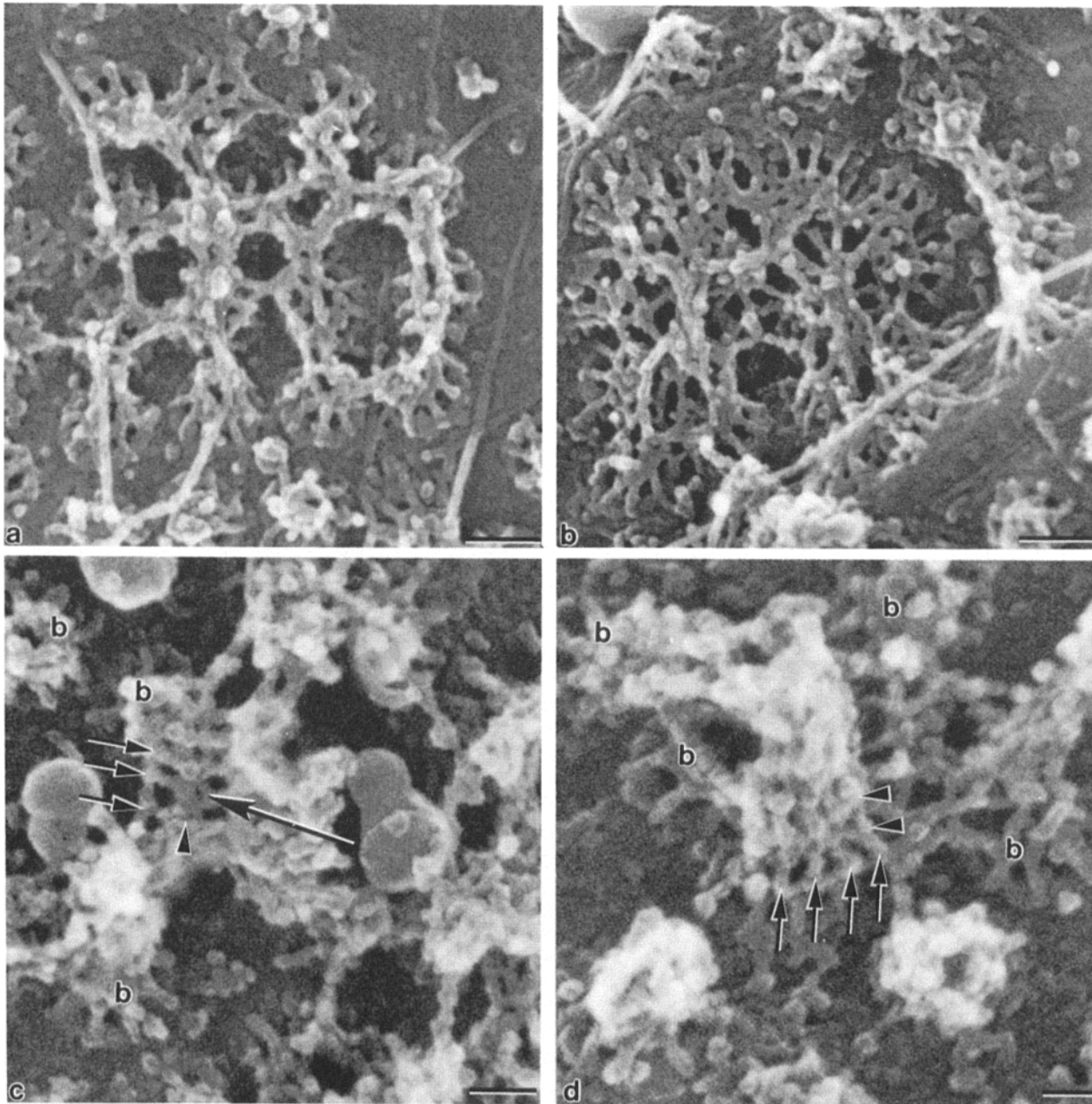


Figure 8. Nucleoplasmic face of quick-isolated *Xenopus* NEs showing fibrous network joining basket rings of close packed NPCs where NE has not stretched (*a* and *b*) and also small, rare NEL patches (*c* and *d*) with thin fibers (*small arrows*), complex elements (*arrowheads*) and branches (*large arrow*). NPC baskets in *c* and *d* are indicated (*b*). Bars *a* and *b* = 100 nm; bars *c* and *d* = 50 nm.

the NEL is a continuous sheet lying over the NPCs and the fragmentary nature of the NEL could be a result of stretching of the NE or of other preparative procedures.

(*b*) *Occurrence of NEL on other NEs.* We have not demonstrated the NEL in any tissue other than oocytes. It is technically difficult to expose the surface of the INM without using harsh procedures except in the very large GV of amphibian oocytes. However, we have attempted to find whether the NEL is specific to *Triturus* or whether it can be found in *Xenopus*. Initial results suggested one reason why the NEL has not previously been observed: it appeared not to be present in *Xenopus*, which is a commonly used organism for NE

studies (Unwin and Milligan, 1982; Aebi et al., 1986; Stewart and Whytock, 1988; Ris, 1989; Jarnik and Aebi, 1991) (although basket connecting fibers have been reported in both metal shadowed and thin sections of isolated NEs [Jarnik and Aebi, 1991]). However, rapid isolation of NEs from *Xenopus* revealed two things: patches of close packed NPCs that were all interconnected by fibers at the level of the basket rings; and fibrous patches whose structure is consistent with a very small piece of NEL. These results indicate that the NEL is not unique to *Triturus*, however more work is required to show whether it is as widespread in *Xenopus*. It may be that retention of *Xenopus* NEL will require refine-

ment of conditions of the buffers used and of the procedures of isolation for its preservation.

NE pockets (Fig. 1) are observed in both *Triturus* and *Xenopus*, although, they are much larger and better retained in *Triturus* during spreading, whereas in spread *Xenopus* NEs, the pockets have usually become flattened out. It is possible that the large pockets in *Triturus* provide a greater reservoir of NE which allows swelling of the GV without stretching of the NE and separation of close packed NPCs. Alternatively, it might suggest that the *Triturus* NE is a more rigid structure.

It is also possible that the NEL in *Xenopus* is lost during separation of the NE from the nucleoplasm, as it has been noted that, of a wide range of amphibian GVs, *Triturus* has the least viscous nucleoplasm (H. MacGregor, personal communication) so any tendency to retain NEL in the peripheral nucleoplasm may be minimized in comparison to *Xenopus* which has a very stiff nuclear gel (Gall et al., 1991).

Although the NEL has only been observed in amphibian oocytes, this is also true for much of the detailed data of NPC, lamina and NE structure, including NPC channels (Hinshaw et al., 1992), spokes, rings (Unwin and Milligan, 1982; Akey, 1989; Reichelt et al., 1990) and baskets (Ris, 1989, 1991; Jarnik and Aebi, 1991; this work) and the square lamina arrays (Aebi et al., 1986; Stewart and Whytock, 1988; Akey, 1989) and much work is required to establish alternative systems in which these structures can be studied and confirmed.

(c) Authenticity of NEL structure proposed. Although highly regular, part of the proposed NEL structure appears surprisingly complex. However, it is possible, if intermediate filament molecules are used as a basic building block, to construct relatively simple models for this structure. This suggests the feasibility of the proposed structure, although it is not presented here as we have no evidence that the NEL is in fact constructed from intermediate filaments.

Suboptimal critical point drying has been blamed for formation of artifactual fibrous networks (Ris, 1985). However, if specimens are fixed sufficiently and solvents are sufficiently dry, these artifacts can be eliminated. Here, we have taken these precautions and obtained consistent results. In addition, it is quite striking that the NPC baskets, which are constructed of fibers of similar appearance to those of the NEL, have a virtually identical appearance in critical point dried preparations (Ris, 1989, 1991; this work) when compared to quick-frozen, freeze-dried preparations (Jarnik and Aebi, 1991), suggesting that both procedures are able to preserve these fragile structures. Future work will be aimed at preservation of the NEL by alternative methods for HRSEM observation.

Thin section TEM results of NEL in isolated GVs support the results seen by HRSEM. In particular the zig-zag nature of the complex element is apparent and this is consistent with being branches of the thin fibers. Thin fibers also have been observed perpendicular to the complex element. Thin sections of whole oocytes provide some evidence for the existence of NEL prior to GV isolation but NEL is not obvious in such sections, which emphasizes one reason why it has not previously been observed.

(d) Interaction of NEL with NPCs and Lamina. Treatment of NEs with Triton X-100 removes the membranes of the NE and reveals another network of fibers that appear at-

tached to the nucleoplasmic coaxial ring of the NPCs. This network is similar to that shown by others (Aebi et al., 1986; Stewart and Whytock, 1988; Akey, 1989) and ourselves in *Xenopus* (not shown) and has previously been described as the nuclear lamina. Immunolabeling studies using antilamin antibodies appear to label near to the INM, rather than the level of the NEL (Stick et al., 1988; Snow et al., 1987). These suggest that the lamina is distinct from the NEL.

The NEL is attached to the NPC basket rings. Around the edges of the NEL patches, this is via fibers that appear to be NEL thin fibers. NPCs beneath the NEL also appear to be attached by similar fibers, although they do not appear to be part of the actual NEL network and may be another "attachment fiber." Basket fibers, NEL thin fibers and attachment fibers all have a similar morphology and it is conceivable that they consist of the same or similar molecules.

(e) Composition of NEL. This is unknown. As mentioned above the structure could possibly be assembled from tetramers of intermediate type filament proteins (Steinert and Roop, 1988), possibly lamin or laminlike proteins. As discussed above, the NEL is unlikely to be the lamina as such. The structure of the NEL has a 36-nm repeat in one direction and 72 nm in the other. The lamina, on the other hand has been described as a simple square array with a cross-over spacing of 52 nm (Aebi et al., 1986) or multiples of 52 nm (Akey, 1989). However the dimensions of the NEL fibers could represent assembled lamin proteins (Aebi et al., 1986; Heitlinger et al., 1991) and the complexity could be organized by posttranslational modifications (Ottaviano and Gerace, 1985; Chelsky et al., 1987; Wolda and Glomset, 1988).

(f) NEL function. The NEL has the superficial appearance of a sieve which could retain large molecular complexes such as hnRNPs before processing (Steitz, 1988). It could also contain receptors and provide a framework for such processing near to the site of export. However, at present this remains entirely speculative.

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