A MODIFIED PROCEDURE FOR THE ISOLATION OF A PORE COMPLEX-LAMINA FRACTION FROM RAT LIVER NUCLEI

NANCY DWYER and GÜNTER BLOBEL

From The Rockefeller University, New York 10021

ABSTRACT

A modified procedure for the isolation of a nuclear pore complex-lamina fraction from rat liver nuclei is described. Evidence is provided that the isolated lamina, a 150-Å thick, proteinaceous structure, apposes the inner nuclear envelope membrane, connecting nuclear pore complexes and surrounding the entire nucleus.

In a previous preliminary report from this laboratory (2) it was shown that nuclear pore complexes, occurring in association with a lamina, can be isolated by subfractionation of rat liver nuclei. This procedure involved preparation of a nuclear envelope fraction by DNase treatment of nuclei (13), followed by solubilization of membranes with Triton X-100 (1) and of residual chromatin with 0.3 M $MgCl_2$ (17). Subsequently, an extensive investigation of each step in this scheme led us to adopt several modifications and it is this modified procedure which we describe here in detail.

MATERIALS AND METHODS

Subfractionation of Isolated Rat Liver Nuclei

A flow diagram for subfractionation of isolated rat liver nuclei to yield a nuclear pore complex-lamina fraction (2) is shown in Fig. 1. Nuclei were prepared from rat liver by the procedure of Blobel and Potter (5); 40 g of liver yielded 1,000–1,500 A_{260} of nuclei; 1.0 A_{260} contained 3.0 × 10^6 nuclei (1). Nuclear envelopes were isolated by a modification of the procedure of Kay et al. (13) (Steps 1 and 2 of Fig. 1). The nuclear envelope fraction, designated D_2p in Fig. 1, was treated with Triton X-100 to solubilize the membrane (Step 3, Fig. 1), yielding a fraction designated D_2Tp. The latter was washed in high salt to solubilize residual chromatin (Step 4, Fig. 1), yielding the pore complex-lamina

fraction, designated D_2TSp. In some experiments the order of Steps 3 and 4 was reversed, i.e. a salt wash, yielding the D_2Sp fraction, was followed by Triton X-100 treatment yielding the D_2STp fraction. In the following, each of the four steps schematically outlined in Fig. 1 is described in detail.

STEP 1 (INCUBATION WITH DNASE AT PH 8.5): A pellet containing 500 A_{260} of nuclei was resuspended by vortexing and the dropwise addition of 5 ml 0.1 mM $MgCl_2$, rapidly followed by the addition of 250 μl of a solution of DNase I (100 μg/ml H_2O, with $E_{280}^{1\%}{}_{cm}$ = 11.0 for DNase) and 20 ml of a solution of 10% sucrose, 10 mM triethanolamine·HCl, pH 8.5, and 0.1 mM $MgCl_2$. The resulting mixture (containing 20 A_{260} of nuclei per ml) was incubated for 15 min at 22°C. After incubation, the mixture was underlaid with 5 ml of a solution of 30% sucrose, 10 mM triethanolamine·HCl, pH 7.5, and 0.1 mM $MgCl_2$, and centrifuged for 10 min at 4°C and at 11,000 rpm (20,000 g-avg) in a swinging bucket rotor (HB-4, DuPont Instruments, Sorvall Operations, Newtown, Conn.), yielding a supernate (D_1s) and a pellet (D_1p).

STEP 2 (INCUBATION WITH DNASE AT PH 7.5): The D_1p fraction was resuspended by vortexing and the dropwise addition of 5 ml (i.e. in one-fifth of the original resuspension volume) of a solution of 10% sucrose, 10 mM triethanolamine HCl, pH 7.5, and 0.1 mM $MgCl_2$. To this suspension, 250 μl of DNase (100 μg/ml) were added. After incubation for 15 min at 22°C, the mixture was underlaid with 5 ml of a solution of 30% sucrose, 10 mM triethanolamine·HCl, pH 7.5, and 0.1 mM $MgCl_2$, and centrifuged as in Step 1,

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STEP 1   D₁s   DNase   D₁p

STEP 2   D₂s   DNase   D₂p

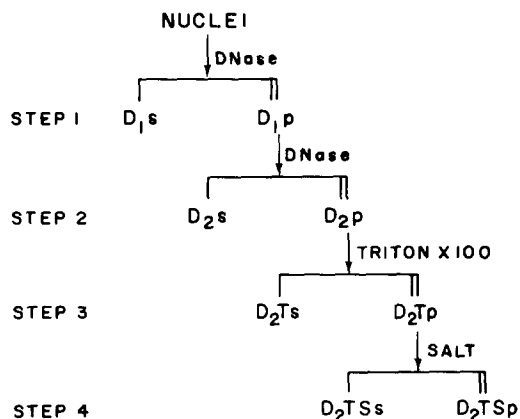STEP 3   D₂Ts   TRITON X 100   D₂Tp

STEP 4   D₂TSs   SALT   D₂TSp

FIGURE 1 Scheme for the subfraction of isolated rat liver nuclei yielding a nuclear pore complex-lamina fraction. A single bar and *s* indicate supernate, double bar and *p* indicate pellet after centrifugation. For details, see Materials and Methods.

yielding a supernate (D₂s) and a pellet (D₂p) of nuclear envelopes.

STEP 3 (TRITON X-100 WASH OF NUCLEAR ENVELOPES): The D₂p pellet was resuspended by vortexing and the dropwise addition of 5 ml of an ice-cold solution of 10% sucrose, 10 mM triethanolamine ·HCl, pH 7.5, and 0.1 mM MgCl₂ 0.5 ml of a solution of 20% wt/vol of Triton X-100 was added. Incubation of the mixture for 10 min in an ice bath, followed by centrifugation (without a sucrose cushion) as in Step 1, yielded a supernate (D₂Ts) and a pellet (D₂Tp).

STEP 4 (SALT WASH OF TRITON X-100 TREATED NUCLEAR ENVELOPES): The D₂Tp fraction was resuspended by vortexing under conditions identical to those in Step 3. To the resulting suspension, 5 ml of a solution of 2.0 M NaCl and 100 mM triethanolamine·HCl, pH 7.5, were added. Incubation of the mixture for 10 min in an ice bath, followed by centrifugation as in Step 1, yielded a supernate (D₂TSs) and a pellet (D₂TSp), the latter representing the pore complex-lamina fraction.

Homogeneous resuspension of the pellet in all four steps is important in order to insure complete reaction of the components. Since our fractionation scheme involves repeated sedimentation, vortexing occasionally was insufficient to disperse remaining clumps, particularly after Steps 3 and 4. However, homogenization by hand using a Potter-Elvehjem homogenizer with a Teflon pestle resulted in homogeneous suspensions without visible clumps. In order to reduce tight packing and facilitate subsequent suspension, centrifugation in Steps 3 and 4 can also be performed by the sucrose cushion techniques as in Step 1 or 2, without significant loss of material.

Electron Microscopy

Samples were fixed in suspension at 0°C and for 30 min with an equal volume of fixative containing 3.4%

glutaraldehyde (prepared from 50% wt/wt, biological grade stock solution, Fisher Scientific Co., Fair Lawn, N. J.) and either 200 mM sodium cacodylate·HCl, pH 7.4, or 50 mM triethanolamine·HCl, pH 7.4. The fixed material was centrifuged at 10,000 g in a Microfuge 152 (Beckman Instruments, Inc., Palo Alto, Calif.). The pellets were postfixed at 0°C for 1 h in 1% OsO₄ in acetate-Veronal buffer, pH 7.4, and stained *en bloc* at 23°C for 1 h with 0.5% uranyl acetate in acetate-Veronal buffer (8). The pellets were then dehydrated in ethanol and embedded in Epon (15). The sections were stained with uranyl acetate (19) and lead citrate (20) before examination in a Siemens Elmiskop 101 at 80 kV.

Negative staining was carried out on unfixed samples with a 2% solution of ammonium molybdate, adjusted to pH 7.0 with NH₄OH (11). Carbon-coated Formvar films were used. Samples were deposited on the grid as a drop and the excess liquid was removed by touching with a filter paper, avoiding complete drying of the film. The grid received several drops of the stain which after ~30 s, was removed by touching with a filter paper. After drying, the grid was viewed as described above.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate of Reduced and Alkylated Proteins

Electrophoresis was performed in 1-mm thick slab gels containing a resolving gel (10–15% acrylamide gradient) and a 5% stacking gel as described by Maizel (16).

PREPARATION OF REDUCED AND ALKYLATED POLYPEPTIDES FOR ELECTROPHORESIS: Nuclei (2 A₂₆₀) or multiple equivalents of nuclear subfractions were precipitated with 2 vol ethanol at −20°C for 12 h. The alcohol precipitate was solubilized during a 15-min incubation at 37°C in 30 µl of a solution of 15% sucrose, 0.02 M Tris·HCl, pH 8.8, 5 mM DTT, 2 mM EDTA, 5% sodium dodecyl sulfate, and traces of bromphenol blue (serving as a tracking dye for electrophoresis). After solubilization, the mixture was incubated in a boiling water bath for 2 min. After cooling to room temperature, 2 µl of a 0.5 M solution of α-iodoacetamide were added and the mixture was incubated again for 1 h at 37°C before being layered into a slot of the slab gel. Rabbit globin, porcine chymotrypsinogen, ovalbumin, bovine albumin and *E. coli* β-galactosidase, reduced and alkylated, were used as standards for molecular weight determinations. After electrophoresis, the slab gel was stained in a solution containing 0.2% Coomassie brilliant blue, 50% methanol, and 10% glacial acetic acid for 2 h and then destained in 50% methanol and 10% acetic acid.

Chemical Analysis of Nuclei and Each Subfraction

After precipitation with cold trichloroacetic acid, analysis was performed by standard techniques for DNA (7), RNA (6), protein (14), and phospholipid (3, 10).

Materials

Bovine pancreatic DNase I, electrophoretically purified, free of ribonuclease, 2,400 Kunitz U/mg was obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Each step in the subfractionation of nuclei leading to isolation of the pore complex-lamina fraction has been monitored by electron microscopy, chemical analysis and polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Figs. 2–5 present electron micrographs of the final pore complex-lamina preparation and of various intermediate fractions. Cross sections of salt-washed nuclear envelopes are shown in Fig. 2 A and B. The material in Fig. 2 A was washed with 500 mM KCl, 50 mM triethanolamine·HCl, pH 7.5, and 5 mM MgCl$_2$ (elimination of Step 3 and modification of Step 4 in Fig. 1), after which most of the ribosomes still remain attached to the outer nuclear envelope membrane. Treatment with higher salt concentration in the absence of MgCl$_2$ led to a removal of ribosomes from the outer nuclear envelope membrane (Fig. 2 B). Both salt conditions result in a near-complete removal of residual chromatin as evidenced by the loss of most of the DNA (from chemical analysis this fraction contains less than 3% DNA) and most of the histone bands on a polyacrylamide gel electropherogram (see Fig. 7, Slot D$_2$Sp). Thus, it can be excluded that the layer of material apposing the inner nuclear membrane in Fig. 2 A and B (referred to as lamina) represents primarily residual chromatin. The electron microscope demonstration of a lamina approximately 150 Å thick in an essentially chromatin-free nuclear envelope fraction is an observation which strongly supports our previous suggestion that the lamina corresponds to a physiological structure, distinct from the inner nuclear envelope membrane.

The membranes can be solubilized by subsequent Triton X-100 treatment while the lamina remains ultrastructurally intact (see Fig. 3). Low, medium, and high magnification electron micrographs of the pore complex-lamina fraction (D$_2$TSp of Fig. 1) are shown in Fig. 3 A–D. At low magnification, the homogeneous nature of this fraction is seen (Fig. 3 A) wherein the lamina with attached pore complexes is readily identifiable. Occasionally, small aggregates of granules (assumed to be contaminants) are observed. At medium magnification (Fig. 3 B) the convoluted lamina with attached pore complexes presents a

spectrum of views of both pore complexes and lamina ranging from tangential (resulting in "frontal" views of pore complexes) to normal (resulting in "lateral" views of pore complexes). Frontal and lateral views of pore complexes are shown at higher magnification in Fig. 3 C and D, respectively. The frontal pore complex images in Fig. 3 C reveal tangential views of the lamina which frequently appears as a network, with a regular substructure.

In sections, it is possible to follow the lamina for several micrometers, suggesting that it is a continuous structure surrounding the whole nucleus. More convincing evidence for this was obtained from examination of the pore complex-lamina fraction with the negative staining technique. As shown in Fig. 4, the pore complex-lamina fraction appears as a partially collapsed and pleated shell with the dimensions of an entire nucleus. In the lower right corner of Fig. 4, this shell is apparently ruptured so that a backfolding reveals a lamina monolayer (lower right-hand corner of Fig. 4) presenting a view of this structure from its nuclear site. A lamina monolayer viewed from this aspect is shown at higher magnification in Fig. 5. The lamina appears to be composed of particles arranged in an irregular network. The appearance of the lamina presented in negative staining could be due to effects of drying which could cause aggregation of its components (compare Fig. 3 C and Fig. 5).

It should be noted that the original procedure for the preparation of the pore complex-lamina fraction involved removal of residual chromatin by 0.3 M MgCl$_2$. Subsequently, however, we found that MgCl$_2$, albeit under more extreme conditions than previously used (i.e., higher concentration and incubation at 37°C), caused disruption of the lamina, leaving at least some pore complexes apparently intact (see Fig. 6 A). A similar fragmentation of the lamina was also observed with other divalent cations, such as Mn^{++} and Ca^{++} (data not shown). On the other hand, incubation in high concentrations of monovalent ions (e.g., 4.0 M LiCl) did not result in lamina disruption. Thus, washing with MgCl$_2$ could lead to a partial fragmentation of lamina, with the fragmented material lost together with the solubilized chromatin during centrifugation. Washing with high concentrations of monovalent ions, on the other hand, avoids such losses.

The lamina is also sensitive to mechanical stress. A mild sonication leads to its fragmentation, leav-
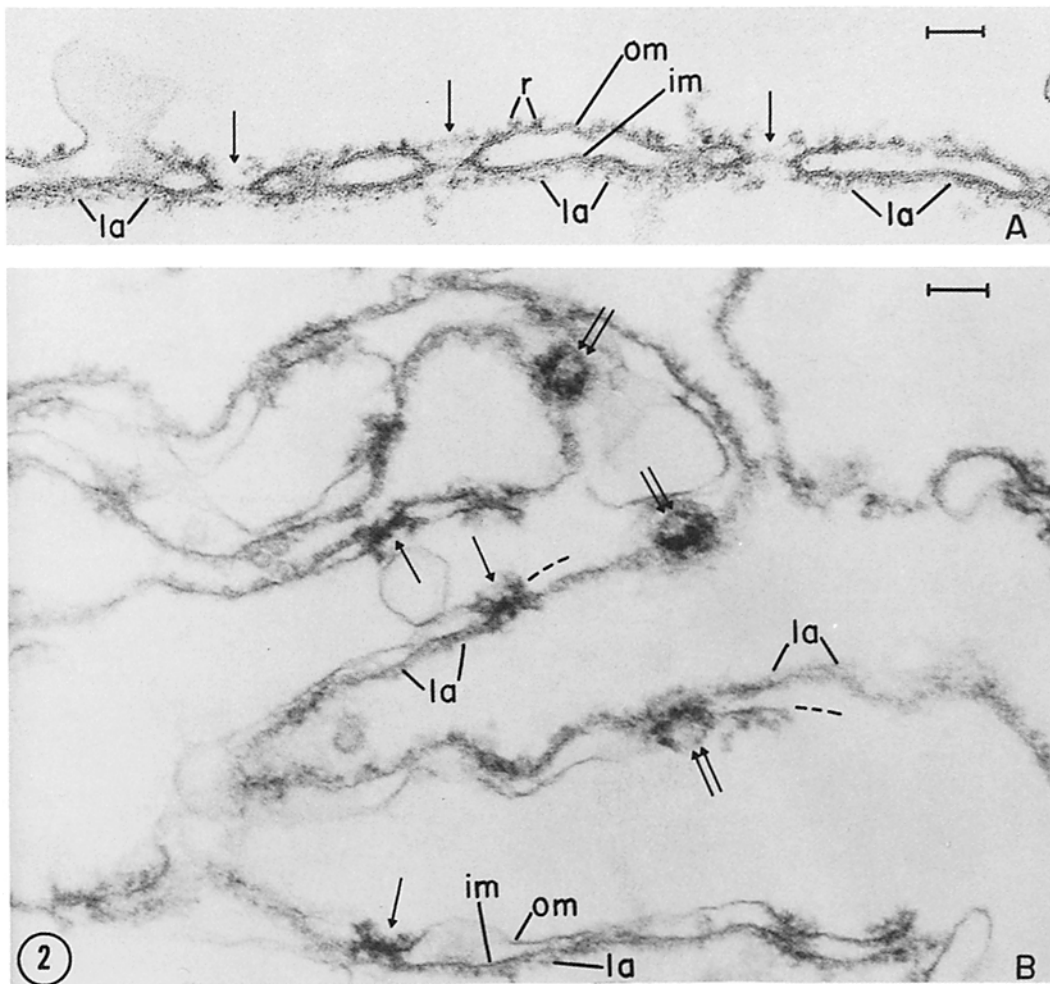
FIGURE 2   Nuclear envelope fraction after a wash with high concentrations of monovalent ions in the presence (A) or absence (B) of MgCl₂. The bars denote 0.1 μm. × 80,000. (A) shows a D₂p fraction (Fig. 1) which was washed with 500 mM KC1, 50 mM triethanolamine·HCl, pH 7.5, and 5 mM MgCl₂. Arrows indicate nuclear pore complexes with arrowheads pointing towards their cytoplasmic aspects. The triple-layered structure of the inner (*im*) and outer (*om*) nuclear membrane with ribosomes (*r*) attached to the latter, as well as a layer of material under the inner membrane, referred to as lamina (*la*), are clearly discernible. (B) shows a D₂Sp fraction, derived from a D₂p fraction, washed with 1.0 M NaCl and 50 mM triethanolamine·HCl, pH 7.5 (see Results). This treatment removed the ribosomes from the other membrane. Both inner (*im*) and outer (*om*) membrane have lost their triple-layered appearance (possibly due to extraction of membrane protein). At some points (indicated by a dashed line), the outer membrane has been torn off. Nuclear pore complexes can be seen either in "lateral" views (indicated by single-bar arrows) or in near "frontal" views (indicated by a double-bar arrow) with arrowheads again pointing toward their cytoplasmic aspects. The lamina (*la*) again appears as a characteristic associate of the inner nuclear membrane.

ing the pore complexes more or less intact (Fig. 6 B).

The composition of various fractions with respect to protein, DNA, RNA, and phospholipids was monitored at all steps of purification. The data obtained with the modified procedure were similar to those previously reported (2). The pore complex-lamina fraction (D₂TSp in Fig. 1) is com-

posed of 95% protein, 3% DNA, and 2% RNA, and does not contain any measurable phospholipid; 2.5% of the nuclear protein is recovered in the pore complex-lamina fraction.

Since the pore complex-lamina fraction consists mostly of protein with three predominating polypeptides (2), it was desirable to analyze the polypeptides present in each step of the subfractionation by polyacrylamide gel electrophoresis in Na dodecyl SO_4 (Fig. 7) in order to obtain semiquantitative recovery data. The analyzed fractions were electrophoresed as multiple equivalents (indicated by numbers in slots of Fig. 7) of the starting material, i.e. of isolated nuclei. Supernates as well as pellets (designated s and p, respectively, in Figs. 1 and 7) were examined in order to follow fractionation in a balance sheet manner. The banding pattern of nuclei (see slot N) is complex, with the histone bands constituting the major polypeptides (indicated by dots in slot N). After the first DNase treatment, about half of the histones are removed and found in the supernate, together with many other polypeptides (slot D_1s). This results in a clearly detectable enrichment of three polypeptides, designated the triplet polypeptides, in the sedimented material (slot D_1p, triplet marked with a bar). The second DNase treatment caused removal of more than half of the remaining histones (compare slot D_2s with D_2p) and further enrichment of the triplet polypeptides. Treatment with Triton X-100 (Step 3, Fig. 1) resulted in the solubilization of a group of minor bands mainly in the 45,000–55,000-mol wt range, probably representing membrane proteins. Almost complete removal of the histone bands (slot D_2TSp vs. D_2TSs) was achieved after the salt wash (see step 4, Fig. 1). The final pore complex-lamina fraction (slot D_2TSp) contains the triplet polypeptides as the major components together with a large number of minor bands, but is essentially free of the histone bands. Reversal of Steps 3 and 4, i.e. a salt wash (slot D_2Ss and D_2Sp) followed by treatment with Triton X-100 (slot D_2STs and D_2STp), yields essentially the same results with regard to the polypeptide banding pattern of the final fraction.

DISCUSSION

The work described in this paper provides a more detailed account including subsequent modifications of a procedure previously published by this laboratory for the subfractionation of isolated rat liver nuclei to obtain a preparation of nuclear pore complexes attached to a lamina (2). A problem which remained largely unresolved by our previous studies was whether the isolated lamina represented a distinct submembranous structure corresponding to a peripheral layer, observed in sections of nuclei and variously referred to as fibrous lamina (9), dense lamella (12), or zonula nucleum limitans (18), or whether it resulted from an artifact of subfractionation. It was conceivable, for instance, that treatment of nuclear envelopes with Triton X-100 resulted in incomplete solubilization of the envelope membrane proteins and that these nonsolubilized proteins in an aggregated form presented the ultrastructural appearance of a 150-Å thick lamina. Our present results, however, are in strong support of our previous conclusion (2), namely that the isolated lamina is a structure distinct from, albeit associated with the membrane, and represents the fractionation equivalent of a peripheral layer beneath the inner membrane of the nuclear envelope. Such a submembranous lamina is clearly discernible in sections of nuclear envelopes which have been salt washed only (Fig. 2) and have not been exposed to Triton X-100. In sections of this fraction, the large amount of material which constitutes the lamina cannot be attributed to residual, DNase-resistant, peripheral heterochromatin, since the salt-washed envelopes not only contain little DNA but also are mostly free of histones. The ultrastructural orientation of the lamina with respect to the pore complexes seen after removal of the envelope membranes by Triton X-100 further supports the notion that it is derived from a submembranous rather than from an intramembranous structure. In so-called "lateral" views of the pore complexes (see Fig. 3 B and 3 D) the lamina is seen to connect with those components of the pore complex which are most proximal to the nuclear interior, the same orientation which is observed in the presence of the membrane (Fig. 2 B). It is clear, however, that our ultrastructural data do not exclude the possibility that the isolated submembranous lamina does contain proteins that are derived from the apposing membrane and remain with the lamina after treatment with Triton X-100.

Our results indicate that the lamina extends over the entire submembranous nuclear surface, in a shell-like fashion (see Fig. 4). Tangential sections of the lamina suggest a regular substructure (Fig. 3 C). Thus, the lamina could represent a polymeric crystalline assembly (comparable, e.g.,
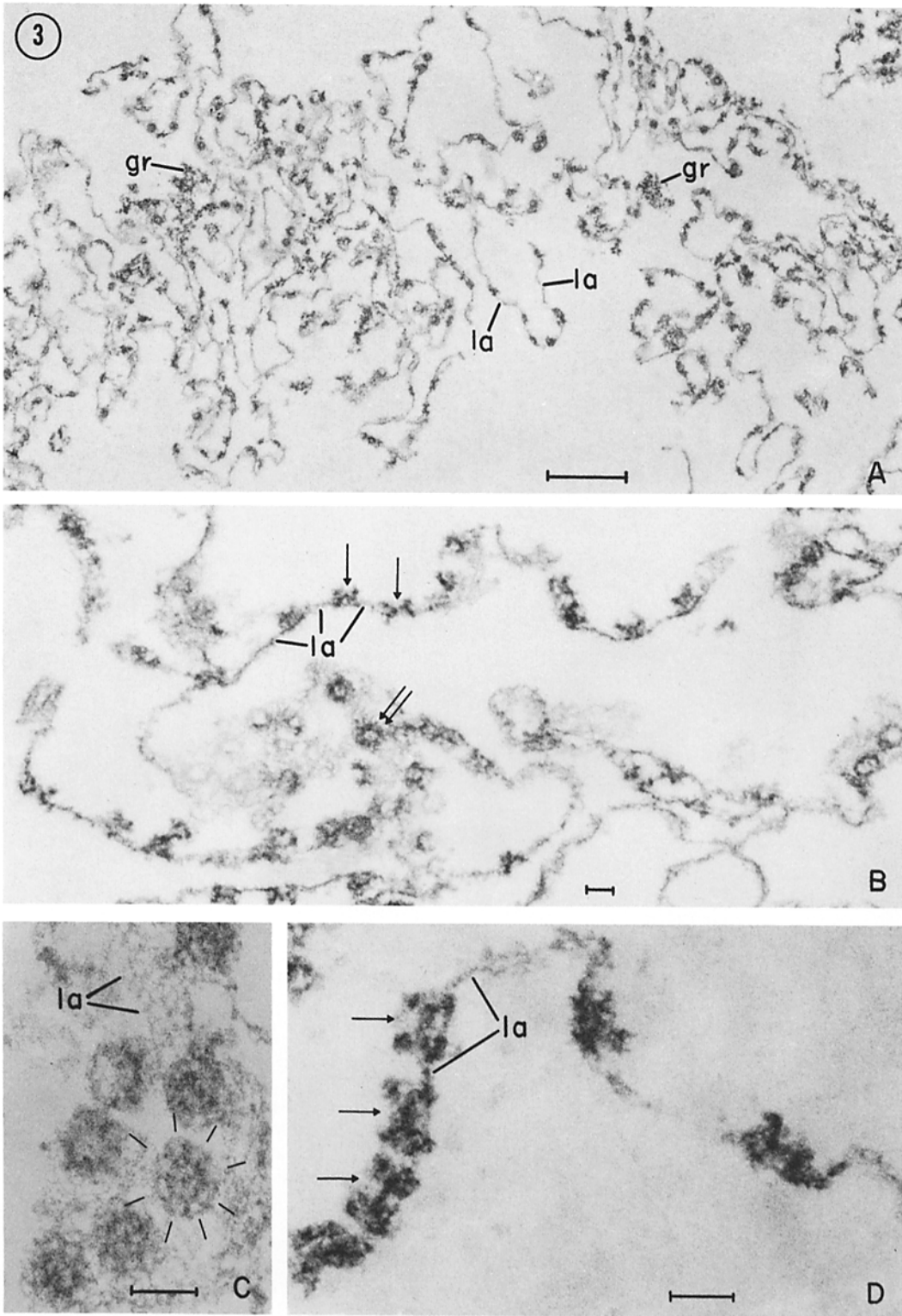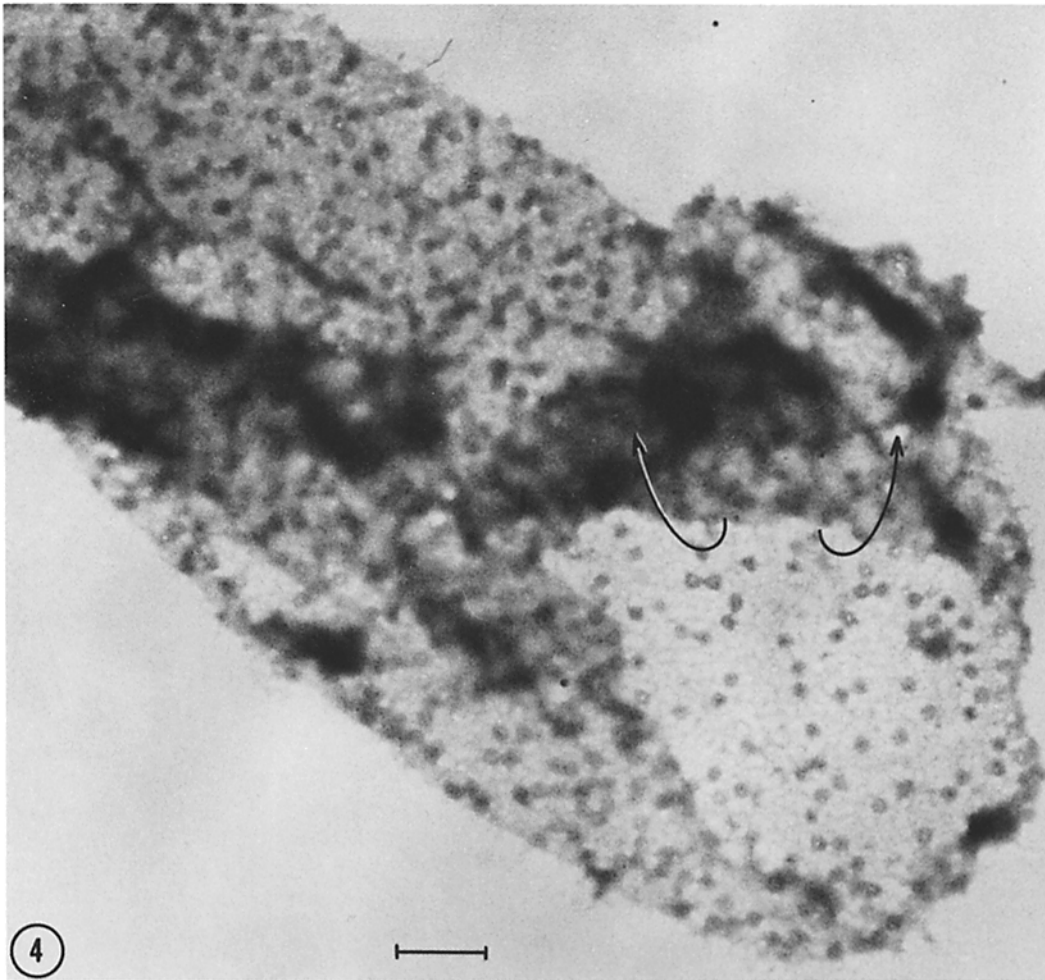
FIGURE 4   Negatively stained pore complex-lamina fraction (see Results). × 24,000. The bar denotes 0.5 μm.

FIGURE 3   Nuclear pore complex-lamina fraction (D₂TSp, Fig. 1) resulting from a successive salt and Triton X-100 treatment of the nuclear envelopes. The low magnification (× 12,000:bar denotes 1.0 μm) survey (A) shows a large number of pore complexes connected by a lamina (la). Occasionally, granules (gr) of uncertain origin can be seen. A medium magnification (× 44,000: bar denotes 0.1 μm) view (B) shows nuclear pore complexes in "lateral" (single arrow) and "frontal" (double arrow) views. Frontal views often reveal the characteristic annular subunits (double arrow). Frontal (C) as well as lateral (D) views of pore complexes are shown at higher magnification (× 100,000: bars denote 0.1 μm). The characteristic eight annular subunits of the nuclear pore complex are clearly recognized in (C) (indicated by spikes). A tangential section of the lamina (la) in (C) suggests a somewhat regular structure resembling a honeycomb. In lateral views of the pore complexes (D), the lamina appears as an ~150 Å thick layer interconnecting the pore complexes at their most nucleus-proximal aspect. Arrowheads point to cytoplasmic opening of the pore complexes.
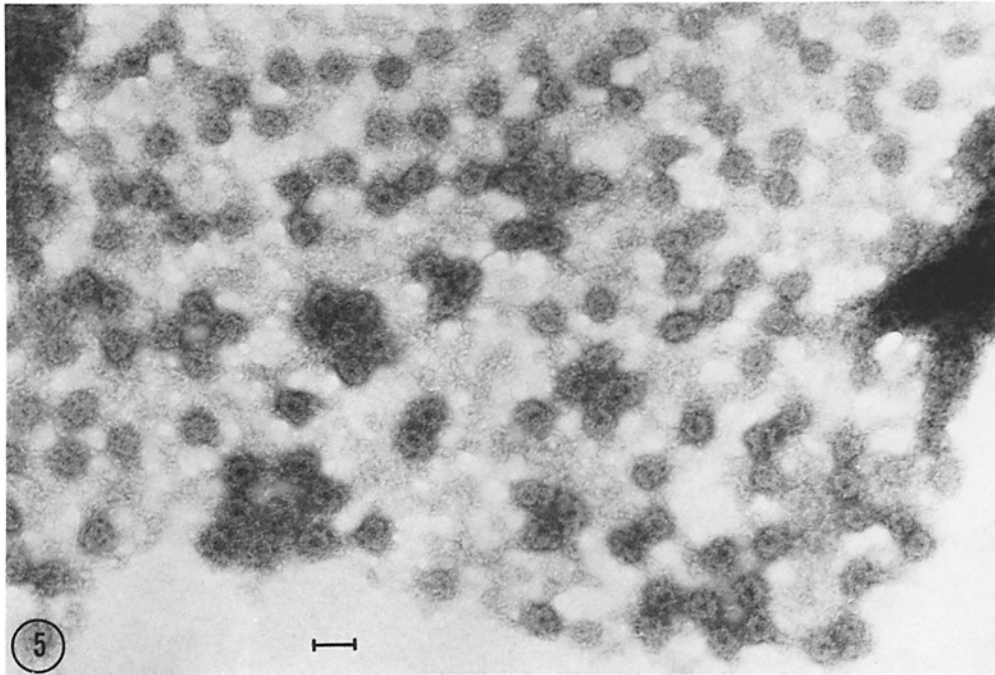
FIGURE 5 Negatively stained pore complex-lamina fraction deposited on the grid as a monolayer. ×
52,000. The bar denotes 0.1 μm.

to actin and myosin filaments or microtubules) composed of a small number of monomeric subunits. Consistent with this notion is the presence of three predominant polypeptides (see Fig. 7, slots D₂STp and D₂TSp) in the pore complex-lamina fraction. This triplet could compose the lamina while the multitude of other minor bands could constitute the protein components of the pore complex proper. However, in the absence of further data, this matter remains unresolved.

It is not clear at present whether the three major polypeptides observed in our pore complex-lamina fraction are identical to three major polypeptides observed by Berezney and Coffey in their "nuclear protein matrix" fraction (4) from rat liver. The reported ultrastructural analysis (4) of this nuclear protein matrix fraction consisted of low magnification electron micrographs revealing structures with the size and shape of a nucleus containing a large amount of amorphous material. Although our subfractionation procedure is similar to that of Berezney and Coffey and therefore could have recovered similar components (i.e. our triplet could be identical to their three major polypeptides), we have been unable so far to identify in our pore complex-lamina fraction any structures

which resemble the extensive nuclear protein matrix structure reported by Berezney and Coffey. However, we cannot exclude the possibility that the lamina extends into the interior of the nucleus constituting the equivalent of a nuclear protein matrix. If the connections between the peripheral lamina and its putative centripetal extensions were, e.g., severed during our subfractionation, they would be absent from our pore complex-lamina fraction. From the polypeptide analysis shown in Fig. 7, this could have occurred only during the preparation of nuclear envelopes by the two incubations with DNase (Steps 1 and 2, Fig. 1); not, however, after salt and detergent treatment (Steps 3 and 4, Fig. 1) since the recovery of the triplet polypeptides is almost complete after these steps (see D₂Ts vs. D₂Tp and D₂TSs vs. D₂TSp in Fig. 7).

The observed fragmentation of the lamina after incubation of the pore complex-lamina fraction in MgCl₂ (see Fig. 6) induced us to modify our original procedure by replacing MgCl₂ with NaCl as a means of removing remaining chromatin. This modification resulted in highly reproducible data as far as recovery, composition, and ultrastructure were concerned. Previously, using a MgCl₂ wash,
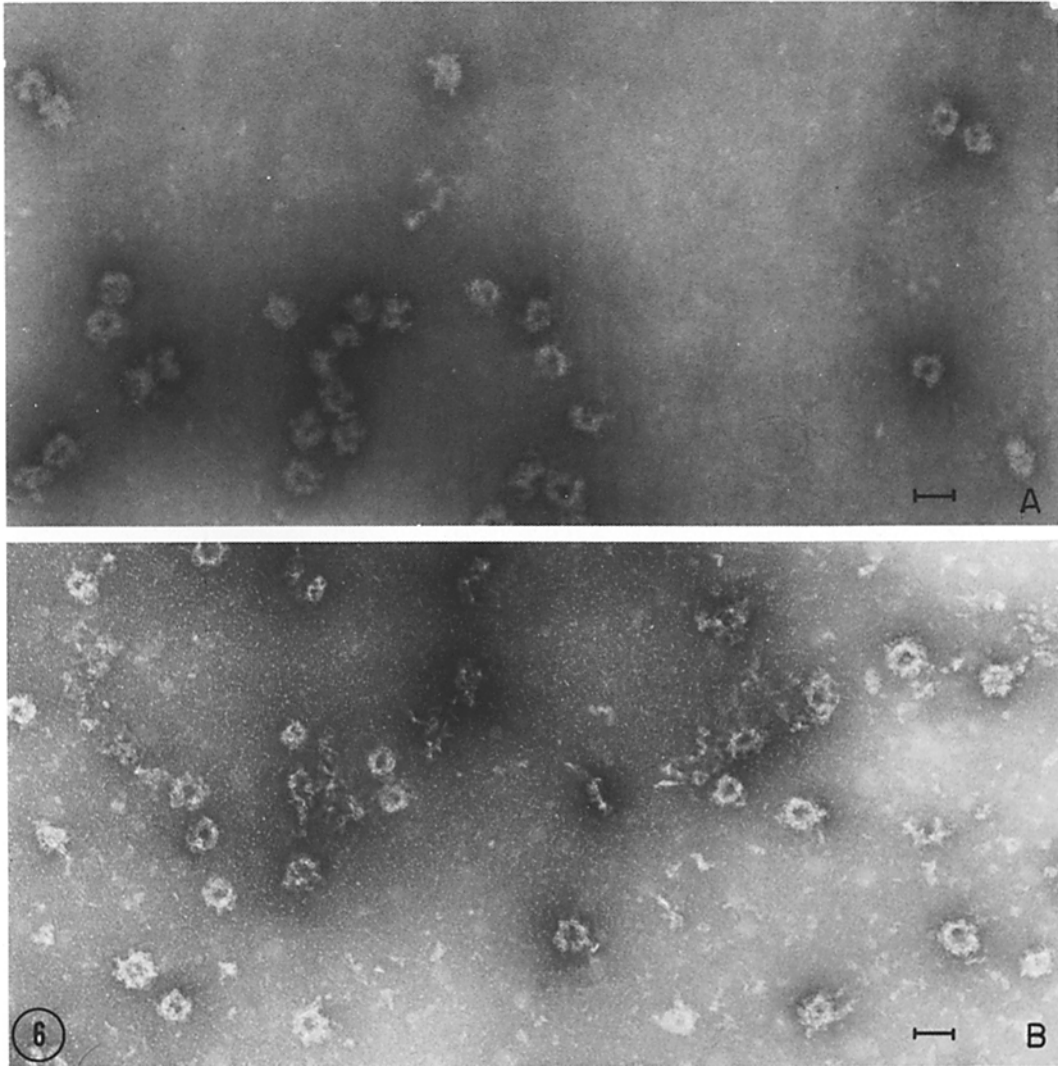
FIGURE 6   Disrupted and negatively stained pore complex-lamina fraction. × 52,000. The bars denote 0.1 μm. (A) Disruption resulted from an incubation of a resuspended D_2TSp fraction in 0.5 M $MgCl_2$ and 50 mM triethanolamine·HCl, pH 7.5, for 10 min at 37°C. (B) Disruption caused by sonication of a D_2TSp fraction resuspended in 500 mM NaCl, 50 mM triethanolamine·HCl, pH 7.5, and 5 mM $MgCl_2$.
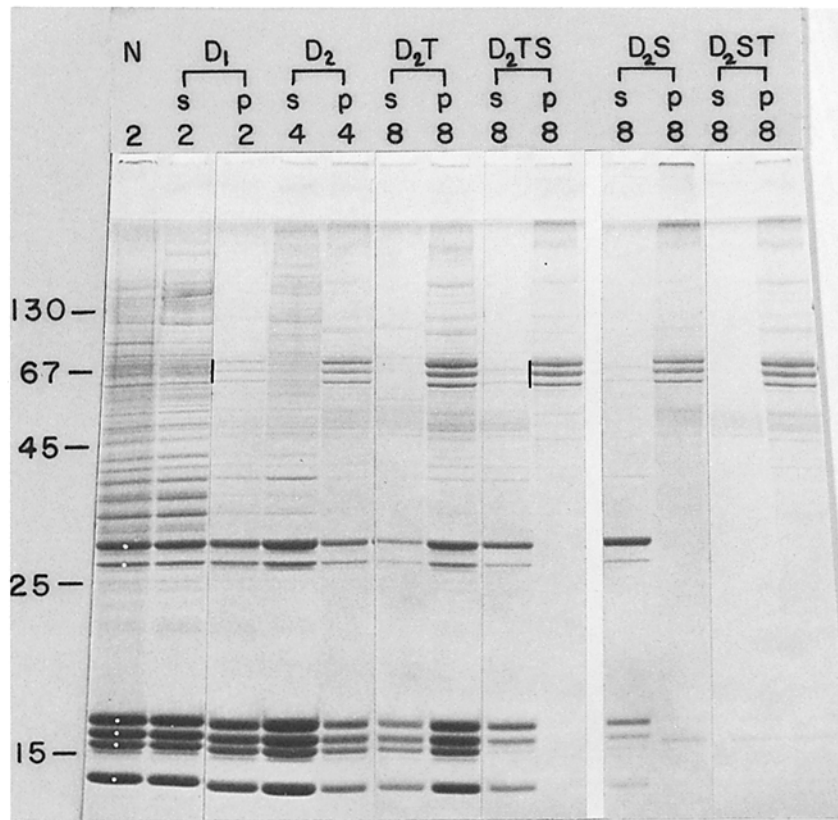
FIGURE 7   Polyacrylamide gel electrophoresis in Na dodecyl SO_4 of reduced and alkylated polypeptides contained in nuclei and subfractions (see Materials and Methods, and Fig. 1). Numbers under N or s and p (s for supernate, p for pellet) refer to $2.0\,A_{260}$ U of nuclei (N), or subfractions derived from 2, 4, and $8\,A_{260}$ U of nuclei, respectively. Numbers to the left of slot N refer to mol wt × 1,000 of standards (see Materials and Methods). Histone bands are indicated by dots. Triplet of bands, characteristic of the pore complex-lamina fraction, is indicated by a vertical bar to the left of slot D_1p and D_2TSp.

we had experienced variable losses of material, most probably resulting from the Mg^{++}-induced lamina fragmentation and incomplete recovery of the components during subsequent centrifugation.

The Mg^{++} (and other divalent cations)-induced preferential disruption of the lamina suggests a possible route for subfractionation of the pore complex-lamina fraction into pore complexes and the components of the disassembled lamina. Work is now in progress to use this and other approaches to achieve this goal.

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