

IMMUNOCYTOCHEMICAL LOCALIZATION OF THE MAJOR POLYPEPTIDES OF THE NUCLEAR PORE COMPLEX-LAMINA FRACTION

Interphase and Mitotic Distribution

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ABSTRACT

This laboratory has previously isolated a fraction from rat liver nuclei consisting of nuclear pore complexes associated with the proteinaceous lamina which underlies the inner nuclear membrane. Using protein eluted from sodium dodecyl sulfate (SDS) gels, we have prepared antibodies in chickens to each of the three predominant pore complex-lamina bands. Ouchterlony double diffusion analysis shows that each of these individual bands cross-reacts strongly with all three antisera. In immunofluorescence localization performed on tissue culture cells with these antibodies, we obtain a pattern of intense staining at the periphery of the interphase nucleus, with little or no cytoplasmic reaction. Electron microscope immunoperoxidase staining of rat liver nuclei with these antibodies labels exclusively the nuclear periphery. Furthermore, reaction occurs in areas which contain the lamina, but not at the pore complexes. While our isolation procedure extracts the internal contents of nuclei completely, semiquantitative Ouchterlony analysis shows that it releases negligible amounts of these lamina antigens. Considered together, our results indicate that these three bands represent major components of a peripheral nuclear lamina, and are not structural elements of an internal "nuclear protein matrix."

Fluorescence microscopy shows that the perinuclear interphase localization of these lamina proteins undergoes dramatic changes during mitosis. Concomitant with nuclear envelope disassembly in prophase, these antigens assume a diffuse localization throughout the cell. This distribution persists until telophase, when the antigens become progressively and completely localized at the surface of the daughter chromosome masses. We propose that the lamina is a biological polymer which can undergo reversible disassembly during mitosis.

KEY WORDS nuclear envelope lamina · chicken antibodies · immunofluorescence microscopy · immunoperoxidase electron microscopy · mitosis · reversible lamina disassembly

The nuclear envelope is a complex eukaryotic organelle, whose biochemistry and cellular physiology are largely undefined at the present (see references 18, 19, and 29 for reviews). It func-

tions to delimit a distinct compartment in the cell (the nucleus), which contains most of the cellular DNA, together with its associated transcriptional and replicative machinery. A characteristic architectural feature of the nuclear envelope is a double membrane structure, consisting of inner and outer nuclear membranes joined at "pores." The nuclear pore occurs in association with material of a regular ultrastructural character, to comprise the "pore complex" (59). Pore complexes are believed to constitute the major passageways of molecular movement between the nucleus and the cytoplasm (18, 19, 29).

The nucleus of numerous eukaryotic cell types on diverse levels of evolution contains a distinct electron-dense layer interposed between the inner nuclear membrane and the peripheral chromatin elements. This structure has been termed the fibrous lamina (17, 21), zonula nucleum limitans (38, 46), dense lamella (27), or lamina (2). In certain lower eukaryotes (6, 14, 24, 45), the fibrous lamina forms a prominent "honeycomb layer" subjacent to the nuclear envelope, occasionally up to 300 nm thick (39, 45). The corresponding ultrastructural entity in mammalian cells is represented by a finely textured layer of thinner dimensions, usually <80 nm in diameter (17, 21, 27, 38, 43, 44, 46). Although many eukaryotic cell types show no visible nuclear lamina, we feel that the functional counterpart may be ubiquitous (2, 15), but often too thin to be recognized by conventional electron microscopy on whole cells.

Most of the functions which have been proposed for the lamina relate to an involvement in nuclear structure. Originally, it was suggested that the fibrous lamina may serve as a skeletal support for the nuclear envelope (11, 39). Recent cell fractionation studies indicate that the lamina may provide an attachment point for nuclear pore complexes in the nuclear envelope (1, 2). In addition, we feel that the lamina may be involved in the large-scale ultrastructural organization of chromatin in the nucleus, possibly by binding defined DNA or chromatin elements. Finally, the results which we present in this paper suggest that the lamina may have a close relationship to the dynamics of the nuclear envelope during the cell cycle.

In this report,¹ we characterize antibodies raised

¹ Most of the data contained in this paper was presented in San Diego, California, at the 17th annual meeting of the American Society for Cell Biology, 15-18 November 1977 (20).

to each of the three prominent polypeptide bands which are components of the pore complex-lamina fraction. These bands, which migrate between 60,000 and 70,000 daltons on SDS gels, are found to cross-react strongly with each other. We have localized the antigens in tissue culture cells by immunofluorescence, and in isolated nuclei by electron microscope immunoperoxidase staining. Our results show that these antigens are located only at the periphery of the normal interphase nucleus, and do not occur in the nuclear interior. Recently, several groups (8, 13) have suggested that the inner membrane-associated lamina is the external component of a structural "nuclear protein matrix," which extends throughout the nuclear interior and contains the same major proteins as the peripheral lamina. Our localization results, however, argue that at least a major portion of the lamina is biochemically distinct from an internal nuclear matrix.

The striking pattern which we observe in immunofluorescence microscopy relating to the cellular redistribution of these antigens during mitosis suggests that the behavior of the lamina is closely related to the disassembly and reconstruction of the nuclear envelope during cell division. Since the isolated rat liver lamina is an insoluble structural entity, we suggest that a major portion of the lamina may be a polymer of these prominent cross-reacting antigens, which undergoes reversible disassembly during mitosis.

MATERIALS AND METHODS

Preparation of Electrophoretically Eluted Protein Bands for Immunization

The pore complex-lamina fraction was obtained as previously described (15), with a slight modification. Since DNase I is unstable in distilled water, it was dissolved instead at a concentration of 1 mg/ml in a buffer consisting of 0.25 M sucrose, 0.05 M triethanolamine-HCl, pH 7.4, 0.025 M KCl, and 0.005 M MgCl₂, and frozen in small aliquots. These aliquots were thawed before use, and were diluted to the final concentrations described (15) for the two DNase digestions.

Apparent molecular weights of the prominent pore complex-lamina bands were estimated by electrophoresis of the pore complex-lamina fraction on a straight 10% sodium dodecyl sulfate (SDS)² gel, with a 5% stacking

² *Abbreviations used in this paper:* HRP, horseradish peroxidase; PBS, Dulbecco's phosphate-buffered saline; PD, Dulbecco's phosphate-buffered saline without MgCl₂ and CaCl₂ containing 0.02% Na₂S₂O₈; PMSF, phenylmethyl sulfonyl fluoride; SDS, sodium dodecyl sulfate;

gel, as described (35). Mobilities (in the 10% separating gel) of the pore complex-lamina bands were compared to those of common molecular weight standards, in a log (molecular weight) vs. mobility plot. For molecular weight standards, we used β -galactosidase, 130,000; phosphorylase A, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; DNase I, 31,000; and chymotrypsinogen, 26,000.

For preparative SDS gel electrophoresis, the pore complex-lamina fraction was solubilized in SDS, reduced, alkylated, and electrophoresed on 10–15% slab gels as previously described (35). Usually, $\sim 1,250 \mu\text{g}$ of protein was applied to a single gel with separating gel dimensions of $30 \times 19 \times 0.2 \text{ cm}$. The 5% stacking gel was polymerized without a slot-forming comb. After electrophoresis, the preparative gels were fixed, stained, and destained (15), and subsequently dried by vacuum after a final wash in 40% methanol containing 5% glycerol. Using scissors, P70, P67, and P60 (see Fig. 1) were separately excised from these dried slab gels. The gel pieces were then swelled in electrode buffer (35) containing 5% SDS for 30 min at room temperature. After draining the liquid from the swollen gel pieces, they were further incubated at 37°C for 2–4 h. Electrophoretic elution in electrode buffer (35) containing 0.1% SDS followed.

For quantitation of eluted protein, samples were precipitated in 20% TCA for 1–2 h on ice. Coomassie Blue stain was extracted with cold 90% acetone containing 0.1 N HCl, and the pellets were solubilized in 1 N NaOH at room temperature before protein analysis by the Lowry method (32).

Before emulsifying antigen samples for immunization, the electrophoretically eluted protein solution was briefly dialyzed against two changes of distilled water to remove electrophoresis salts. 1 vol of protein solution was mixed with 1.2 vol of Freund's adjuvant (complete or incomplete), and 2 M KCl was added dropwise until vigorous mixing with a screwdriver attachment in a motor-driven homogenizer yielded a stable emulsion.

Production and Fractionation of Antisera

Initially, we endeavored to obtain antibodies in rabbits to P70, P67, and P60. In our hands, this species did not yield a strong immune response to these antigens. However, when we immunized chickens with these eluted bands, we obtained comparatively high titer antibodies.

We used female white Leghorn chickens (4–6 mo old) for immunization. Animals were administered ~ 100 – $200 \mu\text{g}$ of emulsified antigen each injection, in a total volume of $< 3 \text{ ml}$. Antigens were injected by an intramuscular route, in both legs and in the breast. We

SuCNM, 0.25 M sucrose, 0.05 M Na cacodylate, pH 7.3, 0.1 M NaCl, 0.005 M MgCl_2 , and 100 U/ml Trasylol.

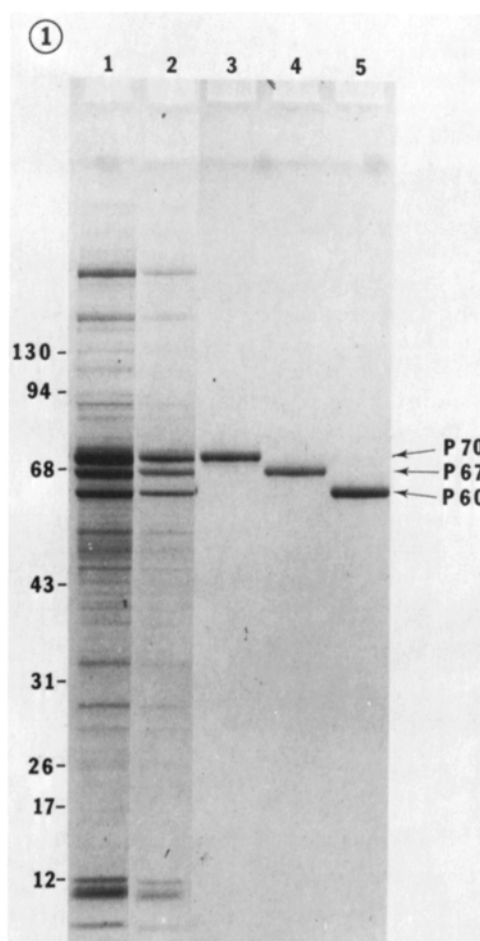


FIGURE 1 SDS polyacrylamide gel electrophoresis of the pore complex-lamina fraction and of eluted protein bands. Protein fractions were electrophoresed on a 7.5–15% linear gradient gel. The pore complex-lamina fraction (lane 1, $50 \mu\text{g}$; lane 2, $20 \mu\text{g}$) contains three prominent bands, which are designated P70, P67, and P60. After separation of reduced and alkylated pore complex-lamina proteins on preparative SDS slab gels, P70, P67, and P60 were electrophoretically eluted. Aliquots were boiled in 5% SDS with 20 mM dithiothreitol for 1 min, and reelectrophoresed (lanes 3, 4, and 5, respectively; 3 – $5 \mu\text{g}$ protein each). Numbers to the left of lane 1 indicate the migration of molecular weight standards, $\pm 1,000$.

employed the following immunization schedule: The first injection was in complete Freund's adjuvant, followed $2\frac{1}{2}$ wk later by a second injection, also in complete Freund's adjuvant. After a period of $3\frac{1}{2}$ wk, a third injection in incomplete Freund's adjuvant was administered. Animals were bled 9 days and 16 days later.

Booster immunizations were given in incomplete Freund's adjuvant at intervals of 1–2 mo. Animals were bled as described above. Sera were stored in the presence of 0.02% NaN₃ at 4°C, or were maintained frozen at –20°C.

To prepare the IgG fraction from chicken sera, we used a procedure involving precipitation of antibodies with Na₂SO₄, followed by gel filtration on Sephadex G-200 (7). After this purification, concentrated antibodies were dialyzed against Dulbecco's phosphate-buffered saline without MgCl₂ and CaCl₂ containing 0.02% NaN₃ (PD). They were stored at –80°C.

Rabbit anti-chicken IgG was obtained in 4–6 kg female New Zealand white rabbits. Chromatographically purified chicken IgG (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) was dissolved in isotonic saline, emulsified with Freund's adjuvant, and injected in a subcapsular location. 3 wk after a primary injection in complete Freund's adjuvant (5 mg protein), animals were given a second injection in incomplete Freund's adjuvant (2.5 mg protein). Bleeding occurred at weekly intervals thereafter, and booster injections in incomplete Freund's adjuvant (2.5 mg protein) were administered as necessary.

For preparation of the IgG fraction from rabbit serum, the latter was dialyzed against 0.01 M potassium phosphate buffer, pH 7.2, and was passed over a column of diethylaminoethyl (DEAE) cellulose equilibrated with the same buffer (all at 4°C). The unbound fraction (containing IgG) was concentrated, dialyzed against PD, and stored at –80°C.

Adsorption of Chicken Anti-P70 against Electrophoretically Eluted P70

Eluted P70 was precipitated with TCA, pelleted, and extracted with acetone-HCl as described above. This material was dissolved in 0.1 M sodium borate, pH 8.5, containing 2.5% SDS by boiling, and coupled to CNBr-activated Sepharose 4B in the same buffer. The coupling reaction yielded a final concentration of ~100 µg of conjugated P70 per 100 µl of packed gel. A small column was made in the tip of a Pasteur pipette, containing ~100 µl of P70-Sepharose 4B conjugate equilibrated with PD. After applying 50 µl of chicken anti-P70 IgG (10 A₂₈₀U/ml) at 4°C, the column was washed with PD. The unbound protein is the "adsorbed" fraction. Bound IgG was eluted with 3 M NaSCN in 0.05 M sodium phosphate, pH 7.4, at room temperature. This eluted protein was immediately desalted on a Sephadex G-25 column in PD.

Ouchterlony Double Diffusion Analysis with Chicken Antibodies

Ouchterlony plates were prepared by pouring 4 ml of molten agarose solution on a 5 × 5 cm glass slide. The agarose solution consisted of 1% agarose, 0.05 M trieth-

anolamine-HCl, pH 7.4, 1.5 M NaCl, and 1% Triton X-100. Sample wells (3 mm internal diameter) had a center-to-center spacing of 9 mm.

For the assay of antibodies to electrophoretically eluted proteins, antigen samples were prepared from pellets of pore complex-lamina material, or from a TCA/acetone precipitate of electrophoretically eluted proteins. Samples were solubilized by boiling for 2 min in 2.5% SDS, 0.02 M triethanolamine-HCl, pH 7.4. After cooling, an equal volume of 10% (wt/vol) Triton X-100 was added, to yield "SDS + Triton" solubilized samples.

Ouchterlony plates were allowed to develop for 30–35 h at room temperature in a humid chamber. They were then washed over a period of at least 3 days in several changes of PD. After a final wash in distilled water, plates were dried by compression under sheets of filter paper. The dried plates were stained briefly with 0.2% Coomassie Brilliant Blue in 50% methanol, 10% acetic acid.

Semiquantitative Ouchterlony analysis was employed to assay for the presence of P70-related antigens in unfractionated nuclei and in the supernate and pellet fractions obtained during preparation of nuclear envelopes by DNase digestion. Aliquots of these fractions were precipitated at 0°C with 10% TCA, diluted with water to 5% TCA, and pelleted. The aliquot consisting of undigested nuclei was first treated with 25 µg/ml of DNase I for 10 min at room temperature before precipitation. Precipitated samples were then solubilized by boiling for 2 min in 2.5% SDS containing 0.5 M triethanolamine-HCl, pH 8.5, cooled, and diluted with an equal volume of 10% Triton X-100. This constitutes the SDS + Triton solubilized material in these experiments. Ouchterlony plates were developed as described above.

If the precipitin reaction for these plates was terminated by washing the plates in PD alone, it was found that a heavy nonspecific precipitate would appear during washing around all antigen wells that initially contained a large quantity of protein. To minimize this problem, plates were first washed for 20 h at room temperature in PD containing 1% Triton X-100, 0.5% SDS, followed by PD alone. This modification greatly diminished the appearance of nonspecific precipitation during washing, without visibly reducing the intensity of specific precipitin lines. Plates were dried and stained as described above.

Indirect Immunofluorescence Staining of Tissue Culture Cells with Chicken Antibodies

Immunofluorescence microscopy was performed on K22 cells, a line derived from an adult Sprague-Dawley rat by Dr. Bernard Weinstein (Columbia University, New York). Cells were maintained in Ham's F12 medium containing 10% fetal calf serum, 75 U/ml penicil-

lin, and 75 $\mu\text{g/ml}$ streptomycin. After trypsinization and plating on cover slips, cells were grown for 48–72 h before each experiment.

In this paper, we present the results of two separate immunofluorescence staining procedures. One (formaldehyde/acetone) (22, 31) provides a substantially stronger fixation than the other (ethanol-acetic acid) (56). In the formaldehyde/acetone procedure, cover slips were rinsed briefly at 37°C in Dulbecco's phosphate-buffered saline (PBS), and fixed in PBS containing 4% formaldehyde for 15 min at room temperature. Cover slips were then immersed in absolute acetone at -20°C for 5 min, and subsequently rinsed well in PD. All following manipulations were conducted at room temperature. Cover slips were drained, and overlaid with chicken antiserum or chicken IgG diluted to an appropriate concentration in a solution containing 90% nonimmune rabbit serum, 10% 3.5 M NaCl. Usually, chicken serum was diluted 1:50 in this solution, while purified chicken IgG was diluted to a final concentration less than or equal to 0.2 $A_{280}\text{U/ml}$ (from a 10–20 $A_{280}\text{U/ml}$ concentrate). After an incubation of 30 min in a humid chamber, cover slips were rinsed well in PD. They were then drained, and overlaid with rhodamine-conjugated rabbit anti-chicken IgG which was diluted 1:6 in nonimmune rabbit serum. To reduce background, the rhodamine-conjugated rabbit antibodies had been previously adsorbed against an SDS-solubilized rat liver homogenate coupled to Sepharose 4B, and before use centrifuged at 100,000 g for 15 min. After an incubation for 30 min in a humid chamber, the cover slips were rinsed in PD, mounted on glass slides over a drop of glycerol:PD diluted 1:1, and examined.

When chicken antiserum or chicken IgG was diluted in PD alone for immunofluorescence staining of cells fixed by the formaldehyde/acetone procedure, we obtained an unacceptably high nonspecific background. This background could be reduced to a satisfactory level by diluting the antibodies in high salt (e.g., 0.5 M NaCl) or in nonimmune rabbit serum. We incorporated both of these conditions for the chicken antibody incubation when using the formaldehyde/acetone fixation.

For the ethanol-acetic acid procedure, rinsed cover slips were fixed for 20–30 min at 0°C in 95% ethanol, 1% acetic acid. After washing in PD, cover slips were drained, and overlaid with chicken antiserum or chicken IgG diluted in PD. They were then incubated for 30 min at room temperature in a humid chamber. After a rinse in PD, cover slips were drained, and overlaid with rhodamine-conjugated rabbit anti-chicken IgG diluted 1:6 in nonimmune rabbit serum. After a further 30-min incubation in a humid chamber, the cover slips were washed in PD and mounted as described above before examination. We observed that this fixation procedure gives a substantially more intense fluorescence staining with the same antibody dilution, compared to the formaldehyde/acetone procedure. There is also a significantly lower background, but a noticeably poorer preservation of cells.

A number of other fixation procedures was tested in addition to the two described in this paper. All gave results identical to the formaldehyde/acetone procedure, with respect to the staining of the lamina antigens. These procedures include: fixation in 4% formaldehyde at room temperature followed by treatment with 1% Triton X-100, fixation in 4% formaldehyde at room temperature, followed by a graded series of acetone solutions at 0°C (acetone:PD 1:1, absolute acetone, acetone:PD 1:1, all steps for 5 min), and fixation in absolute acetone for 5 min at -20°C .

Specimens were examined with a Zeiss photomicroscope III using epifluorescence optics. Photomicrographs were taken with a Ph2 Neofluar 40/0.75 objective, using Kodak Tri X Pan film developed at an ASA rating of 1,200 with Acufine developer. Fluorescence exposure times were 1–3 min.

Indirect Immunoperoxidase Staining of Rat Liver Nuclei for Electron Microscopy

We prepared horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG according to the procedure of Avrameas and Ternyck (4). 20 mg of glutaraldehyde activated HRP was incubated with 6.5 mg of DEAE-purified rabbit anti-chicken IgG antibody, and the resulting conjugate was purified on a 100×2.5 cm Sephadex G-200 column equilibrated with PD. Fractions corresponding to the monomer peak of HRP-rabbit anti-chicken IgG conjugate were pooled, concentrated, and stored at 4°C until use. Our material had an $A_{280}\text{U}/A_{408}\text{U}$ ratio of 2.35.

Rat liver nuclei were obtained as previously described (9). They were suspended in 0.25 M sucrose, 0.05 M triethanolamine, pH 7.4, 0.025 M KCl, and 0.005 M MgCl_2 to a concentration of 20 $A_{280}\text{U/ml}$, and Triton X-100 was added from a 20% (wt/vol) stock to a final concentration of 2%. After a 5-min incubation at 0°C , the nuclei were centrifuged onto cover slips with a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa.). In certain cases, the nuclei were fixed by a 5-min incubation in absolute acetone at -20°C immediately after centrifugation. Nuclei were stored in cold buffer until use.

Cover slips were rinsed in a buffer containing 0.25 M sucrose, 0.05 M sodium cacodylate, pH 7.3, 0.1 M NaCl, 0.005 M MgCl_2 , and 100 U/ml Trasylol (SuCNM), drained, and overlaid with chicken IgG diluted in SuCNM buffer to 0.1 or 0.2 $A_{280}\text{U/ml}$. After a 30-min incubation at room temperature, the cover slips were rinsed in SuCNM for 15 min at room temperature. They were subsequently drained, and overlaid with HRP-conjugated rabbit anti-chicken IgG at a concentration of 0.3 $A_{280}\text{U/ml}$, which had been dialyzed against SuCNM buffer before use. The cover slips were incubated and rinsed as described above. They were then fixed for 15 min at 0°C in 2.5% glutaraldehyde in SuCNM buffer, and rinsed in 0.05 M Tris-HCl, pH 7.6. The diaminobenzidine reaction was conducted for 5 min

at room temperature by incubating cover slips in a solution similar to that of Graham and Karnovsky (23), containing 0.05 M Tris-HCl, pH 7.6, 0.005% H₂O₂, and 0.5 mg/ml diaminobenzidine-HCl. The latter solution was passed through a 45- μ m Millipore filter (Millipore Corp., Bedford, Mass.) before use. This reaction was terminated by rinsing cover slips well in 0.05 M Tris-HCl, pH 7.6. Nuclei were then postfixed in 1% OsO₄ in veronal-acetate buffer, pH 6.2, containing 0.1% CaCl₂ and 0.1% tryptone (53). This fixation mixture was employed to minimize shrinking of nuclei (53) away from a surface layer of diaminobenzidine reaction product during subsequent processing. Nuclei were dehydrated in propylene oxide, and embedded in Epon 812 as described (33). Thin sections (silver-grey) were stained lightly with lead citrate (57) before examination in a Siemens 101 electron microscope.

Materials

We obtained deoxyribonuclease I (electrophoretically purified, bovine pancreas, DN·EP) from Sigma Chemical Co. (St. Louis, Mo.); HRP (RZ = 3.27, 979 U/mg) from Worthington Biochemical Corp. (Freehold, N. J.); tetramethylrhodamine isothiocyanate-conjugated rabbit anti-chicken IgG (heavy and light chains), lot no. 6871, from N. L. Cappel Laboratories; diaminobenzidine hydrochloride, reagent grade, from J. T. Baker Chemical Co. (Phillipsburg, N. J.); Trasylol (Aprotinin) from Mobay Chemical Corp. (New York); DEAE cellulose DE-52 from Whatman Ltd. (Maidstone, Kent, England); Sephadex G-25 medium, Sephadex G-200, and CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals (Piscataway, N. J.); Ham's F12 powdered medium and heat-inactivated fetal calf serum from Grand Island Biological Co. (Grand Island, N. Y.), and Acufine developer from Acufine, Inc. (Chicago, Ill.). All other chemicals were of reagent grade.

RESULTS

The pore complex-lamina fraction is derived from purified rat liver nuclear envelopes by treatment with the detergent Triton X-100 at low ionic strength, followed by washing in 1 M NaCl (15). Hence, the pore complex-lamina material is by definition insoluble under these conditions. This procedure yields a preparation comprised almost exclusively of well-defined nuclear pore complexes interconnected by a 15-nm thick lamina. Ultrastructural studies suggest that the lamina surrounds the entire nucleus, as one continuous macromolecular assembly (15).

Consideration of the structural roles proposed for the lamina (see Introduction) led us to suspect that a major portion of this entity would consist of relatively few polypeptides, arranged in a polymeric fashion. In SDS polyacrylamide gel electro-

phoresis, the pore complex-lamina fraction is resolved into numerous polypeptide components (Fig. 1, lanes 1 and 2). However, ~30–40% of the Coomassie Blue staining mass (determined by densitometric scanning) is located in three major bands, which migrate at 70,000, 67,000, and 60,000 daltons. These are designated P70, P67, and P60, respectively. We regarded these three bands to be likely candidates for major proteins of the lamina, and decided to make antibodies to each.

Production of Antibodies and

Characterization by Ouchterlony Analysis

Reduced and alkylated pore complex-lamina protein was electrophoresed on preparative SDS slab gels. P70, P67, and P60 were separately excised from these gels, and electrophoretically eluted. Each eluted band migrates homogeneously to its original position upon re-electrophoresis (Fig. 1, lanes 3–5). The relative intensities and positions of these three bands are visibly unaltered in electropherograms of nuclear envelopes prepared in the presence of 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 5 mM iodoacetamide, which are potent inhibitors of serine and sulfhydryl-requiring proteases, respectively (data not shown). It therefore seems unlikely that these bands result from *in vitro* proteolytic degradation occurring during preparation of the material.

These three eluted bands were separately used to immunize chickens, and a precipitating antibody response was achieved in each case. Fig. 2 presents a characterization of these three antibodies by Ouchterlony double diffusion analysis. Interestingly, we found that antibodies raised to each of the single bands strongly precipitate all three separate antigens, in addition to the total pore complex-lamina material (Fig. 2a–c). A single discernible precipitin line is obtained by reaction of anti-P70 or anti-P60 antiserum with any of the three eluted antigens (Fig. 2a and c). However, the reaction given by anti-P67 IgG shows that P67 contains two immunologically detectable components, the major³ of which appears related

³ It is important to recognize that this is only an immunological characterization of P67, defined by anti-P67 antibody. Conceivably, the component of P67 which cross-reacts with P70 and P60 may not be biochemically major. However, in all future discussion, the immunologically major component of P67 will be referred to as the "major component."

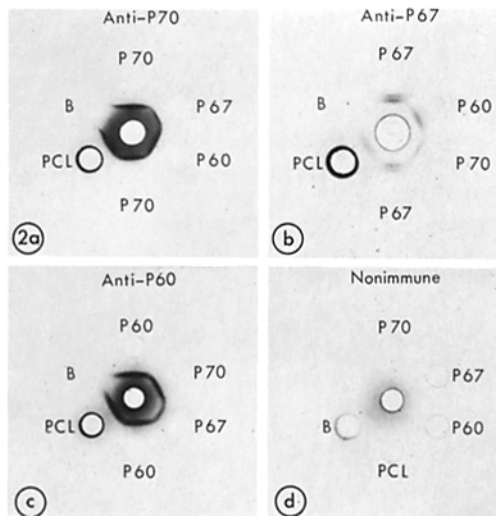


FIGURE 2 Ouchterlony double diffusion plates characterizing antibodies raised to electrophoretically eluted P70, P67, and P60. Antigens were solubilized in SDS + Triton X-100 as described in Materials and Methods. A volume of 20 μ l was added to all peripheral wells, containing: 3–5 μ g of electrophoretically eluted P70, P67, or P60; 20 μ g of the pore complex-lamina fraction (PCL); or solubilizing buffer (B) alone (2.5% Triton X-100, 0.5% SDS, 20 mM triethanolamine HCl, pH 7.4). To the center wells were applied: (a) 20 μ l of anti-P70 antiserum, (b) 40 μ l of anti-P67 IgG (chromatographically purified, 11.0 A_{280} U/ml), (c) 20 μ l anti-P60 antiserum, and (d) 20 μ l of nonimmune chicken serum. Results identical to Fig. 2a and c are obtained when chromatographically purified IgG is used instead of whole antiserum for anti-P70 and anti-P60 antibodies. No precipitate occurs with B alone, or with nonimmune chicken serum (d).

to P70 and P60 (Fig. 2b), i.e., the major anti-P67/P67 precipitin line appears to fuse with the single anti-P67/P70 and anti-P67/P60 precipitin lines. Essentially, each of the three antibody preparations gives a pattern of fused precipitin lines for all antigen pairs, indicating that P70, P60, and a major component of P67 are immunologically very similar or identical.

Immunofluorescence Staining of K22 Cells in Interphase

These antibodies were employed for indirect immunofluorescence localization using a rat liver cell line with epithelial growth characteristics (K22). In Fig. 3a, the staining pattern obtained with anti-P70 antiserum is shown. This antibody

gives an intense perinuclear reaction for all interphase cells. Because of geometric considerations, perinuclear fluorescence labeling presents the appearance of a concentrated “rim” of stain around the circumference of the nucleus, with a less intensely labeled interior. At this level of resolution, there is no evidence of focalized intranuclear⁴ stain. In addition, the cytoplasmic level of anti-P70 fluorescence is equivalent to background (Fig. 3e).

A similar staining pattern is obtained with anti-P67 and anti-P60 antibodies (Fig. 3c and d), except that these reagents show a low level of cytoplasmic reaction. This cytoplasmic fluorescence may relate to minor immunological components of these bands which have a partially cytoplasmic location, or alternatively, to an inherently higher nonspecific background for these antibodies.

The immunofluorescence reaction given by anti-P70 antibody is clearly antigen-specific (Fig. 3e and f). Adsorption of anti-P70 IgG with a P70-Sepharose 4B conjugate results in the loss of all fluorescence staining for the unbound immunoglobulins (Fig. 3e). Furthermore, the bound antibody which is subsequently eluted from this immuno-adsorbent yields a staining pattern indistinguishable from that of the original serum (Fig. 3f).

The immunofluorescence results presented in Fig. 3 were obtained with cells fixed by 4% formaldehyde followed by cold 100% acetone, a procedure which is commonly used for immunofluorescence studies (e.g., references 22 and 31). When K22 cells are fixed by a milder regimen (cold 95% ethanol, 1% acetic acid [56]), an anti-P70 staining pattern identical to that of the formaldehyde fixation is obtained (Fig. 4a). The ethanol-acetic acid procedure would be expected to extract moderate amounts of nuclear components (56), particularly histones (49). Indeed, the phase-contrast image of nuclei in these cells presents an “extracted” appearance (compare Fig. 4b to Fig. 3b). Therefore, with this fixative one

⁴ At electron microscope resolution, the lamina is topographically an “intranuclear” structure, since it is adjacent to the inner nuclear membrane. However, for the purposes of simplicity in all subsequent discussion, the lamina will be considered to occur at the nuclear “surface.” The terms “intranuclear” and nuclear “interior” will be used to designate all areas of the nucleus internal to the lamina.

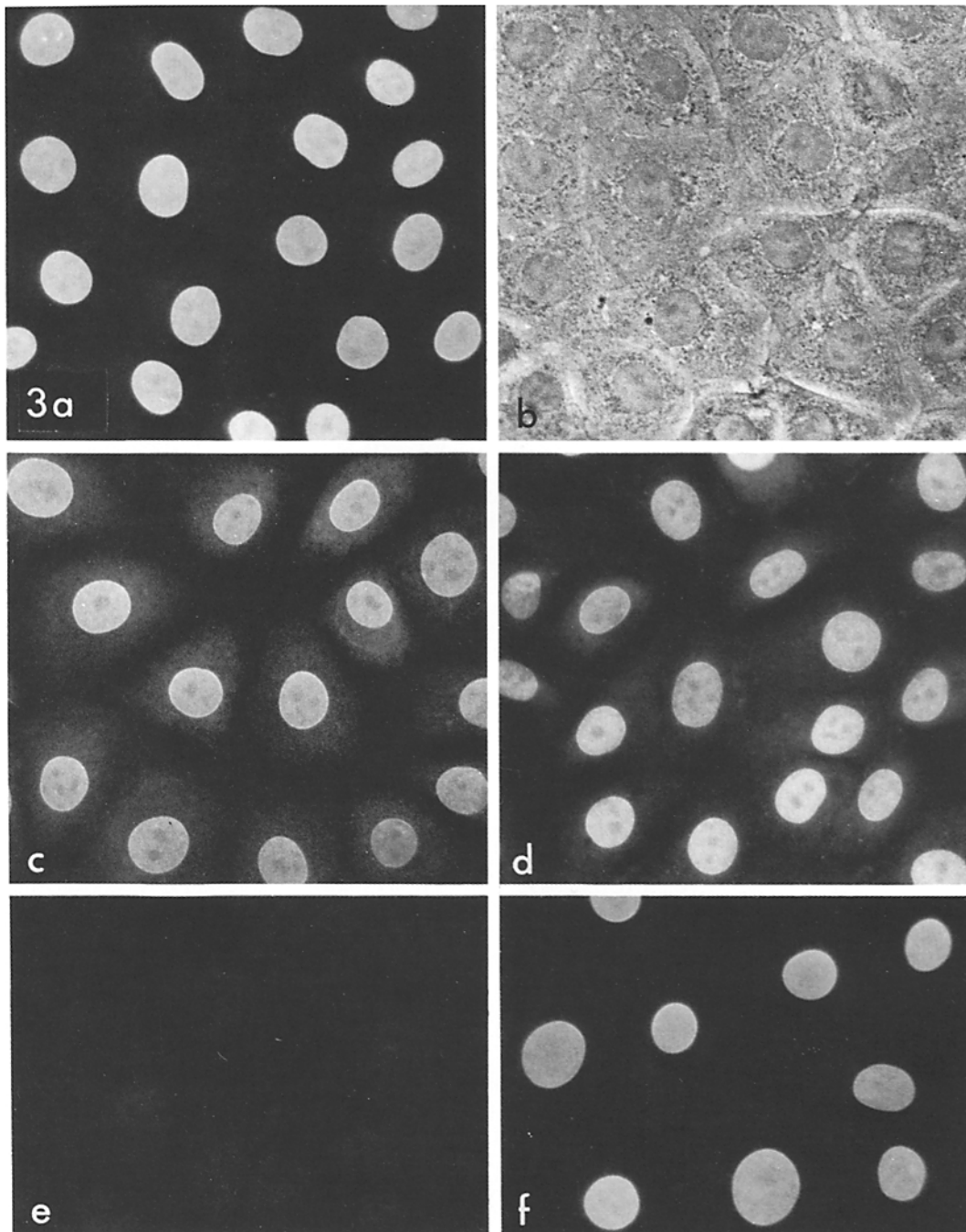


FIGURE 3 Micrographs of K22 cells processed for indirect immunofluorescence localization with chicken antibodies to the major lamina proteins. K22 cells were fixed by the formaldehyde/acetone procedure (see Materials and Methods). Fluorescence micrographs show cells treated with (a) anti-P70 antiserum diluted 1:50, (c) anti-P67 antiserum diluted 1:50, (d) anti-P60 IgG, 0.2 A_{280} U/ml, (e) the unbound fraction from anti-P70 IgG adsorbed on a P70-Sepharose 4B immunoaffinity column, 0.13 A_{280} U/ml, and (f) specific anti-P70 IgG antibodies eluted from a P70-Sepharose 4B immunoaffinity column with NaSCN, 0.01 A_{280} U/ml. Fig. 3b represents the phase-contrast image of the field shown in Fig. 3a. Treatment of cells with either whole serum or chromatographically purified IgG yields the same staining pattern for these three antibodies. Nonimmune serum from chickens of the same age as the immunized animals gives a pattern analogous to Fig. 3e. Bar, 20 μ m. All photographs $\times 480$.

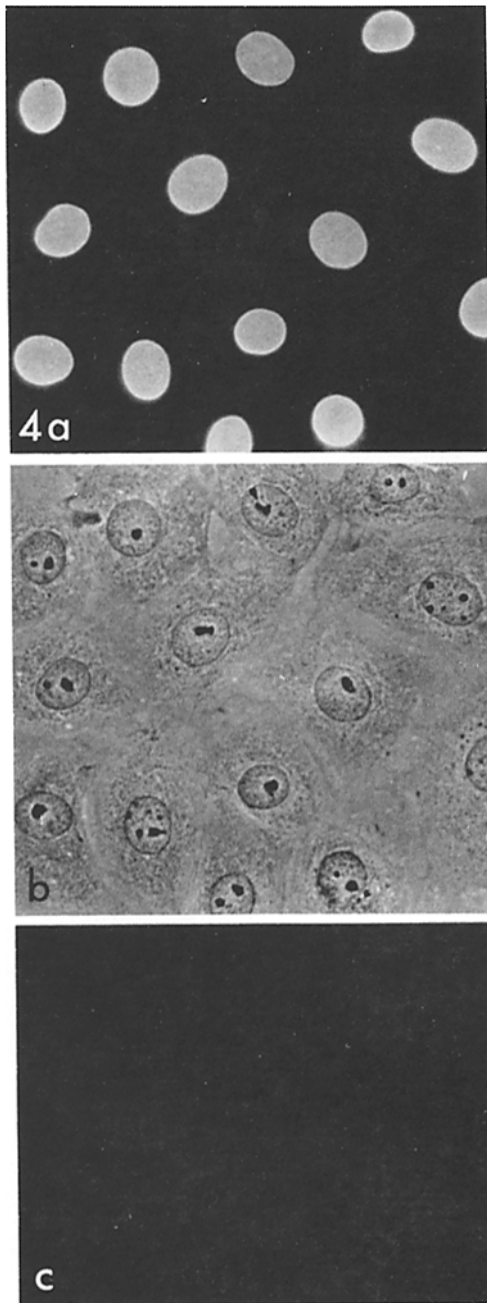


FIGURE 4 Micrographs of K22 cells fixed by the ethanol-acetic acid procedure and subjected to indirect immunofluorescence staining with chicken antibodies. Fluorescence micrographs show cells stained with (a) anti-P70 serum diluted 1:50 and (c) nonimmune chicken serum diluted 1:50. Fig. 4b is the phase-contrast image of the field shown in Fig. 4a. No staining occurs with the

would anticipate greater access of antibodies to intranuclear antigens which are possibly rendered inaccessible by the formaldehyde fixation. However, only perinuclear fluorescence is obtained in this case, without discernible intranuclear staining (Fig. 4a).

Considering these immunofluorescence results together, we conclude that P70, P60, and a major component of P67 are localized at least primarily at the periphery of the interphase nucleus of K22 cells. We have conducted (data not shown) immunofluorescence studies with our antibodies on numerous other types of mammalian cells in tissue culture, having a diverse range of generation times. These include: human fibroblast lines, human adenocarcinoma cells, MDCK cells (a canine kidney line), MBCK cells (a bovine kidney line), primary mouse heart cells, mouse teratoma cells, and CHO cells (a Chinese Hamster ovary line). In all cases, we obtain prominent perinuclear staining with our antibodies, in a pattern comparable to K22 cells. This indicates that these antigens are conserved among mammalian cells, and appear to be a ubiquitous feature of the nucleus.

EM Immunoperoxidase Localization

Immunofluorescence localization of P70, P67, and P60 in K22 cells indicates that these antigens do not occur in the nuclear interior to any substantial degree, at least compared to their concentration at the nuclear surface. However, the question of a possible intranuclear disposition of these proteins can only be clearly resolved by a localization method of higher resolution. Therefore, we have employed electron microscope immunocytochemistry.

We performed indirect immunoperoxidase staining on isolated rat liver nuclei which had been treated with Triton X-100 and centrifuged onto cover slips. Preparations (either acetone-fixed or unfixed) were incubated with chicken antibodies followed by HRP-conjugated rabbit anti-chicken IgG, and were subsequently processed for electron microscope immunoperoxidase localization in thin sections.

A representative low magnification survey of acetone-fixed nuclei stained with anti-P70 IgG is shown in Fig. 5a. A copious amount of the

nonimmune chicken serum. Bar, 20 μ m. All photographs \times 480.

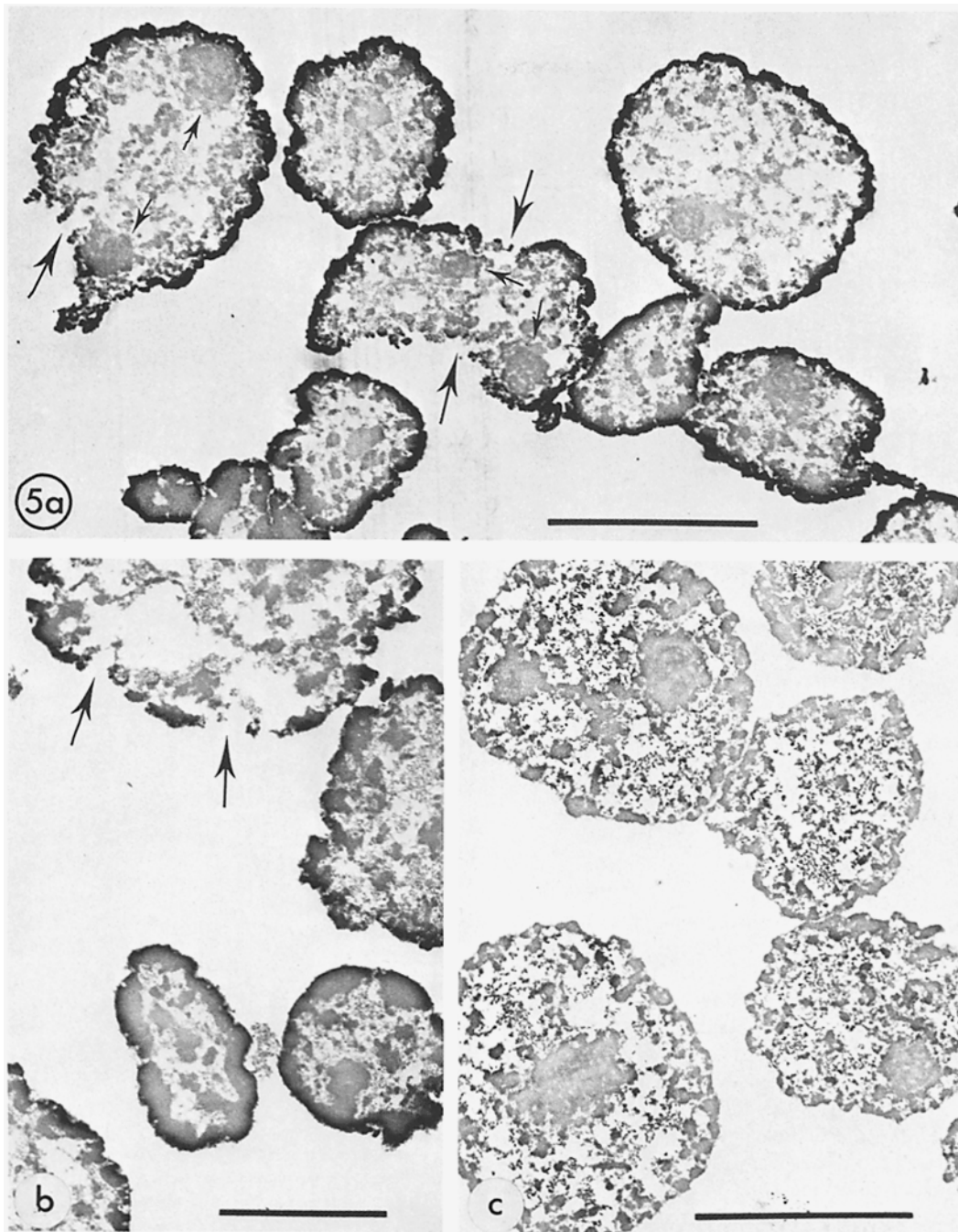


FIGURE 5 Electron micrographs of isolated rat liver nuclei stained by an indirect immunoperoxidase technique to localize the major pore complex-lamina proteins. Isolated nuclei were treated with Triton X-100, centrifuged onto coverslips, and either fixed with cold acetone (*a* and *c*), or maintained in an unfixed state (*b*). They were stained with (*a*) anti-P70 IgG, (*b*) anti-P67 IgG, or (*c*) nonimmune chicken IgG, all at $0.2 A_{280} U/ml$. In Fig. 5*a* and *b*, exclusively perinuclear reaction is obtained, even for nuclei which have been obviously fractured open before the staining procedure (large arrows). However, reaction product does not occur at peripheral areas of fractured nuclei from which the lamina has been severed (e.g. bottom-most large arrow in Fig. 5*a*). No structures in the interior or these broken nuclei show reaction product, including nucleoli (small arrows in Fig. 5*a*). Slight intranuclear nonspecific background is given by the nonimmune chicken IgG which was used in Fig. 5*c*, emphasizing that under our conditions all nuclei are permeable to antibodies, whether obviously fractured or not. Although the lamina is located at the surface of these Triton-treated nuclei, peroxidase reaction product diffuses from the initial site of reaction (42), producing a gradient of stain in the peripheral heterochromatin subjacent to the lamina. All bars, $5 \mu m$. (*a*) $\times 6,000$; (*b*) $\times 4,800$; and (*c*) $\times 6,250$.

electron-dense peroxidase reaction product is deposited in a layer around the surfaces of all nuclei. However, there is a complete absence of reaction product in the nuclear interior. Because this pattern is found for nuclei which have been obviously fractured open before fixation and antibody incubations (Fig. 5a, large arrows), the absence of intranuclear staining cannot be attributed to a lack of penetration of antibodies into the nucleus. The perinuclear reaction occurring with anti-P70 IgG is antigen-specific, since it is absent in preparations treated with nonimmune chicken IgG (Fig. 5c), as well as in material stained with anti-P70 IgG which was previously adsorbed with a P70-Sepharose 4B conjugate (data not shown). An identical pattern to the P70 localization is achieved when unfixed nuclei are reacted with anti-P67 IgG (Fig. 5b), or with anti-P60 IgG (data not shown).

In general, with this immunocytochemical technique we obtain only perinuclear reaction for all three antibodies, whether the nuclei are fixed or unfixed. We therefore conclude that on the level of sensitivity of this approach, all three of these antigens are located exclusively at the periphery of the interphase rat liver nucleus, and do not occur internally.

In nuclei washed with Triton X-100, it has been shown that nuclear pore complex protrude prominently from the nuclear surface (1), appearing as short columnlike structures. Fig. 6 shows a medium magnification view of an unfixed, Triton-washed nucleus stained with anti-P70 IgG by the immunoperoxidase procedure. Pore complexes (designated by arrowheads) are clearly visible in this sample, and can be seen in a variety of tilted images with respect to the plane of the thin section. It is apparent that little or no reaction product is concentrated at the pore complexes. However, stain occurs densely at immediately adjacent areas of the nuclear surface, which contain the lamina. The pattern presented in Fig. 6 was also observed for anti-P67 and anti-P60 antibodies (data not shown). We conclude, therefore, that on the level of sensitivity of our assay, these proteins are either not present, or not concentrated in the pore complex in an immunologically reactive form. Instead, they are localized in areas of the Triton-treated nuclear surface which contain the lamina. Because these antigens collectively constitute ~30–40% of the SDS gel staining mass of the pore complex-lamina fraction, we suggest that P70, P67, and P60 are the major structural components of the lamina.

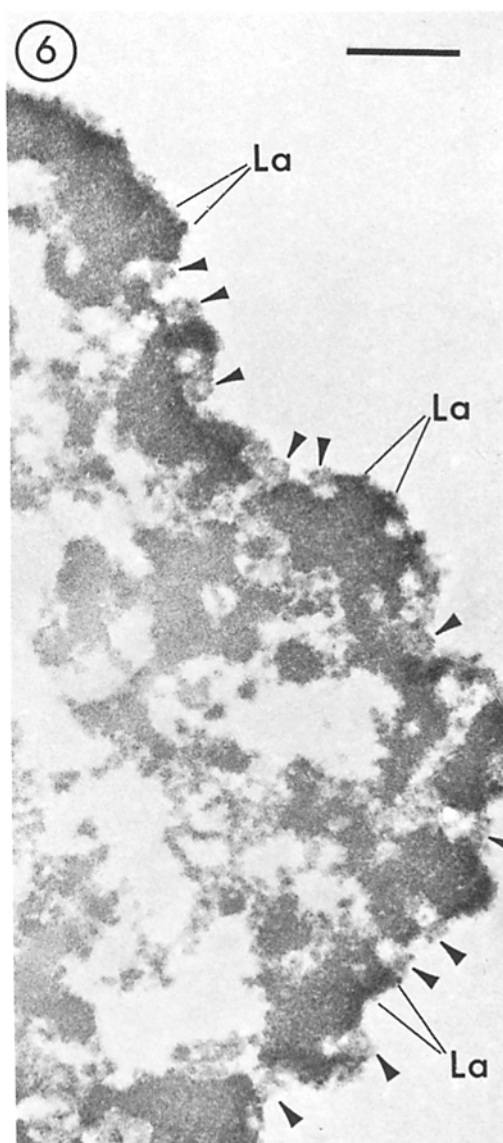


FIGURE 6 Medium magnification electron micrograph of a rat liver nucleus stained with anti-P70 IgG by an indirect immunoperoxidase technique. This preparation was washed with Triton X-100, and (in an unfixed state) treated with anti-P70 IgG, 0.1 A_{280} U/ml. In this case, a lower concentration of chicken IgG was used than for the staining shown in Fig. 5, to diminish the magnitude of the peroxidase reaction. This would minimize diffusion of the reaction product (42), and increase the resolution of the localization. The pore complexes (arrowheads) which protrude prominently from the surface of this nucleus show no reaction. However, dense reaction product is deposited at the adjacent lamina (La). Bar, 0.5 μ m. \times 30,000.

Semiquantitative Ouchterlony Analysis to Monitor Lamina Antigen Loss during Preparation of Nuclear Envelopes

It could be argued that immunoperoxidase staining of nuclei with our three antibodies does not detect any P70-related antigens which extend centripetally to form an intranuclear matrix (8, 13) because these putative intranuclear antigens are "masked" in an immunologically unreactive form. We consider this argument to be unlikely, since P70-related antigens are strongly reactive at the periphery of the nucleus. Furthermore, intranuclear reaction is not achieved with any of three independently derived antibodies, in either fixed or unfixed nuclei, which are both accessible to the penetration of antibodies. However, to support the results of our electron microscope immunocytochemistry, we have invoked a second approach that is independent of these possible objections.

The pore complex-lamina fraction is derived from a nuclear envelope preparation which is obtained by two successive digestions of nuclei with DNase (15). Semiquantitative SDS gel analysis demonstrates (15) that P70, P67, and P60 are not extracted from our nuclear envelope fraction during treatment with Triton X-100 and 1 M NaCl. Therefore, any postulated loss of these proteins must occur before this stage. Because the two DNase digestions completely remove the structural contents at the nucleus (28), these treatments would be expected to extract lamina antigens if they existed in an internal nuclear matrix.

We have examined the supernatant (extracted) fraction of our two DNase digestions (D_1 and D_2) for the presence of the major lamina antigens using a semiquantitative Ouchterlony double diffusion analysis. Because the assayed material was completely solubilized in SDS before analysis, any masked intranuclear antigens would now be rendered accessible to precipitation by antibodies. Fig. 7a presents a standard "curve" for the Ouchterlony reaction of anti-P70 with D_2p (purified nuclear envelope fraction). The P70-related antigens in as little as 0.1 equivalents of D_2p material can be visualized as a precipitate in this system. Moreover, progressively increasing precipitate intensities are seen for correspondingly increasing amounts of D_2p antigen.

The outer wells of the plates in Fig. 7b and c represent a balance sheet describing the recovery of P70 antigens at the D_1 and D_2 digestion steps,

respectively. Comparing the standards in Fig. 7a to the precipitate given by $4D_1s$ (Fig. 7b), it is apparent that approximately two-tenths equivalents of the P70 antigens are found in the supernate of the first DNase digestion. In other words, $\sim 0.2 \div 4 = 5\%$ of the P70 antigens are extracted at this step. Undetectable amounts of P70 antigens are present in D_2s (Fig. 7c). As a control, addition of defined quantities of D_2p to the D_1s and D_2s fractions before precipitation and SDS solubilization gives the expected defined increases in precipitate intensities (Fig. 7b and c). This indicates that no inhibition of the precipitin reaction of P70-related antigens in the D_1s and D_2s fractions occurs under our assay conditions.

Therefore, it appears that very small quantities of P70-related antigens are extracted from nuclear envelopes during the DNase digestion steps. We consider it likely that these extracted antigens relate to fragments of nuclear envelopes which are too small to pellet during centrifugation, rather than to actual intranuclear proteins. In conclusion, this semiquantitative Ouchterlony analysis provides additional evidence for a strictly peripheral nuclear location of the major lamina polypeptides.

Immunofluorescence Staining of K22 Cells during Mitosis

In higher eukaryotes, the nuclear envelope is disassembled during cell division (18, 19, 29). This process occurs in mitotic prophase, when nuclear membranes usually disintegrate to form vesicles and bilamellar cisternae which become essentially indistinguishable from components of the endoplasmic reticulum (19, 29, 48, 51). At the same time, nuclear pore complexes disappear as ultrastructural entities (19, 29). A new nuclear envelope is reconstructed in late anaphase, by a process involving aggregation and coalescence of membranous vesicles and cisternae on the surfaces of the highly condensed daughter chromosomes (10, 16, 29, 41, 48, 51). In most cell types, the chromosomes become fused into a continuous mass at this time, facilitating construction of a single membrane-bounded nuclear compartment.

A question of fundamental interest is the disposition of the lamina proteins during mitosis, particularly in relation to nuclear envelope disassembly and reconstruction. Figs. 8 and 9 present a series of immunofluorescence and corresponding phase-contrast micrographs of K22 cells at progressive stages of cell division. These cells were

stained with anti-P70 antiserum to follow the fate of the major lamina antigens.

Electron micrographs of higher eukaryotic cells in early prophase show that the nuclear surface becomes involuted at this stage, in areas opposed to the forming mitotic spindle (41, 51). Although the parallel nuclear membranes become markedly

undulatory at this time (5, 51, 55), they often remain substantially continuous. A K22 cell in early prophase is shown in Fig. 8a and b. Two distinct involutions of the perinuclear lamina fluorescence are apparent (Fig. 8a), corresponding to indentation in the nucleus visible in the phase-contrast image of the same cell (Fig. 8b, arrows). This picture is reminiscent of separating asters producing invaginations in the prophase nuclear surface. Although the cell in Fig. 8a is unrounded, a uniform diffuse fluorescence is apparent in its cytoplasm. This presents a marked contrast to the lack of anti-P70 cytoplasmic reaction in interphase cells (Fig. 3a).

A cell in mid-prophase is shown in Fig. 8c and d. While no clearly defined nuclear compartment is visible in phase contrast (Fig. 8d), focalized (but discontinuous) lamina fluorescence is present in areas of the condensing chromosomes (Fig. 8c). This focalized lamina staining may correspond to large fragments of the dispersing nuclear envelope (10, 41, 51), but seems to be concentrated in areas of the forming mitotic spindle (arrows in Fig. 8d). In addition, there is a considerable amount of uniform fluorescence throughout the rest of the cell.

By prometaphase (Fig. 8e), most fluorescence in the cell is diffusely located, except for a small amount of concentrated stain (presumably) near the center of the forming mitotic spindle. This residual antigen concentration becomes dispersed a short time later (Fig. 8g). From this period until

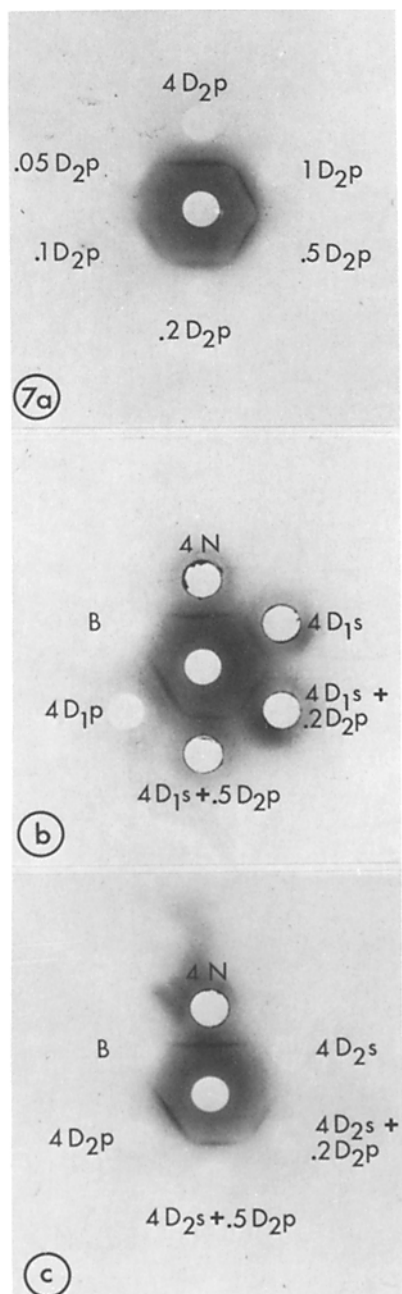


FIGURE 7 Ouchterlony double diffusion plates presenting a semiquantitative analysis of extraction of the lamina antigens occurring during preparation of nuclear envelopes. Rat liver nuclei (*N*) were digested at two successive steps with pancreatic DNase I according to our standard procedure (17). After the first (D_1) digestion at pH 8.5, centrifugation yields a supernate (D_{1s}) and a pellet (D_{1p}). The latter is digested a second time at pH 7.4 (D_2) and centrifuged, to give a supernate (D_{2s}) and the final preparation of nuclear envelopes (D_{2p}). Antigen fractions were precipitated with TCA and solubilized in SDS + Triton X-100 as described in Materials and Methods. Numbers refer to the equivalents of material contained in each outer well; e.g., digestion of 4 $A_{280}U$ of nuclei (4*N*) at the first DNase step yields after centrifugation 4*D*_{1s} and 4*D*_{1p}. 20 μ l of solution were applied to each well. Anti-P70 antiserum was added to the center well of all plates. Approximate quantities of protein are 4*N*, 400 μ g; 4*D*_{1s}, 330 μ g; 4*D*_{1p}, 70 μ g; 4*D*_{2s}, 25 μ g; and 4*D*_{2p}, 45 μ g. B represents the solubilization buffer alone.

telophase, the highly condensed mitotic chromosomes are distinctly visible as dark structures by virtue of an absence of fluorescence reaction throughout their structure (Fig. 8, *g*, *i*, and *k*). The lamina antigens remain dispersed through metaphase (Fig. 8*i*) and mid-anaphase (Fig. 8*k*) stages.

A striking redistribution of these antigens begins to occur in late anaphase, when reconstruction of a nuclear envelope takes place around the fused daughter chromosomes (10, 16, 29, 41, 48, 51). As shown in Fig. 9*a*, a noticeable concentration of fluorescence staining appears at the surfaces of these late anaphase chromosome masses, in the midst of the homogeneous cytoplasmic fluorescence. There is marked enhancement of this perichromosomal fluorescence by telophase (Fig. 9*c*), but the cytoplasmic reaction remains prominent. In mammalian cells at comparable stages of telophase, electron microscopy reveals that the nuclear envelope is largely or completely reformed (16, 41, 51). The cell in Fig. 9*e* has almost completed cytokinesis. Compared to the previous stage, there is an intensification of the (now) perinuclear fluorescence, and a diminution in the cytoplasmic antigen reaction. All cytoplasmic staining has disappeared by early G1 (Fig. 9*g*). It is apparent that the progressive decrease in cytoplasmic localization of these lamina antigens from telophase to early G1 stages (Fig. 9*c*, *g*, and *e*) occurs simultaneously with a rapid increase in the size and surface of the daughter nuclei (Fig. 9*d*, *f*, and *h*).

In summary, these immunofluorescence results indicate that on the level of resolution of the light microscope, the lamina becomes progressively and completely dispersed in mitotic prophase. The major lamina proteins remain diffusely localized throughout metaphase and anaphase stages, until the time of reformation of the nuclear envelope. At this point, there occurs a gradual redistribution of the lamina antigens in the cell, to a highly focalized position at the surface of the reforming nucleus. We observe that considerable amounts of the lamina antigens occur in a cytoplasmic location in telophase. Electron microscopy performed on cells at comparable stages of mitotic progression shows that the nuclear envelope has been completely reconstructed at this point. The gradual increase in fluorescence at the surface of the reforming nucleus from late anaphase to early G1 occurs coordinately with a diminution in the level

of the diffuse cytoplasmic reaction. This indicates that the lamina proteins are recycled for formation of the new nuclear envelope.

When mitotic K22 cells are examined after fixation in cold 95% ethanol, 1% acetic acid, we observe an anti-P70 fluorescence pattern similar to the staining obtained with formaldehyde-fixed cells (data not shown). However, the mitotic chromosome structure is notably less well-preserved in this case. With either fixative, we find no evidence that the lamina proteins occur in the mitotic chromosome structure during metaphase and anaphase. Therefore, it seems unlikely that these antigens are components of a postulated "mitotic chromosome scaffold" (3). The same dynamic pattern of redistribution of the major lamina proteins during cell division has been observed for all mammalian cell types which we have henceforth examined by immunofluorescence (see above).

DISCUSSION

A fibrous layer apposed to the nucleoplasmic surface of the inner nuclear membrane was first described for a number of invertebrate cell types (e.g., references 6, 11, 24, 39, 45). Subsequently, Fawcett reported the occurrence of an analogous "fibrous lamina" in a number of vertebrate cells (17), and it has become evident that the presence of a nuclear lamina is widespread among various mammalian cell types (21, 27, 38, 43, 44, 46).

There are many classes of mammalian cells in which a nuclear lamina is not discernible by electron microscopy. This category includes rat hepatocytes. However, in preparations of isolated rat liver nuclear envelopes from which the adjacent heterochromatin has been removed, a thin lamina-like structure is distinctly visible in apposition to the inner nuclear membrane (15). We feel that this is the equivalent of the thicker layers observed in other mammalian cells, because the lamina is clearly not a cellular structure with an invariant dimension. When it is visible in intact cells by conventional electron microscopy, the lamina can exhibit a broad range of thicknesses among different cell types (e.g., references 27, 38, and 46). Moreover, its dimension can dynamically change in a single class of cells (21, 43, 44). Although it is often not apparent as a distinct ultrastructural entity, we suggest that the lamina may be a ubiquitous component of the eukaryotic nuclear envelope.

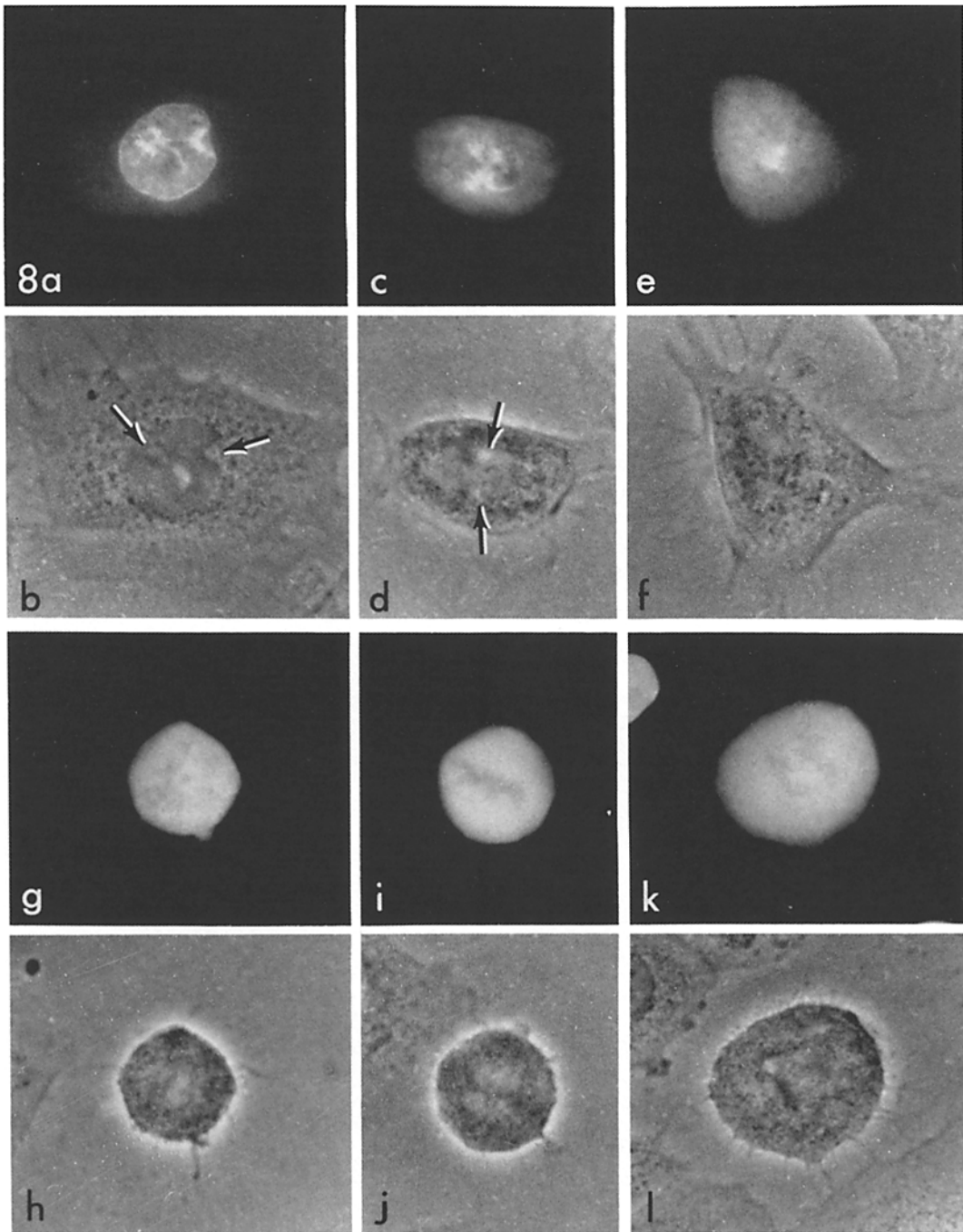


FIGURE 8 Micrographs of mitotic K22 cells stained by an indirect immunofluorescence procedure with anti-P70 antiserum. Progression from prophase to anaphase stages of cell division. Cells were fixed with formaldehyde/acetone, and incubated with a 1:50 dilution of anti-P70 antiserum. Fluorescence micrographs are shown in Fig. 8*a, c, e, g, i, and k*. The corresponding phase-contrast images are presented in Fig. 8*b, d, f, h, j, and l*, respectively. This mitotic progression shows the gradual disaggregation of the lamina which occurs through early prophase (*a*), mid-prophase (*c*), and prometaphase (*e* and *g*). The lamina antigens remain diffusely localized through metaphase (*i*) and anaphase (*k*). Arrows in Fig. 8*b* and *d* indicate the presumed poles of the forming mitotic spindle. Bar, 20 μm . All magnifications $\times 750$.

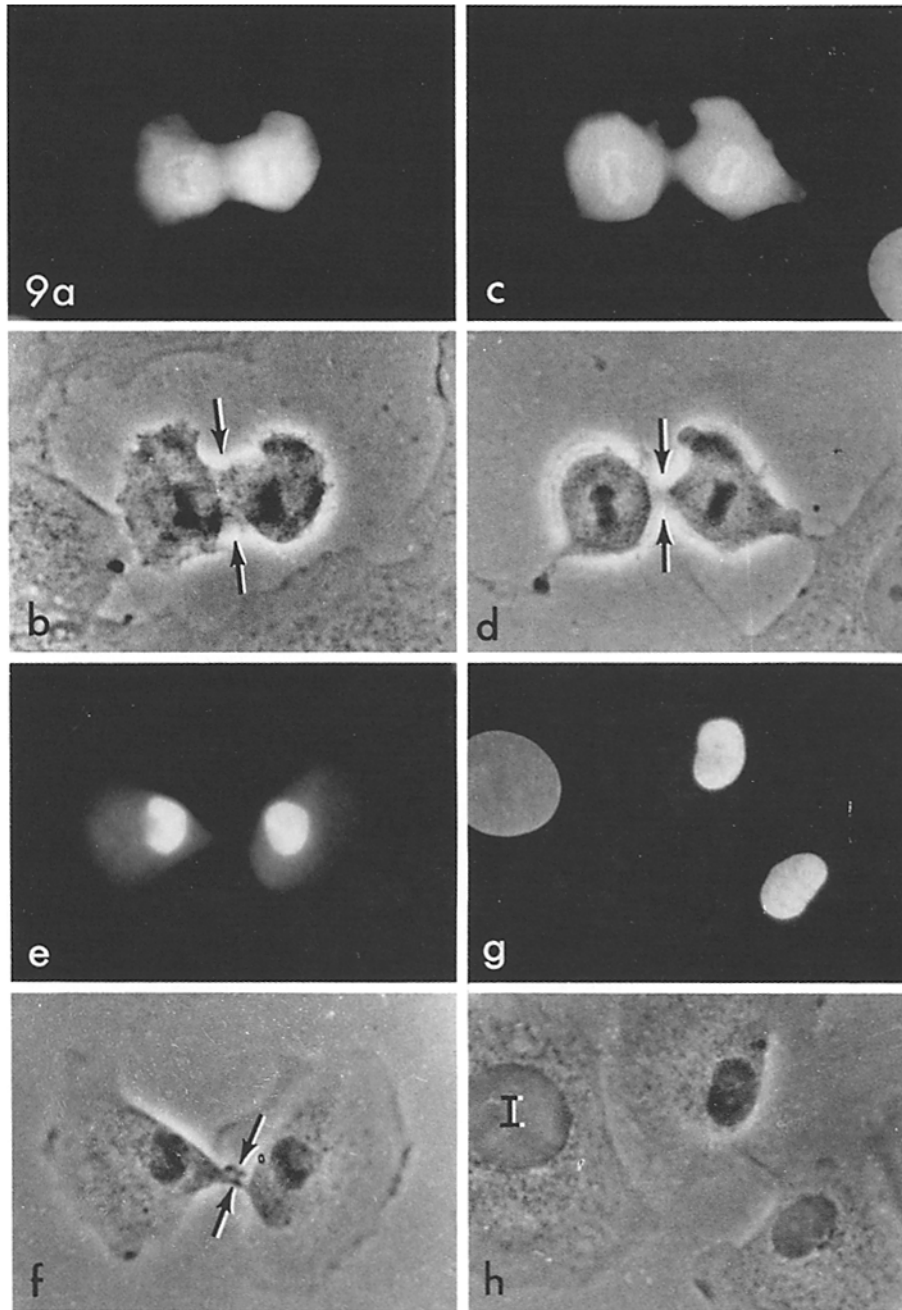


FIGURE 9 Micrographs of mitotic K22 cells stained by an indirect immunofluorescence procedure with anti-P70 antiserum. Progression from late anaphase to early G1 stages. Cells were fixed and stained as described in Fig. 8. Fluorescence images appear in Fig. 9a, c, e, and g. The corresponding phase-contrast micrographs are shown in Fig. 9b, d, f, and h. This series depicts the gradual reappearance of the perinuclear lamina, which occurs through late anaphase (a), mid-telophase (c), late telophase (e), and early G1 (g). Arrows in Fig. 9b, d, and f indicate the progressive stages of cytokinesis that are represented by these three cells. The early G1 daughter nuclei in Fig. 9h still have moderately pycnotic chromatin, and a much smaller diameter than the adjacent interphase (I) nucleus. Bar, 20 μm . All magnifications $\times 750$.

Immunological Analysis and Localization of the Major Lamina Polypeptides

In this paper, we describe investigations conducted with chicken antibodies prepared to the three major polypeptide bands of the rat liver pore complex-lamina fraction. We observe that P70, P60, and a major component of P67 cross-react strongly with each other in Ouchterlony double diffusion analysis, behaving as immunologically very similar or identical. We feel that the simplest explanation for this observation is that P60 and the cross-reacting component of P67 are proteolytic products of P70. This interpretation is supported by cyanogen bromide peptide mapping of P70 and P60 (preliminary data). However, our results with protease inhibitors suggest that degradation does not occur during our isolation procedure. If P60 and P67 arise from P70 by *in vivo* proteolytic cleavage, it is possible that these cleavages are physiologically meaningful to the structure or function of the lamina.

Using our three antibody preparations, we have performed electron microscope immunoperoxidase localization on rat liver nuclei. These studies demonstrate that the three polypeptides are located exclusively at the periphery of the interphase nucleus, in areas which contain the lamina. They do not occur in a concentrated fashion at the pore complex. The fact that ~30–40% of the pore complex-lamina material consists of three immunologically related polypeptides which are located in the lamina strengthens our earlier suggestion (15) that the lamina is primarily a polymer of these proteins. However, we emphasize that there are many less prominent polypeptides in the pore complex-lamina fraction. A number of these may also be functionally important components of the lamina, relating to its interaction with chromatin, the inner nuclear membrane, or pore complexes (see below).

Recently, a number of diverse procedures has been employed to obtain subnuclear fractions containing structural components of the nucleus (8, 13, 15, 26, 40, 50, 60). These techniques have involved the use of high ionic strength solution (8, 13, 15, 26, 40, 50, 60), nonionic detergents (8, 13, 15, 50, 60), digestions by DNase (8, 13, 15, 26, 40, 60) and RNase (8, 13, 60), and low ionic conditions (8, 13, 15, 60), in various combinations. In many cases (8, 13, 26, 40, 60), fractions are obtained which contain an internal "nuclear matrix" in apparent attachment to a peripheral

lamina-like component. These matrix fractions are devoid of most DNA, but can contain substantial amounts of RNA (40), depending on the technique employed.

Berezney and Coffey (8), and Comings and Okada (13) obtain a nuclear protein matrix structure from rodent liver which contains three predominant polypeptides in the ~60,000–70,000 dalton region on SDS gels. It seems likely that these three bands correspond to the major polypeptides of the pore complex-lamina fraction, considering the similarities between our procedure (15) and the technique which is used by these two groups (8).⁵ These investigators propose (8, 13) that the internal nuclear matrix, as well as the peripheral lamina to which it is attached, contain these three prominent polypeptides.

Electron microscopy of the pore complex-lamina preparation indicates conclusively the intranuclear components are not present in our fraction to any significant degree (15). Specifically, because almost all of the material in this preparation consists of a 15-nm layer to which clearly defined nuclear pore complexes are attached at frequent intervals, the material necessarily derives from the nuclear envelope (15). However, we previously could not exclude the possibility that hypothetical centripetal extensions of the lamina were severed during our isolation procedure, and lost during a purification step (15).

The results of the present study provide strong evidence that the three prominent pore complex-lamina bands occur only at the periphery of the nucleus (in the lamina), and not in the interior. In addition to our immunoperoxidase data, this is shown by results from an independent approach. Semiquantitative Ouchterlony analysis demonstrates that the major lamina antigens are extracted to a negligible degree during our preparation of nuclear envelopes by DNase digestion, a procedure that completely releases the interior nuclear contents. These results imply that at least a major portion of the nuclear lamina is a biochemically separate entity from an internal nuclear matrix. The polypeptide nature of the nu-

⁵ One notable difference between the two techniques is that our procedure (15) includes a pH 8.5 DNase digestion of nuclei, in addition to the pH 7.4 digestion used by the others (8). This more alkaline pH condition might be expected to facilitate extraction of intranuclear ribonucleoprotein structures, based on the work of others (34, 36).

clear matrix is presently undefined. Whether its composition will resemble the very heterogeneous polypeptide pattern of ribonucleoprotein complexes stable in high salt and low ionic strength (47), or whether it will contain a few characteristic polypeptides remains to be determined.

Involvement of the Lamina in Interphase Nuclear Structure

The lamina may be an important element in the structural organization of the interphase nucleus, by virtue of its relationship to other components of the nuclear surface. We postulate three important functional interactions for the lamina, involving the nuclear pore complex, the inner nuclear membrane, and elements of the chromatin (see Fig. 10).

Ultrastructural studies of the isolated rat liver pore complex-lamina fraction indicate that the nuclear pore complex is attached in a topologically

specific fashion to the lamina (15). It therefore seems possible that the lamina is important for the structural stability and/or assembly of the pore complex in the interphase nucleus. Furthermore, studies performed on rat liver nuclear envelopes demonstrate that the lamina has a morphologically and biochemically tight interaction with the inner nuclear membrane (15). While the major lamina polypeptides are stably associated with nuclear membranes at low ionic strength and in the presence of 1 M NaCl (15), these proteins can be eluted from nuclear envelopes with 1 M MgCl₂, 6 M urea, and 0.1 N NaOH (data not shown). Therefore, they operationally behave as peripheral membrane proteins (54). We propose that the association of the lamina with the nuclear envelope is mediated by an integral membrane protein(s) unique to the inner nuclear membrane. Such an association could be functionally important for compartmentalization of newly synthesized lamina proteins during interphase. It may also be involved in the

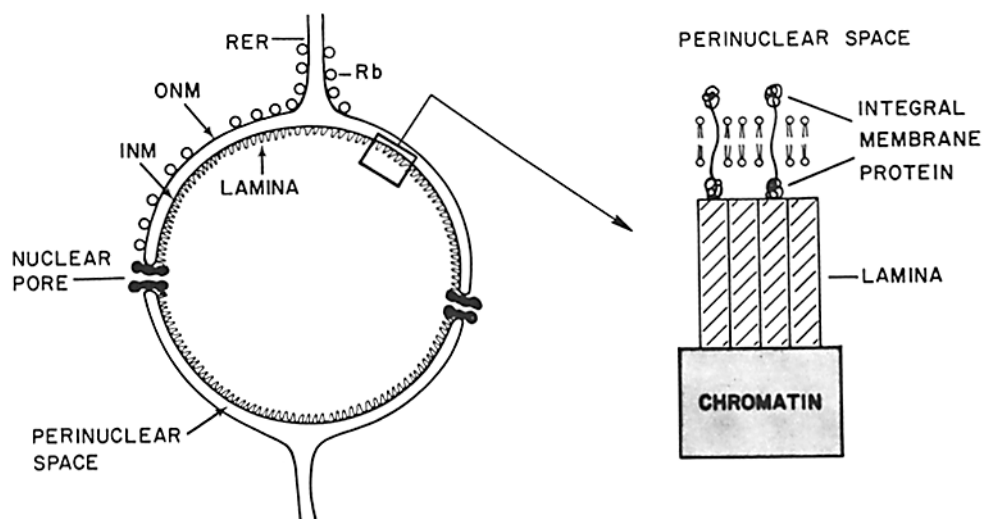


FIGURE 10 Schematic diagram describing the surface topography of a typical eukaryotic nucleus. Left: The nucleus is encircled by two approximately parallel membranes, the inner (INM) and outer nuclear membrane (ONM). These structures are joined at the nuclear pores, which contain the architecturally elaborate material of the pore complex. The outer nuclear membrane has attached ribosomes (Rb), and occurs in morphological continuity with the rough endoplasmic reticulum (RER). Through this structural relationship, a luminal continuity exists between the perinuclear space and the cisternae of the rough endoplasmic reticulum. The lamina occurs immediately subjacent to the inner nuclear membrane and encircles the entire nucleus. Pore complexes are associated with the lamina on their nucleoplasmic side. Right: An enlarged view of the enclosed region shown on the left depicts the close relationship which exists between the inner membrane, the lamina, and the underlying chromatin. The lamina is represented as a polymeric structure of similar subunits, involved in a tight binding of the inner nuclear membrane and of the adjacent chromatin. The association of the lamina with the inner membrane is depicted to occur through an integral membrane protein, presumably unique to the inner nuclear membrane.

reconstruction of the nuclear envelope at the conclusion of cell division (see below).

Circumstantial evidence suggests that nuclear chromatin is tightly associated with some component of the inner nuclear membrane (18, 19, 29). In cells with a morphologically substantial lamina (i.e., 20–80 nm), a finely textured layer physically separates the peripheral nuclear chromatin from the inner nuclear membrane bilayer. This makes interaction of chromatin with inner membrane lipoprotein structures topologically impossible. We suggest that the lamina is the component of the inner membrane which mediates binding of nuclear chromatin, in instances where the lamina is not morphologically apparent, as well as in cells with a thick lamina. Clearly, the interaction of specific regions of chromosomes with a constituent of the nuclear envelope could confer a macroscopic degree of organization to the nucleus (12, 58).

Lamina during the Cell Cycle

The surface area of the nuclear envelope increases throughout the cell cycle, as well as the number and density of nuclear pores (37). Because the lamina is a peripheral protein component of the inner nuclear membrane, we would expect its mass to increase in a balanced fashion with the rest of the nuclear envelope during interphase growth. Immunofluorescence microscopy shows that the major lamina polypeptides are not detectable in cytoplasmic areas of interphase tissue culture cells. This suggests that the cytoplasmic concentration of these polypeptides is low during interphase, and that any newly synthesized proteins are rapidly incorporated into the existing lamina structure from their cytoplasmic site of synthesis. Such an interpretation is consistent with the solubility properties of the isolated rat liver lamina, which indicate that an insoluble (polymeric) form is highly favored.

During cell division, the location of the major lamina polypeptides undergoes dramatic changes. This first occurs in prophase, when the antigens gradually shift from their focalized interphase position, to a distribution that is completely diffuse throughout the cell at the resolution of light microscopy. This change occurs simultaneously with the disassembly of the nuclear envelope. A diffuse localization persists through metaphase and anaphase, until the period in late anaphase when reconstruction of the nuclear envelope takes place. A reversal of the prophase

pattern is observed from this time until late telophase, whereby the lamina antigens progressively accumulate at the surface of the daughter chromosome masses. We observe that the major lamina polypeptides do not totally disappear from a cytoplasmic location until after the nuclear envelope has completely reformed. Telophase temporally coincides with a period of rapid growth for the newly assembled nuclear envelope, when substantial decondensation of the mitotic chromosomes occurs. During the late mitotic stages, the coordinated disappearance of the major lamina antigens from a cytoplasmic localization, together with their accumulation at the surfaces of the reforming daughter nuclei offer strong evidence that these antigens are recycled for reconstruction of the new nuclear envelopes.

The isolated rat liver lamina is a relatively insoluble structure, consisting primarily of a single antigenic species which may be arranged in a polymeric array. We propose that the redistribution of the lamina antigens which occurs during mitotic prophase may result from a hypothetical disassembly of a polymeric lamina. Such an event could be caused by a reversible modification (possibly enzymatic) of the major lamina polypeptides. We would envision lamina disassembly as a necessary, but not sufficient, condition for the disruption of nuclear membrane morphology in prophase. According to this mechanism, reassembly of the lamina would occur in late anaphase and telophase, possibly by a reversal of a hypothetical modification of the major lamina proteins. The process of lamina reassembly could be centrally involved in the reconstruction of a new nuclear envelope at the daughter chromosome surfaces, considering the interactions which we propose for the lamina in the interphase nucleus (Fig. 10).

In contrast to higher eukaryotes, a pattern of complete nuclear envelope disassembly during mitosis does not occur for many lower eukaryotic species (18). In these cases, nuclear envelope disruption may be either partial (e.g., *Physarum polycephalum* [25, 52]), or absent (e.g., dinoflagellates [30]). Furthermore, pore complexes usually remain ultrastructurally intact in these situations (18, 25, 30, 52). We suggest that these species may have a nuclear lamina structure analogous to that of the higher eukaryotes, but that lamina disassembly is incomplete or absent during cell division.

Employing cell fractionation studies on populations of CHO cells, we have recently obtained evidence in support of a putative lamina depolymerization during mitosis in mammalian cells (L. Gerace and G. Blobel, manuscript in preparation). For either metaphase, early G1, or exponentially growing interphase CHO cells, anti-(rat liver) P70 antibody specifically immunoprecipitates three polypeptides which approximately comigrate on SDS gels with rat liver P70, P67, and P60. While these antigens occur exclusively in the nuclear/mitochondrial pellet of interphase and early G1 cell homogenates, they are found predominantly in a postmicrosomal supernate of metaphase cell homogenates. Hence, when the lamina is morphologically disrupted during mitosis as visualized by immunofluorescence microscopy, the major lamina polypeptides are not membrane-associated, but occur in a presumably soluble form. The biochemical mechanism of lamina depolymerization during mitosis is currently being investigated in our laboratory.

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