Characterization of the Major Polypeptides of the Rat Liver Nuclear Envelope*

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Rat liver nuclear envelopes have been isolated using a newly developed method. Purified nuclei were digested with DNase I and RNase A and then extracted with 1.6 M NaCl in the presence of a reducing agent. This method, which is rapid and simple, yielded predominantly intact nuclear envelope spheres. After correction for phospholipid recovery (40–50%), these nuclear envelopes contained 13, 3, and <1% of the total nuclear protein, RNA, and DNA, respectively.

Electrophoretic analysis of these nuclear envelopes revealed a marked enrichment for three polypeptides with apparent molecular masses of 69, 67, and 62 kDa (previously designated lamins A, B, and C, respectively). By two-dimensional isoelectric focusing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, lamin A could be resolved into at least four components (pI 6.8–7.2) and lamin C into at least three components (pI 6.8–7.2), while lamin B migrated as a single species (pI 5.7). Unidimensional proteolytic mapping of these polypeptides established that the major charged species with a particular molecular weight were structurally homologous. Two-dimensional tryptic maps revealed extensive sequence homology between lamins A and C, while lamin B had an apparently unrelated sequence.

When nuclear envelopes were prepared in the absence of reducing agents, analysis by two-dimensional nonreducing/reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that approximately half of lamin B was present as a disulfide-cross-linked 220-kDa oligomer. Disulfide-cross-linked homopolymers (apparent molecular mass, 160–420 kDa) of lamin A were also observed in small amounts. Despite its sequence homology with lamin A, lamin C did not form appreciable amounts of disulfide-cross-linked oligomers. Moreover, the oligomers of lamins A and B were absent when nuclei are isolated at pH 7.4 in the presence of specific sulfhydryl-blocking reagents (N-ethylmaleimide or S-methylmethane thiosulfonate) or at pH 2.5 in citric acid. Thus it appears that disulfide bonds are formed during the nuclear envelope isolation procedure. Nonetheless, the ability to form these intermolecular cross-links suggests the presence of regularly arranged polymeric arrays, the monomers of which contain juxtaposed sulfhydryl groups within the intact nuclear envelope.

According to current models (1–3), the nuclear envelope consists of two concentric 7.5–10-nm membranes separated by a 40–60-nm perinuclear space. Both the membranes and the perinuclear space are spanned by the nuclear pore complexes, which in turn are embedded in a proteinaceous, detergent- and salt-insoluble lamina situated between the inner nuclear membrane and the peripheral chromatin.

As analyzed by one-dimensional polyacrylamide gel electrophoresis, three major 60–70-kDa polypeptides, previously termed lamins A, B, and C, comprise 25–50% of the total rat liver nuclear envelope protein (4–6). These polypeptides have been localized to the lamin (6) and to the pore complex (7). The structural relationships between these three polypeptides remains unclear. Based on one-dimensional (8) or two-dimensional (9) proteolytic maps, it has been suggested that the 62- and 69-kDa polypeptides have homologous sequences while the 67-kDa polypeptide is unrelated. In contrast, we have previously observed that two-dimensional tryptic maps of all three polypeptides were similar (10, 11). In addition, Gerace et al. (6) have observed that antibodies prepared against isolated 69-kDa polypeptide cross-react with both 67- and 62-kDa polypeptides; Krohne et al. (7) have observed that antibodies prepared against isolated 67-kDa polypeptide cross-react with 62 and 69-kDa polypeptides. For all of these analyses, polypeptides were separated and isolated on the basis of molecular weight and without regard to the possibility that several different polypeptides might be contained in each molecular weight class. It is therefore not clear whether previous authors were dealing with individual polypeptides or heterogeneous mixtures of polypeptides.

A second point of controversy is the presence or absence of intermolecular disulfide cross-links between the major lamina polypeptides. Lam and Kasper (8) and Maul and Avdalovic (13) have noted the presence of large amounts of disulfide-cross-linked homo-oligomers of lamins A and B when nuclear envelope polypeptides from mammalian liver and clam oocytes were analyzed by two-dimensional nonreducing/reducing SDS-PAGE. Coupled with recent results (14–17) which suggest the reutilization of premitotic nuclear envelope constituents in the construction of the postmitotic nuclear envelope, these observations have led to the speculation that...
reduction and formation of disulfide bonds might be involved in the reversible depolymerization and polymerization of the nuclear envelope (see also Ref. 18). Shelton and Cochran (19), however, were able to demonstrate similar quantities of disulfide-cross-linked oligomers in avian erythrocyte nuclear envelopes only when disulfide bond formation was promoted by treatment with o-phenanthroline/copper. This observation calls into question the importance of disulfide bonds for lamin stability during interphase.

In the present study, we have modified previous procedures (20) to develop a rapid and convenient method for the isolation of rat liver nuclear envelopes (see Miniprint).2 Using this method, we have characterized the three major 60-70-kDa polypeptides of the nuclear envelope. Two-dimensional high-resolution IEF/SDS-PAGE reveals at least four major 69-kDa species (pl 6.8-7.2). Unidimensional proteolytic mapping established that the major 69-kDa species were structurally homologous (lamin A), as were the major 62-kDa species (lamin C). Two-dimensional tryptic mapping revealed extensive sequence homology between lamins A and C, while lamin B was apparently unrelated. Two-dimensional nonreducing/reducing SDS-PAGE indicated the presence of disulfide-cross-linked homopolymers of lamins A and B. These intermolecular disulfide bonds, however, appeared to result from manipulation of nuclei in vitro and not to reflect the intrinsic state of the nuclear envelope polypeptides in vivo.

MATERIALS AND METHODS

Materials.—Nonidet P-40 was from Bethesda Research Laboratories. N-Ethylmaleimide was from K & K. S-Methylmethane thiosulfonate was from Aldrich. Iodoacetamide and iodoacetate were from Sigma. Guanidine hydrochloride was from Heico. β-Mercaptoethanol, electrophoresis grade urea, and all reagents for electrophoresis were from Bio-Rad. Trypsin (1-1-tosylamido-2-phenylethyl chloromethyl ketone-treated) was from Worthington. Chloramine-T was from British Drug Houses. Sodium [""II]iodide (specific activity, 13-17 mCi/g) was from New England Nuclear.

Buffers.—Alkylation buffer consisted of 6 M guanidine hydrochloride, 250 mM Tris-HCl (pH 8.5 at 22° C), and 10 mM EDTA. SDS sample buffer was composed of 4 M deionized urea, 2% (w/v) sodium dodecyl sulfate, 50 mM Tris-HCl (pH 6.8 at 22° C), and 1 mM EDTA.

Isolation of Nuclei and Nuclear Envelopes—Except as indicated, all steps were performed as described in detail in the Miniprint.

Isolation of Nuclei and Nuclear Envelopes in the Presence of Sulphydrol Blocking Reagents—All steps were performed at 4° C. 10 mM iodoacetamide, N-ethylmaleimide, or S-methylmethane thiosulfonate was added to the buffers used for steps 1-3 of the nuclear isolation procedure (see Miniprint). After step 4, nuclei were resuspended at a concentration of 1 × 10^6/ml in STM/PMSF buffer again containing the indicated sulphydryl-blocking reagent (10 mM). After a one h incubation, the nuclei were sedimented at 800 × g for 10 min and washed three times with STM buffer (no sulphydryl-blocking reagent). Nuclear envelopes were then prepared as described above except that β-mercaptoethanol was omitted from step B.

Isolation of Nuclei and Citric Acid—Nuclei were isolated by steps 1-5 (Miniprint) except that 50 mM citric acid (pH 2.5) replaced 50 mM Tris-Cl, 5 mM MgSO_4, in all buffers.

Electrophoresis.—SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (21). Samples for two-dimensional reducing/nonreducing SDS-PAGE were, immediately after isolation, solubilized for 1 h at 22° C in alkylating buffer containing 140 mM iodoacetamide. They were then dialyzed successively (2 passes of 100 volumes each) into 6 M urea and into 0.1% (w/v) SDS, lyophilized, and solubilized in SDS sample buffer. Electrophoresis in the nonreducing dimension was performed in 3-mm tube gels consisting of a 5% (w/v) acrylamide resolving gel (9 cm high) and a 3% (w/v) stacking gel (0.5 cm high). After electrophoresis for 4 h at 1 mA/gel, these tube gels were equilibrated for 1 h at 21° C with 1.25% (v/v) β-mercaptoethanol in SDS sample buffer. Electrophoresis in the second (reducing) dimension utilized a 0.75-mm thick slab gel with a 10% (w/v) acrylamide resolving gel and a 5% (w/v) stacking gel (1.5 cm high).

Samples for IEF/SDS-PAGE were, immediately after isolation,
reduced and solubilized for 12-14 h at 22° C in 140 mM β-mercaptoethanol in alkylation buffer. Iodoacetamide was then added to a final concentration of 140 mM. After 1 h, samples were dialyzed at 4° C against 100 volumes of 6 M freshly deionized urea containing 50 mM Tris-HCl (pH 7.4 at 4° C) and 2% (w/v) Nonidet P-40 and then against 3 changes of 6 M freshly deionized urea containing 2% (w/v) Nonidet P-40. Samples were then warmed to 22° C. Crystalline urea (electrophoretic grade) was added to saturation, and pH 3.5-10 ampholytes (LKB) were added to a final concentration of 2% (w/v). Gels for isoelectric focusing were prepared as described by O'Farrell (22) except that the gels contained 2% (w/v) pH 3.5-10 ampholytes and twice the specified amount of ammonium persulfate. Samples from 1-2 × 10⁸ nuclei were applied to 10-cm tube gels and electrophoresed for 6000 V-h (22). Equilibration with SDS sample buffer and electrophoresis in the second dimension were performed as described above.

Proteolytic Mapping of Lamins—One-dimensional analysis of partial Staphylococcus aureus V-8 protease digests was performed as described by Cleveland et al. (23) with the following exception: 0.75-mm thick polyacrylamide gels consisted of a 6-cm high 15% (w/v) acrylamide-resolving gel and a 5-cm high 5% (w/v) stacking gel. After completion of the electrophoresis, gels were stained with silver as described by Merril et al. (24).

For two-dimensional tryptic peptide mapping, reduced and alkylated lamins A, B, and C were isolated from two-dimensional IEF/SDS-polyacrylamide gels as described below. The isolated lamins were solubilized in 0.1% (w/v) SDS and iodinated for 10 min at 20° C by sequential addition of sodium [¹²⁵I]iodide (100 μCi) and chloramine-T (final concentration, 250 μg/ml) as described by Greenwood and Hunter (25). The individual iodinated proteins were repurified by electrophoresis in polyacrylamide gels (21) containing 7.5% (w/v) acrylamide. Radioactive bands were located by autoradiography, sectioned from the gels, lyophilized, and rehydrated in 0.5 M ammonium bicarbonate (1 ml/band). The rehydrated bands were pulverized by passage through a stainless steel screen. Trypsin (20 μg/band) was added and digestion was allowed to proceed for 14 h at 20-22° C. Two hours after addition of a second aliquot (10 μg/band) of trypsin, polyacrylamide fragments were removed from the suspension by filtration through glass-fiber filters. The peptide-containing filtrate was lyophilized, resuspended in water, and relyophilized twice. The resulting peptide preparations were resuspended in peptide electro-

FIG. 4. Tryptic maps of lamins A, B, and C and mixtures A + C and A + B. Lamins were isolated and radiolabeled as described under "Materials and Methods." After exhaustive digestion with trypsin, the polypeptides were subjected to electrophoresis (left to right) followed by chromatography (bottom to top). A number of tryptic peptides of lamin A (a-o) have been lettered for reference. Lamin C contains a subset (a, b, e-k, m, and o) of these tryptic peptides as well as two apparently unique polypeptides (1 and 2). Mixing experiments (A + C) confirms the apparent homology between lamins A and C. Peptides 1 and 2 are indicated to confirm the presence of lamin C in the mixture. Lamin B yields a dissimilar set of tryptic peptides. Mixing experiments (A + B) confirm the lack of homology between lamins A and B. The peptides of lamin A have been identified by letter.

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...phoresis (see below) and subjected to two-dimensional separation (electrophoresis-chromatography) on microcrystalline cellulose thin-layer plates as described elsewhere (26). Peptide electrophoresis and chromatography buffers contained butanol, pyridine, acetic acid, and water in the ratios 2:1:1:36 and 97:75:15:60, respectively. Individual 125I-labeled peptides were detected by autoradiography.

Isolation and Analysis of Lamins—Two-dimensional IEF/SDS-PAGE of the nuclear envelope protein was performed as described above with the following exception: SDS-polyacrylamide gels for the second dimension contained the reversible cross-linker bisacrylylcysteine (27). After electrophoresis, staining with Coomassie Brilliant Blue R-250 and destaining, areas of the gels containing each of the lamins were excised. The gels were solubilized in β-mercaptoethanol, and the polypeptides were recovered by chromatography on hydroxyapatite.³

Amino acid analyses of the S-carboxymethylated proteins were performed on a Durrum D-500 automatic amino acid analyzer (Di- onex) using single column methodology (28). Duplicate samples were hydrolyzed in vacuo for 24 h at 110°C in 2.0 ml of 6 N HCl containing 20 µl of 5% (w/v) phenol. Threonine and serine were corrected upward 5% and 10%, respectively, for destruction during acid hydrolysis. Tryptophan was not determined. Amino sugars were analyzed as described (28) except that duplicate samples were hydrolyzed in vacuo for 6 h at 100°C in 4 N HCl.

RESULTS

We have developed a convenient and rapid method for the isolation of nuclear envelopes from rat liver nuclei (see Miniprint). This method, which involves the digestion of isolated nuclei with DNase I and RNase A followed by extraction with 1.6 M NaCl under conditions which minimize or reverse the formation of intermolecular disulfide bonds, yields intact nuclear envelope spheres (Fig. 1) which contain 13, 3, and <1% of the total nuclear protein, RNA, and DNA, respectively (Table I). One-dimensional SDS-PAGE reveals the virtual absence of histones and a marked enrichment for a small subset of nuclear polypeptides (Fig. 2). Particularly prominent are three polypeptide bands with apparent molecular masses 69, 67, and 62 kDa.

Isoforms of the 69-kDa and 62-kDa Polypeptides—Two-dimensional IEF/SDS-PAGE of reduced, alkylated nuclear envelope polypeptides (Fig. 3A) indicates the presence of at least four major 69-kDa polypeptides with apparent pI 6.8–7.2 (Fig. 3A, spots 1–4) and at least three major 62-kDa polypeptides with apparent pI 6.8–7.2 (Fig. 3A, spots 7–9). In contrast, a single major 67-kDa polypeptide with apparent pI 5.7 (Fig. 3A, spot 5/6) is apparent. These observations are in substantial agreement with previous results (9, 17).

In order to determine the relationship of the various polypeptides in the 62- and 69-kDa ranges, individual spots were excised from two-dimensional gels and subjected to unidimensional proteolytic mapping (Fig. 3B). The major 69-kDa polypeptides were found to have indistinguishable proteolytic degradation products (Fig. 3B, lanes 1–4) indicating structural homology. Similarly, the major 62-kDa polypeptides were found to have indistinguishable degradation products (Fig. 3B, lanes 7–9), a result which indicates that these 62-kDa polypeptides are likewise structurally homologous. These isoelectric variants will be collectively termed lamin A (69 kDa) and lamin C (62 kDa) using the nomenclature of Cerace et al. (6).

The single 67-kDa polypeptide will be termed lamin B.

Relationship between Lamins A, B, and C—The analysis shown in Fig. 3B indicates that the unidimensional proteolytic map of lamin B is substantially different from unidimensional maps of the lamins A and C. The results presented in Fig. 3B also show differences in the unidimensional maps of lamins A and C above 30 kDa. On the other hand, we and others (see Introduction) have suggested that lamins A and C have extensive sequence homology.

To further study the structural relationships between the three lamins, two-dimensional tryptic peptide mapping was performed. Lamins A, B, and C were separated by two-dimensional IEF/SDS-PAGE using bisacrylylcystine-cross-linked gels for the second dimension. After staining with Coomassie Brilliant Blue, regions of the gel corresponding to lamins A, B, and C were excised and solubilized. The isolated lamins were recovered, iodinated, and exhaustively digested with trypsin. Analysis of the iodinated tryptic peptides (Fig. 4) indicates that lamin C contains a subset of the iodinated peptides of lamin A, while lamin B contains a totally different set of peptides. Thus, lamins A and C share extensive sequence homology, while lamin B has an unrelated sequence.

Amino Acid Composition of Lamins A, B, and C—The amino acid compositions of the isolated lamins are presented in Table III. Despite our inability to demonstrate sequence homology between lamin B and the other lamins, the composition of lamin B is remarkably similar to that of lamins A and C.

The lamins are apparently nonglycosylated proteins. The absence of both glucosamine and galactosamine indicates that the lamins are devoid of asparagine-linked and serine/threonine-linked oligosaccharide side chains.

Disulfide-cross-linked Oligomers of Lamins A and B—While some workers (8, 13) have found the lamins to be present in isolated nuclear envelopes as disulfide-cross-linked homopolymers, others have reported that the lamins are present as monomers (17). In view of this controversy, we have reinvestigated the presence of intrinsic intermolecular disulfide bonds between the lamins.

Rat liver nuclear envelopes were prepared as described above except that β-mercaptoethanol was omitted from the procedure. When these nuclear envelopes were analyzed on one-dimensional SDS-polyacrylamide gels under reducing conditions, polypeptide bands with apparent molecular masses 67 and 69 kDa were present in approximately equal proportions.

Table III

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<tr>
<th>Amino acid</th>
<th>Lamin A</th>
<th>Lamin B</th>
<th>Lamin C</th>
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<tr>
<td>GalNH₂</td>
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*Threonine and serine were corrected upward 5% and 10%, respectively, for destruction during acid hydrolysis.

* Determined as S-carboxymethylcysteine.

ND, not determined.

Fig. 5. Two dimensional nonreducing/reducing SDS-PAGE. Structures resistant to treatment with nucleases and 1.6 M NaCl were isolated in the absence of reducing agents. A, one-dimensional SDS-PAGE was performed in the presence (left lane) or absence (right lane) of β-mercaptoethanol. Note the presence of a major polypeptide (arrowhead, apparent molecular mass, 220 kDa) present in the nuclear envelopes under nonreducing conditions (right lane) but not under reducing conditions (left lane). The decrease in this polypeptide under reducing conditions is accompanied by an increase in a 67-kDa polypeptide. Molecular mass markers (kilodaltons) at left refer to gel A. B-E, SDS-polyacrylamide gel electrophoresis was performed under nonreducing conditions in the first dimension (direction of migration from left to right). After the tube gels were reduced, SDS-polyacrylamide gel electrophoresis was performed from top to bottom. B, when no sulfhydryl-blocking agent was present during isolation of rat liver nuclei a number of oligomers were evident. The numbers 1-8 are located directly above the oligomeric species to which they refer. C, the addition of N-ethylmaleimide during the course of nuclear isolation resulted in a marked diminution in the amount of oligomers. D, the addition of iodoacetamide during the course of nuclear isolation did not diminish the amounts of oligomers. Gels B-D were loaded with samples derived from equal numbers of starting nuclei. E, oligomers were not discernible when rat liver nuclei were isolated in citric acid (see "Materials and Methods"). Molecular mass markers (kilodaltons) at right and across top refer to gels B-E.

amounts (Fig. 5A, left lane). In contrast, the same samples analyzed prior to reduction (Fig. 5A, right lane) showed diminished quantities of 67-kDa polypeptide and the concomitant appearance of a major new species with apparent molecular mass of ~220 kDa indicated by the arrowhead in Fig. 5A.

To further analyze the oligomeric state of the lamins, nuclear envelopes prepared in the absence of β-mercaptoethanol were subjected to two-dimensional nonreducing/reducing SDS-polyacrylamide gel electrophoresis. As is shown in Fig. 5B, at least eight disulfide-cross-linked oligomers were present as indicated by their migration below the diagonal. In additional to the major 220-kDa oligomer of lamin B (spot 1, Fig. 5B), several oligomers of lamin A (spots 2-5), and much smaller amounts of oligomers with approximate monomer-molecular masses 220 (spot 6), 72 (spot 7), and 62 kDa (spot 8) were evident. Unidimensional proteolytic digests confirmed that spot 1 consisted of lamin B and spots 3-5 consisted of lamin A (not shown—see also Ref. 8). The absence of a second component of differing molecular weight vertically aligned with the major oligomeric species 1, 3, and 4 indicates that these are disulfide-cross-linked homopolymers. Their apparent molecular masses (150-220 kDa before reduction) suggest that these oligomers are at least trimeric.

It is important to note that a large number of nuclear envelope polypeptides, e.g. those in the 42-60-kDa range, are not seen as disulfide-cross-linked oligomers in Fig. 5B. This result suggests that the presence of disulfide-cross-linked oligomers is not a result of fortuitous cross-linking of polypeptides during the solubilization and electrophoresis procedures, but instead represents the orientation of lamins in oligomeric arrays which can be cross-linked within the intact nuclear envelope.

Disulfide Cross-links Arise during Manipulation of Nuclei in Vitro—To assess the possibility that the disulfide bonds described above were formed during the isolation of nuclear envelopes, rat liver nuclei were isolated in the presence of
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sulfhydryl-blocking reagents and subjected (in the absence of β-mercaptoethanol) to the same series of digestions and extractions used to prepare nuclear envelopes. When nuclei were isolated in the presence of the hydrophobic sulfhydryl-blocking reagent N-ethylmaleimide (Fig. 5C) or S-methylmethane thiosulfonate (not shown—identical with Fig. 5C), disulfide-cross-linked oligomers of lamins A and B were essentially absent. Because nuclear envelopes isolated in the presence of these blocking agents do not contain sulfide-cross-linked oligomers, lamins A and B must be present in a "noncross-linked" state at some point during the nuclear envelope-isolation procedure.

The effect of hydrophobic sulfhydryl-blocking reagents was not limited to nuclear envelopes from rat liver. When rat kidney nuclei were isolated in the absence of sulfhydryl-blocking reagents, oligomers of lamins A and B were readily discernible in the nuclear envelope fraction (not shown—similar to Fig. 5B). When kidney nuclei were isolated in the presence of N-ethylmaleimide, the oligomers were again absent (not shown—similar to Fig. 5C).

Interestingly, not all sulfhydryl-blocking reagents have the same effect. When rat liver nuclei were isolated in the presence of the more hydrophilic sulfhydryl-blocking reagents iodoacetamide (Fig. 5D) or iodoacetate (not shown—identical with Fig. 5D), the amounts of cross-linked oligomers subsequently found in the nuclear envelope fraction were similar to those found in control preparations (Fig. 5B). This observation suggests that the cross-linkable sulfhydryl groups on lamins A and B are located in a nonpolar environment where they can readily react with hydrophobic blocking reagents but not hydrophilic reagents.

Are Disulfide Cross-links an Intrinsic Feature of Lamins A and B?—The absence of sulfide cross-linked oligomers of lamins A and B after isolation of nuclei in buffers containing N-ethylmaleimide (Fig. 5C) or S-methylmethane thiosulfonate suggests that lamins A and B are present in the intact nucleus in a noncross-linked state. An alternative interpretation, however, is that the disulfide-cross-linked oligomers exist in vivo, are reduced by glutathione which gains access to the nuclear compartment after homogenization of the tissue, and are trapped in the reduced state by N-ethylmaleimide and S-methylmethane thiosulfonate. Two additional experiments appear to rule out this latter interpretation. 1) Rat liver nuclei were isolated in citric acid at pH 2.5. Under these conditions, reduction of cross-links by glutathione would be expected to be minimal, yet no sulfide-cross-linked oligomers were observed (Fig. 5E). 2) Mixing experiments failed to detect reduction of disulfide bonds at pH 7.4. Nuclei isolated in the absence of blocking reagents (and containing sulfide-cross-linked oligomer) were added to fresh rat liver and reisolated in the presence of S-methylmethane thiosulfonate. When nuclear envelopes were subsequently isolated, sulfide-cross-linked oligomers were observed (data not shown—similar to Fig. 5B). Thus conditions encountered during nuclear isolation did not result in reduction of sulfide cross-links once they are formed. This result, coupled with the absence of intermolecular disulfide bonds in nuclear envelopes prepared in the presence of hydrophobic sulfhydryl-blocking reagents (see above), leads to the conclusion that the intermolecular disulfide cross-links are not an intrinsic structural feature of the nuclear envelope, but result, instead, from manipulations during the isolation procedure in vitro.

DISCUSSION

A point of current uncertainty regarding the structure of the nuclear envelope is the relationship of the major 60-70-kDa polypeptide species (the lamins) to each other. Lam and Kasper (8) and Shelton et al. (9) have reported that the 62- and 69-kDa polypeptides have similar one- and two-dimensional proteolytic maps, while the 67-kDa polypeptide has a unique proteolytic map. In contrast, Gerace and Blobel (17) and Shaper et al. (11) have reported that a nuclear envelope polypeptide with apparent molecular mass 67 kDa cross-reacts with antibodies to the 62- and 69-kDa polypeptides.

In all of these analyses, it has been assumed that the multiple components of the 62- and 69-kDa polypeptides resolved by IEF/SDS-PAGE were structurally related. By high resolution proteolytic mapping of individual spots excised from two-dimensional IEF/SDS gels, we have established this point (Fig. 3). It should be noted, however, that the nuclear envelope also contains many minor polypeptides in the 60-75-kDa range (Fig. 3A), all of which have proteolytic maps which differ from those of the major polypeptides. Thus the terms lamin A, lamin B, and lamin C should be reserved for the polypeptides characterized in the present study.

The basis for the charge heterogeneity of lamins A and C is currently unknown. In view of the observation (17, 29-31) that the lamins from a variety of species are phosphorylated proteins, the charge heterogeneity might reflect differing degrees of phosphorylation. Phosphorylation of the lamins has been implicated in depolymerization of the lamina during metaphase (17) and in transport of ribonucleic acid across the rat liver nuclear envelope during interphase (29). On the other hand, the presence of multiple components of lamins A and C during isoelectric focusing does not necessarily indicate structural heterogeneity. As Cann (32) has demonstrated, multiple components can reflect the presence of various ampholyte-macromolecular complexes formed by the interaction of ampholytes with a homologous polypeptide. Whether the charge heterogeneity of lamins A and C described in the present study reflects intrinsic structural heterogeneity or artificial interaction with ampholytes remains to be determined.

Our two-dimensional maps (Fig. 4) confirm that the 125I-labeled tryptic peptides of the major 62-kDa polypeptide comprise a subset of the 125I-labeled tryptic peptides of the major 69-kDa polypeptide. In contrast, the 67-kDa polypeptide contains a unique set of 125I-labeled tryptic peptides. Hence it appears that lamin B is a unique gene product.

Whether lamin A and lamin C are derived from a single gene (by pre- or post-transcriptional mechanisms) or from two closely related genes remains to be determined.

In view of these results, how is one to interpret the results of Gerace et al. (6) and Krohne et al. (12) as well as our own preliminary experiments (10, 11) indicating that polypeptides with apparent molecular masses 62, 67, and 69 kDa all share a set of 125I-tryptic peptides? Recent experiments indicate that the isolation of nuclear envelopes in the absence of the serine-esterase inhibitor phenylmethylsulfonyl fluoride results in envelopes which contain new polypeptide species of apparent molecular mass 67 kDa and apparent pi 6.8-7.2. These species, which appear to be derived from the 69 kDa lamin A by limited proteolysis in vitro, are absent when nuclei and nuclear envelopes are isolated in the presence of PMSF (Fig. 3A). These proteolytically-derived 67-kDa species might well represent the 67-kDa polypeptides which have previously been observed to cross-react with antibodies to lamin A. Gerace and Blobel (17) have reported similar observations in Chinese hamster ovary cells. These results underscore the need for the isolation of nuclear envelopes under conditions which minimize proteolytic degradation. (see "Discussion" in the Miniprint).

A second point of uncertainty is the presence of disulfide-
cross-linked homopolymers in nuclear envelope preparations. While some authors (8, 13) have reported that the disulfide cross-links are an intrinsic feature of the nuclear envelope, others have reported that the lamins are present as "monomers" (17) unless disulfide cross-links are deliberately induced (19). In the present communication, we have reported that rat liver and kidney nuclear envelopes isolated at pH 7.4 in the absence of sulfhydryl-blocking agents contain a prominent disulfide-cross-linked homopolymer of lamin B and smaller amounts of several homopolymers of lamin A. In contrast, little if any lamin C is recovered in disulfide-cross-linked oligomers (see also Ref. 8). In view of the structural homology between lamins A and C (Fig. 4), the diminished ability of lamin C to form disulfide-cross-linked homopolymers might reflect the absence of a sulfhydryl-containing molecular domain which is involved in the cross-linking. Consistent with this view, lamin C contains considerably less cysteine than lamin A (Table III). On the other hand, we have not ruled out the possibility that lamin C is oriented in the nuclear envelope in such a way that its sulfhydryl groups are not available to form intermolecular sulfhydryl cross-links.

The disulfide cross-links described above are not observed when nuclei are isolated at pH 2.5 in citric acid or at pH 7.4 in the presence of the hydrophobic sulfhydryl-blocking reagents N-ethylmaleimide or N-methylmethane thiosulfonate. Thus the disulfide bonds appear to form during nuclear envelope isolation. The inability of the hydrophilic sulfhydryl-blocking reagents iodoacetamide and iodoacetate to prevent disulfide bond formation suggests that the sulfhydryl groups which form the intermolecular disulfide bonds are located in a hydrophobic environment, either in hydrophobic patches on the surfaces of neighboring polypeptides or perhaps in the hydrophobic core of the inner nuclear membrane.

We have been unable to demonstrate that the intermolecular disulfide bonds which stabilize the homopolymers are an intrinsic feature of the nuclear envelope. The ability to isolate nuclear envelopes which lack these cross-links indicates that these covalent bonds are not essential for the stability of the nuclear envelope during interphase. Nonetheless, the ability to form these disulfide cross-links indicates the presence of juxtaposed sulfhydryl groups on neighboring polypeptides in the nuclear envelopes. Thus, these polypeptides are not present as true monomers, but instead as noncross-linked polymeric arrays in the nuclear envelope.

The inability of the lamins to form disulfide-cross-linked heteropolymers (see also Refs. 8, 13, 19) is striking. While this might reflect the absence of suitably spaced groups, it might also reflect the absence of juxtaposition between lamins A and B. One could envision, for example, fibers (33-35) composed of lamin A and other fibers composed of lamin B. Further studies are needed to more clearly define the topological relationships of the lamins to each other.

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REFERENCES


Additional references are found on p. 2719.
Nuclear Envelope Polypeptides

Scott R. Kaufman, Wade Gibson and Joel M. Shapiro

Study of the mammalian nuclear envelope requires the isolation of this structure in a highly purified form in which it is suitable for subsequent characterization. Several techniques are currently available, but none of these offers the advantages of high purity and yield that are provided by methods developed for isolation of the nuclear envelope. The major advantage is the relative purity and yield of nuclei obtained by these methods, which provide a source of nuclei that are more suitable for isolation of nuclear envelope than are those obtained by other methods. The method described here provides a simple and efficient method for isolation of highly purified nuclear envelopes that can be used in subsequent studies of nuclear envelope structure and function.

In this method, the nuclear envelope is isolated from intact cells or tissues by a combination of mechanical and chemical treatments. The nuclear envelope is first detached from the nuclear matrix by gentle agitation and subsequent incubation with a mild detergent. The nuclei are then released from the cell by a combination of high-speed centrifugation and digestion with a protease. The released nuclei are then purified by a series of centrifugation and dialysis steps, resulting in a highly purified preparation of nuclear envelope proteins.

Materials

1. Nuclear extract buffer: 250 mM sucrose, 50 mM Tris-HCl (pH 7.4 at 4°C), 5 mM MgCl₂, 0.5 mM EDTA, 0.5% Nonidet P-40.
2. Nuclear isolation buffer: 220 mM sucrose, 50 mM Tris-HCl (pH 7.4 at 4°C), 5 mM MgCl₂, 450 mM KCl, 0.5 mM EDTA, 0.5% Nonidet P-40.
3. Nuclear extract buffer: 250 mM sucrose, 50 mM Tris-HCl (pH 7.4 at 4°C), 5 mM MgCl₂, 0.5 mM EDTA, 0.5% Nonidet P-40.

Methods

1. Preparation of nuclear envelope: Intact cells or tissues are resuspended in nuclear extract buffer, and nuclei are isolated by a combination of high-speed centrifugation and digestion with a protease. The isolated nuclei are then purified by a series of centrifugation and dialysis steps.
2. Purification of nuclear envelope: The purified nuclear envelope is then further purified by a series of centrifugation and dialysis steps to remove debris and nonspecific proteins.

Results

1. Analysis of nuclear envelope: The isolated nuclear envelope is analyzed by SDS-PAGE and Western blotting to identify the major proteins present in the nuclear envelope.
2. Function of nuclear envelope: The functional properties of the isolated nuclear envelope are studied by measuring its ability to maintain the integrity of the nuclear membrane and to regulate nuclear transport.

Figure 1: ISOLATION OF NUCLEAR ENVELOPE

A. Nuclear envelopes isolated as described in MATERIALS and METHODS appear morphologically intact, as expected. B, At higher magnification, the isolated nuclear envelopes show some loss of material, possibly due to degradation of nuclear matrix proteins. C, At higher magnification, the isolated nuclear envelopes show some loss of material, possibly due to degradation of nuclear matrix proteins.
Critical variables in the isolation procedure

Several aspects of the isolation procedure are critical. Digestion with Phaeo A was performed in order to facilitate the removal of the nucleolus in 1.6 M NaCl. When digestion with Phaeo A was omitted, residual components of the nucleolus remained discernible (Fig. 1B). On the other hand, the addition of d-mercaptoethanol during the first or second extraction with 1.6 M NaCl was required to quantitatively remove d-mercaptoethanol was lost, variable amounts of granular non-nuclear intercular material were adsorbed (Fig. 1D). This non-nuclear intercular material was not seen if nuclear envelopes were isolated from nuclei which had been treated with d-mercaptoethanol from HClO4. These observations suggest that the role of d-mercaptoethanol is to reduce intermolecular disulfide bonds which sparsely form during the isolation and subsequent manipulation of the nuclei (see also ref. 20).

Purity of the nuclear envelopes

To ascertain the level of contamination by extranuclear material, the nuclear envelopes were assayed for a number of enzymes which are characteristic of various cytoplasmic fractions. Results of such an analysis (Table I) are comparable to those previously reported (reviewed in 5). Because this analysis compares purified nuclear envelopes with crude mitochondria, lysosomes, etc., it tends to overestimate the contamination in the nuclear envelope fraction. Nonetheless, less than 1% of the protein in the nuclear envelope fraction results from contamination by cytoplasmic organelles.

Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Succinate Dehydrogenase</th>
<th>Acid Phosphatase</th>
<th>Lactate Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear Envelope</td>
<td>0.2</td>
<td>0.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>12.0</td>
<td>15.6</td>
<td>1.4</td>
</tr>
<tr>
<td>27,000 x g pellet</td>
<td>2.5</td>
<td>8.6</td>
<td>15.0</td>
</tr>
<tr>
<td>Microsomes</td>
<td>1.1</td>
<td>0.3</td>
<td>1.9</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>0.8</td>
<td>0.7</td>
<td>18.0</td>
</tr>
</tbody>
</table>

Fractions were isolated and assayed as described in MATERIALS AND METHODS. Data are expressed as units of product formed/mg protein. Similar results were obtained in three additional experiments.

Yield and gross biochemical composition

Previous results from a number of laboratories (29,48,58) have indicated that 90% or the nuclear phospholipid is located in the nuclear envelope. One can therefore ascertain the yield of nuclear envelopes by estimation of the recovery of phospholipid. When nuclei were extracted as described above, 44 ± 12 (n = 12) of the phospholipid was recovered. This is similar to yields reported previously (38,41).

In Table II the gross biochemical compositions of nuclear and nuclear envelopes are compared. Data are expressed as mg of protein, DNA, or RNA per mg phospholipid and are similar to those obtained by others (38,47,49,51). From these data, one can also calculate that the nuclear envelopes contained 11.8, 29.9, and 31% of the total nuclear protein. (See legend to Table II.)

Table II

<table>
<thead>
<tr>
<th>Component</th>
<th>Nuclear Envelope</th>
<th>Nuclear Envelope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein/Phospholipid (mg/mg)</td>
<td>6.2 ± 0.7</td>
<td>1.6 ± 0.12</td>
</tr>
<tr>
<td>RNA/Phospholipid (mg/mg)</td>
<td>0.36 ± 0.08</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>DNA/Phospholipid (mg/mg)</td>
<td>2.5 ± 0.4</td>
<td>0.01 ± 0.00</td>
</tr>
</tbody>
</table>

Nuclei and nuclear envelopes were isolated as described in MATERIALS AND METHODS. Duplicate aliquots were stored at -20°C until analyzed for protein, RNA, DNA, and phospholipid. Values are means ± S.E. of 12 independent experiments. If one assumes that all of the DNA and RNA found in the nuclear envelope is associated with the nuclear envelope (see 48,56), then the recovery of nuclear components in the nuclear envelope fraction can be derived by dividing each value in the right-hand column by the corresponding value in the left-hand column. That the nuclear envelopes contain 11.8, 29.9, and 31% of the total nuclear protein, DNA, and RNA, respectively. In addition, the nuclear envelopes contain small amounts of neutral carbohydrates which amount to approximately 5% of their total dry weight (see also 48).


**Figure 2.** SDS-PAGE PHOTOS \textit{Electrophoreis.} Nuclear envelopes prepared as described in MATERIALS AND METHODS were extracted for 15 minutes at 4°C with 1% (w/v) Triton X-100 in 50 mM Tris buffer. The pellet (membrane) was collected by centrifugation at 16,000 g for 30 minutes. The Triton-soluble material was collected by precipitation with 50% (v/v) trichloroacetic acid and washed with acetone before solubilization in SDS-sample buffer containing 1% (v/v) mercaptoethanol. Electrophoresis was performed by the method of Laemmli ([27] using 5-15% slab gels with a 5% stacking gel. Samples from equal numbers of starting nuclear envelopes were applied to each well. A, whole nuclear envelopes; B, Triton-soluble nuclear envelope polysaccharides; C, lamina. Molecular masses in kDa are shown as a left. A indicates the region of migration of histone H1A, H1B, H2A, H2B, and H4 in parallel wells containing whole nuclei.

**Discussion.**

The technique described above can be used to isolate highly purified nuclear envelopes in yields of >90%. The technique depends upon the combination of 7M LiCl and 1 M NaCl to remove DNA and histones, Phase A and 3 M NaCl to remove components of the nucleolus, and reducing agents to break disulfide bonds, which otherwise prevent the complete extraction of intranuclear components. Contamination by non-nuclear membranous material is minimized by two cycles of density sedimentation of the nuclei prior to their extraction. The nuclear envelopes obtained by this method have biochemical properties which are similar to those reported for nuclear envelopes prepared by other techniques. In particular, the nuclear envelopes contain (after correction for phosphorolytic recovery) 13% of the total nuclear protein, 3% of the total DNA RNA, and 1% of the total DNA. These results are similar to those of previous authors (39, 40, 41, 42).

When compared to current techniques used to isolate nuclear envelopes, this new method has a number of advantages. First, all of the nuclear envelopes are obtained in the form of intact spheres which reflect the quantity of the nuclei from which they were isolated. Moreover, the nuclear envelopes have not been subjected to the various procedures which assess the contamination by intranuclear components. The large size of the nuclear envelopes allows their rapid sedimentation at 40,000g and facilitates analysis of nuclear envelope functions. In addition, the technique described in the present study is rapid (4 h). Partly of the nuclear envelopes is not sacrificed, however, for analysis of marker enzymes (Table I) reveals levels of contamination by extranuclear material which is similar to that reported when nuclear envelopes are prepared by other techniques (39, 40, 41). Other rapid techniques (42) yield nuclear envelopes which are contaminated by substantial amounts of chromatin as indicated by the levels of DNA contamination. In contrast, the technique developed in the present study yields nuclear envelopes substantially free of chromatin as indicated by the absence of chromatin in the histone fraction. The present technique also avoids the addition of complex polysaccharides such as heparin (43) or dextran sulfate which could complicate the analysis of endogenous nuclear envelope carbohydrates (39, 40, 41, 42). Finally, the present technique involves steps which can all be carried out at 4°C in the presence of inhibitors of proteolysis. This, coupled with the rapidity of the technique, should enable the isolation of nuclear envelopes in which degradation of the nuclear envelope component has been minimized.

**References.**

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