The early flowering and longer span of male activity, highly male-biased sex ratio, and high male-flower density under poor light regime suggest that, in this species selection favours males to ensure availability of sufficient pollen to every female flower irrespective of the light conditions under which it grows. The greater number of females favoured under high light regime is probably to minimize resource constraint for developing seeds.

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A novel cross-linking technique to study nuclear lamina–membrane interactions

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Protein cross-linking studies using various bifunctional reagents can provide information about the organization of complex proteinaceous structures. We have studied the association of the nuclear lamina with membrane proteins using bifunctional cross-linkers of the bis-(imidoester) class. Analysis of cross-linked products by two-dimensional diagonal gel electrophoresis demonstrates that the lamins are closely associated with three nuclear membrane proteins (54, 50 and 45 kDa) in intact nuclear envelopes. This interaction does not occur when the envelope organization is disrupted with detergent or urea.

THE inner surface of the nuclear envelope in a eukaryotic cell is closely associated with a filamentous network or lamina, composed of intermediate filament-like proteins called the lamins[1–4]. The lamins may be classified into two major types: the A-type, which get solubilized during mitosis[5,6], and represented by lamins A and C in most cells; and the relatively insoluble B-type lamin. Lamin B is strongly associated with the nuclear membrane[7] and remains bound to membrane vesicles during nuclear-envelope disassembly at mitosis and subsequent reformation of the envelope[5]. The biochemical basis of interaction of the lamina with the inner nuclear membrane is not clearly understood. Recent studies with purified lamin B and lamin-depleted nuclear membranes suggest that lamin B is anchored to the membrane via a 58-kDa receptor protein in avian and yeast cells[8–10].

In the present study, we have adopted a direct approach to look at the interactions of the lamina with nuclear membrane proteins in mammalian cells by chemical cross-linking studies with purified nuclear envelopes using bifunctional imidoesters and analysis of cross-linked products by two-dimensional diagonal gel electrophoresis.

Nuclear envelopes were isolated from purified mouse liver nuclei by Kaufmann's procedure[11], and characterized in detail by biochemical and morphological criteria as described earlier[12]. Nuclear envelopes were obtained as intact, double-membrane vesicles, similar in size to nuclei and devoid of intranuclear and cytoplasmic contaminants. Envelopes were fractionated with (i) 8 M urea, or (ii) 2% Triton X-100 and low or high concentrations of salt (20 mM or 300 mM KCl) or 4 M urea by published methods[8,13,14].

In a typical cross-linking reaction, intact or extracted nuclear envelopes (~100 μg protein) were incubated with 5 mM dimethyl suberimidate (DMS; Pierce Chemicals, USA) for 30 min at 30°C in 100 μl of 100 mM triethanolamine·HCl, pH 8.0 (refs. 15–17). The reaction was quenched with excess glycine and the samples separated by SDS–polyacrylamide gel electrophoresis (8% polyacrylamide gels)[18]. These conditions were found to be suitable after standardization with respect to concentration of cross-linking reagent (0.5–20 mM) and time (15 min–24 h). Under these conditions, up to 10% of cross-linked higher-molecular-mass species were detectable for a known tetrameric protein, alcohol dehydrogenase, the remainder being mostly products of addition of several molecules of cross-linker to monomers of the protein. Experiments were also carried out with dimethyl pimelimidate (DMP) and dimethyl adipimidate (DMA) (which form shorter bridges) under similar conditions. Samples of cross-linked proteins (in triplicate) were separated by SDS–polyacrylamide gel electrophoresis. One lane was stained with Coomassie blue and the second lane was treated with methylamine exactly as described[16]. The treated lane and the third, untreated lane were separated in the second dimension by SDS–polyacrylamide gel electrophoresis (8% polyacrylamide gels). The two-dimensional gels were stained by the more sensitive

silver-staining technique[19], which was applied subsequent to or as an alternative to staining with Coomassie blue.

Cross-linking of highly purified, intact nuclear envelopes with DMS gives rise to extensive cross-links, as indicated by the high-molecular-mass (~200 kDa) material seen in the first-dimension gel in Figure 1, *a*. Treatment of the first-dimension gel with methylamine followed by electrophoresis in the second dimension (Figure 1, *b*) indicate the presence of proteins that were originally cross-linked as spots off the diagonal, and give a major set of spots in the range 70–67, 62, 54, 50 and 45 kDa. A second set of spots is also seen at 70–67 and 62 kDa. The spots at 70–67 and 62 kDa migrate to positions identical to those of lamins A, B and C respectively in the first-dimension gel. Slight cross-linking between the 54 and 45 kDa proteins is also observed but it is not significant enough to suggest that these proteins are associated with each other. Although some spots are also observed just below the diagonal, their molecular masses do not indicate that they were cross-linked initially and so they may have arisen through methylamine cleavage of the non-cross-linked proteins in the first-dimension gel, which is known to occur[16]. Visible cross-linking with DMA or DMP was not observed with intact envelopes.

To confirm that the 70–67- and 62-kDa spots are the nuclear lamins, envelopes were extracted with 8 M urea to preferentially solubilize the lamins, leaving membranous material in the high-speed pellet[8]. Cross-linking of proteins in the 8-M-urea supernatant with DMS (or DMA or DMP) gave a faint set of spots in the range 70–67 and 62 kDa, corresponding to the lamins, and did not give spots at 54, 50 and 45 kDa (see Figure 2). This indicates that the interaction between the lamins and the 54-, 50- and 45-kDa proteins is lost upon urea extraction of envelopes, and suggests that the 54-, 50- and 45-kDa proteins may be integral membrane proteins. Their membrane location was confirmed as follows.

When envelopes were extracted with Triton/low-salt or Triton/high-salt buffers to solubilize membrane proteins, and the supernatants subjected to cross-linking with DMS, no significant cross-linking was discernible (data not shown). Extraction of envelopes with Triton/urea buffer (which also partially disrupts the lamina) followed by cross-linking of proteins in the supernatant, gave cross-linked spots at 70–67 and 62 kDa (Figure 3). However, no spots were seen at 54, 50 and 45 kDa, suggesting that the interaction of the lamins with integral membrane proteins seen in intact envelopes is lost when the envelope architecture is disrupted with detergent.

Our data demonstrate that (i) the nuclear lamins are closely associated with three membrane proteins (54, 50 and 45 kDa) in intact nuclear envelopes; (ii) this association does not occur when the envelope organization is disrupted, either by solubilizing the membrane with detergent or by extracting the lamina with 8 M urea. The requirement for a definite length of cross-linker bridge, viz. 11 Å for DMS, and the absence of visible cross-linking of intact nuclear envelopes with reagents forming shorter bridges (8.6 Å for DMA and 9.2 Å for DMP) are further evidence for a specific interaction between the lamins and their membrane-binding sites. Our observations on the cross-linking of the lamins to each other is consistent with the available data on the strong interactions between the different lamins. Recent reports have suggested that lamin B in avian and yeast cells is anchored to the membrane via a

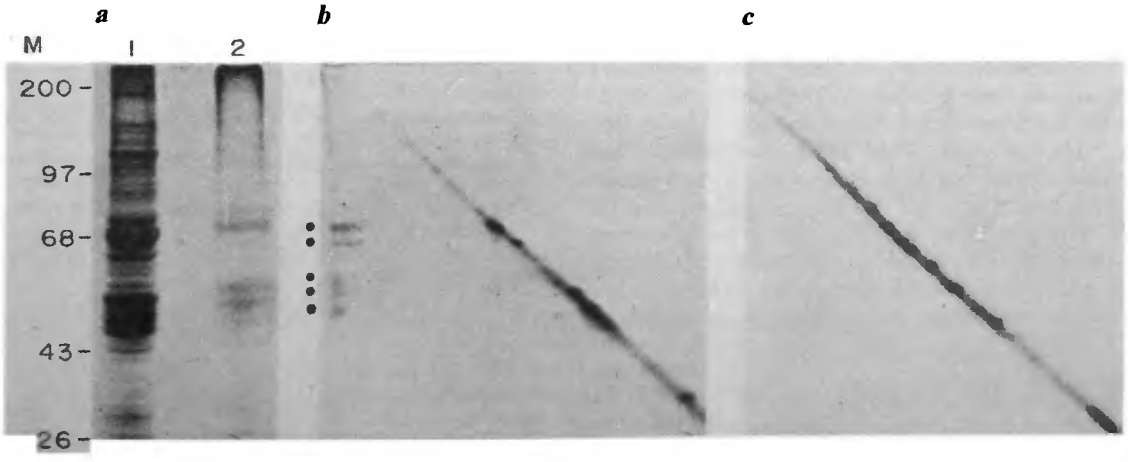

Figure 1. Cross-linking of intact nuclear envelopes with DMS. *a*, First-dimension gel: lane 1, intact envelope proteins; lane 2, cross-linked envelope proteins. *b*, Second-dimension gel, after methylamine treatment. Bold dots indicate cross-linked polypeptides. *c*, Untreated second-dimension gel, molecular mass markers (M) are myosin (200 kDa), phosphorylase *b*, (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), α-chymotrypsinogen (26 kDa).

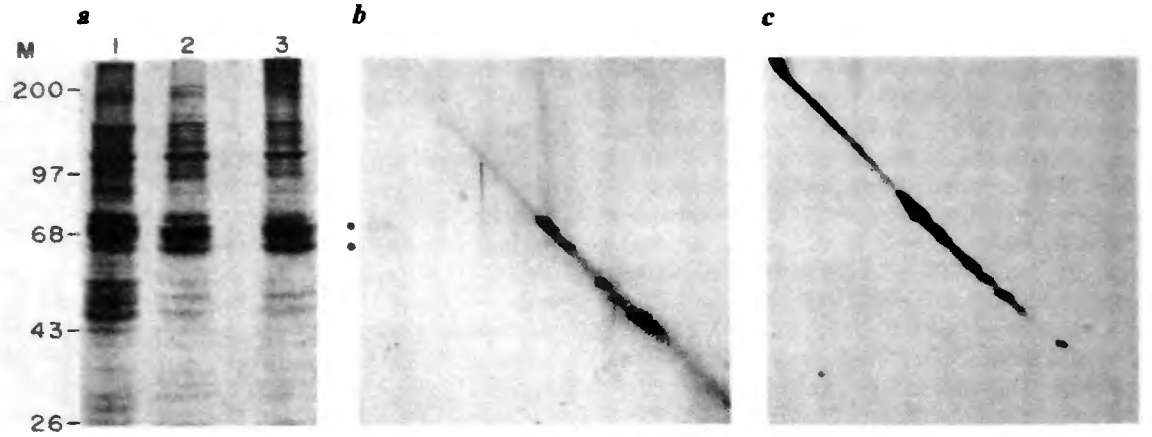

Figure 2. Cross-linking of proteins solubilized with 8 M urea. *a*, First-dimension gel: lane 1, intact envelope proteins; lane 2, supernatant after 8 M urea extraction of envelopes; lane 3, cross-linked proteins from urea supernatant. *b*, Second-dimension gel, after methylamine treatment. *c*, Untreated second-dimension gel; molecular mass markers are as in legend to Figure 1.

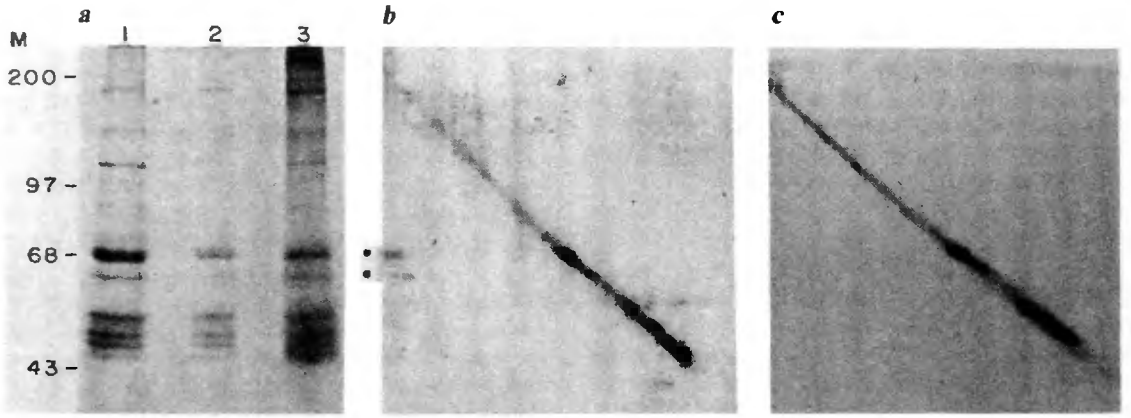

Figure 3. Cross-linking of proteins solubilized with Triton/urea buffer. *a*, First-dimension gel: lane 1, intact envelope proteins; lane 2, supernatant after Triton/urea extraction of envelopes (contains half the amount of protein in lane 3); lane 3, cross-linked proteins from Triton/urea supernatant. *b*, Second-dimension gel, after methylamine treatment. *c*, Untreated second-dimension gel; molecular mass markers are as in legend to Figure 1.

58-kDa membrane receptor protein[8,9]. Our preliminary evidence on binding assays with labelled lamin B and nuclear envelopes from mouse liver nuclei suggests that the molecular mass of the lamin B receptor in the mouse system is 54 kDa (S. Pandey and V. K. Parnaik, unpublished results), and this may be similar to the 54-kDa membrane-binding protein that we have observed in this study. Further studies on these proteins and their exact sites of attachment to the different lamins should be feasible once purified proteins are available.

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