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# Heterotypic and homotypic associations between the nuclear lamins: Site-specificity and control by phosphorylation

**(affinity chromatography/blot assays/protein–protein interaction/synthetic peptide)**

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ABSTRACT **Using purified components in affinity chromatography and blot binding assays, we have found that rat liver lamins A, B, and C can associate in homotypic and heterotypic fashions. Heterotypic A–B and C–B complexes are unusually stable and involve the common amino-terminal domain of lamins A and C, but not their helical "rod" domain. A synthetic peptide, comprising the first 32 amino acid residues of lamins A and C, is able to fully compete with the intact molecules for binding to lamin B. Conversely, heterotypic A–C associations and homotypic A–A and C–C interactions appear significantly weaker than A/C–B binding and do not involve the lamin A and C amino-terminal domain. Homotypic B–B complexes are not formed to any considerable extent unless isolated lamin B subunits are "superphosphorylated" *in vitro* with protein kinase A. However, when lamins A and C are similarly modified, no changes in their binding specificity can be detected. These data suggest that the nuclear lamina, unlike other multicomponent intermediate filaments, constitutes a nonobligatory heteropolymer. They also indicate that cAMP-dependent phosphorylation of interphase lamin B could cause remodeling of the lamina and establishment of homopolymeric domains.**

The nuclear lamina is an anastomosed fibrous meshwork linking the nucleoplasmic surface of the inner nuclear membrane (for reviews see refs. 1 and 2). This structure, situated at the nucleoplasm–cytoplasm interface, is thought to serve diverse integration functions such as the attachment of chromatin to the nuclear envelope (3, 4), the anchorage of cytoplasmic intermediate filaments to the nucleus (5, 6), and the stabilization of the nuclear membrane, in analogy to the plasma membrane skeleton (7). The molecular composition of the lamina is tissue-specific (8, 9) and its constituents, the so-called nuclear lamins, are distinct proteins structurally related to intermediate filament subunits (10–15).

In previous studies we noticed that certain lamin subunits form complexes even at high urea concentrations (5). In the present report, we examine in detail the site-specificity of such lamin–lamin associations and investigate some properties of lamin complexes.

## MATERIALS AND METHODS

**Protein-Chemical Procedures.** Ion-exchange resins (DE-AE-celluloses DE52 and DE53) were obtained from Whatman (Whatman Paper, Maidstone, Kent, England). Rat liver lamins were isolated and radioiodinated as described (5). For "superphosphorylation" of the lamins, the purified proteins [lamins A/C ( a mixture) at 0.07 mg/ml and lamin B at 0.12 mg/ml] were first dialyzed against 30 mM NaCl/15 mM Tris·HCl, pH 7.0/0.3 mM $MgCl_2$/1 mM dithiothreitol. Then [$\gamma$-$^{32}$P]ATP (adenosine 5′-[$\gamma$-$^{32}$P]triphosphate, 200 $\mu$Ci, 5000 Ci/mmol, New England Nuclear, Boston, MA; 1 Ci = 37 GBq) and nonlabeled ATP were added to the samples to give a final ATP concentration of 0.2 mM. The reactions were initiated by introducing 900 units of the catalytic subunit of protein kinase A (Sigma). After 1-hr incubation at room temperature, the samples were dialyzed extensively against 150 mM NaCl/15 mM Tris·HCl, pH 7.3/2 mM $MgCl_2$/1 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride/0.1% Tween 20. Limited proteolysis was performed by incubating the lamins with chymotrypsin at a 250:1 ratio (wt/wt), at room temperature and for various time periods. Affinity matrices were constructed by chemically coupling purified lamin B to Affi-Gel 15, and lamins A and C, or their N-terminal peptide, to Affi-Gel 10 (derivatized agarose; Bio-Rad. The coupling of intact lamins was done in 7 M urea/100 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]/1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride at pH 7.4. The synthetic lamin A/C peptide, referred to as $L_{1-32}$, was coupled in the above medium without urea. In general, the affinity matrices contained 50–200 $\mu$g of immobilized protein per ml of agarose beads.

**Assays.** Solid-phase binding assays, involving electrophoretically separated polypeptides as substrates and radiolabeled lamins as probes, were exactly as described (6). In the case of the blot shown in Fig. 4*C* (with the iodinated synthetic peptide), washing time and volume were reduced to 1 hr and 400 ml, respectively. Chromatographic assays were performed as described (5).

**Other Procedures.** One-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis was performed according to Laemmli (16), and protein concentrations were determined according to Lowry *et al.* (17). Electropherograms shown in this article are based on 10% polyacrylamide gels unless stated otherwise. All autoradiograms presented here have been printed in reverse contrast.

## RESULTS

**Lamins A and C Interact Directly with Lamin B.** When a mixture of $^{125}$I-labeled lamins ($^{125}$I-lamins) A/C and unlabeled bovine serum albumin was applied under physiological conditions of ionic strength to a lamin B affinity matrix, the tracer, but not the carrier protein, was quantitatively retained by the column. Subsequent elution with 8 M urea (Fig. 1*A*, lanes 1 and 2) released all the bound material. Likewise, columns consisting of lamin A/C-agarose bound $^{125}$I-lamin B (Fig. 1*A*, lanes 5 and 6); $^{125}$I-lamins A/C also bound to this column but to a lesser extent (lanes 3 and 4).

To confirm these results by another method, we used a binding assay (6) whereby proteins fractionated by $NaDodSO_4$/polyacrylamide gel electrophoresis are quantita-

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Abbreviation: $^{125}$I-lamin, $^{125}$I-labeled lamin.

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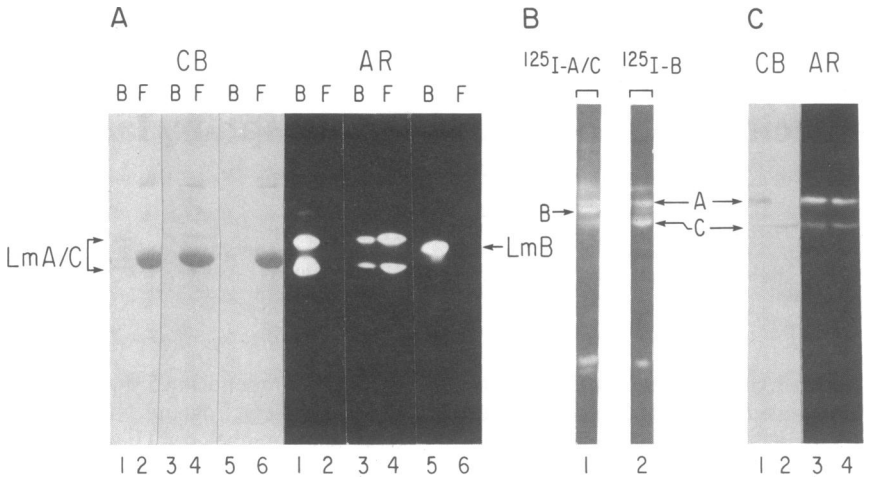


FIG. 1. Detection of lamin complexes formed *in vitro*. (*A*) Affinity chromatography assays. Samples of $^{125}$I-lamins A/C (199,000 cpm/$\mu$g; final concentration, 1.5 $\mu$g/ml) or $^{125}$I-lamin B (248,000 cpm/$\mu$g; 1 $\mu$g/ml) were passed through lamin-agarose columns containing ≈80 $\mu$g of unlabeled purified lamins A/C or B in buffer A (150 mM NaCl/10 mM Tris·HCl, pH 7.3/2 mM $MgCl_2$/1 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride containing bovine serum albumin at 25 $\mu$g/ml). Fractions from the flowthrough (lanes F) or from the bound (lanes B) material eluted with buffer B (8 M urea/10 mM Tris·HCl, pH 8.0/4 mM EDTA/1 mM dithiotheitol/0.2 mM phenylmethylsulfonyl fluoride) were collected, concentrated, and analyzed electrophoretically. Lanes: 1 and 2, $^{125}$I-lamins A/C passed through a lamin B column; 3 and 4, $^{125}$I-lamins A/C passed through a lamin A/C column; 5 and 6, $^{125}$I-lamin B passed through a lamin A/C column. Coomassie blue-stained profiles are shown on the left (CB) and autoradiographic profiles on the right (AR). The positions of the lamins A, B, and C are indicated by arrows. (*B*) Solid-phase binding assays. Water-washed nuclear envelopes from rat liver were extracted with 7 M urea. The extracts were fractionated electrophoretically and probed with $^{125}$I-lamins A/C (lane 1) or with $^{125}$I-lamin B (lane 2). Autoradiographic profiles are shown. (*C*) Detection of homotypic and heterotypic interactions between lamins A and C. Material contained in the lamin A or C bands of blots similar to the one shown in *B* was extracted from the nitrocellulose strip by 2% $NaDodSO_4$/6 M urea and reelectrophoresed. Lanes: 1 and 3, material extracted from the lamin A band; 2 and 4, material extracted from the lamin C band. The gels were stained for protein (lanes 1 and 2, CB) or autoradiographed (lanes 3 and 4, AR).

tively transferred to nitrocellulose filters, renatured, and probed directly with radioactive tracers. When polypeptides of urea extracts of (water-washed) rat liver nuclear envelopes were incubated with $^{125}$I-lamins A/C, the tracers reacted with lamin B and to a lesser extent with lamins A and C, although the stoichiometry of the three lamins in the probed preparation was 1:1:1 (Fig. 1*B*, lane 1). When $^{125}$I-lamin B was used as a probe, much less of the tracer bound to lamin B, whereas the bands corresponding to lamins A and C were heavily labeled (Fig. 1*B*, lane 2). These observations supported the affinity-chromatography results, indicating a strong binding of lamins A and C to lamin B, a weaker binding of lamins A and C to themselves, and a barely detectable binding of lamin B to itself.

To distinguish whether the binding of the A/C probe to the A and C bands was due to an interaction of A with A and C with C (homotypic association), or of A with C and C with A (heterotypic association), the binding assay was repeated with blots of purified lamins A and C. After an incubation with the mixture of $^{125}$I-lamins A/C, the bands corresponding to lamin A or lamin C on the electropherogram were first identified with the aid of an autoradiogram. The protein contained in each band, together with the bound radioactive tracers, were then recovered from the nitrocellulose strip by extraction with 2% $NaDodSO_4$/6 M urea and reelectrophoresed. The profiles (Fig. 1*C*) demonstrated that the two radiolabeled lamins bound to both homotypic and heterotypic species.

**Stability and "Melting" of Heterotypic Complexes.** Because of the coextraction of the three lamins by treatment of nuclear envelopes with urea (13), and because of the previously demonstrated existence of a stable lamin A–B complex that resisted dissociation in 6 M urea (5), we decided to examine the stability of lamin–lamin complexes in urea solutions, as has been done with the cytokeratins (18). In a pilot experiment, $^{125}$I-lamins A/C were loaded onto a lamin B-agarose column and tested for binding in the presence of 4 M urea. It was found that the immobilized lamin B retained the tracers quantitatively (Fig. 2*A*), as had been observed under physiological conditions (compare with Fig. 1*A*, lanes 1 and 2). Binding in 4 M urea exhibited also the same site-specificity as the binding in physiological salt (see below).

Based on this observation, a "melting" experiment was done as follows: a 4 M urea extract of nuclear envelopes was applied to a weak anion-exchanger (DEAE-cellulose DE52) in 4 M urea at low salt (10 mM Tris·HCl) and pH 7.3; then, without changing the pH or the ionic strength of the medium, a 4–8 M urea gradient was applied to the column. A sharp coelution of lamins A/C at precisely 7.35 M urea was observed (Fig. 2*B*), indicating that the association of lamins A/C with lamin B is abolished at this urea concentration. At pH 7.3 the lamins A/C did not bind to the DE52 anion-exchanger in the absence of lamin B (Fig. 2*C*), but they did bind to a stronger anion exchanger (DE53 at pH 8.0), from which they were eluted with 35–50 mM NaCl (S.D.G., unpublished data). In a variation of this experiment, the urea extract was loaded onto DE52 in 8 M urea. Under these conditions, all of the lamins A and C were recovered in the column flowthrough and the first wash fractions, whereas elution of lamin B required a salt gradient (Fig. 2*D*).

**Site-Specificity in the Interactions of Lamins A and C with Lamin B.** To study the site-specificity of lamin–lamin interactions, isolated and radiolabeled lamins A and C were digested with chymotrypsin under controlled conditions in order to generate subfragments that could be tested for binding. As could be predicted from the sequence of the two proteins (10, 11), this treatment yielded a major 43-kDa peptide corresponding to their common "rod" domain, which was then further digested into smaller fragments (time course depicted in Fig. 3*A*). Affinity chromatography assays done with a digest of radiolabeled lamins A/C at physiological salt or, alternatively, in 4 M urea, revealed that none of the major fragments were able to bind to immobilized lamin B (Fig. 3 *B* and *C*). From these results it was concluded that the helical portion of lamins A and C is not primarily involved in their interactions with lamin B.

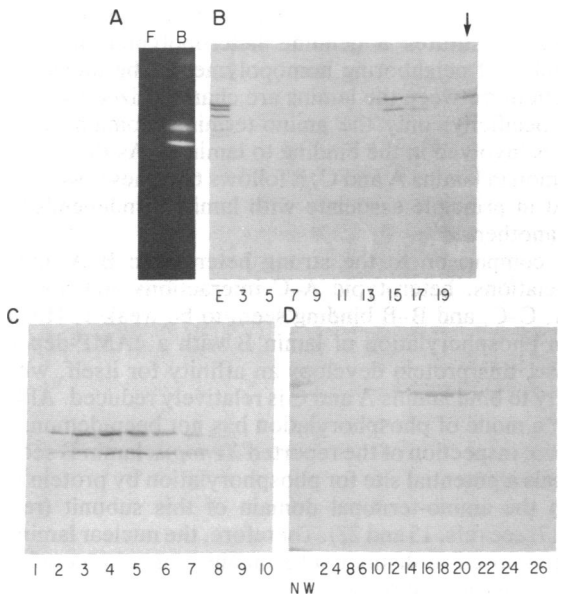


FIG. 2. Stability and melting of heterotypic complexes. (*A*) Affinity chromatography of $^{125}$I-lamins A/C loaded on a lamin B-agarose column in buffer C (4 M urea/10 mM Tris·HCl, pH 7.3/0.1 mM EDTA/1 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride) and eluted with buffer B (see Fig. 1*A* legend). Flowthrough (lane F) and bound (lane B) fractions were analyzed by electrophoresis followed by autoradiography. (*B*) Chromatographic elution profile of lamins A, B, and C when a 4 M urea extract of nuclear envelopes (lane E) was applied to a DEAE-cellulose column in buffer C (as in *A*) and eluted with a gradient of 4–8 M urea in buffer C, followed by 0.5 M NaCl in the same buffer. The various fractions (numbers below lanes) are shown after electrophoresis (7.5% gel) and Coomassie blue staining. Note the sharp coelution of lamins A and C without any change in the ionic conditions and the elution of lamin B by a "knock" with 0.5 M NaCl (arrow). (*C*) An experiment, similar to the one shown in *B*, with isolated lamins A and C. The two lamins were loaded at 4 M urea (buffer C) and the column was washed with the same buffer (see *A* and *B*). Note that the proteins were recovered in serial fractions of the flowthrough and the wash (7.5% gel). (*D*) Chromatography of lamins A, B, and C performed as in *B* except that the proteins were applied to the anion-exchanger in 8 M urea/buffer C. Note that lamins A and C did not bind (lane N shows the material recovered in the flowthrough and lane W the material collected in the column wash), whereas lamin B bound and was eluted with a 0–100 mM NaCl gradient in 8 M urea/buffer C.

These data suggested that the lamin B-binding site of lamins A and C might be located either at their amino-terminal domain or at the region extending from the end of the helical rod domain to their carboxyl termini. To differentiate between these possibilities, we synthesized a peptide that contained the 32 amino-terminal residues of lamins A and C (i.e., 32 of the 33 residues of the amino-terminal nonhelical domain. The synthetic peptide ($L_{1-32}$) was then tested for its ability to compete with isolated lamins A and C for binding to lamin B. First, $^{125}$I-lamins A/C were allowed to bind to a lamin B-agarose column; Subsequent "elution" with $L_{1-32}$ effectively displaced the bound tracers (Fig. 4*A*). To determine whether the observed displacement was due to binding of $L_{1-32}$ to lamin B rather than to some type of interaction with lamins A and C, we prepared a peptide-agarose column and applied $^{125}$I-lamin B to it. The tracer was quantitatively retained by the immobilized peptide (Fig. 4*B*). We also probed electrophoretically separated lamins A, B, and C with $^{125}$I-labeled $L_{1-32}$. The iodinated probe bound selectively to lamin B and not to lamins A and C (Fig. 4*C*). The apparent lack of interaction between the synthetic peptide and the intact lamins A and C suggested, moreover, that this segment is not primarily involved in A–C, C–C, or A–A associations.

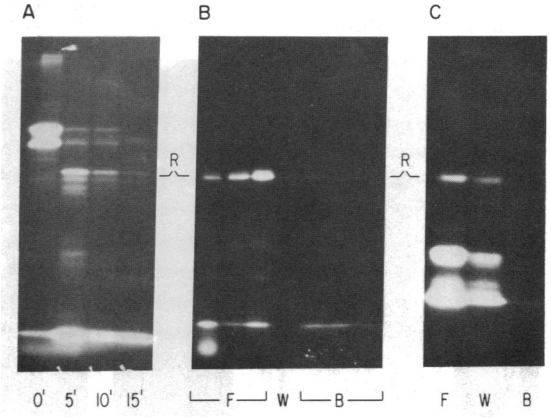


FIG. 3. Assessment of binding of lamin A and C fragments to lamin B. (*A*) Protease-digestion time course. $^{125}$I-lamins A/C were digested with chymotrypsin for 0, 5, 10, or 15 min. (*B*) Affinity chromatography assay of a 5-min digest of $^{125}$I-lamins A/C on a lamin B affinity column. Serial fractions of the flowthrough (lanes F), the last wash (lane W), and the material recovered after elution with buffer B (lanes B) are shown. (*C*) The same type of assay as in *B*, except in the presence of 4 M urea (buffer C, see Fig. 2*A* legend). Material bound to the column was eluted with buffer B containing 1% $NaDodSO_4$. Autoradiographic profiles are shown in all lanes. The position of the lamin A and C helical fragment (rod domain) is indicated (R).

**Role of Phosphorylation.** The heterotypic interactions so far described seemed to be significantly stronger than homotypic binding. This was surprising in view of other reports indicating that a single lamin species constitutes the full lamin complement in a number of cell types (14, 19). Suspecting that phosphorylation of interphase lamins may be a factor

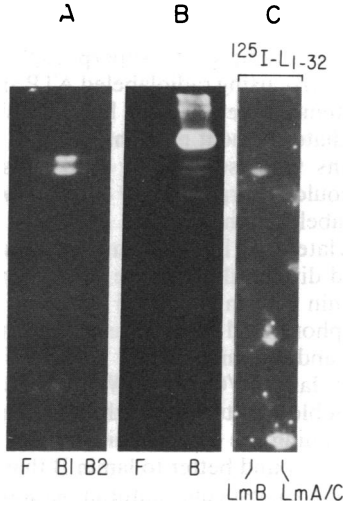


FIG. 4. Binding of a synthetic peptide containing the amino-terminal domain of lamins A and C to lamin B. (*A*) Displacement of radiolabeled lamins A and C from a lamin B-agarose column by an excess of the synthetic peptide $L_{1-32}$. Lane F, lamins A/C recovered in the flowthrough; lane B1, lamins A/C eluted with 2 mg of $L_{1-32}$ in buffer A; lane B2, lamins A/C that remained in the column after $L_{1-32}$ elution and were removed by buffer B/1% $NaDodSO_4$. (*B*) Direct binding of $^{125}$I-lamin B to an $L_{1-32}$-agarose column as detected by affinity chromatography (executed as in Fig. 1*A* with a column containing 800 $\mu$g of the synthetic peptide). Flowthrough (lane F) and bound (lane B) fractions were analyzed by electrophoresis in a 15% acrylamide gel. Note that lamin B was quantitatively retained by the immobilized peptide. (*C*) Direct binding of $^{125}$I-labeled $L_{1-32}$ to lamin B as detected by the solid-phase binding assay. Lanes: LmB, purified lamin B; LmA/C, purified lamins A and C. An autoradiogram is shown. The assay was as described in *Materials and Methods* and in Fig. 1 legend.

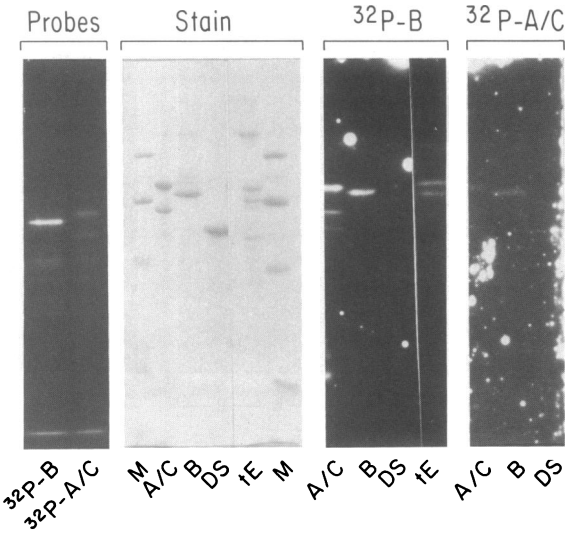


FIG. 5. Detection of lamin complexes with $^{32}$P-labeled lamins. Purified rat liver lamins were phosphorylated with [$\gamma$-$^{32}$P]ATP and protein kinase A. These preparations were then used to probe different lamin fractions that had been electrophoresed and blotted as in Fig. 1*B*. (*Probes*) $^{32}$P-labeled lamin B and lamins A/C after electrophoresis and autoradiography. There was a certain degree of proteolysis due to the long handling of the samples. (*Stain*) Electropherograms stained with Coomassie blue. Lane A/C, purified rat liver lamins A and C; lane B, lamin B; lane tE, urea extract of turkey nuclear envelopes (prepared as in ref. 5) containing lamins A and B; lane DS, purified chicken desmin; lanes M, molecular mass markers (from top to bottom, 97.4, 66.2, 42.7, and 31 kDa). (*$^{32}$P-B* and *$^{32}$P-A/C*) Autoradiograms of blots corresponding to the preparations depicted in *Stain*, after incubation with $^{32}$P-labeled lamin B and $^{32}$P-labeled lamins A/C, respectively. The gels represented here were of slightly different sizes.

regulating the degree of their heterotypic versus homotypic associations, we attempted to superphosphorylate the nuclear lamins *in vitro*, using radiolabeled ATP and the catalytic subunit of protein kinase A, which has been shown to act on other intermediate filament proteins (20–22). We found that all three lamins were suitable *in vitro* substrates for this enzyme and could incorporate $^{32}$P (Fig. 5, *Probes*).

When $^{32}$P-labeled lamin B was incubated with purified, nonphosphorylated rat liver lamins, we observed that the probe behaved differently than the corresponding nonphosphorylated lamin B form examined before: it readily reacted with nonphosphorylated lamin B as well as nonphosphorylated lamin A, and it bound slightly better to lamin A than to lamin C (Fig. 5, lanes A/C and B). When the same tracer was incubated with blots of turkey erythrocyte nuclear envelope extracts, containing the avian lamins A and B at a stoichiometry of 2:1, it bound better to lamin B than to lamin A, as evidenced by the almost equal autoradiographic signals of the A and B bands (lanes tE). No binding to purified desmin (lanes DS) or to erythrocyte vimentin (lanes tE) was noticed under these conditions. When $^{32}$P-labeled lamin A/C was tested with similar substrates, no major change in its binding behavior was seen (i.e., it still bound better to lamin B than to itself; Fig. 5, lanes A/C and B). Thus, it appeared that the phosphorylation of lamin B by protein kinase A did affect its pairing preference, whereas the same modification did not influence the binding specificity of lamins A and C.

## DISCUSSION

Using affinity chromatography and solid-phase binding assays, we have shown that lamins A and C bind directly to lamin B. Recently, *in vitro* synthesized *Xenopus* lamin B has been shown to bind to rat liver lamins A and C in a solid-phase assay (15). Together, these data suggest that the nuclear lamina constitutes a genuine heteropolymer and not an assembly of neighboring homopolymers. The molecular interactions between the lamins are characterized by a unique site-specificity: only the amino-terminal domain of lamins A/C is involved in the binding to lamin B. As this domain is common in lamins A and C, it follows that these two proteins could in principle associate with lamin B independently of one another.

In comparison to the strong heterotypic B–A and B–C associations, heterotypic A–C interactions and homotypic A–A, C–C, and B–B binding seem to be weaker. However, upon phosphorylation of lamin B with a cAMP-dependent kinase, this protein develops an affinity for itself, while its ability to bind lamins A and C is relatively reduced. Although such a mode of phosphorylation has not been demonstrated *in vivo*, inspection of the reported *Xenopus* lamin B sequence reveals a potential site for phosphorylation by protein kinase A in the amino-terminal domain of this subunit (residues 14–17; see refs. 15 and 22). Therefore, the nuclear lamina may be composed of distinct subdomains, some homopolymeric and some heteropolymeric, depending on the phosphorylation state of the assembled lamins at interphase. This prediction would conflict with potential models requiring the three lamins to occur uniformly throughout the lamina at a 1:1:1 stoichiometry. Although uniformity of the lamina is a reasonable assumption because the lamins, like the cytokeratins (18, 22), do show a certain "pairing preference" for heterotypic species (as demonstrated above), the detectably different binding affinities in heterotypic A–B, C–B, and A–C complexes and the mere existence of homotypic species do not favor a strictly (obligatorily) heterotypic model.

In support of both homotypic and heterotypic interactions, we have found that in some mammalian tissues, the ratio of the three lamins is clearly nonstoichiometric (H. J. Worman, I. Lazaridis, G.B., and S.D.G., unpublished data). Moreover, a number of cell types have been seen to express and assemble only a single lamin species (14, 19). Thus, in contrast to the cytokeratin paradigm, the nuclear lamina assembly appears to be a more "degenerate" process, allowing for both homotypic and heterotypic subunit interactions.

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