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Biogenesis of the nuclear lamina: *In vivo* synthesis and processing of nuclear protein precursors

(lamin precursors/topogenesis/nucleocytoplasmic communication)

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ABSTRACT **Utilizing antibodies against lamins A, B_1, and B_2, we have studied the biogenesis of the nuclear lamina in chicken embryo fibroblasts. (Lamins B_1 and B_2 have been identified recently as structurally distinct "lamin B" proteins.) We demonstrate that, unique among the nuclear proteins studied to date, lamin A is synthesized as a higher molecular mass precursor. A short-lived higher molecular mass variant ($t_{1/2} \approx 3$ min) accompanying the mature-size protein was also detected in the case of lamin B_2 biosynthesis, but no precursor was found for lamin B_1. By combining pulse–chase experiments with subcellular fractionation, we provide evidence that synthesis of lamin proteins occurs on free polysomes; subsequently, the newly synthesized proteins become rapidly associated with a crude nuclear fraction. The lamin A precursor is processed within the nucleus with a half-time of about 30 min. Concomitantly, lamin proteins acquire a characteristic resistance to detergent extraction, suggesting their insertion into a submembraneous protein network. The described biogenetic pathway involving precursor synthesis and processing is very unusual for nuclear proteins; it may have interesting implications for the mechanisms of transport and assembly of poorly soluble nuclear proteins.**

The nucleoplasmic surface of the inner nuclear membrane is lined by a proteinaceous layer commonly referred to as the nuclear lamina (1–3). The lamina is presumed to represent a karyoskeletal element influencing the shape and integrity of the nuclear envelope. In addition, it provides attachment points for interphase chromatin and hence may influence basic cellular functions, including DNA replication and gene transcription (4–8). The quantitatively major components of the nuclear lamina are proteins of molecular mass around 70 kDa (3); in particular, three proteins, called lamins A, B, and C, have been described in mammalian and avian tissues (9, 10). Most recently, we have demonstrated the existence of two structurally distinct "lamin B" proteins, lamins B_1 and B_2; lamin B_1 appears to represent the quantitatively predominant lamin B protein in mammals, whereas lamin B_2 predominates in birds (to be reported elsewhere). Here, utilizing antibodies against lamins A, B_1, and B_2, we have studied the biogenesis of the nuclear lamina.

MATERIALS AND METHODS

Cell Culture and Antibodies. Chicken embryo fibroblasts were cultured as described (11). For metabolic labeling, monolayers were washed and incubated for 30 min in methionine-free medium. Then, they were incubated in methionine-free medium containing 4% fetal calf serum and [^{35}S]methionine at 50 μCi/ml (1 Ci = 37 GBq) for overnight labeling or 200 μCi/ml for pulse-labeling. To chase incorporated [^{35}S]methionine, labeled cell monolayers were quickly washed twice and incubated at 37°C in medium containing 5 mM unlabeled methionine. The production and characterization of antilamin antibodies will be described elsewhere.

Cell Fractionation. Monolayers were washed twice with ice-cold 8 mM Na_2HPO_4/1.5 mM KH_2PO_4/138 mM NaCl/4 mM KCl and once with homogenization buffer (10 mM Tris·HCl, pH 7.4/140 mM KCl/1.5 mM $MgCl_2$/0.1 mM phenylmethylsulfonyl fluoride/5 mM iodoacetamide/aprotinin at 50 μg/ml). Cells were then taken up in 750 μl of homogenization buffer and broken by Dounce homogenization (20 strokes). Cell lysis was routinely monitored by phase-contrast microscopy and was always >95%. The homogenate was centrifuged at 1000 × *g* for 8 min to yield a crude nuclear pellet and a cytoplasmic fraction. The cytoplasmic fraction was further fractionated by centrifugation for 30 min at 100,000 × *g* to produce a particulate fraction and a postmicrosomal supernatant. In some experiments the first low-speed centrifugation step was omitted and the homogenate was fractionated directly into a particulate pellet and a postmicrosomal supernatant by high-speed centrifugation (30 min, 100,000 × *g*). For fractionation in the presence of Triton X-100, detergent was included in the homogenization buffer at 1% (wt/vol) and cells were spun on a Vortex for 10 sec instead of the Dounce homogenization.

Immunoprecipitation and Peptide Mapping. Prior to immunoprecipitation from subcellular fractions, the fractions were adjusted to equal volumes and brought up to 1% Triton X-100/1% deoxycholate/0.1% $NaDodSO_4$/140 mM KCl/0.1 mM phenylmethylsulfonyl fluoride/aprotinin at 50 μg/ml. Immunoprecipitation was then carried out exactly as described (11). One-dimensional peptide mapping was carried out according to Cleveland *et al.* (12). Polypeptides were resolved by $NaDodSO_4$/PAGE and visualized by fluorography.

RESULTS

Characterization of Newly Synthesized Lamin Proteins. Characterization of the antilamin antibodies used here will be reported elsewhere. Briefly, as shown by immunoprecipitation from overnight labeled cells, our rabbit antilamin A/B_2 serum recognized lamins A and B_2 (Fig. 1*A*, lane 1) but did not react with lamin B_1; it also did not react with any protein corresponding to lamin C, presumably because lamin C is scarce or absent in chicken embryo fibroblasts. Lamin B_2 was precipitated by monoclonal antibody E-3 (Fig. 1*A*, lane 3), whereas lamin B_1 was precipitated by monoclonal antibody L-5 (Fig. 1*A*, lane 5). To study newly synthesized lamin proteins, immunoprecipitation experiments were carried out after a 5-min pulse-labeling of fibroblasts. Under these conditions, the rabbit antilamin A/B_2 serum precipitated three proteins of approximately 71, 67, and 66 kDa (Fig. 1*A*, lane 2). E-3 precipitated two proteins of 67 and 66 kDa (Fig. 1*A*, lane 4), whereas L-5 precipitated only one protein of 68

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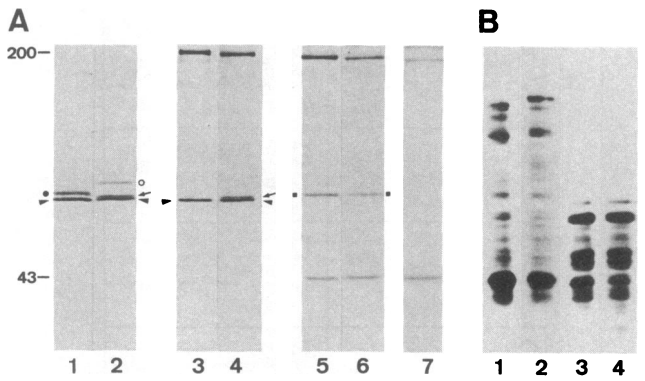

FIG. 1. Characterization of newly synthesized lamin proteins. (*A*) Immunoprecipitation of lamin proteins from chicken embryo fibroblasts. Cells were labeled with [^{35}S]methionine for 16 hr (lanes 1, 3, 5, and 7) or 5 min (lanes 2, 4, and 6). Antibodies used for immunoprecipitation were a rabbit antilamin A/B_2 serum (lanes 1 and 2), monoclonal antilamin B_2 antibody E-3 (lanes 3 and 4), monoclonal antilamin B_1 antibody L-5 (lanes 5 and 6), and normal mouse IgG (lane 7). Proteins were resolved by 8% NaDodSO$_4$/PAGE and visualized by fluorography. The proteins migrating at 200 and 43 kDa were nonspecifically precipitated and most probably correspond to myosin heavy chain and actin, respectively. ○, Purported lamin A precursor (71 kDa); ●, mature lamin A (69 kDa); arrow, 67-kDa lamin B_2 variant; arrowhead, lamin B_2 (66 kDa); ■, lamin B_1 (68 kDa). (*B*) Peptide maps of mature lamin proteins and purported lamin precursors. Immunoprecipitated lamin proteins were excised from gels and partially digested with 150 ng of V8 protease (12). Lane 1, mature lamin A; lane 2, purported lamin A precursor; lane 3, mature lamin B_2; lane 4, 67-kDa lamin B_2 variant together with mature lamin B_2.

kDa (Fig. 1*A*, lane 6). Fig. 1*A*, lane 7, shows an immunoprecipitation using normal mouse IgG for control.

V8 protease digestions of the 69-kDa lamin A protein (Fig. 1*B*, lane 1) and the 71-kDa protein (Fig. 1*B*, lane 2) produced very similar peptide patterns, although some size differences are visible in the region of the largest peptides. This indicates that the 71-kDa protein is closely related to lamin A but possibly may contain an additional peptide. The 67- and 66-kDa lamin B_2 proteins precipitated from pulse-labeled fibroblasts could not be efficiently separated and therefore were digested with V8 protease as a doublet. The peptide pattern generated from this doublet (Fig. 1*B*, lane 4) was indistinguishable from the peptide map of lamin B_2 (Fig. 1*B*, lane 3). Digestion of the 67-kDa protein thus produced exclusively lamin B_2-related peptides, whereas no high molecular mass fragments indicative of lamin A were generated.

The results of these peptide mapping experiments implicated the 71- and 67-kDa proteins as precursors for lamins A and B_2, respectively. This possibility was investigated by pulse-chase experiments. Fig. 2*A* shows immunoprecipitation of lamin B_2 proteins by E-3. When fibroblasts were labeled with [^{35}S]methionine for only 3 min and then immediately solubilized for immunoprecipitation, two bands at 67 and 66 kDa were observed (Fig. 2*A*, lane 1). However, when the 3-min pulse was followed by a 2-min chase with unlabeled methionine, only trace amounts of the 67-kDa protein remained detectable (Fig. 2*A*, lane 2), and after a 5-min chase period the 67-kDa variant had completely disappeared (Fig. 2*A*, lane 3). These results are consistent with a precursor–product relationship between the 67-kDa variant and the 66-kDa lamin B_2 protein. However, because the short half-life of the 67-kDa variant made accurate quantitations impossible, we cannot exclude the possibility that the 67-kDa variant might have been degraded rather than being converted to the 66-kDa lamin B_2.

Fig. 2*B* shows the results of a similar pulse–chase experiment using the rabbit antilamin A/B_2 serum for immunoprecipitation. After a 5-min pulse period both putative precursors were readily visible at 71- and 67-kDa together with mature lamin B_2 at 66 kDa (Fig. 2*B*, lane 1). As expected from the previous results (Fig. 2*A*), the 67-kDa lamin B_2 variant was no longer detectable after the first chase period of 10 min (Fig. 2*B*, lane 2). The lamin A precursor was considerably more stable (Fig. 2*B*, lanes 2–5): about equal amounts of lamin A precursor and mature protein were precipitated after a chase period of 30 min (Fig. 2*B*, lane 4), whereas most of the lamin A precursor had been trimmed to the mature-size lamin A after a 1-hr chase (Fig. 2*B*, lane 5). A control immunoprecipitation using the rabbit preimmune serum is shown in Fig. 2*B*, lane 6.

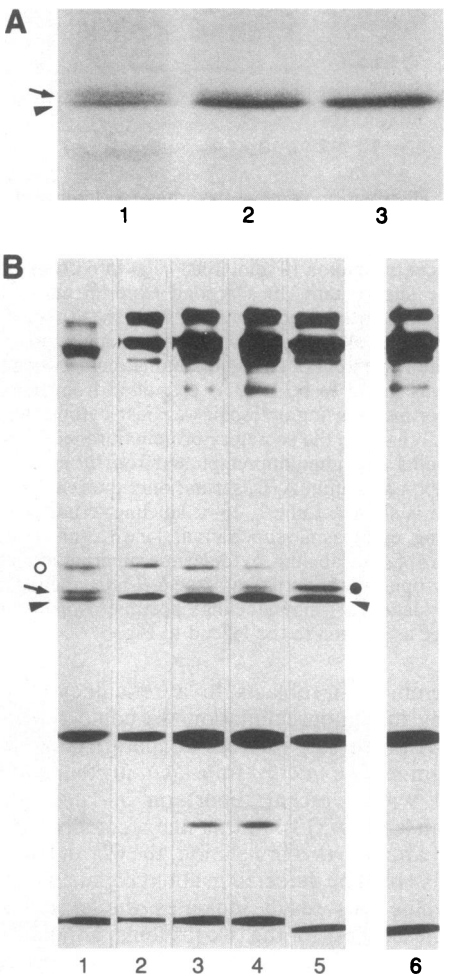

FIG. 2. Kinetics of lamin precursor processing. (*A*) Fibroblasts were labeled with [^{35}S]methionine for 3 min and either solubilized directly (lane 1) or incubated in chase medium for 2 min (lane 2) or 5 min (lane 3) before solubilization. Proteins were immunoprecipitated with monoclonal antibody E-3. Only the region of the fluorograph is shown that contains the bands corresponding to the 67-kDa lamin B_2 variant (arrow) and the mature lamin B_2 (arrowhead). (*B*) Fibroblasts were labeled with [^{35}S]methionine for 5 min and either solubilized directly (lane 1) or incubated in chase medium for 10 (lane 2), 20 (lane 3), 30 (lane 4), or 60 min (lanes 5 and 6) before solubilization. Proteins were immunoprecipitated by rabbit antilamin A/B_2 serum (lanes 1–5) or preimmune serum (lane 6). Symbols are as defined in the legend to Fig. 1.

By isoelectric focusing (not shown) we found that the lamin A precursor is slightly more basic than mature lamin A. In contrast, the 67-kDa lamin B_2 variant is more acidic than mature lamin B_2; however, phosphorylation is unlikely to account for the difference between the 67- and 66-kDa proteins, because the migration of the 67-kDa variant on polyacrylamide gels was not affected by alkaline phosphatase treatment. We have also translated poly(A)$^+$ RNA from

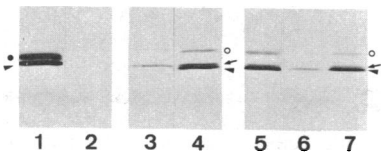

FIG. 3. Distribution of newly synthesized lamins in subcellular fractions. Fibroblasts were labeled with [^{35}S]methionine and broken up by Dounce homogenization. They were then fractionated by low-speed centrifugation (8 min, 1000 × *g*) into crude nuclei and a cytoplasmic supernatant. In a second experiment, a cytoplasmic supernatant was further fractionated by high-speed centrifugation (30 min, 100,000 × *g*) into a microsomal fraction and a postmicrosomal supernatant. The extent of cross-contamination between the various fractions was found to be low, as estimated from immunoprecipitation experiments using antibodies against cytoplasmic aspartate aminotransferase and the secretory protein fibronectin (not shown). Lamin proteins were immunoprecipitated from the different fractions with the rabbit antilamin A/B_2 serum; only the relevant part of the fluorograph is shown. Lane 1, 16-hr labeling, crude nuclei; lane 2, 16-hr labeling, cytoplasmic supernatant; lane 3, 5-min labeling, crude nuclei; lane 4, 5-min labeling, cytoplasmic supernatant; lane 5, 7-min labeling, cytoplasmic supernatant; lane 6, 7-min labeling, microsomal fraction; lane 7, 7-min labeling, postmicrosomal supernatant. Symbols are as defined in the legend to Fig. 1.

chicken embryo fibroblasts in a reticulocyte lysate (not shown): by immunoprecipitation we could readily identify the lamin A precursor, whereas no mature lamin A could be detected among the *in vitro* translated proteins; this result is consistent with a recent report on *in vitro* synthesis of mammalian lamin A (13). Rather unexpectedly, however, we found that after *in vitro* translation, the 67- and 66-kDa lamin B_2 proteins could be detected in about equal amounts. Taken at face value this result indicates the existence of two separate messengers for the two proteins, although it remains possible that one protein is converted to the other even in the cell-free lysate.

Nuclear Uptake and Lamina Insertion. When cells were labeled for 16 hr and fractionated by homogenization and low-speed centrifugation into a crude nuclear pellet and a postnuclear supernatant, lamin proteins were quantitatively found in the nuclear fraction in the mature form (Fig. 3, lane 1); no lamins could be detected in the postnuclear supernatant (Fig. 3, lane 2). However, most of the newly synthesized lamins were found in the postnuclear supernatant (Fig. 3, lane 4); no lamin B_2 variant and only a small amount of lamin A precursor could be detected in the crude nuclear pellet (Fig. 3, lane 3). Newly synthesized but apparently mature lamin B_2 was present in the crude nuclear pellet and the postnuclear fraction.

In a second type of experiment, one-half of a low-speed supernatant was used directly for immunoprecipitation (Fig. 3, lane 5), whereas the other half was further fractionated by a high-speed centrifugation (100,000 × *g*, 30 min). Almost all of the lamin A precursor, the 67-kDa lamin B_2 variant, and mature lamin B_2 were found in the postmicrosomal supernatant (Fig. 3, lane 7), whereas only trace amounts of material were detectable in the microsomal fraction (Fig. 3, lane 6). The distribution of lamin B_1 among these subcellular fractions roughly paralleled the distribution of lamins A and B_2 (not shown). These results suggest that lamin proteins are synthesized on free rather than membrane-bound ribosomes.

To investigate the kinetics of the transition of newly synthesized lamin proteins from a soluble to a particulate compartment, cells were metabolically labeled for 7 min and fractionated after different chase time periods into a high-speed supernatant and particulate material. The content of lamin proteins in the two fractions was then analyzed by immunoprecipitation (Fig. 4 *A* and *A'* and *B* and *B'*). After a 7-min pulse most of the lamin A precursor and the mature

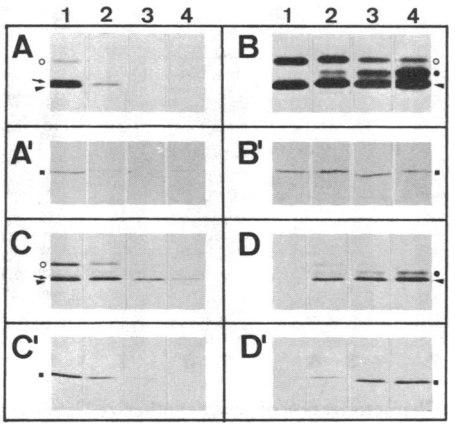

FIG. 4. Kinetics of nuclear uptake and lamina insertion. Fibroblasts were labeled with [^{35}S]methionine for 7 min and fractionated either immediately (lanes 1) or after chase periods of 15 (lanes 2), 30 (lanes 3), and 60 min (lanes 4). For fractionation, cells were either broken up by Dounce homogenization (*A* and *A'* and *B* and *B'*) or lysed in the presence of 1% Triton X-100 (*C* and *C'* and *D* and *D'*). They were then centrifuged for 30 min at 100,000 × *g* to yield a cytoplasmic supernatant (*A* and *A'*) and a particulate fraction (*B* and *B'*) or, alternatively, a Triton X-100-soluble (*C* and *C'*) and a Triton X-100-resistant fraction (*D* and *D'*) Lamin proteins were immunoprecipitated with the rabbit antilamin A/B_2 serum (*A–D*) or monoclonal antilamin B_1 antibody L-5 (*A'–D'*). Only the relevant parts of the fluorographs are shown. Symbols are as defined in the legend to Fig. 1.

lamin B_2 were already in the particulate fraction (Fig. 4*B*, lane 1), whereas some lamin A precursor, all of the 67-kDa lamin B_2 variant, and some mature lamin B_2 were found in the postmicrosomal supernatant (Fig. 4*A*, lane 1). No 67-kDa lamin B_2 variant was ever detected within the crude nuclear fraction, not even on shorter exposure of the gels (Fig. 4*B*, lane 1; see also Fig. 3, lane 3). Already after a 15-min chase period only trace amounts of lamin proteins could be found in the postmicrosomal supernatant (Fig. 4*A*, lane 2), and after 30- or 60-min chase periods no lamin proteins were soluble any more (Fig. 4*A*, lanes 3 and 4). As can be seen from Fig. 4 (*A'* and *B'*), the transition of lamin B_1 from the soluble to the particulate compartment roughly paralleled the transitions of lamins A and B_2.

The well-established resistance of lamin proteins to extraction of nuclei by a variety of agents, including nonionic detergents, is believed to reflect the existence of a lamin network apposed to the nucleoplasmic surface of the inner nuclear membrane (2). To determine the time course of acquisition of this extraction resistance, we carried out pulse–chase experiments as described above, but this time cells were fractionated in the presence of 1% Triton X-100. As can be seen from comparison of Fig. 4 *C* and *C'*, lanes 1, with Fig. 4 *D* and *D'*, lanes 1, virtually all of the lamin proteins labeled during a 7-min pulse were extractable with Triton X-100. With increasing chase time periods, the lamin A precursor slowly disappeared from the Triton X-100-soluble fraction (Fig. 4*C*, lanes 2–4), but only trace amounts of precursor could be recovered in a Triton X-100-resistant form (Fig. 4*D*, lanes 2–4). The distribution of mature lamin A was roughly inverse to the distribution of its precursor: in Triton X-100 extracts mature lamin A could be detected only in trace amounts (Fig. 4*C*, lanes 2–4), but with longer chase periods it accumulated in the particulate fractions (Fig. 4*D*, lanes 2–4). Allowing for some minor cross-contamination of the fractions, these results suggest that the processing of the lamin A precursor is accompanied by the acquisition of resistance to Triton X-100 extraction. Lamins A and B_2 (Fig.

4 *C* and *D*) as well as B_1 (Fig. 4 *C'* and *D'*) acquired resistance to detergent extraction with very similar kinetics.

DISCUSSION

We show that chicken lamin A is synthesized as a protein with an apparently higher molecular mass. In addition, we have detected a higher molecular mass variant of lamin B_2, but we have not observed any precursor in the case of lamin B_1. Though the molecular nature of the short-lived 67-kDa lamin B_2 variant remains elusive, results of pulse–chase experiments, comparative peptide mapping, and *in vitro* translation are all consistent with lamin A being synthesized with a polypeptide extension. Synthesis of lamin proteins presumably occurs on free polysomes. Subsequently, the 67-kDa lamin B_2 variant disappears with a half-time of about 3 min, and lamin proteins become rapidly associated with a particulate fraction, a process most probably reflecting their uptake into nuclei. The kinetics of this transition are consistent with the time course of nuclear uptake determined for bulk nuclear proteins (14). The lamin A precursor is processed within the nucleus with a half-time of about 30 min. With very similar kinetics, the lamin proteins acquire the characteristic resistance to detergent extraction, a property that probably reflects their insertion into the preexisting submembraneous lamina.

The postulated removal of a purported lamin A precursor moiety during the transit of the newly synthesized protein to its final intranuclear destination would be reminiscent of signal peptide removal as it occurs during protein secretion or in the case of protein import into mitochondria or chloroplasts (15–17). Though the mechanisms operating to ensure selective accumulation of nuclear proteins still remain to be elucidated (18–20), it is clear that nuclear translocation processes are not in general accompanied by irreversible removal of topogenic protein sequences (20–22). The stability of any karyophilic information may in fact represent a prerequisite for reutilization of nuclear proteins after cell division. Reutilization of lamin proteins after mitosis has been demonstrated (9); moreover, during mitosis lamin A was reported to exist as a soluble monomeric protein (9). This result suggests that mature lamin A is competent to reassociate with the reforming nuclear envelope in telophase. There is good evidence that the dissociation of lamin proteins during mitosis is regulated by increased phosphorylation (9, 23). However, because extensive phosphorylation of lamin proteins may represent an important element contributing to the disintegration of the nuclear envelope (9, 23), it appears that the corresponding kinase activity should be restricted to mitosis and meiosis. The solubility of newly synthesized lamin proteins during interphase would then have to be controlled by alternative mechanisms. Consistent with this view, Ottaviano and Gerace found no evidence for phosphorylation of newly synthesized lamin proteins (24). Therefore, the lamin A precursor moiety might substitute for phosphorylation and prevent premature oligomerization in the cytoplasm. The lamin A precursor might also function in mediating the association of newly synthesized lamins with the nuclear envelope or their insertion into the lamina. Thus, although the lamin A precursor moiety may not represent a karyophilic signal in strict analogy to signal peptides (15–17), it may be related to proper intracellular targeting in an indirect way. Alternatively, it cannot be excluded that lamin A precursor processing might activate as yet unidentified enzymatic functions.

While the present study was being completed, evidence for a lamin A precursor was obtained in mammalian cells (3, 13). However, Gerace *et al.* report that the conversion of the putative lamin A precursor to mature lamin A occurs in a Triton X-100-resistant pellet (3), whereas in our hands, the lamin A precursor clearly is extractable by detergent, and only mature lamin A has acquired resistance to detergent extraction. No precursor has been reported for mammalian lamin B_1, consistent with our present results. Finally, the 67-kDa lamin B_2 variant described here had not been detected in the studies involving mammalian cells; this is not surprising, because lamin B_2 in mammalian species represents a minor component (to be reported elsewhere). With respect to the functional significance of the 67-kDa variant of chicken lamin B_2, it is possible that its transient expression might have some influence on the assembly of newly synthesized lamin proteins. Along similar lines, transient protein expression has been proposed recently to control the assembly and topogenesis of the erythrocyte membrane skeleton (25, 26).

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