The Role of CaaX-dependent Modifications in Membrane Association of *Xenopus* Nuclear Lamin B3 during Meiosis and the Fate of B3 in Transfected Mitotic Cells

Iris Firmbach-Kraft and Reimer Stick

Institut für Biochemie und Molekulare Zellbiologie, Abt. Enzymchemie, Universität Göttingen, D-37073 Göttingen, Federal Republic of Germany

Abstract. Recent evidence shows that the COOH-terminal CaaX motif of lamins is necessary to target newly synthesized proteins to the nuclear envelope membranes. Isoprenylation at the CaaX-cysteine has been taken to explain the different fates of A- and B-type lamins during cell division. A-type lamins, which loose their isoprenylation shortly after incorporation into the lamina structure, become freely soluble upon mitotic nuclear envelope breakdown. Somatic B-type lamins, in contrast, are permanently isoprenylated and, although depolymerized during mitosis, remain associated with remnants of nuclear envelope membranes. However, *Xenopus* lamin B3, the major B-type lamin of amphibian oocytes and eggs, becomes soluble after nuclear envelope breakdown in meiotic metaphase.

Here we show that *Xenopus* lamin B3 is permanently isoprenylated and carboxyl methylated in oocytes (interphase) and eggs (meiotic metaphase). When transfected into mouse L cells *Xenopus* lamin B3 is integrated into the host lamina and responds to cell cycle signals in a normal fashion. Notably, the ectopically expressed *Xenopus* lamin does not form heterooligomers with the endogenous lamins as revealed by a coprecipitation experiment with mitotic lamins. In contrast to the situation in amphibian eggs, a significant portion of lamin B3 remains associated with membranes during mitosis. We conclude from these data that the CaaX motif-mediated modifications, although necessary, are not sufficient for a stable association of lamins with membranes and that additonal factors are involved in lamin-membrane binding.

THE nuclear lamina is a major skeletal component of the eukaryotic nucleus. It is composed of intermediate filament proteins, the lamins, that are arranged as a network of 10 nm filaments (2, 22, 43). Lamin filaments line the nucleoplasmic surface of the inner nuclear membrane and, at least in amphibian oocytes, form a strikingly regular orthogonal meshwork (2). Besides its structural role in maintaining nuclear envelope integrity, the lamina provides attachment points for interphase chromatin and is important for DNA replication (7, 44, 46; for review see references 25, 47, 48).

Based on sequence comparison and biochemical data lamins can be classified as either A- or B-type lamins (10, 30, 31, 50, 60, 61, 68). Analysis of the gene structure of A- and B-type lamins in conjunction with their pattern of expression in vertebrates led to the assumption that B-type lamins represent the ancestral type of lamins and that A-type lamins derived therefrom by acquisition of an extra exon (20, 61).

During mitotic and meiotic cell division, when the nuclear envelope breaks down, lamin filaments are depolymerized into oligomers (8, 24, 63). The reversible polymerization is effected by a transient hyperphosphorylation of lamins by the universal cell cycle protein kinase complex $p32^{cdc2}$ (29, 51, 69).

Two topogenic sequences are necessary to target newly synthesized lamins to the inner nuclear membrane, a nuclear localization signal and the COOH-terminal CaaX motif (C = cysteine, a = aliphatic, X = any amino acid) (32, 34, 37, 41). Such motifs are found at the COOH termini of several cytoplasmic as well as secreted proteins. Examples are the yeast mating type pheromones, the *ras* oncogene proteins, and small GTP-binding proteins. Notably, some nuclear proteins including all known members of the lamin protein family with the exception of human lamin C (for review see reference 53) and a recently described lamin of *Drosophila* (9) also have CaaX boxes. The CaaX tetrapeptide is the substrate for a series of posttranslational modifications including isoprenylation of the cysteine via a thioether bond and proteolytic removal of the last three amino acids, followed by carboxyl methylation of the resulting COOH-terminal cysteine residue (53). There are two types of prenyl groups, C15 farnesyl and, more abundant, C20 geranylger-

Address all correspondence to Dr. Stick, Institut für Biochemie und Molekulare Zellbiologie, Abt. Enzymchemie, Universität Göttingen, Humboldtallee 23, D-37073 Göttingen, FRG.

anyl, that can be linked to CaaX-proteins. The nature of the attached substituent is dependent on specific sequence information in the carboxyl terminus of the protein reflecting the substrate specificity of distinct types of isoprenyl transferases involved in this process (52, 53). *Ras* proteins and lamins have been shown to be farnesylated (11, 72). The CaaX-dependent modifications result in an increased hydrophobicity of the carboxyl terminus and may explain the affinity of prenylated proteins for membranes. Point mutations within or deletion of the CaaX motif have demonstrated that these four carboxyl terminal amino acids and the modifications they mediate are essential for the integration of newly synthesized lamins into the nuclear lamina structure (32, 34, 37). Isoprenylation has also been taken to explain the different fates of A-type and B-type lamins during cell division (34). During mitotic nuclear envelope breakdown A-type lamins are dispersed throughout the cytoplasm as soluble oligomers, whereas B-type lamins, although depolymerized, remain associated with remnants of the nuclear envelope membranes (24, 60). This has been explained by the fact that B-type lamins remain permanently isoprenylated while A-type lamins lose the COOH-terminal modifications shortly after incorporation into the lamina by an additional proteolytic processing event (5, 6, 70). In contrast to B-type lamins in somatic cells, lamin B3, the major lamin of the amphibian oocytes, is soluble rather than membrane associated in the egg cytosol after meiotic nuclear envelope breakdown (8, 46, 59, 63). While this is reminiscent of the fate of mitotic A-type lamins, comparison of primary sequences clearly classifies *Xenopus* lamin B3 as a B-type lamin (60; for lamin nomenclature see reference 61). In addition, previous experiments gave no indication for a lamin A-like proteolytic maturation of B3 (60).

We have studied the posttranslational modifications of *Xenopus* lamin B3 in oocytes (interphase) and in eggs (meiotic metaphase) and we have compared the subcellular distribution of B3 in these stages with that in transfected mouse cell lines expressing *Xenopus* lamin B3. We show here that lamin B3, similar to somatic B-type lamins, is permanently isoprenylated in oocytes and eggs. In variance to mitotic B-type lamins (14, 15) it remains carboxyl methylated throughout the egg maturation process. In transfected somatic mouse cells a significant portion of B3 remains associated with membranes during mitosis. Taken together, these results show that the CaaX motif-mediated modifications alone, although necessary for membrane targeting, are not sufficient for a stable association of lamins with membranes.

Materials and Methods

Isolation of Oocyte Nuclei and Preparation of Egg Extracts

Germinal vesicles (GVs)[1], nuclear envelopes, and nuclear contents were isolated manually as described previously (62). Nuclear envelope fractions shown in Fig. 3 were in addition extracted with 2 M NaCl, 100 mM Tris-HCl, pH 7.5, and washed with 1% Triton X-100, 100 mM Tris-HCl, pH 7.5, before immunoprecipitation.

For fractionation experiments of GVs several buffers were used with identical results. Either equal volumes of 2× Triton X-100 buffer (2% Triton X-100, 100 mM Tris-HCl, 300 mM NaCl adjusted to pH 7.4, 0.5 mM PMSF, 0.5% Trasylol (Bayer, Leverkusen, FRG)) were added to the GV suspension. After 10-min incubation at 4°C the samples were spun in a microcentrifuge for 15 min. Supernatants were transferred to a new tube and respun. The pellets were resuspended in 1× Triton X-100 buffer and also spun once more. Alternatively, the extraction of GVs was done in extraction buffer (see below) or nuclear isolation medium (63). For the preparation of egg extracts the matured oocytes were washed in extraction buffer (80 mM glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 20 mM Hepes, adjusted to pH 7.5, 0.5 mM PMSF (1)). 50 to 100 eggs in extraction buffer (10 μl/egg) were centrifuged at 10,000 *g* in a HB-4 swing-out rotor in a Sorvall RC-5B centrifuge (Dupont, Newton, CT) for 10 min. The resulting pellet was washed in extraction buffer and pellet and supernatant fraction were recentrifuged at 10,000 *g* before a 1 h spin at 200,000 *g* in a RP55S-217 swing-out rotor in a Hitachi himac CS120 centrifuge. Recentrifugation of the 200,000 *g* supernatant under the same conditions gave identical results in comparison to a single high-speed centrifugation.

Gel Electrophoresis and Immunoblotting

Proteins were separated on 10% polyacrylamide gels according to Laemmli (38). ^{3}H- and ^{35}S-labeled proteins were detected after fixing the gel in 10% acetic acid for 30 min followed by fluorography with Amplify (Amersham, Braunschweig, FRG) for 20 min. For Western blotting, proteins were electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Dassel, FRG), or polyvinylidene difluoride membranes (Diagen GmbH, Düsseldorf, FRG) in a Bio-Rad Trans-Blot cell (Bio-Rad, Richmond, CA). Polyvinylidene difluoride membranes were processed for chemiluminescent detection with primary mouse monoclonal antibodies and secondary alkaline phosphatase-coupled antibodies according to manufacturer's instructions (Western Light kit, Tropix, Bedford, MA). Nitrocellulose membranes were incubated as described previously (60) and antibody binding visualized with the Amersham ECL kit (Amersham) using a peroxidase-coupled secondary antibody.

Immunoprecipitation and Coprecipitation Assay

All immunoprecipitations were carried out with magnetic MP450 Dynabeads precoated with anti-mouse IgG antibodies (Dynal, Oslo, Norway). The beads were incubated under constant agitation with anti-B3 mouse monoclonal antibodies at 4°C for 12 h. They were then washed five times in PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) and resuspended in immunoprecipitation buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 20 mM Tris-HCl pH 7.2, 150 mM NaCl, 2 mM EDTA, 20 mM methionine, 0.1 mM PMSF, 0.5% Trasylol (Bayer, Leverkusen, FRG)). If necessary lamins were solubilized in 0.4% SDS, 0.05 M Tris-HCl, pH 7.4, 0.1 M NaCl, 2 mM EDTA, and boiled for 5 min. The solution was brought to a final concentration of 3% Triton X-100 and an equal volume of 2× immunoprecipitation buffer was added. The antigen solution was then preincubated with Dynabeads precoated with anti-mouse IgGs for 30 min. After removal of the beads, anti-B3-coated beads were added and incubated at 4°C for 12 h with constant rotation. The antigen loaded beads were washed five times with immunoprecipitation buffer and the antigen was removed from the beads by boiling for 5 min in SDS-gel sample buffer.

For the coprecipitation assay immunoprecipitations were carried out as described above with the omission of SDS at any stage of the protocol.

Antibodies

L6-5D5 is a monoclonal murine antibody against *Xenopus laevis* lamins B2 and B3 described by Stick (60). PKB8 is a monoclonal murine antibody generated against human lamins A, B, and C (36).

In Vitro Translation

Synthetic lamin B3 RNA was transcribed from a T7 promoter using an in vitro transcription kit according to manufacturer's instructions (Stratagene, La Jolla, CA). 1/20 of the reaction was used for in vitro translation in a rabbit reticulocyte lysate (Promega, Madison, WI) in the presence of [^{35}S]methionine (60 μCi/50 μl reaction (≥1,000 Ci/mmol)) (Amersham) or [^{3}H]mevalonolactone (50 μCi/50 μl reaction (10–30 Ci/mmol)) (NEN Dupont, Wilmington, DE). The translation reaction was incubated at 30°C for 1 h. Samples were either snap frozen in liquid nitrogen or immediately processed for immunoprecipitation.

1. *Abbreviation used in this paper*: GV, germinal vesicle.

Biosynthetic Labeling of Xenopus laevis Oocytes and Eggs

Oocytes were surgically removed and defolliculated in 2 mg (522 U)/ml collagenase (Worthington Biochem. Corp., Freehold, NJ) in 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes, adjusted to pH 7.5 with constant agitation at room temperature for 2–3 h. After extensive washing in the above described buffer, stage 5 and 6 oocytes were selected. Staging of oocytes was according to Dumont (21). They were then kept in modified Barth solution (84 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca[NO_3]_2$, 0.41 mM $CaCl_2$, 7.5 mM Tris-HCl, pH 7.4, 100 U/ml Penicillin, 100 μg/ml Streptomycin) at 18°C. In all labeling experiments 10 μl of modified Barth solution per oocyte were used. For labeling with [^{35}S]methionine (Amersham) 10 μCi/oocyte were added for 16 h. [^{3}H]mevalonolactone (NEN Dupont) in the presence of 50 μM Lovastatin (gift from Dr. M. Stapff, Merck, Sharp & Dohme, München, FRG) was administered at a concentration of 0.5 μCi/oocyte for 48 h. [^{3}H]methylmethionine (NEN Dupont) was added at 1–10 μCi/oocyte for 16 h. After the appropriate incubation period, nuclei were either manually removed or oocytes were incubated with progesterone (Sigma Immunochemicals, St. Louis, MO) at 2 μg/ml for 2 to 4 h. Matured oocytes (eggs) were scored according to the appearance of the maturation spot at the animal pole. In each experiment ~5% of the obtained eggs were dissected as described in "Isolation of Oocyte Nuclei" to verify the absence of a GV.

Methylation Assay

Determination of carboxyl methylation was carried out as described by Chelsky (13) with minor modifications. Briefly, after labeling of oocytes with [^{3}H]methyl-methionine (NEN Dupont) at a concentration of 1–10 μCi/oocyte (100 Ci/mmol) for 16 h, immunoprecipitation with the lamin-specific monoclonal antibody L6-5D5 and SDS-PAGE followed by fluorography and exposure to X-ray film, the radioactive bands corresponding to B3 were excised from the gel and transferred to 0.5 ml microfuge tubes. 200 μl of 1 M NaOH were added and the open tubes were immediately put into 7 ml scintillation vials (scint.vial I) containing 2.5 ml Aquasafe300 (Zinser Analytic, Maidenhead, Berks, UK). After 24-h incubation at room temperature with the vial lids closed tightly, the microfuge tubes were removed and their contents transferred to a new scintillation vial (scint.vial II). 1 ml 30% H_2O_2 was added to vial II and the vials were incubated at 65°C overnight. 4-ml scintillation fluid was added to scint.vial II and the samples I and II counted in a Packard Liquid Scintillation Analyzer for 2 h each. The radioactivity in vial I reflects the ^{3}H contained in carboxyl methyl groups, whereas counts in vial II indicate the amount of [^{3}H]methionine incorporated into the polypeptide chain. To determine the counting efficiency a defined amount of ^{3}H label was added to each sample as an internal standard and counting was repeated. After correcting the values for counting efficiency, the number of [^{3}H]carboxyl methyl groups per polypeptide chain was calculated, assuming that the initiation methionine had been cleaved off.

Transfection of Mouse L tk⁻ Cells and Detergent Solubilization Assay

For transfection experiments the complete cDNA sequence coding for lamin B3 (4,689 bp, which includes eight nucleotides of 5′-untranslated region and 2,921 bp of 3′-untranslated region) (60) was subcloned into the EcoRI site of the phTRcDEX vector (kindly provided by Dr. G. Kitten, University of Iowa, Iowa City, IA). Transcription is driven by the human transferrin receptor promotor and 3′ polyadenylation is ensured by an SV-40 polyadenylation signal. Both transfection and detergent solubilization experiments were carried out as described by Kitten and Nigg (34).

Immunocytochemistry

Transfected mouse L cells were plated on sterile coverslips 24 h before fixation in 50% methanol/50% acetone for 2 min. After several washes in PBS, the lamin specific mouse monoclonal antibody L6-5D5, diluted 1:100 in PBS with 10% BSA, was added for 2 h. The cells were extensively washed in PBS, followed by a 30-min incubation with anti-mouse FITC-conjugated antibodies (Dianova, Hamburg, FRG). Coverslips were dipped into a 1 μg/ml solution of 4′,6′-diamidino-2-phenyl-indol-dihydrochloride (Boehringer, Mannheim, FRG), mounted in Elvanol (Hoechst, Frankfurt/Main, FRG) and viewed in a Zeiss Axioplan microscope equipped with epifluorescence optics and a MC100 camera (Zeiss, Oberkochen, FRG).

Results

Xenopus Lamin B3 Is Soluble During Meiotic Metaphase

Lamin B3 is the major lamin constituent of the amphibian oocyte nucleus (35, 62). In interphase lamin B3 is stably integrated into the nuclear envelope lamina. Upon egg maturation, in the course of lamina disassembly, lamin B3 disperses throughout the egg cytoplasm as soluble oligomers (8, 46, 59, 63). In attempts to rigorously prove the subcellular distribution of lamin B3 we have repeated the fractionation experiments with more sensitive immunodetection methods. Mature eggs (in meiotic metaphase I) were homogenized in a buffer that stabilizes extracts in mitosis (1) and then fractionated by differential centrifugation under conditions that had previously been shown to separate membrane vesicles from soluble proteins (65). Both fractions were immunoprecipitated with a lamin specific monoclonal antibody. In parallel, manually isolated oocyte nuclei (germinal vesicles) were fractionated using a variety of buffers including the one used for egg fractionation. Since lamins are insoluble in oocyte nuclei, fractionation was done by low-speed centrifugation (see Materials and Methods). All four fractions were then subjected to Western blot analysis with the same monoclonal antibody used for the immunoprecipitation. The combination of these two immunodetection methods allows detection of unlabeled proteins facilitating the analysis of steady state conditions in oocytes which have a very long growth period (months). The majority of lamin B3 is found in the soluble fraction of eggs under these conditions (Fig. 1, lane *5* versus lane *3*) while in oocyte nuclei B3 is completely insoluble (Fig. 1, lane *1* versus lane *2*). A band just visible after the exposure time shown in Fig. 1 was detected when one tenth of the immunoprecipitate used in lane *5* was loaded (lane *4*). From the amount of egg extract analyzed and the detection limit of our immunodetection method, it can be estimated that more than 95% of the egg lamin is in the soluble fraction. This is in agreement with previously published data (8, 66).

Xenopus Lamin B3 is Isoprenylated In Vitro

We next asked whether the posttranslational modifications of B3 resemble those described for B-type lamins or whether B3 is subject to an additional processing event characteristic for A-type lamins (6, 34, 67, 70). We first analyzed isoprenylation of lamin B3 in vitro. Synthetic RNA encoding *Xenopus* lamin B3 was translated in a reticulocyte lysate either in the presence of [^{35}S]methionine or [^{3}H]mevalonic acid, the biosynthetic precursor of isoprene derivatives. The translation products were immunoprecipitated with a lamin specific monoclonal antibody and subjected to SDS gel electrophoresis. In vitro synthesized lamin B3 readily incorporates a derivative of mevalonic acid (Fig. 2, lane *2*). Lamin B3 translated in the presence of [^{35}S]methionine migrates as two closely spaced bands (Fig. 2, lane *1*). Only the faster migrating band had incorporated [^{3}H]mevalonic acid (compare Fig. 2, lanes *1* and *2*). Taken together, the in vitro results show that lamin B3 becomes isoprenylated after in vitro synthesis and that the modified form of B3 can be distinguished from the unmodified form by SDS gel electrophoresis.

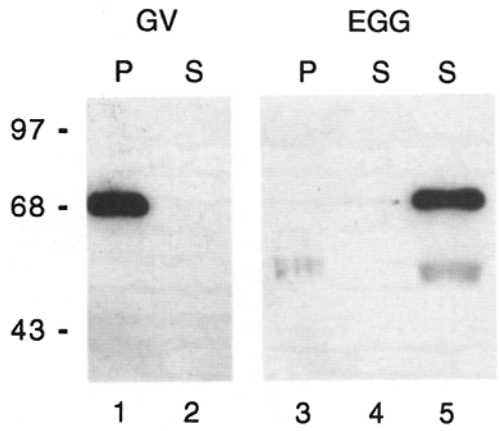

Figure 1. Lamin B3 becomes soluble upon meiotic egg maturation. Shown are Western blots depicting the solubilization characteristics of *Xenopus laevis* lamin B3 in oocytes (lanes *1* and *2*) and eggs (lanes *3–5*). Isolated germinal vesicles (*GV*) and eggs were fractionated as described in Materials and Methods. Fractions were either directly processed for Western blotting (equivalents of 25 GVs were loaded; lane *1* and *2*) or immunoprecipitated with the lamin specific monoclonal antibody L6-5D5 (lanes *3–5*). To check the sensitivity of our detection system different amounts of egg equivalents were loaded. Lane *3* shows the equivalent of 120 eggs (100%), lane *4* 12 eggs (10%), and lane *5* 108 eggs (90%). Proteins were visualized by chemiluminescence with the same anti-lamin monoclonal antibody and a secondary peroxidase conjugated antibody. Exposure time was 20 s. Whereas lamin B3 is insoluble in interphase nuclei, >95% of B3 becomes soluble during meiotic metaphase. The faint bands in lanes *3* and *5* migrating at approximately 50 kD are due to cross reactivity with IgG heavy chains used in the immunoprecipitation. The size of molecular mass markers is given in kD at left.

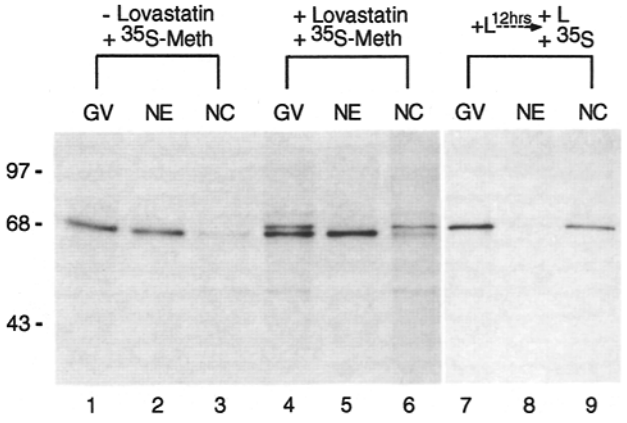

Figure 3. Isoprenylation is necessary for the incorporation of lamin B3 into the nuclear lamina. *Xenopus laevis* oocytes were biosynthetically labeled with [^{35}S]methionine in the absence (lanes *1–3*) or presence of Lovastatin (*L*, lanes *4–9*), an inhibitor of mevalonate synthesis. Results shown in lanes *7–9* were from oocytes preincubated with inhibitor for 12 h before labeling with [^{35}S]methionine. After 16 h of labeling nuclei were manually isolated and either processed directly (*GV*, lanes *1*, *4*, and *7*) or separated into nuclear envelope (*NE*, lanes *2*, *5*, and *8*) and nuclear content (*NC*, lanes *3*, *6*, and *9*). Lamin B3 was immunoprecipitated with the lamin specific monoclonal antibody L6-5D5. Samples were analyzed by SDS-PAGE and fluorographed followed by exposure to X-ray film for 1 d (lanes *1–6*) or 3 d (lanes *7–9*), respectively. The faster migrating isoprenylated form of B3 localizes exclusively to the nuclear envelope (lanes *2* and *5*) whereas under conditions of complete isoprene starvation, lamin B3 remains entirely in the karyoplasma (lane *9*). In lanes *4–6* conditions were chosen which allow simultaneous detection of the unprocessed and isoprenylated form of lamin B3. The size of molecular mass markers is given in kD at left.

Isoprenylation of Lamin B3 Is Necessary for Incorporation into the Nuclear Envelope Lamina

The importance of the CaaX motif for correct targeting of lamins has previously been shown by site directed mutagenesis (32, 34, 37). To analyze the role of isoprenylation of the endogenous lamin B3 in *Xenopus* oocytes we have used an inhibitor of mevalonic acid synthesis, Lovastatin, to prevent isoprenylation (33). Oocyte proteins were labeled with [^{35}S]methionine after preincubation with the inhibitor to exhaust the isoprenoid pool completely. Alternatively, inhibitor was administered only during the labeling period to allow a certain extent of isoprenylation. After labeling, oocyte nuclei were manually isolated, separated into nuclear envelope and nuclear content and processed for immunoprecipitation with a lamin specific monoclonal antibody. As a control, total unfractionated nuclei were analyzed in parallel. The gel electrophoretic conditions allowed to distinguish between processed (faster migrating) and unprocessed (slower migrating) form of B3. Under the labeling conditions applied (constant labeling without chase) newly synthesized B3 is detected nearly exclusively in the nuclear envelope of untreated oocytes (Fig. 3, lane *2*) whereas in the nuclear content only a very minor fraction is detected (Fig. 3, lane *3*). Only the processed form of B3 is present in these nuclei. When Lovastatin was administered during the labeling period the distribution of both the processed and the unprocessed form of B3 can be analyzed in the same nuclei. The unprocessed form is found exclusively in the nuclear content (Fig. 3, lane *6*), whereas the processed form appears only as a faint band in the nuclear content with the majority located in the nuclear envelope (Fig. 3, compare lanes *5* and *6*). The ratio of processed lamin B3 in the nuclear envelope fraction versus the nuclear content is approximately the same in Lovastatin and control oocytes (Fig. 3, compare lanes *2* and *3* with lanes *5* and *6*). In a third experiment cellu-

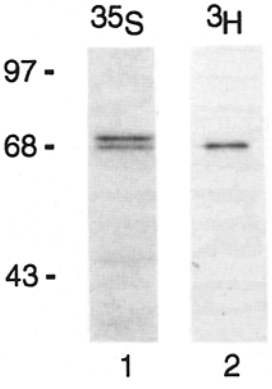

Figure 2. Lamin B3 can be isoprenylated in vitro. Synthetic B3 mRNA was in vitro translated in a reticulocyte lysate in the presence of [^{35}S]methionine (lane *1*) or [^{3}H] mevalonolactone (lane *2*). B3 was immunoprecipitated with the lamin specific monoclonal antibody L6-5D5 and analyzed by SDS-PAGE. Proteins were visualized by fluorography and exposed to X-ray film for 1 (lane *1*) or 7 d (lane *2*). Comparison of the doublet in lane *1* to the single band in lane *2* indicates that about half of the B3 protein present is processed into the isoprenylated form which migrates faster in our gel system. The size of molecular mass markers is given in kD at left.

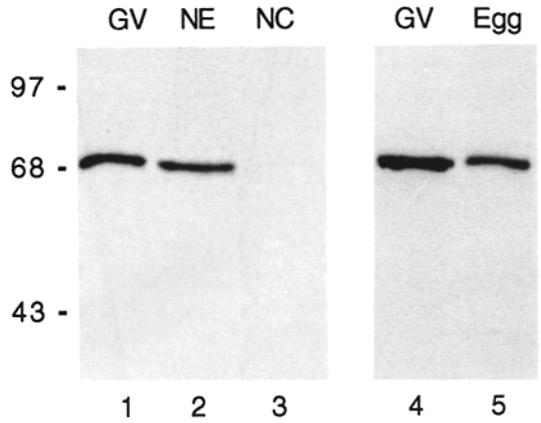

Figure 4. Isoprenylation of lamin B3 in *Xenopus laevis* oocytes and eggs. *Xenopus laevis* oocytes were incubated in [^{3}H]mevalonolactone in the presence of Lovastatin, an inhibitor of mevalonate synthesis. This results in the incorporation of [^{3}H]labeled farnesyl moieties into lamin B3. After 16 h oocyte nuclei were either manually removed (laens *1–4*), separated into nuclear envelope (*NE*, lane *2*) and nuclear content (*NC*, lane *3*) or oocytes were matured into eggs by addition of progesterone (lane *5*). Proteins were immunoprecipitated with the lamin specific monoclonal antibody L6-5D5, separated by SDS-PAGE, visualized by fluorography and exposure to X-ray film for 10 (lanes *1–3*) and 7 (lanes *4* and *5*) d. Comparison of equivalent numbers of GVs (lane *4*) and matured eggs (lane *5*) does not reveal a significant change in the degree of B3 isoprenylation. The isoprenylated form of B3 localizes exclusively to the nuclear envelope in oocytes (lane *2*; see also Fig. 3). The size of molecular mass markers is given in kD at left.

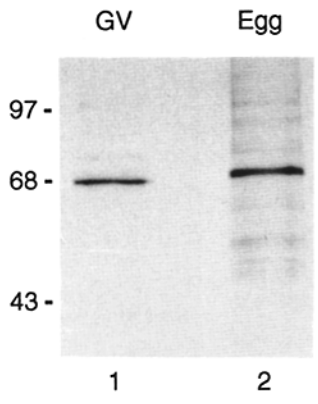

Figure 5. Lamin B3 is carboxyl methylated in oocytes and eggs. *Xenopus laevis* oocytes were incubated in [^{3}H]methyl-methionine. After 16 h progesterone was added to 50% of the oocytes to mature them to eggs. B3 was immunoprecipitated from manually isolated nuclei (lane *1*) or eggs (lane *2*) using the lamin specific monoclonal antibody L6-5D5. After SDS-PAGE analysis and exposure to X-ray film, labeled bands corresponding to B3 were cut out of the gel and radioactivity of the carboxyl methyl groups was determined with the vapor phase methyl release assay as described in Materials and Methods. Values are listed in Table I. The size of molecular mass markers is given in kD at left.

lar isoprenoids were completely depleted by a 12-h incubation with Lovastatin before the labeling period. Only the unprocessed form of lamin B3 is detected (Fig. 3, lane *7*) and it is found exclusively in the nuclear content (Fig. 3, compare lanes *8* and *9*). Thus, inhibition of protein prenylation completely blocks integration of newly synthesized lamin B3 into the oocyte nuclear envelope.

Lamin B3 Is Isoprenylated in Oocytes and Eggs

To monitor isoprenylation of lamin B3 in vivo we cultured oocytes for a long period of time (two days) in the presence of [^{3}H]mevalonic acid under conditions where endogenous synthesis of isoprenoids was inhibited by Lovastatin. Under these conditions oocytes were viable for several days; they showed an unaltered protein synthesis capacity as monitored by incorporation of [^{35}S]methionine and could be induced to egg maturation by treatment with progesterone. As shown in Fig. 4, lamin B3 immunoprecipitated from total oocyte nuclei or isolated nuclear envelopes is readily labeled by [^{3}H]mevalonic acid (Fig. 4, lanes *1*, *2*, and *4*). No isoprenylated B3 is detected in nuclear contents after the prolonged labeling period with [^{3}H]mevalonic acid (Fig. 4, lane *3*).

After meiotic nuclear envelope breakdown lamin B3 is freely soluble in the egg cytoplasm (see Fig. 1). To see whether the dissociation of lamin B3 from the envelope membranes is accompanied by loss of the isoprenyl moiety we followed the fate of [^{3}H]mevalonic acid–labeled lamin B3 after egg maturation. Eggs that had undergone germinal vesicle breakdown, monitored by the appearance of the maturation spot, were selected, fractionated into a soluble and a pellet fraction and processed for immunoprecipitation as described above. In control experiments B3 was immunoprecipitated from nuclei of oocytes that were incubated in parallel in the absence of hormone. Under these conditions lamin B3 is detected in the soluble fraction but not in the pellet fraction of eggs (Fig. 4, lane *5*; results of the pellet fraction not shown). Although the yield of immunoprecipitations from total egg supernatants was always somewhat lower than from isolated nuclei, these results clearly show that isoprenylation persists throughout the egg maturation process.

Lamin B3 Is Carboxyl Methylated in Oocytes and Eggs

Isoprenylation of the COOH-terminal cysteine in CaaX motif proteins is followed by proteolytic removal of the last three amino acids and carboxyl methylation of the resulting COOH-terminal cysteine residue (for review see reference 53). For B-type lamins in somatic cells it has been shown that this is the only site of carboxyl methylation and that this methylesterification seems to be cell cycle dependent (14). According to previous reports Lamin B is demethylated during mitotic metaphase (15). To test whether this holds also for meiotic lamin B3 we assayed carboxyl methylation of B3 in oocytes (interphase) and eggs (meiotic metaphase). For these experiments oocytes were labeled with [^{3}H]methyl-methionine and half of the oocytes were matured to eggs by addition of progesterone. In Fig. 5 [^{3}H]methyl-methionine–labeled lamin B3 from oocytes (lane *1*) and total egg soluble

Table I. Methyl Groups/Lamin Molecule

	GV	Egg	GV/Egg
Experiment 1	0.6	1.0	0.6
Experiment 2	0.7	0.5	1.4
Experiment 3	1.3	1.3	1.0

Degree of carboxyl methylation in oocytes (*GV*) and eggs (*Egg*). In three independent experiments the stoichiometry of the carboxyl methyl groups of lamin B3 in oocytes and eggs was determined by the vapor phase methyl release assay as described in Materials and Methods. The ratio of labile to nonlabile radioactivity was determined and multiplied by the number of methionine residues in Lamin B3 (11). In Experiment 3 ten times the amount of [^{3}H]methyl-methionine was used to obtain higher absolute values in scintillation counting.

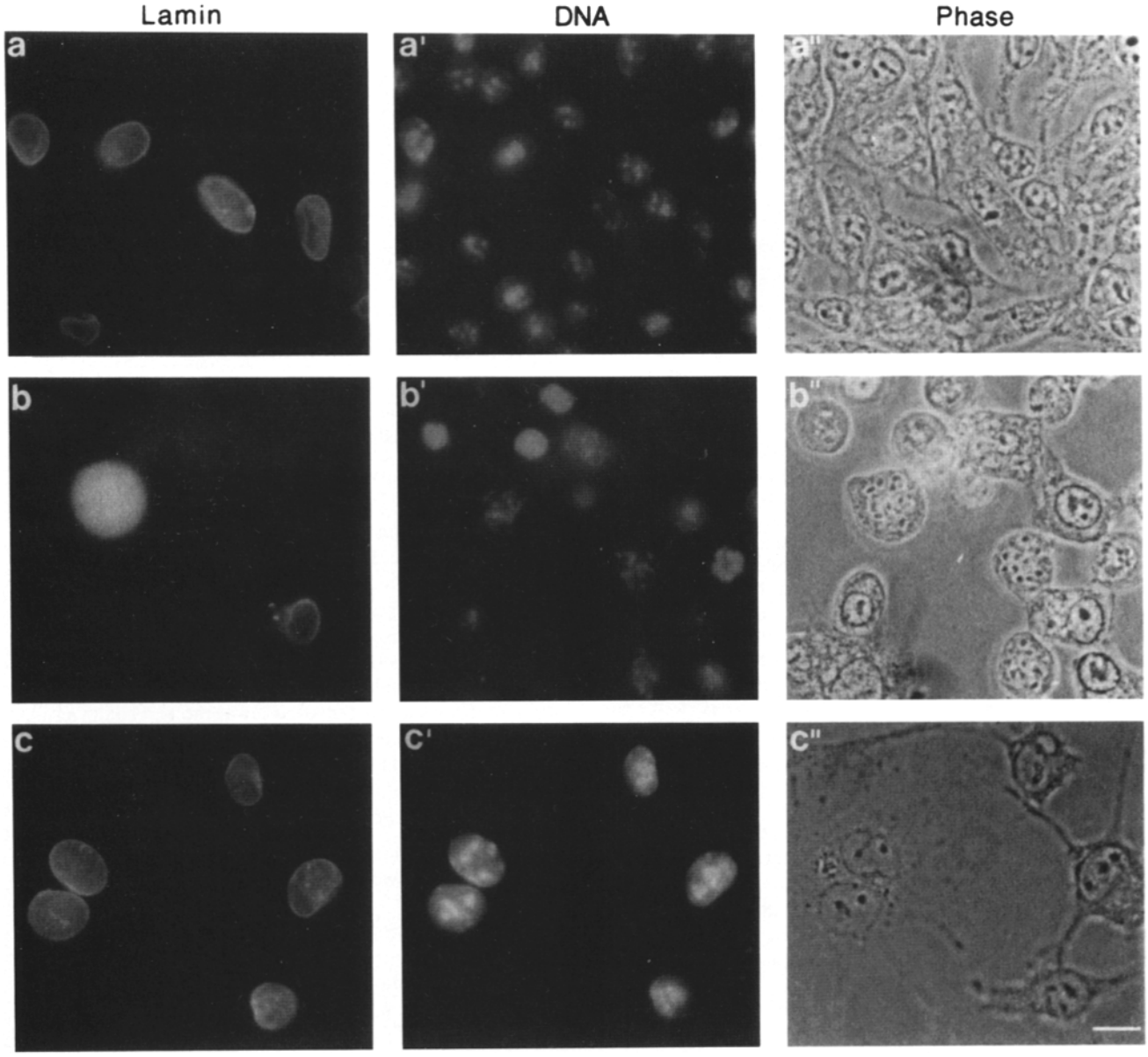

Figure 6. Indirect immunofluorescence analysis of *Xenopus laevis* lamin B3 in transfected mouse L cells. The cDNA encoding lamin B3 under the control of a transferrin-receptor promoter was transfected into mouse L cells as described in Materials and Methods. Cells plated on glass coverslips were fixed and probed with the lamin specific monoclonal antibody L6-5D5, followed by a FITC-conjugated secondary antibody (*a–c*). DNA was visualized by staining with 4',6'-diamidino-2-phenyl-indol-dihydrochloride (*a'–c'*). The corresponding phase images are shown in *a''–c''*. Panels *a/a'/a''* and *b/b'/b''* show transiently transfected cells in interphase (*a/a'/a''*) and in mitosis (*b/b'/b''*). *c/c'/c''* is an example of a stably transfected cell line generated as described in Materials and Methods. The *Xenopus* lamin B3 shows a distribution typical for lamins when transfected in mouse L cells; that is, confined to the nuclear periphery during interphase and distributed throughout the cytosol during mitosis. Bar, 10 μm.

fraction (lane 2) is shown after immunoprecipitation. To determine the extent of carboxyl methylation versus labeling of methionine residues in the polypeptide chain radiolabeled bands were excised from the gel and subjected to a vapor phase methyl release assay (13). The stoichiometry of the carboxyl methyl groups was in the range of about one carboxyl methyl group per lamin B3 molecule and was unaltered between oocytes and eggs (Table I). Therefore, lamin B3 seems not to be demethylated during meiotic lamina disassembly to an extend reported for B-type lamins in mitotic cells.

Lamin B3 Is Associated with Mitotic Membranes When Transfected into Mouse L Cells

The results obtained so far give no obvious clue how to explain the different fates of the oocyte lamin B3 and the somatic lamins B1 and B2 during nuclear envelope breakdown. Both mitotic as well as meiotic lamins are permanently isoprenylated and persistence of the carboxyl methylation in meiosis should increase membrane affinity of the prenylated lamin rather than weaken membrane interaction. To test whether the dissociation of lamin B3 from meiotic envelope

membranes is a property of lamin B3 itself rather than being specific for the developmental stage, we analyzed mitotic lamin distribution in mouse cells transfected with *Xenopus* lamin B3. A mammalian expression vector containing the full length *Xenopus* lamin B3 coding sequence (for details see Materials and Methods) was constructed and transfected into mouse L cells. The antibodies available discriminated between endogenous and transfected lamins thereby greatly facilitating the analysis. Both transiently transfected cells as well as subcloned stably expressing cell lines were analyzed and gave corresponding results. The amounts of *Xenopus* lamin expressed in transiently transfected cells varied considerably. However, irrespective of this variation lamin B3 localized correctly to the nuclear envelope membrane in interphase cells (Fig. 6, *a* and *c*) and became distributed throughout the whole cytoplasm during mitosis (Fig. 6 *b*). Biochemical analysis revealed that the ectopically expressed lamin B3 was resistant to extraction with non ionic detergent and high salt concentrations in interphase (results not shown) and was completely solubilized after treatment with Triton X-100 in mitotic cells (Fig. 7, lane *3* and *4*). Therefore, we can conclude that lamin B3 interacts with the endogenous mouse lamina in a normal fashion and that its mitotic disassembly and reassembly is under normal cell cycle control.

To investigate the state of the lamins during mitosis by cell fractionation, transfected mouse cells were arrested in mitosis by treatment with nocodazole. Cells were homogenized in hypotonic buffer. To one half of the homogenate Triton X-100 was added to dissolve lipid membranes. Particulate fractions were then separated from soluble fractions by centrifugation and fractions were subjected to SDS gel electrophoresis and immunoblotting (for details see Materials and Methods). The monoclonal antibody used to detect mouse lamins (PKB8 (36)) reacts with A-type lamins (A and C) and B-type lamins in mouse but does not cross react with *Xenopus* lamin B3. The mitotic mouse lamins partitioned as previously described for mammalian and bird lamins (Fig. 7, lanes *5–8*) (24, 34, 64). The fractionation of *Xenopus* lamin B3 was analyzed in the same manner using a monoclonal antibody that is specific for lamin B3 in this cellular system. Remarkably, a significant portion of lamin B3 (~50%) sedimented with mitotic membranes in homogenates that had not been treated with detergent (Fig. 7, lane *1*). This fraction of B3 was completely soluble when membrane lipids had been solubilized with Triton X-100 (Fig. 7, lanes *3* and *4*). To exclude the possibility that the partition of lamin B3 in mitotic mouse cells is caused by heterooligomer formation with endogenous B-type lamins a co-precipitation experiment was carried out. Transfected mitotic cells were homogenized in the presence of Triton X-100 to solubilize all membrane bound lamins. Lamin B3 was immunoprecipitated with a B3 specific antibody under conditions where lamin oligomers are stable. The immunoprecipitate was then split into two parts and these were subjected to Western blot analysis with two different antibodies. One tenth of the precipitate was probed with the same antibody that had been used for the immunoprecipitation of B3 (Fig. 8, lane *2*). In parallel an aliquot of the unfractionated cell homogenate (Fig. 8, lane *1*) and the supernatant after immunoprecipitation (Fig. 8, lane *3*) was analyzed. In this way the extent of immunoprecipitation could be monitored. The remaining nine tenths of the immunoprecipitate were probed with a monoclonal antibody specific for the endogenous mouse lamins A, B, and C (Fig. 8, lane *5*). The absence of any mouse lamins in the immunoprecipitate obtained with the anti-lamin B3 antibody rules out the possibility that heterooligomers between endogenous lamins and the ectopically expressed *Xenopus* lamin had formed. Thus it can be concluded that ectopically expressed *Xenopus* lamin B3, like the endogenous B-type lamins, has the ability to remain associated with nuclear envelope derived membranes in mitosis. Furthermore, the clear partition of the endogenous lamins A/C and lamin B into the supernatant and pellet fraction respectively (Fig. 7, lanes *5* and *6*) indicates that heterooligomer formation between endogenous A- and B-type lamins does not occur to a significant extent during mitosis.

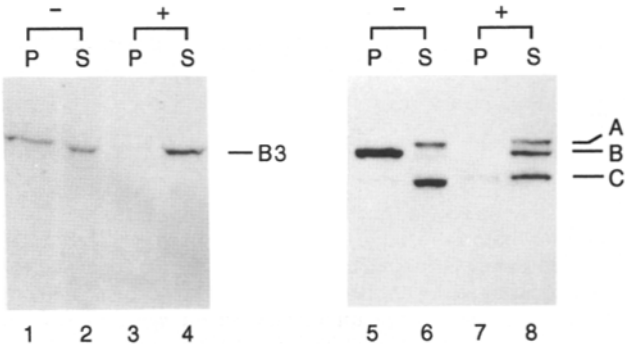

Figure 7. Fractionation of mitotic lamins. Stably B3-expressing mouse L cells were arrested in mitosis by addition of nocodazole. Mitotic cells were harvested by mechanical shake-off, homogenized in the presence (+, lanes *3*, *4*, *7*, and *8*) or absence (−, lanes *1*, *2*, *5*, and *6*) of Triton X-100 and separated by centrifugation into a pellet (*P*) and supernatant (*S*) fraction. Western blots were carried out and proteins were visualized by incubation with the lamin specific monoclonal antibody L6-5D5 (lanes *1–4*) or the monoclonal antibody PKB8, which reacts with the endogenous mouse lamins A, B, and C, but does not crossreact with the transfected lamin B3. Chemiluminescence was used for detection using alkaline phosphatase conjugated secondary antibodies (exposure time 30 min). Mouse lamin B stays membrane associated and mouse lamins A/C become soluble during mitosis. *Xenopus* lamin B3 is found in the pellet and the supernatant fraction. In control experiments membrane lipids were solubilized by addition of Triton X-100. Under these conditions all lamins are found in the supernatant fraction (lanes *4* and *8*).

Discussion

We have analyzed the posttranslational modifications of *Xenopus* lamin B3 in oocytes (meiotic interphase) and eggs (meiotic metaphase). We find that lamin B3, like somatic B-type lamins, is isoprenylated and carboxyl methylated in interphase and that association of newly synthesized lamin B3 with the nuclear envelope membranes requires isoprenylation. These modifications persist during meiotic nuclear envelope breakdown when lamin B3 becomes freely soluble in the egg cytosol. Furthermore, by stably expressing *Xenopus* lamin B3 in mammalian cells, we have demonstrated that lamin B3 is able to associate with mitotic nuclear envelope membranes. As discussed below these findings indicate that the CaaX motif–mediated modifications, although necessary

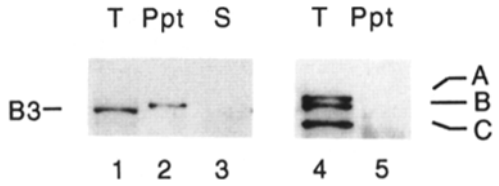

Figure 8. Lamin B3 does not form oligomers with endogenous lamins during mitosis. Mitotic B3-expressing mouse L cells were harvested and homogenized as described in Fig. 7. Lamin B3 was immunoprecipitated with the lamin specific monoclonal antibody L6-5D5 in immunoprecipitation buffer containing no SDS. After SDS-PAGE samples were processed for Western blotting. Blots were either probed with L6-5D5 (lanes *1–3*) detecting transfected *Xenopus* lamin B3 or with the monoclonal antibody PKB8 (lanes *4* and *5*), reacting with the endogenous mouse lamins A, B, C, but not with the transfected lamin B3. Proteins were visualized with a peroxidase conjugated secondary anti-mouse antibody using chemiluminescence (exposure time 1 min). In lanes *1* and *4* an aliquot of the total cell homogenate (*T*) before immunoprecipitation was loaded for control. 10% of the immunoprecipitate was loaded in lane *2*, 90% in lane *5*. The effectiveness of the procedure is shown in lane *3* where the B3-depleted supernatant (*S*) after immunoprecipitation was loaded. The absence of mouse lamins in the immunoprecipitate (lane *5*) indicates that the transfected *Xenopus* lamin B3 does not form heterooligomers with the endogenous lamins A, B, or C under the described conditions. Due to different amounts of protein loaded in lane *1* (total cell lysate) and lane *2* (immunoprecipitate) lamin B3 shows a slightly different mobility.

for membrane targeting, are not sufficient for stable membrane association.

The importance of the CaaX motif-dependent modifications for targeting newly synthesized lamins to the nuclear envelope and for stable integration into the nuclear lamina structure has been demonstrated by several experimental approaches (32, 34, 37). The first three steps of these CaaX motif-mediated modifications are identical for both A- and B-type lamins including *Xenopus* lamin B3 (this study). They include isoprenylation, proteolytic processing, and carboxyl methylation following the scheme worked out for *ras* proteins (for review see reference 53). The increased hydrophobicity of the COOH termini introduced by these modifications has been taken to explain the affinity of prenylated proteins for membranes (for reviews see references 16, 18). In the case of nuclear lamins a nuclear localization signal in addition to the CaaX motif is necessary for directing newly synthesized lamins to the nuclear lamina (32). Due to the lipophilic modifications at the CaaX-cysteine, lamins would concentrate at the inner nuclear membrane where filament formation would be initiated. According to this model specific targeting of lamins should critically depend on the time when lamin isoprenylation occurs relative to nuclear transport since the addition of a lipophilic modification before nuclear entry would bear the potential of misrouting lamins to cytoplasmic membranes. It is not yet known, however, when lamins are isoprenylated. Interpretation of kinetic studies favors the view that CaaX box processing of lamins is a cytoplasmic event (34). Since the substrate specificity of the farnesyl/protein transferase is confined to the four amino acids of the CaaX motif lamin isoprenylation could indeed by carried out by a cytoplasmic transferase (52). On the other hand, studies involving inhibitors of isoprenylation point to an intra nuclear isoprenylation (42). Whether nuclear isoprene transferases exist is presently unknown.

While B-type lamins retain their lipid modifications lamin A is subject to an additional processing event that takes place concomitant or shortly after incorporation into the lamina (19, 26, 39, 40). In the course of this maturation process 18 amino acids are cleaved from the COOH terminus (70) and thereby the isoprenoid moiety is lost from the mature lamin A. The transient prenylation of A-type lamins has been taken to explain the fate of A-type lamins during mitosis (34). When the lamina depolymerizes in mitotic prophase, lamin A oligomers are released from nuclear membranes and become freely soluble in the cytosol (24, 64). Consequently, the mechanism by which mitotic lamin A is retargeted to the nuclear envelope at the end of mitosis must be different from the one operating for newly synthesized lamin A.

B-type lamins, in contrast, are permanently isoprenylated and this holds also true for the meiotic lamin B3 of *Xenopus* oocytes and eggs (this study). However, while lamins B1 and B2 remain associated with remnants of the mitotic membranes after depolymerization of the lamina (24, 64), lamin B3 is released from nuclear envelope membranes in the course of meiotic germinal vesicle breakdown. The only difference we noticed so far in the modifications of mitotic and meiotic B-type lamins is the stoichiometry of carboxyl methylation. While for somatic B-type lamins a mitosis-specific demethylation has been reported (15) carboxyl methylation of depolymerized lamin B3 in amphibian eggs remains unchanged. However, this difference cannot readily explain the fate of meiotic lamin B3 since methylesterification of a carboxyl group increases hydrophobicity of a protein and consequently should strengthen rather than weaken its membrane affinity. Therefore, other factors must be considered to explain the difference in the subcellular distribution of mitotic and meiotic B-type lamins.

The experiments reported here show that lamin B3 when expressed in somatic cells by transfection is able to stably associate with mitotic membranes indicating that the lamin B3 polypeptide itself does not contain sequences that prevent membrane association. Therefore, we can conclude that isoprenylation and carboxyl methylation alone are not sufficient to mediate stable membrane association of lamins.

Similar observations have been made with *ras* proteins which associate with cytoplasmic membranes. Isoprenylation and carboxyl methylation have been shown to be required for membrane association and full biological activity of *ras* proteins (54; for review see reference 53), but these modifications are not sufficient for stable membrane binding (27). Additional palmitoylation of sites in close proximity to the CaaX isoprenylation or an additional plasma membrane targeting signal consisting of a polybasic domain that also acts in combination with the CaaX motif are necessary for stable membrane binding of *ras* proteins (27, 28). None of these additional modifications or targeting signals are found in the presently known lamins with the notable exception of an alternative splice variant of *Xenopus* lamin B3, namely lamin B3b (20). The last alternatively spliced exon of lamin B3b, only 12-amino acids long, encodes an additional cysteine residue adjacent to the CaaX-cysteine that might serve as a palmitoylation site. Moreover, B3b contains a stretch of six basic amino acids similar to the one found in p21Ki-*ras*

(B) (27). The amino acid sequences of these alternatively spliced exons are: B3a: SHQSVDPSCSIM; B3b: TKRRKKKCCSVS. The RNA of the variant B3b is present in oocytes at very low abundance (20). Currently, however, we do not know whether this splice form is expressed at the protein level in oocytes and whether it shows stronger membrane binding than lamin B3 lacking these sequence motifs.

Any additional factor, however, that would confer a general increase in membrane affinity could not account for the specific targeting of isoprenylated lamins from the cytosol to the inner nuclear membrane at the end of meiosis. Lamina formation in meiotic telophase, as studied in a cell free system from *Xenopus* eggs, is a late event (46). In a first step, membrane vesicles are targeted to the surface of chromatin by the interaction of nonlamin membrane receptor(s) with chromatin (45, 56, 65, 71). These vesicles fuse and provide a scaffold for assembly of nuclear pore complexes. Transport of lamin B3 into the nucleus then occurs through the pores (46). In HeLa cells it has been observed that reassembly of lamins A, B, and C into the nuclear lamina begins only during late telophase (12). As demonstrated for newly synthesized lamins, prenylation might also be required for the retargeting of lamin B3 in meiotic telophase, but prenylation alone would not be sufficient to explain the correct relocalization to the inner nuclear membrane. Possible candidates that could mediate the specific membrane binding of lamins are integral membrane proteins that may function as lamin B receptors (4, 49, 55, 58, 73, 74). These receptors are located in the inner nuclear membrane and might either interact directly with lamin polypeptides itself or indirectly via the farnesyl moiety. The alternate fates of B-type lamins after lamina disassembly in mitosis and meiosis respectively could possibly be explained by a different modulation of the receptor–lamin interaction. Indeed, phosphorylation might be one type of modification that could influence these interactions. In vitro studies show that phosphorylation of the chicken lamin B receptor can lower its binding to lamin B (3, 17) and recent findings show that the receptor is associated with a kinase in vivo (57).

Recently Foisner and Gerace (23) have described integral membrane proteins of the nuclear envelope that interact in vitro with lamins and chromosomes. Some of these lamina-associated polypeptides specifically bind to both A- and B-type lamins, while another protein specifically associates with lamin B1. Interestingly, these lamina-associated proteins are phosphorylated during mitosis, and in vitro phosphorylation of one of the proteins inhibits its binding to both lamin B1 and chromosomes.

Nuclear organization in amphibian oocytes differs from that in somatic cells. The oocyte chromosomes are located in the central part of the nucleus and are not in contact with the nuclear envelope. The oocyte lamina is formed by an orthogonal meshwork consisting only of a single layer of intermediate filaments (2). Future analysis will reveal whether the lamin-associated proteins present in amphibian oocytes are homologous to those described above. Alternatively, oocytes might express specialized lamin receptor(s) that function in organizing these giant nuclei.

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