Analysis of Nuclear Lamin Isoprenylation in *Xenopus* Oocytes: Isoprenylation of Lamin B3 Precedes Its Uptake into the Nucleus

Iris Firmbach-Kraft and Reimer Stick

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

Abstract. Protein prenylation is a posttranslational modification involving the covalent attachment of a prenyl lipid to a cysteine at or near the COOH terminus of a protein. It is required for membrane localization and efficient function of a number of cytoplasmic as well as nuclear proteins including the proto-oncogenic and activated forms of *Ras*. Farnesylation in conjunction with a nuclear localization signal has been shown to be necessary to target newly synthesized nuclear lamins to the inner nuclear envelope membrane. It is, however, not clear where in the cell isoprenylation of nuclear lamins takes place. In this study we describe in vivo and in vitro experiments on the isoprenylation of the *Xenopus* oocyte nuclear lamin B3. We show by kinetic analysis that newly synthesized lamins are isoprenylated in the cytosol of oocytes before uptake into the nucleus. From our data it can be concluded that isoprenylation of lamins in the nucleus, as it is observed under certain conditions of isoprene starvation, represents a default pathway rather than the physiological situation. We further analyzed the capacity of isolated nuclei to carry out isoprenylation of B3. Our results are in line with a dual localization of a protein farnesyltransferase in the cytosol and nuclei of amphibian oocytes. Implications for the possible functions of a nuclear protein farnesyltransferase as well as possible mechanisms of the selective inhibition of farnesylation of cytoplasmic proteins by peptidomimetics are discussed.

EUKARYOTIC polypeptides with certain types of cysteine-containing carboxy-terminal sequence motifs are candidates for posttranslational modifications including isoprenylation, proteolytic cleavage, and methyl esterification. The major class of these proteins contains a COOH-terminal CXXX tetrapeptide (C = cysteine, X = generally aliphatic). Most prenylated proteins are modified by attachment of either a 15-carbon farnesyl or a 20-carbon geranylgeranyl group in a thioether linkage to the cysteine residue. Proteins that contain these sequence motifs include the *Ras* proteins and many other small G proteins, the heterotrimeric large G proteins, the retinal cGMP phosphodiesterases, most nuclear lamins, and several fungal mating pheromones (for review see references 9, 10, 40).

For many of these proteins the importance of prenylation for their function has been shown (3, 8, 19, 22, 29). The precise mechanism, however, by which these modifications exert their function is not clear at present. Isoprenylation and carboxyl methylation result in an increased hydrophobicity of the carboxyl terminus (4). This has been taken to explain the affinity of many prenylated proteins for membranes. In other cases the primary effect of prenylation seems to be a strengthening of protein-protein interaction that can be observed even in the absence of membranes (15). For several of the membrane bound proteins such as *Ras* proteins and B-type lamins it has been shown, that the CXXX motif mediated modifications, although necessary, are not in themselves sufficient for a tight association with membranes, and that additional factors are involved in membrane binding (13, 17, 18). Membrane receptor proteins, that recognize the isoprenyl and methyl groups might be involved in targeting such proteins to membranes.

Several enzymes, termed protein prenyltransferases, which catalyze the attachment of a prenyl moiety to proteins, have been identified (for review see 6, 9, 40). Depending on their substrate specificity they are classified as either farnesyl or geranylgeranyl protein transferases. Protein farnesyltransferases are soluble enzymes. Their substrate specificity is confined to the four amino acids of the CXXX motif. Farnesylation of all the above mentioned proteins could therefore be carried out by a single cytosolic enzyme. Proteins carrying the same CXXX-dependent modifications are located in different membrane compartments, therefore additional factors must be responsible for the specific targeting of these proteins. In the case of *Ras* proteins efficient binding to the plasma membrane has been shown to depend

Address all correspondence to R. Stick, Institut für Biochemie und Molekulare Zellbiologie, Abt. Entwicklungsbiochemie, Universität Göttingen, Humboldtallee 23, D-37073 Göttingen, FRG. Ph.: (49) 551-39 82 73. Fax: (49) 551-39 59 60.

on either an additional modification, i.e., palmitoylation of another cysteine, or the presence of a polybasic domain in close proximity to the isoprenylated COOH terminus (17, 18). The translocation of the transducin $\beta\gamma$ complex from the cytosol to the rod outer segment membranes might be achieved by the CXXX-dependent modifications of the γ subunit that increases the interaction with membrane associated transducin α and rhodopsin (15, 36). Mutational analysis has shown that targeting of lamins to the inner nuclear membrane requires in addition to the CXXX-mediated modifications a nuclear localization signal (21, 26, 28, 31). It is, however, not known whether newly synthesized lamins are isoprenylated in the cytoplasm before entering the nucleus or whether nuclear entry precedes the modification reactions. In the latter case isoprenylation would take place within the nucleus and due to the lipophilic modifications lamins would be concentrated at the inner nuclear membrane where filament formation would be initiated.

We have analyzed isoprenylation of nuclear lamin B3 in *Xenopus* oocytes. We show here by kinetic analysis that newly synthesized lamins are isoprenylated in the cytosol before uptake into the nucleus and that isoprenylation of lamin B3 can be achieved in vitro by incubation with cytosol of enucleated oocytes. However, in vitro experiments demonstrate that isoprenylation of lamins can also occur in isolated nuclei and evidence is given that these in vitro prenylated lamins are integrated into the lamina structure. These results are discussed in the light of the recent finding of the dual localization of the protein farnesyltransferase in cytosol and nuclei (44) and pose the question of the possible function of a nuclear protein farnesyltransferase.

Materials and Methods

Biosynthetic Labeling of Xenopus laevis Oocytes

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 (12). 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. Preincubation with Lovastatin was done at 50 μM. Lovastatin was a gift from Dr. M. Stapff (Merck, Sharp & Dohme, München, FRG). To make a 10 mM stock solution 90 mg Lovastatin was dissolved by heating to 55°C in 1.8 ml ethanol, 0.9 ml of a 0.6 N NaOH solution, and 18 ml H_2O were added and incubated at room temperature for 30 min. pH was adjusted to 8.0 with HCl and the solution was brought to a final volume of 22.5 ml with H_2O. This stock solution was kept at −20°C (25). In all labeling experiments 10 μl of modified Barth solution per oocyte were used. For labeling with [^{35}S]methionine (Amersham, Braunschweig, FRG) 10 μCi/oocyte were added. [^{3}H]Mevalonolactone (NEN Dupont, Wilmington, DE) in the presence of 50 μM Lovastatin was administered at a concentration of 0.5 μCi/oocyte. After the appropriate incubation period, nuclei were manually removed. To inhibit protein synthesis oocytes were incubated in 200 μg/ml cycloheximide (Sigma Chem. Co., St. Louis, MO).

Isolation and Manipulation of Oocyte Nuclei

Oocyte nuclei were isolated manually from collagenase treated oocytes as described previously (46).

For the isolation of nuclei under mineral oil (38) oocytes were transferred to small plastic weight boats and the buffer was carefully drained with a piece of filter paper. The oocytes were then covered with mineral oil (M5904, Sigma Chem. Co.). Nuclei were manually isolated and the enucleated oocytes were removed from the oil with forceps. Application of [^{3}H]farnesylpyrophosphate (22.5 Ci/mmol; NEN Dupont, Wilmington, DE) was carried out with microcapillaries (Clark Electromedical Instruments, Reading, UK) with the help of a microsyringe. Droplets of less than 10 ηl were applicated. After the incubation nuclei were removed from the oil with a Gilson pipette equipped with a flattened tip.

For high salt extraction nuclei were transferred into a microcentrifuge tube and 2 volumes of 2 M NaCl, 100 mM Tris-HCl, pH 7.4, were added. After 5 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 the same buffer and also spun once more.

Preparation of Cytoplasmic Extract

Oocytes were manually enucleated in 83 mM KCl, 17 mM NaCl, 10 mM Tris-HCl, pH 7.2. The enucleated oocytes were transferred into a microcentrifuge tube, excess buffer was removed and the oocytes were homogenized by pipetting up and down with a yellow tip. The cytoplasmic homogenate was used without further fractionation. Lamin B3 was isolated with magnetic immunobeads from isolated nuclei of Lovastatin-treated [^{35}S]methionine-labeled oocytes. Labeling conditions were as described above. Immunobeads loaded with lamin B3 (equivalent to 10 nuclei) were incubated with cytoplasmic extract (equivalent to 10 oocytes) in the presence of 22 pmole farnesylpyrophosphate (NEN Dupont, Wilmington, DE) for 16 h at 18°C. After the incubation the beads were washed and processed as described for immunoprecipitation experiments.

Gel Electrophoresis and Fluorography

Proteins were separated on 10% polyacrylamide gels according to Laemmli (30). For the detection of ^{3}H- and ^{35}S-labeled proteins, gels were fixed in 10% acetic acid for 30 min, incubated in Amplify (Amersham, Braunschweig, FRG) for 20 min, dried, and exposed to x-ray film.

Immunoprecipitation

The lamin specific monoclonal murine antibody L6-5D5 (45), used in all experiments, recognizes *Xenopus laevis* lamins B3 and to a lesser extent B2 in immunoblots. In immunoprecipitations, however, this antibody is specific for lamin B3, there is no precipitation of B2 to any significant degree.

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-lamin monoclonal antibody 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). Nuclei and high salt pellet fractions 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. Enucleated oocytes were homogenized in 10 μl extraction buffer/oocyte (80 mM glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 20 mM Hepes, adjusted to pH 7.5, 0.5 mM PMSF) and an equal volume of 2× immunoprecipitation buffer was added. The antigen solutions were then preincubated with Dynabeads precoated with anti-mouse IgGs for 30 min. After removal of the beads, anti-lamin-coated beads were added and incubated at 4°C for 12 h with constant rotation. The antigen loaded beads were washed five times with imunoprecipitation buffer and the antigen was removed from the beads by boiling for 5 min in SDS-gel sample buffer.

Results

To analyze the isoprenylation kinetics of newly synthesized nuclear lamins we have chosen amphibian oocytes as experimental system. Amphibian oocytes express lamin B3 as their major lamin constituent (27, 46) and B3 is readily radiolabeled in vivo by either [^{35}S]methionine or [^{3}H]mevalonic acid, the biosynthetic precursor of isoprene derivatives

(13). Furthermore, nuclear and cytoplasmic fractions can easily be obtained by manual enucleation of oocytes, facilitating kinetic analysis of nuclear uptake of lamin polypeptides. For this analysis we used a monoclonal antibody (L6-5D5) that specifically immunoprecipitates *Xenopus* lamin B3 but not lamin B2, which has recently been shown to be expressed in oocytes as a minor component (32) (see also Materials and Methods). We have shown previously that immunoprecipitation in conjunction with SDS-gel electrophoresis allows to distinguish between the unprocessed and the isoprenylated form of lamin B3 (13).

Isoprenylation of Nuclear Lamin B3 Occurs in the Cytoplasm before Nuclear Uptake

To determine the time when isoprenylation of newly synthesized lamins occurs relative to nuclear uptake we carried out a kinetic analysis of lamin prenylation in oocytes. Oocytes were labeled in medium containing [^{35}S]methionine. After 1 h the medium was changed and [^{35}S]methionine was replaced by an excess of unlabeled methionine. Oocytes were manually enucleated at different times during the chase period and lamin B3 was immunoprecipitated from both the cytoplasm and the nuclei, followed by SDS-PAGE analysis. Fig. 1 shows the results of such a pulse chase experiment. After the labeling period of 1 h > 90% of the radiolabeled lamin B3 is still found in the cytoplasm (Fig. 1, lane *2*). Both forms of B3, the isoprenylated faster migrating and, less abundant, the unmodified slower migrating form of B3 are present in the cytoplasm (Fig. 1, lane *2*). After a 5 h chase period, and probably before that time, the newly synthesized lamin B3 is completely converted to the isoprenylated form (Fig. 1, lanes *3* and *4*). Nuclear entry of lamin B3 is slower; accumulation of the fully processed form within the nucleus is observed over the entire chase period. Quantification of the results by phosphoimaging revealed, that: (*a*) the [^{35}S]-methionine-labeled lamin B3 is stable throughout this period, and that (*b*) B3 had accumulated about 16-fold in the nucleus after 24 h. The observed rate of nuclear accumulation of lamin B3 is within the broad range of other oocyte nuclear proteins. Moreover, in nuclei only isoprenylated lamin is detected. From these results it can be concluded that isoprenylation of newly synthesized nuclear lamins occurs in the oocyte cytoplasm and precedes the uptake of these proteins into the nucleus.

In line with these findings is the observation that isoprenylated lamin B3, radiolabeled by its isoprene moiety, can be immunoprecipitated from the cytoplasm of oocytes that had been metabolically labeled with [^{3}H]mevalonic acid (Fig. 2, lane *2*). Comparison of immunoprecipitates

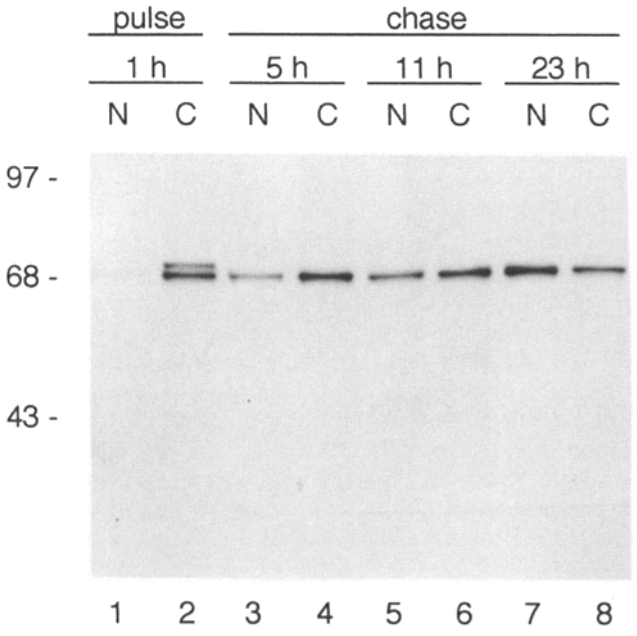

Figure 1. Lamin B3 is isoprenylated in the cytoplasm before uptake into the nucleus. Oocytes were biosynthetically labeled with [^{35}S]methionine for 1 h, [^{35}S]methionine was then replaced by unlabeled methionine and nuclei were isolated immediately after the pulse-labeling period (*1 h*) and after 5, 11, and 23 h of chase respectively. Lamin B3 was detected after immunoprecipitation and SDS-PAGE by fluorography for 3 d. The immunoprecipitate of 40 nuclei (*N*, lanes *1, 3, 5, 7*) or 40 enucleated oocytes (*C*, and *2, 4, 6, 8*), respectively, were loaded per lane. The size of molecular mass markers is given in kD at left.

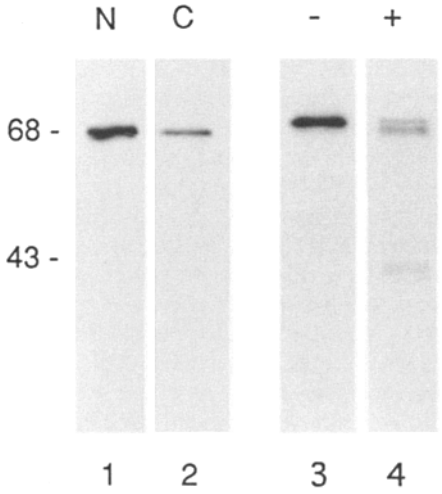

Figure 2. Analysis of lamin isoprenylation in oocyte cytoplasm (Lanes *1* and *2*) Lamin B3, radiolabeled in its isoprene group, can be isolated from oocyte cytoplasm. Oocytes were incubated in Lovastatin and [^{3}H]mevalonic acid for 48 h. Nuclei were isolated manually and lamin B3 was immunoprecipitated from nuclei and enucleated oocyte cytoplasm. B3 precipitated from 25 nuclei (lane *1*) and 100 oocyte cytoplasm equivalents (lane *2*) was analyzed by SDS-PAGE followed by fluorography. (Lanes *3* and *4*) In vitro isoprenylation of lamin B3 by incubation in extracts of enucleated oocytes. For preparation of the extract see Materials and Methods. Unmodified [^{35}S]methionine-labeled lamin B3, equivalent to 10 oocyte nuclei, was isolated from mevalonic acid starved oocytes by immunoprecipitation with magnetic beads. The B3 loaded beads were incubated with farnesylpyrophosphate for 16 h either in the absence (lane *3*) or presence of oocyte cytoplasmic extract, equivalent to 10 oocytes, (lane *4*). They were then washed and processed for immunoprecipitation as described. Proteins were analyzed by SDS-PAGE and visualized by fluorography. Exposure to x-ray film was for 14 d (lanes *1* and *2*) and 16 h (lanes *3* and *4*) respectively. Note that recovery from samples incubated with cytoplasm (lane *4*) was always lower than from controls (lane *3*). The size of molecular mass markers is given in kD at left.

from nuclei (lane *1*) and cytoplasm (lane *2*) shows that only a small fraction of fully processed lamin B3 can be detected in the cytoplasm (for gel loading amounts see legend to Fig. 2). Moreover, we tested crude cytoplasmic extracts of enucleated oocytes for their ability to prenylate nuclear lamin B3 in vitro. Unmodified, [^{35}S]methionine-labeled lamin B3 was isolated from nuclei of mevalonic acid starved oocytes by immunoprecipitation with magnetic beads (for details see Materials and Methods). These lamin B3–loaded beads were incubated in cytoplasmic extracts in the presence of farnesylpyrophosphate, the direct precursor of the farnesyl moiety. Although incubation with cytoplasmic extract reproducibly lowered the recovery of lamin B3, it resulted in the appearance of a faster migrating band in SDS-PAGE, which corresponds to the isoprenylated form of B3 (Fig. 2, lane *4*), as compared to an untreated control sample (Fig. 2, lane *3*). This experiment shows that nuclear lamins can be isoprenylated in vitro by cytoplasmic protein farnesyltransferases of *Xenopus* oocytes.

Evidence for a Nuclear Protein Isoprenyltransferase Activity

The analysis of the processing pathway of prelamin A in somatic cells has been interpreted in favor of the view that prelamin A undergoes its posttranslational modifications in the nucleus rather than in the cytosol (33). Consistent with this view both subunits of protein farnesyltransferase have been detected in nuclei of CHO cells by indirect immunofluorescence techniques (44). We had previously shown that treatment of oocytes with Lovastatin results, like in other cells, in the accumulation of non-isoprenylated lamin polypeptides in the nucleoplasm (13). We therefore asked whether these lamin polypeptides can be processed upon replenishing the isoprenoid pools by exogenous administration of mevalonic acid. To follow the fate of lamin B3 in this experiment oocytes were first preincubated for 12 h in Lovastatin to exhaust the endogenous isoprenoid pool. Proteins were then radiolabeled with [^{35}S]methionine for 12 h. The medium was changed and [^{35}S]methionine was chased out by incubation with an excess of unlabeled methionine. Mevalonic acid was added to the medium and incubation was continued for another 12 h (Fig. 3, lane *2*). To one part of the oocytes cycloheximide was added to inhibit de novo protein synthesis (Fig. 3, lane *3*). Control oocytes were incubated in the absence of mevalonic acid and cycloheximide (Fig. 3, lane *1*). At the end of the incubation time nuclei were manually isolated and lamin B3 was processed by immunoprecipitation and SDS-PAGE as described above. As can be seen in Fig. 3 replenishing of isoprenoid pools leads to conversion of the unprocessed form of B3, that had been accumulated in the nucleoplasm, to the faster migrating, isoprenylated form (compare Fig. 3, lanes *2* and *1*). This conversion also occurs when protein synthesis is blocked by cycloheximide (Fig. 3, lane *3*). The simplest explanation of these results would be, that under these experimental conditions lamin isoprenylation takes place within the nucleus. The unmodified lamin B3 in the nucleoplasm is, however, soluble (see below) and it can therefore not be ruled out that it shuttles back into the cytosol. After addition of mevalonic acid it might be isoprenylated in the cytoplasm before reentering the nucleus.

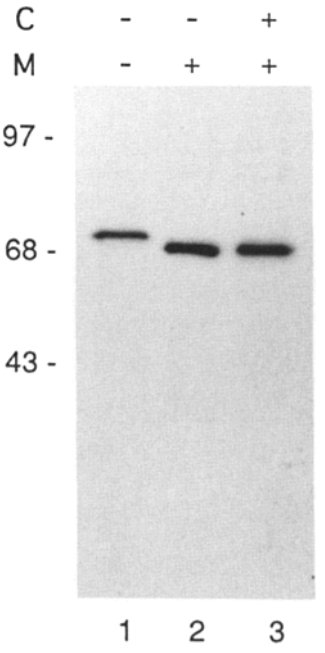

Figure 3. Isoprenylation of lamin B3 in oocyte nuclei in vivo. Oocytes were incubated in Lovastatin for 12 h, proteins were then radiolabeled with [^{35}S]methionine in the presence of Lovastatin for 12 h. The oocytes were transferred into fresh medium containing an excess of unlabeled methionine and were incubated for another 12 h. Mevalonic acid (*M* +) was then added and oocytes were incubated for another 12 h either in the absence (*C* −) (lane *2*) or presence (*C* +) of cycloheximide (lane *3*), or oocytes were processed directly, omitting the incubation with mevalonic acid (M −) (lane *1*). Nuclei were isolated manually and lamin B3 was detected after immunoprecipitation and SDS-PAGE by fluorography. Exposure to x-ray film was for 5 h. The size of molecular mass markers is given in kD at left.

Lamins Can Be Isoprenylated in Isolated Nuclei

To further investigate the possibility of shuttling of unmodified lamin B3, we carried out an in vitro farnesylation experiment with isolated nuclei. The outline of this experiment is explained in Fig. 4. Depletion of endogenous isoprenoids and labeling of proteins was carried out as in the previous experiment, oocytes were then transferred into mineral oil and nuclei were manually isolated free of cytoplasm and kept under oil. Nuclei isolated in this manner remain metabolically active for prolonged periods of time. They faithfully carry out protein im- and export and are active in RNA synthesis (38). To test the ability of the nuclei to process lamin B3, microdroplets containing farnesylpyrophosphate were added to individual nuclei. After incubation for 16 h lamin B3 was isolated by immunoprecipitation and analyzed by SDS-PAGE as described above. The results of two independent experiments are shown in Fig. 5 (lanes *1–3* and *4–6*, respectively). It is evident from the gel electrophoretic comparison of lamin B3 isolated from untreated oocyte nuclei (Fig. 5, lane *1*) to B3 isolated from oocyte nuclei incubated with farnesylpyrophosphate, that B3 can be processed to the mature form in isolated nuclei (Fig. 5, lanes *3* and *4*). This processing is dependent on the addition of farnesylpyrophosphate (Fig. 5, lanes *3* and *4*) since without any additions (lane *2*) or addition of buffer alone (lane *5*) B3 remains unprocessed. Incorporation of the farnesyl moiety can be demonstrated directly by incubation of nuclei, isolated from unlabeled oocytes, with [^{3}H]farnesylpyrophosphate (Fig. 5, lane *6*).

Isoprenylation is a prerequisite for the incorporation of lamin polypeptides into the nuclear lamina structure. Lamins form intermediate type filaments that are resistant to extraction with buffers containing high salt concentrations (1, 16, 46). To see whether this process can occur in isolated

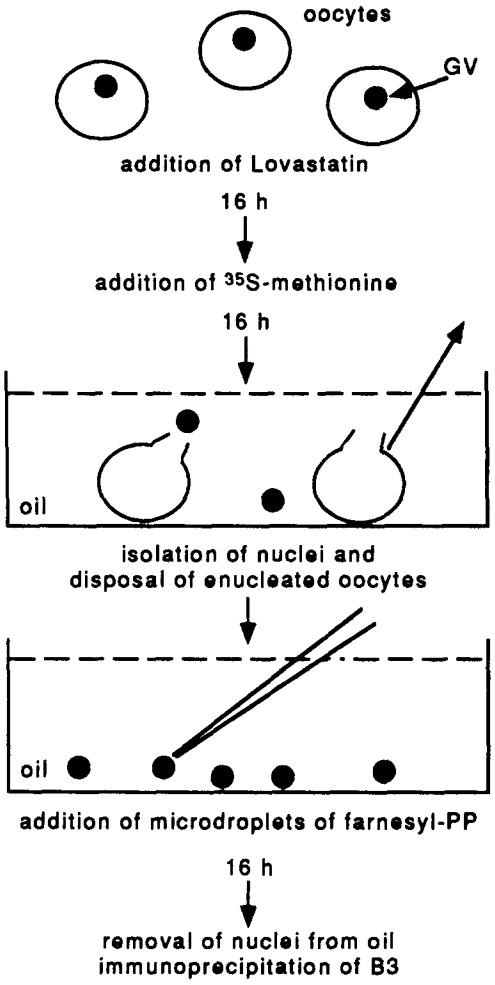

Figure 4. Isoprenylation of lamin B3 in isolated oocyte nuclei. Scheme of the experimental design. Oocytes were preincubated in Lovastatin for 16 h and proteins were radiolabeled with [^{35}S]methionine in the presence of Lovastatin for 16 h. The oocytes were then transferred into mineral oil and nuclei (*GV*) were manually isolated. After addition of microdroplets of farnesylpyrophosphate (*farnesyl-PP*) to the nuclei, incubation was continued for 16 h followed by immunoprecipitation of lamin B3, gel electrophoretic analysis and detection by fluorography.

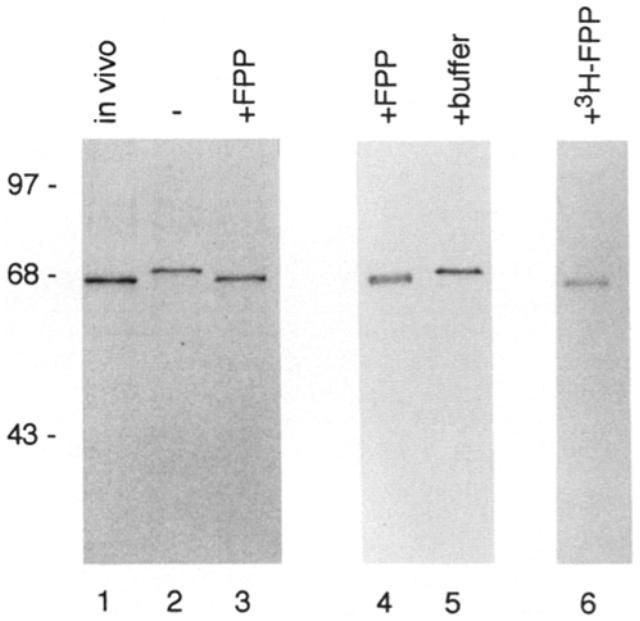

Figure 5. Detection of lamin B3 isoprenylated in isolated nuclei as outlined in Fig. 4. (Lane *1*) Isoprenylated lamin B3 from control oocytes; (lane *2*) unprocessed B3 from isolated nuclei of Lovastatin treated oocytes with no additions (-). (Lanes *3* and *4*) Lamin B3 immunoprecipitated from isolated nuclei of Lovastatin treated oocytes after addition of farnesylpyrophosphate (+FPP); (lane *5*) B3 from isolated nuclei of Lovastatin treated oocytes after addition of buffer alone (+ buffer). Results of two independent experiments are shown in lanes *1–3* and *4–5*, respectively. For the experiment shown in lanes *1–3* five nuclei were processed per lane, for the experiments shown in lanes *4–5* 10 nuclei were processed per lane. The same type of experiment was carried out with unlabeled nuclei. Isoprenylation of lamin B3 was directly demonstrated by adding microdroplets of [^{3}H]farnesylpyrophosphate to the nuclei (lane *6*). 30 nuclei were processed for this experiment. Exposure times were 2 d for lanes *1* to *3*, 1 d for lanes *4* and *5*, and 17 d for lane *6*. The size of molecular mass markers is given in kD at left.

nuclei we transferred nuclei that had been incubated with farnesylpyrophosphate as described above into high salt buffer and fractionated them by centrifugation into a high salt–resistant pellet and a supernatant fraction. While in the absence of farnesylpyrophosphate only a minute amount of B3 is found in the pellet fraction (compare Fig. 6, lanes *2* and *3*), a significant amount of B3 becomes high salt resistant after in vitro farnesylation (Fig. 6, lanes *5* and *6*). This indicates that the farnsylated lamin polypeptides are incorporated into the existing lamin filaments in isolated nuclei. While in vitro farnesylation of lamin B3 is carried out with high efficiency (see Fig. 5) the conversion of B3 into a high salt–resistant fraction is not complete. This might be explained by the fact, that in contrast to the enzymatic isoprenylation reaction, incorporation of B3 into the lamina structure is a relatively slow process and not all nuclei might be fully functional over the long in vitro incubation period.

Discussion

In this study we report on the isoprenylation of nuclear lamin B3 in *Xenopus* oocytes. We have used in vivo kinetic analysis to determine when after de novo synthesis lamin B3 is isoprenylated relative to its uptake into the nucleus. We further analyzed the capacity of isolated nuclei to carry out isoprenylation of B3 and its integration into the lamina structure.

Kinetic Analysis of Lamin B3 Isoprenylation

Two topogenic sequences are responsible for targeting nuclear lamins to the inner nuclear membrane, the nuclear localization signal and the COOH-terminal CXXX motif that is the major determinant of a series of posttranslational modifications including isoprenylation, proteolytic processing, and carboxyl methylation (21, 26, 28, 31, 44, 47). While it is clear that both signals are necessary for correct targeting of lamins (21) it is not known how the action of the two signals is concerted, i.e., whether isoprenylation occurs in the cytoplasm prior to nuclear uptake, or whether lamins are first transported into the nucleoplasm where they are then

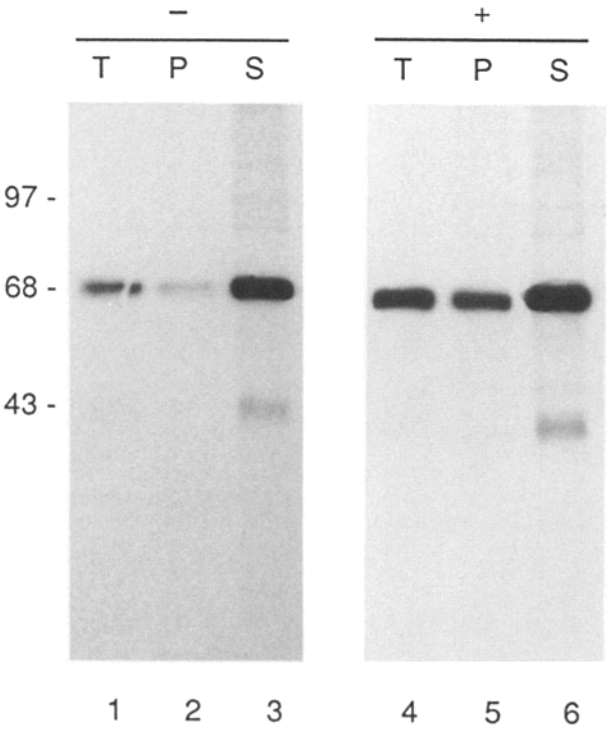

Figure 6. Lamin B3, isoprenylated in isolated nuclei, is incorporated in the lamina structure. Oocytes and isolated nuclei were processed as outlined in Fig. 4. After the incubation of the isolated nuclei in the presence (+) (lanes *4–6*) or absence (-) (lanes *1–3*) of farnesylpyrophosphate, nuclei were either processed directly for immunoprecipitation with the lamin-specific monoclonal antibody L6-5D5 (lanes *1* and *4*) (*T*) or transferred into a buffer containing high salt and fractionated by centrifugation into a high salt resistant pellet fraction (*P*) (lanes *2* and *5*) and a supernatant fraction (*S*) (lanes *3* and *6*) before immunoprecipitation. The following numbers of nuclei were processed in each lane: lanes *1* and *4*, 5 nuclei; lanes *2* and *3*, 15 nuclei; lanes *5* and *6*, 25 nuclei. Lamin B3 was detected after immunoprecipitation and SDS-PAGE by fluorography. Exposure to x-ray film was for 1 d. The size of molecular mass markers is given in kD at left.

modified by a nuclear protein farnesyltransferase. The latter pathway would prevent the potential misrouting of nuclear lamins to cytoplasmic membranes due to the addition to the lipophilic modifications before nuclear entry. In fact, mutant lamins that lack a nuclear localization signal show a non-homogeneous cytoplasmic distribution after transfection into cultured cells, suggesting that they might be associated with cytoplasmic endomembranes (20, 21). Experiments that could discriminate between the two possibilities have so far been inconclusive. While interpretation of kinetic data favor the view that lamin isoprenylation is a cytoplasmic event (26), studies involving inhibitors of isoprenoid synthesis have been taken as evidence that lamins undergo post-translational processing in the nucleus rather than in the cytosol (33, 44). The kinetic analysis presented here clearly shows that isoprenylation of lamin B3 is a much faster event than nuclear uptake and that non-isoprenylated B3 does not enter the nucleus to a significant extent (Fig. 1). The most straightforward interpretation of these results is, that, under normal conditions, isoprenylation of lamin B3 occurs in the cytoplasm and that nuclear uptake is dominant over association of isoprenylated B3 with cytoplasmic membranes. This raises the question whether newly synthesized lamins are freely soluble in the cytosol or whether they become transiently associated with cytoplasmic membranes on their way to the nuclear pore complexes. Membrane association might influence the kinetics of nuclear uptake. The observed rate of nuclear accumulation of lamin B3 is within the broad range of rates of other, non-prenylated, oocyte nuclear proteins and therefore does not give a clue toward one or the other possibility. A direct comparison of the nuclear uptake kinetics of isoprenylated and mutant non-isoprenylated lamin might give hints whether transient membrane binding is involved in nuclear migration of CXXX-modified lamins.

It has been shown in several experimental systems that protein isoprenylation is not sufficient to confer membrane attachment (7, 13, 17) and that other factors must be involved in a tight membrane binding of isoprenylated proteins (18). Several integral membrane proteins of the inner nuclear membrane have been characterized that interact with lamins (2, 14, 34, 37, 42, 43, 49). One of these contains several membrane spanning domains of unknown function (48). It has been speculated that these hydrophobic domains might interact with the farnesyl groups of lamins. This protein might act as a lamin farnesyl receptor (20, 35) and might determine the specific association of lamins with the inner nuclear membrane.

In vitro studies with synthetic peptides have shown that the substrate requirements of purified protein farnesyltrasferase are confined to the CXXX tetrapeptide (39). It is therefore likely that the same cytosolic enzyme carries out the isoprenylation of cytoplasmic proteins like *Ras*, cGMP phosphodiesterase α and nuclear lamins. Our observation that immunopurified lamin B3 can be isoprenylated by crude cytoplasmic extracts of enucleated oocytes is in line with this assumption.

Isoprenylation of Lamins in Nuclei

Our finding that lamins are isoprenylated in the cytoplasm before entry into the nucleus contradicts the conclusion drawn from the analysis of the processing pathway of lamin A in cell culture cells (33). In these experiments the cellular isoprene pools were depleted by treatment of the cells with Lovastatin to facilitate the analysis of isoprenylation. In isoprene depleted cells lamins accumulate in the nucleoplasm. Upon replenishment of isoprene pools by administration of mevalonic acid, lamins are rapidly isoprenylated and incorporated into the nuclear lamina structure. This has been taken as evidence for a nuclear localization of lamin processing (33, 44). We report identical results in experiments with Lovastatin-treated oocytes (Fig. 3) ruling out differences in the experimental systems. Nucleoplasmic lamin B3 is isoprenylated and incorporated into the lamina after addition of mevalonic acid to isoprene starved oocytes (Fig. 3; 13). The isoprenylation is also observed when protein synthesis is blocked by cycloheximide (Fig. 3). However, nuclear accumulation of non-prenylated lamins does not occur to a significant extend under normal conditions (Fig. 1), it rather represents a default pathway in isoprene starved cells. Moreover, although the above mentioned observations suggest that isoprenylation can take place in the nucleus they cannot be taken as a firm proof. It might be possible that shuttling of non-isoprenylated lamins occurs be-

tween the nucleoplasm and the cytoplasm. Shuttling has been shown for nuclear proteins that do not tightly bind to an intranuclear component (5, 41). However, the in vitro experiments with isolated oocyte nuclei, described in this study, suggest that isoprenylation might indeed take place in the nucleus. This interpretation is supported by the demonstration of both subunits of protein farnesyltransferase in the nucleus of mammalian cell culture cells by immunofluorescence methods (44). However, both methods of detection, i.e., monitoring the enzymatic activity in isolated nuclei described here, and the localization by immunofluorescence microscopy (44) are indirect. A firm proof of the presence of a protein farnesyltransferase in the cell nucleus has to await the isolation and biochemical characterization of the enzyme(s).

Whether cytosolic and nuclear protein farnesyltransferases will represent distinct proteins remains to be determined. Sequences for the α and β subunits of these enzymes have been determined for several species (for review see references 6, 9, 40). There is no obvious nuclear localization signal of the polybasic SV-40 T antigen (24) or the bipartite nucleoplasmin type (11) in either of these sequences, posing the question of the mechanism of nuclear localization of these polypeptides.

The existence of a nuclear protein farnesyltransferase could explain the different sensitivity of nuclear lamins versus other (cytoplasmic) proteins to a specific class of protein farnesyltransferase inhibitors, the benzodiazapine peptidomimetics. It has been found that isoprenylation of the Triton X-100–insoluble lamins is much less impaired by benzodiazapine peptidomimetics than the isoprenylation of Triton soluble proteins like *Ras* and other cytoplasmic proteins (23). In light of our findings this could be explained if newly synthesized lamins, that are normally isoprenylated in the cytoplasm, enter the nucleus unmodified, similarly as in isoprene starved cells. They might then be isoprenylated in the nucleoplasm either due to a different accessibility of the peptidomimetics to the nuclear compartment or due to a different sensitivity of the nuclear protein farnesyltransferase to the inhibitors. Since these substances have been shown to be potent inhibitors of oncogenic *Ras* in animal cells, it will be worth learning more about their specific action. Due to the ease with which amphibian oocytes can be isolated and manipulated, *Xenopus* oocytes might be a suitable biological system to undertake a more detailed biochemical characterization of nuclear protein farnesyltransferases as well as to study the accessibility of peptidomimetic protein farnesyltransferase inhibitors to nuclei.

Since our studies have shown that nuclear isoprenylation of lamins is a default pathway rather than a reflection of the normal in vivo situation, the question of the in vivo function of nuclear protein farnesyltransferase(s) is raised. The *Xenopus* oocyte nucleus contains several other polypeptides in addition to lamin B3 that are radiolabeled upon incubation of oocytes with [^{3}H]mevalonic acid (our unpublished observation). A nuclear enzyme might therefore act on other nuclear CXXX motif proteins. Alternatively, as shown for other modifications, protein function might be modulated by deisoprenylation and reisoprenylation. So far, however, no loss of isoprenyl groups has been observed in mammalian *Ras* proteins (19) and B-type nuclear lamins (13). If an unmodified COOH-terminal cysteine residue would be regenerated by deisoprenylation of a mature isoprenylated protein, its reisoprenylation would depend on an enzyme with a different substrate requirement than the CXXX-dependent protein farnesyltransferase (for discussion see reference 9). Such enzymes might be expected to have different sensitivity to peptidomimetics that were designed to inhibit CXXX-dependent protein farnesyltransferases.

This paper is dedicated to Peter Hausen on the occasion of his 60th birthday.

Most of this work was carried out during our stay in the laboratory of Dr. H. Jäckle (Max-Planck-Institute for Biophysical Chemistry, Göttingen, FRG). We would like to thank him for his hospitality and generous support. We appreciate the support of Dr. K. Weber (Max-Planck-Institute for Biophysical Chemistry, Göttingen, FRG) and Dr. T. Pieler (this institute). We thank Dr. M. Stapff (Merck, Sharp & Dohme, München, FRG) for a gift of Lovastatin, I. Gajewski (Max-Planck-Institute for Biophysical Chemistry, Göttingen, FRG), and A. Misgaiski (this institute) for photographic work.

This work has been supported by the Deutsche Forschungs Gemeinschaft (DFG grant Sti 98/4-3; 4-4).

Received for publication 11 October 1994 and in revised form 20 December 1994.

References

1. Aebi, U., J. Cohn, L. Buhle, and L. Gerace. 1986. The nuclear lamina is a meshwork of intermediate filaments. *Nature (Lond.)*. 323:560–564.
2. Bailer, S. M., H. M. Eppenberger, G. Griffiths, and E. A. Nigg. 1991. Characterization of a 54-kD protein of the inner nuclear membrane: evidence for cell cycle-dependent interaction with the nuclear lamina. *J. Cell Biol.* 114:389–400.
3. Beck, L. A., T. J. Hosick, and M. Sinensky. 1990. Isoprenylation is required for the processing of the lamin A precursor. *J. Cell Biol.* 110: 1489–1499.
4. Black, S. D. 1992. Development of hydrophobicity parameters for prenylated proteins. *Biochem. Biophys. Res. Commun.* 186:1437–1442.
5. Borer, R. A., C. F. Lehner, H. M. Eppenberger, and E. A. Nigg. 1989. Major nucleolar proteins shuttle between nucleus and cytoplasm. *Cell.* 56:379–390.
6. Brown, M. S., and J. L. Goldstein. 1993. Mad bet for rab. *Nature (Lond.)*. 366:14–15.
7. Butrynsky, J. E., T. L. Z. Jones, P. S. Backlund, Jr., and A. M. Spiegel. 1992. Differential isoprenylation of carboxy-terminal mutants of an inhibitory G-protein α-subunit: neither farnesylation nor geranylgeranylation is sufficient for membrane attachment. *Biochemistry.* 31:8030–8035.
8. Casey, P. J., P. A. Solski, C. J. Der, and J. E. Buss. 1989. p21^{ras} is modified by a farnesyl isoprenoid. *Proc. Natl. Acad. Sci. USA.* 86:8323–8327.
9. Clarke, S. 1992. Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. *Annu. Rev. Biochem.* 61:355–386.
10. Cox, A. D., and C. J. Der. 1992. Protein prenylation: more than just glue? *Curr. Opin. Cell Biol.* 4:1008–1016.
11. Dingwall, C., and R. A. Laskey. 1991. Nuclear targeting sequences—a consensus? *Trends in Biochem. Sci.* 16:478–481.
12. Dumont, J. N. 1972. Oogenesis in *Xenopus laevis* (Daudin). Stages of oocyte development in laboratory maintained animals. *J. Morphol.* 136: 153–179.
13. Firmbach-Kraft, I., and R. Stick. 1993. 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. *J. Cell Biol.* 123: 1661–1670.
14. Foisner, R., and L. Gerace. 1993. Integral membrane proteins of the nuclear envelope interact with lamins and chromosomes, and binding is modulated by mitotic phosphorylation. *Cell.* 73:1267–1279.
15. Fukada, Y., T. Takao, H. Ohguro, T. Yoshizawa, T. Akino and Y. Shimonishi. 1990. Farnesylated γ-subunit of photoreceptor G protein indispensable for GTP-binding. *Nature (Lond.)*. 346:658–660.
16. Gerace, L., and G. Blobel. 1982. Nuclear lamina and the structural organization of the nuclear envelope. *Cold Spring Harbor Symp. Quant. Biol.* 46:967–978.
17. Hancock, J. F., K. Cadwallader, and C. J. Marshall. 1991. Methylation and proteolysis are essential for efficient membrane binding of prenylated p21$^{K\text{-}ras(B)}$. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:641–646.
18. Hancock, J. F., H. Paterson, and C. J. Marshall. 1990. A polybasic domain or palmitoylation is required in addition to the CaaX motif to localize p21^{ras} to the plasma membrane. *Cell.* 63:133–139.
19. Hancock, J. F., A. I. Magee, J. E. Childs, and C. J. Marshall. 1989. All *ras* proteins are polyisoprenylated but only some are palmitoylated. *Cell.*

57:1167–1177.
20. Hennekes, H., and E. A. Nigg. 1994. The role of isoprenylation in membrane attachment of nuclear lamins. A single point mutation prevents proteolytic cleavage of the lamin A precursor and confers membrane binding properties. *J. Cell Sci.* 107:1019–1029.
21. Holtz, D., R. A. Tanaka, J. Hartwig, and F. McKeon. 1989. The CaaX motif of lamin A functions in conjunction with the nuclear localization signal to target assembly to the nuclear envelope. *Cell.* 59:969–977.
22. Itho, T., K. Kaibuchi, T. Masuda, T. Yamamoto, Y. Matsuura, A. Maeda, K. Shimizu, and Y. Takai. 1993. The post-translational processing of *ras* p21 is critical for its stimulation of mitogen-activated protein kinase. *J. Biol. Chem.* 268:3025–3028.
23. James, G. L., J. L. Goldstein, M. S. Brown, T. E. Rawson, T. C. Somers, R. S. McDowell, C. W. Crowley, B. K. Lucas, A. D. Levison, and J. C. Marsters, Jr. 1993. Benzodiazepine peptidomimetics: potent inhibitors of *Ras* farnesylation in animal cells. *Science (Wash. DC).* 260:1937–1942.
24. Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear location. *Cell.* 39: 499–509.
25. Kita, T., M. S. Brown, and J. L. Goldstein. 1980. Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in livers of mice treated with mevinolin, a competitive inhibitor of the reductase. *J. Clin. Invest.* 66:1094–1100.
26. Kitten, G. T., and E. A. Nigg. 1991. The CaaX motif is required for isoprenylation, carboxyl methylation and nuclear membrane association of lamin B2. *J. Cell Biol.* 113:13–23.
27. Krohne, G., M.-C. Dabauvalle, and W. W. Franke. 1981. Cell type-specific differences in protein composition of nuclear pore complex-lamina structures in oocytes and erythrocytes of *Xenopus laevis*. *J. Mol. Biol.* 151:121–141.
28. Krohne, G., I. Waizenegger, and T. H. Höger. 1989. The conserved carboxy-terminal cysteine of nuclear lamins is essential for lamin association with the nuclear envelope. *J. Cell Biol.* 109:2003–2013.
29. Kuroda, Y., N. Suzuki, and T. Kataoka. 1993. The effect of posttranslational modifications on the interaction of *Ras* with adenylyl cyclase. *Science (Wash. DC).* 259:683–686.
30. Laemmli, U. K. 1970. Clevage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
31. Loewinger, L., and F. McKeon. 1988. Mutations in the nuclear lamin proteins resulting in their aberrant assembly in the cytoplasm. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2301–2308.
32. Lourim, D., and G. Krohne. 1993. Membrane-associated lamins in *Xenopus* egg extracts: identification of two vesicle populations. *J. Cell Biol.* 123:501–512.
33. Lutz, R. J., M. A. Trujillo, K. S. Denham, L. Wenger, and M. Sinensky. 1992. Nucleoplasmic localization of prelamin A: implications for prenylation-dependent lamin A assembly into the nuclear lamina. *Proc. Natl. Acad. Sci. USA.* 89:3000–3004.
34. Meier, J., and S. D. Georgatos. 1994. Type B lamins remain associated with the integral nuclear envelope protein p58 during mitosis: implications for nuclear reassembly. *EMBO (Eur. Mol. Biol. Organ.) J.* 13: 1888–1898.
35. Nigg, E. A., G. T. Kitten, and K. Vorburger. 1992. Targeting lamin proteins to the nuclear envelope: the role of CaaX box modifications. *Biochem. Soc. Trans.* 20:500–504.
36. Ohguro, H., Y. Fukada, T. Takao, Y. Shimonishi, T. Yoshizawa, and T. Akino. 1991. Carboxyl methylation and farnesylation of transducin γ-subunit synergistically enhance its coupling with metarhodopsin II. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3669–3674.
37. Padan, R., S. Nainudel-Epszteyn, R. Goitein, A. Fainsod, and Y. Gruenbaum. 1990. Isolation and characterization of the *Drosophila* nuclear envelope otefin cDNA. *J. Biol. Chem.* 265:7808–7813.
38. Paine, P. L., M. E. Johnson, Y.-T. Lau, L. J. M. Tluczek, and D. S. Miller. 1992. The oocyte nucleus isolated in oil retains *in vivo* structure and functions *BioTechniques.* 13:238–246.
39. Reiss, Y., S. J. Stradley, L. M. Gierasch, M. S. Brown, and J. L. Goldstein. 1991. Sequence requirement for peptide recognition by rat brain $p21^{ras}$ farnesyl:protein transferase. *Proc. Natl. Acad. Sci. USA.* 88:732–736.
40. Schafer, W. R., and J. Rine. 1992. Protein prenylation: genes, enzymes, targets, and functions. *Annu. Rev. Genet.* 30:209–234.
41. Schmidt-Zachmann, M. S., C. Dargemont, L. C. Kühn, and E. A. Nigg. 1993. Nuclear export of proteins: the role of nuclear retention. *Cell.* 74:493–504.
42. Senior, A., and L. Gerace. 1988. Integral membrane proteins specific to the inner nuclear membrane and associated with the nuclear lamina. *J. Cell Biol.* 107:2029–2036.
43. Simos, G., and S. D. Georgatos. 1992. The inner nuclear membrane protein p58 associates *in vivo* with a p58-kinase and the nuclear lamins. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4027–4036.
44. Sinensky, M., K. Fantle, M. Trujillo, T. McLain, A. Kupfer, and M. Dalton. 1994. The processing pathway of prelamin A. *J. Cell Sci.* 107: 61–67.
45. Stick, R. 1988. cDNA cloning of the developmentally regulated lamin LIII of *Xenopus laevis*. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3189–3197.
46. Stick, R., and G. Krohne. 1982. Immunological localization of the major architectural protein associated with the nuclear envelope of the *Xenopus laevis* oocyte. *Exp. Cell Res.* 138:319–330.
47. Vorburger, K.,G. T. Kitten, and E. A. Nigg. 1989. Modification of nuclear lamin proteins by a mevalonic acid derivative occurs in reticulocyte lysates and requires the cysteine residue of the C-terminal CXXM motif. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:4007–4013.
48. Worman, H. J., C. D. Evans, and G. Blobel. 1990. The lamin B receptor of the nuclear envelope inner nuclear membrane: a polytopic protein with eight potential transmembrane domains. *J. Cell Biol.* 111:1535–1542.
49. Worman, H. J., J. Juan, G. Blobel, and S. D. Georgatos. 1988. A lamin B receptor in the nuclear envelope. *Proc. Natl. Acad. Sci. USA.* 85: 8531–8534.