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Nucleoplasmic localization of prelamin A: Implications for prenylation-dependent lamin A assembly into the nuclear lamina

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ABSTRACT **The synthesis of the nuclear lamina protein lamin A requires the prenylation-dependent processing of its precursor protein, prelamin A. Unlike p21^{ras}, which undergoes similar initial posttranslational modifications, maturation of lamin A results in the proteolytic removal of the prenylated portion of the molecule. We have used an *in vitro* prenylation system to demonstrate the nature of the prenyl substituent on prelamin A to be a farnesyl group. Further, the *in vitro* farnesylation of prelamin A requires an intact cysteine-aliphatic-aliphatic-other (CAAX) amino acid sequence motif at its carboxyl terminus. The effect of blocking the prenylation of prelamin A on its localization and assembly into the nuclear lamina was investigated by indirect immunofluorescence. Expression of wild-type prelamin A in lovastatin-treated cells showed that nonprenylated prelamin A accumulated as nucleoplasmic particles. Upon addition of mevalonate to lovastatin-treated cells, the wild-type lamin A was incorporated into the lamina within 3 hr. Expression of a mutant lamin A in which the carboxyl-terminal 21 amino acids were deleted resulted in a lamin molecule that was directly assembled into the lamina. These results indicate that the carboxyl-terminal peptide of prelamin A blocks its proper assembly into the nuclear lamina and that the prenylation-initiated removal of this peptide can occur in the nucleus.**

The nuclear lamina is a polymeric protein structure that lines the inner nuclear membrane. In most mammalian cells, it consists of three major proteins, lamins A–C (for review, see ref. 1). Mature lamin A is synthesized from a larger precursor protein (2, 3) and lacks the carboxyl-terminal 18 amino acids predicted by the cDNA sequence (4). Conversion of the lamin A precursor, prelamin A, to lamin A is dependent upon the isoprenylation of prelamin A (5). Prelamin A is an example of a class of proteins terminating in the sequence cysteine-aliphatic-aliphatic-other (CAAX), which is prenylated at the consensus cysteine (for reviews, see refs. 6 and 7). This motif, shared by such proteins as p21^{ras} and the a-type mating factor of *Saccharomyces*, entrains a series of posttranslational processing steps. These include prenylation at the CAAX cysteine with either a 15-carbon (farnesyl) or 20-carbon (geranylgeranyl) isoprenoid followed by proteolytic removal of the A-A-X amino acids and carboxyl methylation of the now terminal cysteine. Evidence has been reported that the B-type lamins also undergo these prenylation-dependent processing reactions (8–10). However, specific steps for the processing of prelamin A have not been directly demonstrated. Activities capable of catalyzing the carboxyl-terminal processing of a-factor and p21^{ras} *in vitro* have been described (11, 12). For p21^{ras}, these activities were localized to the cytosolic and microsomal compartments (12, 13).

Prelamin A and the yeast a-factor share an additional reaction subsequent to these carboxyl-terminal processing events. Both are subject to an endoproteolytic cleavage of their respective precursor proteins (2–4, 14, 15). In the case of a-factor, the biologically active entity is the carboxyl-terminal prenylated peptide, which is ultimately secreted. However, for prelamin A, removal of the carboxyl-terminal (4, 16) prenylated peptide (5) gives rise to the nonprenylated mature lamin A.

Here we demonstrate that nonprenylated prelamin A accumulates as nucleoplasmic inclusions in cells blocked in prenylation with the hydroxymethylglutaryl-CoA reductase inhibitor lovastatin. Accumulated prelamin A can serve as a precursor for conversion to lamin A (5). We show that nucleoplasmic prelamin A becomes rapidly associated with the lamina in response to mevalonate treatment. We interpret this finding as indicative of a nuclear localization for the prenylation-dependent processing of prelamin A. We also present evidence that the carboxyl-terminal peptide of prelamin A acts to block rather than facilitate normal assembly of lamin A. This observation suggests that the prenylation dependence of prelamin A processing may have regulatory implications.

MATERIALS AND METHODS

Plasmids. Wild-type prelamin A cDNA cloned into the *Eco*RI (5′)–*Bam*HI (3′) site of pUC19 (17) was a gift from N. Chaudhary (Rockefeller University). The prelamin A sequence was subcloned into the *Eco*RI–*Bam*HI site of pGEM-3Z (Promega). A mutant prelamin A sequence in which the CAAX-box cysteine is changed to methionine was excised from pHLA-C661:M (18), a gift from F. McKeon (Harvard Medical School), with *Eco*RI and *Xba* I and ligated into the *Eco*RI/*Xba* I site of pGEM-3Z. *In vitro* transcription from these plasmids followed the protocol provided with the pGEM-3Z cloning vector (Promega).

For transient transfection studies, the plasmid pHLA, containing the full-length prelamin A cDNA, also a gift of F. McKeon, and the plasmid pHLA-C661:M were used. Both plasmids are derived from the constitutive expression vector pECE (19).

To obtain the plasmid pMMLAΔ21 (Fig. 1), the prelamin A sequence was obtained from pUC19/lamin A, and the 1030-nt *Bst*EII (nt 2061)–*Spe* I (nt 3091) fragment containing the coding region for the carboxyl-terminal 21 amino acids of prelamin A was removed by partial *Bst*EII digestion. This truncated sequence was inserted into a vector in which dexamethasone-regulated expression is under control of the mouse mammary tumor virus promoter, pSP64 MMTV-vimentin (20), which was obtained from R. Evans (University of Colorado Health Sciences Center, Denver). The size of the 3′ end of the construct was verified by restriction digestion with *Nco* I and *Bam*HI.

Cells and Transfections. CHO-K1 cells were cultured in Ham's F12 medium supplemented with 5% (vol/vol) fetal

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Abbreviation: CAAX, amino acid sequence cysteine-aliphatic-aliphatic-other.

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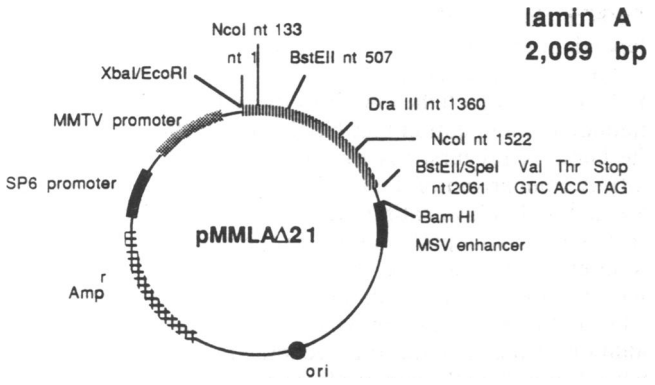

FIG. 1. Dexamethasone-inducible expression vector of truncated prelamin A. The pMMLAΔ21 construct shown was constructed as described in *Materials and Methods* and expresses a mature lamin A missing the carboxyl-terminal Arg-Ser-Tyr as shown. MMTV, mouse mammary tumor virus; ori, origin; Amp^r, ampicillin-resistance gene; MSV, murine sarcoma virus; nt, nucleotide; bp, base pairs.

bovine serum. Cells were transfected by the Lipofectin method (21) as described by the manufacturer (GIBCO/BRL). Transient expression of wild-type (pHLA) and mutant (pHLA-C661:M) prelamin A was examined 14–16 hr after removal of Lipofectin. Stable transfectants with pMMLAΔ21 were selected by cotransfection with pSV2-neo (22), a gift of P. Berg (Stanford University) and selection for G418 (Sigma) resistance (300 μg/ml). G418-resistant mutants were screened for dexamethasone-inducible lamin A expression by indirect immunofluorescence. Expression of the truncated lamin A protein was verified by Western blot analysis. Following 16 hr of induction with dexamethasone, nuclear matrix proteins were isolated essentially as described (23) and resolved by SDS/PAGE (see conditions in ref. 5). After electrophoresis, proteins were electrotransferred onto reinforced nitrocellulose (MSI) and probed with 1E4 (24), a human species-specific mouse monoclonal antibody (1:500 dilution), followed by horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (NEN/DuPont) (1:50 dilution). The blotting procedure and visualization with ECL Western reagent were per manufacturer's instructions (Amersham).

***In Vitro* Translation and Protein Prenylation.** Synthetic mRNAs were translated in pretreated reticulocyte lysate as described by the supplier (Promega). Incubations were conducted for 60 min at 30°C in the presence of [^{3}H]mevalonolactone (NEN/DuPont; 24 Ci/mmol; 1 Ci = 37 GBq) at 0.5 mCi/ml or [^{35}S]methionine/[^{35}S]cysteine (ICN; Tran^{35}S-label; 10 mCi/ml) at 0.8 mCi/ml and were stopped by the addition of an equal volume of 2× SDS/PAGE sample buffer. Raney nickel treatment of gel-purified [^{3}H]mevalonate-labeled prelamin A and gas chromatography of the pentane-extractable product were as described (25). Farnesyl- and geranylgeranylcysteine methyl ester standards were synthesized according to Kamiya *et al.* (26) by using cysteine methyl ester (Sigma) and the appropriate isoprenol (Kuraray) as starting material.

Indirect Immunofluorescence. Cells were plated on sterile glass coverslips and rinsed with phosphate-buffered saline (PBS). Transient transfectants were fixed with 1% formaldehyde in PBS for 5 min at room temperature and permeabilized by three washes with 0.1% Nonidet P-40 in PBS. Stable transfectants were fixed with ethanol/acetic acid (20:1, vol/vol) at room temperature for 5 min. Primary antibodies specific for lamins A and C were LS-1 (27), a human autoantiserum (diluted 1:200) crossreactive between mammalian species, and 1E4 (24) (diluted 1:1000) (see above). The secondary antibody used with LS-1 was fluorescein-conjugated anti-human (Meloy Laboratories) (diluted 1:64). The secondary antibody used with 1E4 was either rhodamine-conjugated sheep anti-mouse (Cappel Laboratories) (diluted 1:125) or fluorescein-conjugated goat anti-mouse (Organon Teknika–Cappel) (diluted 1:200).

Subcellular Localization of Prelamin A. CHO-K1 cells were plated at 50% confluency into 150-mm plates on day 0. On day 1 the cells were labeled with [^{35}S]methionine/[^{35}S]cysteine (Tran^{35}S-label; 10 mCi/ml) at 20 μCi/ml in medium with or without 12.5 μM lovastatin. After an overnight incubation (17 hr), the cells were washed with ice-cold PBS and scraped into 10 mM Tris·HCl, pH 7.4/140 mM KCl/1.5 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride (1.5 ml per plate). The cells were homogenized by 50 strokes in a tight-fitting Dounce homogenizer. Cell disruption was judged at >90% by phase-contrast microscopy. The homogenate was centrifuged at 1000 × *g* for 10 min at 4°C. The postnuclear supernatant was removed with a pipet and the nuclear pellet was washed once with PBS/1 mM phenylmethylsulfonyl fluoride (1.5 ml per plate). Lamin A was immunoprecipitated from the subcellular fractions with LS-1 lamin A/C antibody and resolved by SDS/PAGE (5). Labeled proteins were visualized by fluorography with Amplify (Amersham).

RESULTS

Prelamin A Is Farnesylated *in Vitro*. The prenylation of prelamin A has been demonstrated (28). Analysis of the *in vivo* prenyl substituent on prelamin A is problematic because the modified portion of the molecule is rapidly cleaved following prenylation (3–5). We have shown that the *in vitro* prenylation of Ha-Ras and Rab-1b by labeled mevalonate in reticulocyte lysates reflects prenylation specificities observed in whole cells (25). Labeling of prelamin A by mevalonate in an *in vitro* translation system has been described (16). To determine the nature of the prenyl substituent on prelamin A, prelamin A mRNA obtained by *in vitro* transcription was translated in the presence of labeled mevalonate in a reticulocyte lysate system. After isolation of the labeled protein by gel electrophoresis and electroelution, the prenyl group was reductively cleaved from the protein with Raney nickel catalyst and analyzed by radio-gas chromatography. The results (Fig. 2) demonstrate that prelamin A is derivatized with farnesyl by an *in vitro* system and suggests that prelamin A is farnesylated *in vivo*. *In vitro* translation

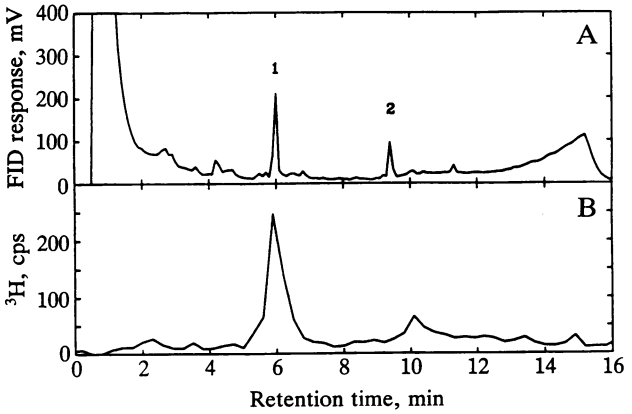

FIG. 2. Farnesyl modification of prelamin A *in vitro*. [^{3}H]Mevalonate-labeled prelamin A was prepared by *in vitro* translation and was treated with Raney nickel to release the isoprenoid moiety. Pentane-extractable products were analyzed by gas chromatography. (*A*) Flame ionization detection (FID) of the products from synthetic farnesylcysteine (peak 1) and geranylgeranylcysteine (peak 2) methyl ester. (*B*) In-line radioactivity detection of the product from [^{3}H]mevalonate-labeled prelamin A.

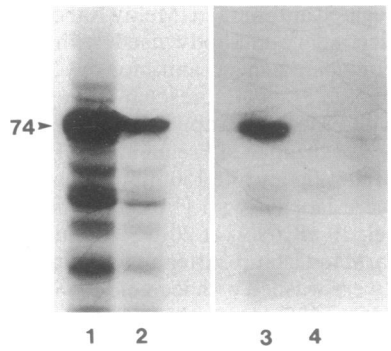

FIG. 3. *In vitro* prenylation of prelamin A requires the CAAX cysteine. Prelamin A mRNA was translated in reticulocyte lysate supplemented with [^{35}S]methionine (lanes 1 and 2) or [^{3}H]mevalonate (lanes 3 and 4). Lanes 1 and 3, wild-type prelamin A; lanes 2 and 4, prelamin A mutant with the CAAX cysteine changed to methionine. Exposure times were 3 hr (lanes 1 and 2) and 24 hr (lanes 3 and 4). Molecular size (kDa) is indicated at left.

and prenylation of the γ subunit of the heterotrimeric guanine nucleotide-binding proteins result in specific geranylgeranylation of the newly synthesized protein (data not shown), which reflects the nature of the *in vivo* substituent (29, 30).

To determine whether the farnesylation of prelamin A occurs at the cysteine of the carboxyl-terminal CAAX sequence, we compared the incorporation of labeled mevalonate into prelamin A translated from mRNAs derived from wild-type cDNA or from a previously described mutant prelamin A clone that encodes a methionine in place of the cysteine (18). The absence of incorporation of label from mevalonate into the mutant prelamin A (Fig. 3) is consistent with farnesylation of the wild-type prelamin A at the appropriate CAAX-box cysteine.

Accumulation of Prelamin A in the Nucleus of Lovastatin-Treated Cells. Treatment of cultured cells with lovastatin, which blocks protein prenylation, results in the accumulation of prelamin A at the expense of mature lamin A (5). The observed block in processing of lamin A is analogous to the accumulation of mating-factor precursor in *Tremella mesenterica* and *Saccharomyces* cells in which isoprenoid biosynthesis is inhibited (31, 32). In both cases, prenylation-dependent endoproteolysis is blocked.

It has also been reported that upon expression of a mutant prelamin A in which the CAAX cysteine has been replaced by methionine, a mutation that blocks prenylation (see Fig. 3), the lamin A precursor is found as nucleoplasmic particles (18). A comparison of the localization of wild-type prelamin A expressed in lovastatin-treated cells (Fig. 4 *C* and *D*) with the CAAX mutant prelamin A (Fig. 4 *E* and *F*) shows that the nonprenylated wild-type prelamin A also accumulates as nucleoplasmic particles. In the case of the lovastatin-treated cells, if the block in prenylation is circumvented by the addition of mevalonate, the accumulated prelamin A is localized entirely to the lamina after only 3 hr of incubation with mevalonate (Fig. 4 *G* and *H*). The time course of prelamin A incorporation into the lamina upon mevalonate addition correlates with the previously published time course of prelamin A processing to mature lamin A in lovastatin-treated cells subjected to a mevalonate chase (5). Therefore, the proper assembly of the nuclear prelamin A into the lamina is concomitant with its prenylation-dependent processing to mature lamin A. Since mevalonate-starved cells are in a G_0 cell cycle arrest and will not begin to enter S phase for at least 11 hr after mevalonate supplementation (33), this lamin processing is occurring in an interphase nucleus.

To determine whether prelamin A accumulates in the nucleus even at physiological levels of lamin A expression, we determined the subcellular distribution of prelamin A in lovastatin-treated nontransfected CHO-K1 cells. The results (Fig. 5) show that prelamin A, as well as mature lamin A, is found in the nuclear pellet.

Assembly of a Carboxyl-Terminal 21-Amino Acid Deletion Mutant of Prelamin A into the Nuclear Lamina. To test whether the carboxyl-terminal peptide of prelamin A prevents its proper assembly into the lamina, we prepared an expression construct missing the carboxyl-terminal 21 amino acid codons of prelamin A, pMMLAΔ21, and tested whether the resulting truncated protein was competent for assembly into the lamina. Upon dexamethasone induction of CHO-K1 cells, stable transfectants with pMMLAΔ21 express the expected 72-kDa protein, which can be visualized on a Western blot with anti-human lamin A/C antibody (Fig. 6).

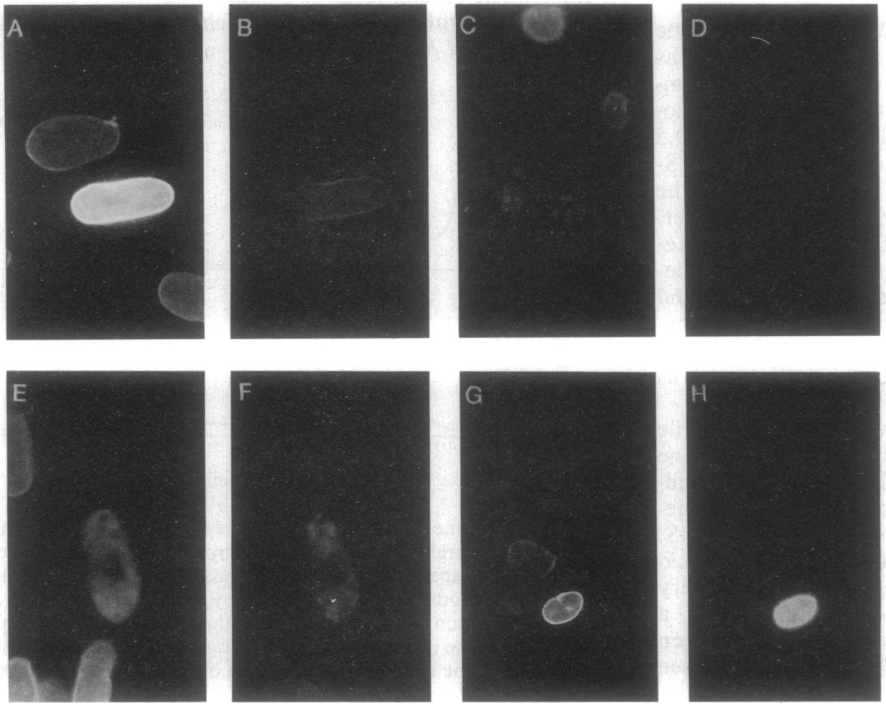

FIG. 4. Effect of inhibition of prelamin A prenylation on its nuclear localization. CHO-K1 cells were transfected with human prelamin A clones and the expressed protein was visualized by indirect immunofluorescence with LS-1, nonspecies-specific anti-lamin A/C (*A*, *C*, *E*, and *G*), and 1E4, human-specific anti-lamin A/C (*B*, *D*, *F*, and *H*) antisera. (*A* and *B*) Wild-type prelamin A. (*C* and *D*) Wild-type prelamin A in the presence of 12.5 μM lovastatin. (*E* and *F*) Prelamin A mutant with the CAAX cysteine changed to methionine. (*G* and *H*) Wild-type prelamin A in cells treated as in *C* and *D* and then incubated with 1 mM mevalonate for 3 hr.

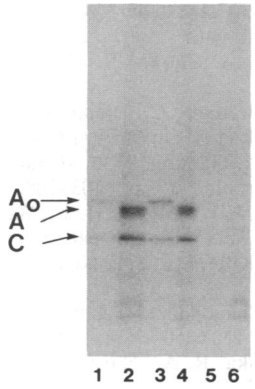

FIG. 5. Subcellular localization of endogenously expressed prelamin A. CHO-K1 cells were incubated overnight in medium supplemented with [^{35}S]methionine in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of 12.5 μM lovastatin. Lamin A was immunoprecipitated with LS-1 anti-lamin A/C antiserum from whole cell homogenates (lanes 1 and 2), or nuclei (lanes 3 and 4), or postnuclear (1000 × *g*) supernatants (lanes 5 and 6) and resolved by SDS/PAGE. Proteins were identified by comparison to molecular weight markers. A_o, prelamin A; A, mature lamin A; C, lamin C. Exposure time was 14 days.

Immunofluorescence of induced cells shows that this lamin A molecule does localize to the nuclear lamina (Fig. 7), consistent with the hypothesis that the carboxyl-terminal peptide of prelamin A blocks proper assembly of this protein. Expression and assembly of the truncated prelamin A into the lamina can be seen in all cells as early as 4 hr after induction (data not shown), which indicates that assembly does not require the cells to go through mitosis.

DISCUSSION

Nuclear localization of the lamin proteins appears to be independent of their carboxyl-terminal CAAX sequence; however, the association of the lamins with the nuclear envelope requires this motif (18, 34). In this report we directly demonstrate that prelamin A is farnesylated at the CAAX-box cysteine. Therefore this posttranslational modification most likely plays a role in the association of prelamin A with the nuclear membrane.

Prelamin A differs from the B-type lamins in that during the prenylation-dependent maturation of this molecule, an 18-amino acid carboxyl-terminal peptide bearing the isoprenoid substituent is removed. In the absence of this processing, blocked either by site-directed mutation (Fig. 4 and ref. 18)

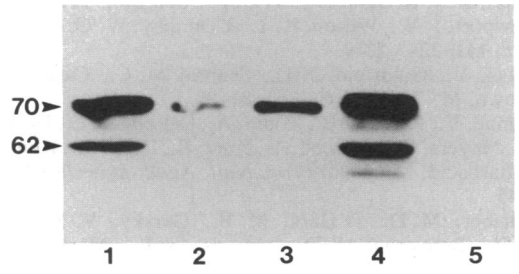

FIG. 6. Dexamethasone-induced expression of truncated prelamin A. Stable transfectants of CHO-K1 cells with pMMLAΔ21 were induced with dexamethasone for 16 hr. The nuclear lamins were isolated and Western blot analysis was conducted with anti-human lamin A/C antibody 1E4. Nuclear lamin proteins from untreated (lane 1) or 12.5 μM lovastatin-treated (lane 4) HeLa cells were used as controls. Dexamethasone-induced expression of pMMLAΔ21 in the absence (lane 2) or presence (lane 3) of 12.5 μM lovastatin is shown. Lane 5, transfectants that were not induced with dexamethasone.

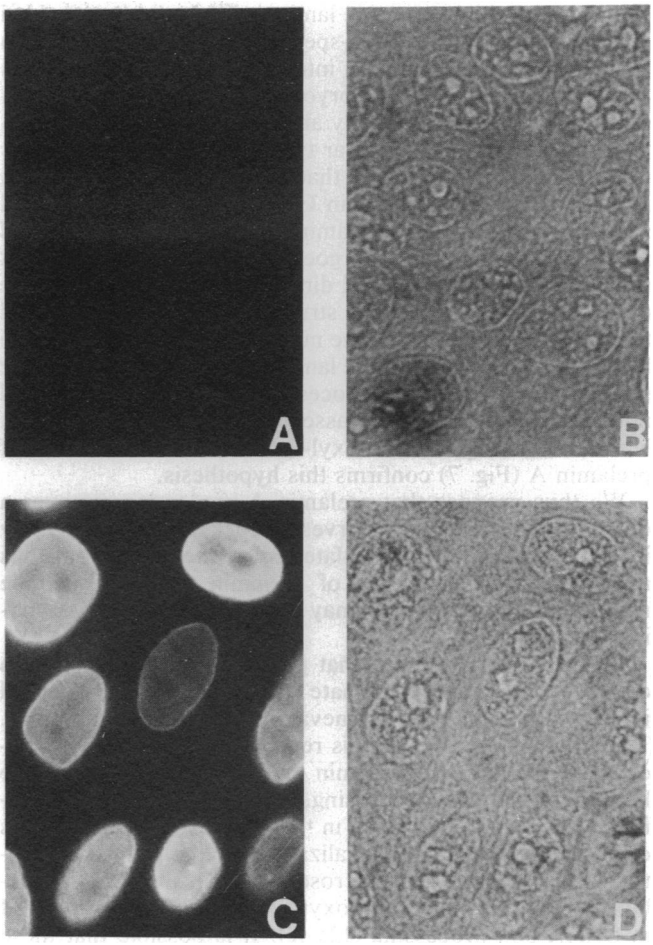

FIG. 7. Assembly of a truncated prelamin A into the nuclear lamina. Stable transfectants of CHO-K1 cells with pMMLAΔ21 (Fig. 5) were induced with dexamethasone for 20 hr and the subcellular distribution of the 21-amino acid carboxyl-terminal deletion mutant of prelamin A was visualized by indirect immunofluorescence with anti-human lamin A/C antibody 1E4 (*C*). Phase-contrast micrograph of the same field of cells is shown for comparison (*D*). No fluorescence is seen after staining cells not treated with dexamethasone (*A*). The field of cells shown in *A* visualized by phase-contrast microscopy is also shown (*B*). (×650.)

or by treatment with lovastatin (Fig. 4), proper assembly of lamin A into the nuclear lamina is prevented. In cells expressing nonprenylated human prelamin A (see Fig. 4 *C* and *E*), it is difficult to detect the host-cell lamin A. We cannot, therefore, distinguish between a total block of incorporation of nonprenylated prelamin A into the lamina and improper assembly of the protein resulting in a collapse of the preexisting lamina. However, we have found by indirect immunofluorescence that the host-cell lamin B remains properly localized to the nuclear periphery in cells expressing the nonprenylated mutant prelamin A (data not shown), which is consistent with the host-cell lamina remaining intact.

Why is prenylation-dependent protein processing required for assembly of mature lamin A into the nuclear lamina? The answer is not immediately obvious. During mitosis, the lamina is disassembled and mature lamin A is depolymerized to oligomers by a phosphorylation-mediated process (2, 35). This mature preexisting lamin A then reassembles into the lamina of mitotic cells (36). *In vitro* reassembly of the lamina can take place directly on chromatin (37), suggestive of a mitosis-specific pathway for assembly of mature lamin A into the lamina. In unpublished studies, we found that the assembly of the truncated prelamin A into the lamina occurs in our permanent transfectants in as little as 4 hr after induction.

Thus, it appears that mature lamin A need not be assembled into the lamina by a mitosis-specific pathway but rather can be transported through the interphase nuclear membrane. Similarly, the *Xenopus* embryonic lamin protein L_{III} is assembled into the lamina only after nuclear envelope formation and activation of nuclear transport (38). This *Xenopus* lamin is similar to lamin A in that it is soluble in a mitotic cell. It is also relevant that lamin C, which has an amino acid sequence identical to the amino-terminal 566 amino acid residues of lamin A, undergoes no prenylation-dependent maturation and is integrated directly into the lamina. These observations argue that the structural information required for lamin A transport into the nucleus and assembly into the lamina is available in mature lamin A and, therefore, that the synthesis and subsequent processing of the precursor should not be required for lamin A assembly. Assembly of a mutant lamin A missing the carboxyl-terminal 21 amino acids of prelamin A (Fig. 7) confirms this hypothesis.

We thus propose that prelamin A processing removes a domain of the protein that serves to block its proper assembly into the lamina. We speculate that either the anomalous nucleoplasmic localization of prelamin A or the peptide released during processing may have some regulatory significance.

Previous work showed that prelamin A, accumulated in cells in response to mevalonate starvation, is rapidly chased into mature lamin A upon mevalonate supplementation (5). The studies described in this report, demonstrating the nuclear localization of prelamin A, are consistent with the hypothesis that this processing, including the farnesyltransferase reaction, takes place in the nucleus. Previous results demonstrate a cytosolic localization for the $p21^{ras}$ farnesyltransferase (39–41) and microsomal localization of the carboxyl methylation and carboxypeptidase activities involved in CAAX-box processing (12, 42). It is possible that these activities have dual localization or that isozymes exist for these activities.

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