Faculty Publications, Biological Sciences

Biological Sciences

Incorporation of a Product of Mevalonic Acid Metabolism Into Proteins of Chinese Hamster Ovary Nuclei

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Recommended Citation

Isoprenylation Is Required for the Processing of The Lamin A Precursor

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Abstract. The nuclear lamina proteins, prelamin A, lamin B, and a 70-kD lamina-associated protein, are posttranslationally modified by a metabolite derived from mevalonate. This modification can be inhibited by treatment with (3-R,S)-3-fluoromevalonate, demonstrating that it is isoprenoid in nature. We have examined the association between isoprenoid metabolism and processing of the lamin A precursor in human and hamster cells. Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by mevinolin (lovastatin) specifically depletes endogenous isoprenoid pools and inhibits the conversion of prelamin A to lamin A. Prelamin A processing is also blocked by mevalonate starvation of Mev-1, a CHO cell line auxotrophic for mevalonate. Moreover, inhibition of prelamin A processing by mevinolin treatment is rapidly reversed by the addition of exogenous mevalonate. Processing of prelamin A is, therefore, dependent on isoprenoid metabolism. Analysis of the conversion of prelamin A to lamin A by two independent methods, immunoprecipitation and two-dimensional nonequilibrium pH gel electrophoresis, demonstrates that a precursor-product relationship exists between prelamin A and lamin A. Analysis of R,S-[5-^{3}H(N)]mevalonate–labeled cells shows that the rate of turnover of the isoprenoid group from prelamin A is comparable to the rate of conversion of prelamin A to lamin A. These results suggest that during the proteolytic maturation of prelamin A, the isoprenylated moiety is lost. A significant difference between prelamin A processing, and that of p21^{ras} and the B-type lamins that undergo isoprenylation-dependent proteolytic maturation, is that the mature form of lamin A is no longer isoprenylated.

THREE major polypeptides termed lamins A, B, and C (14, 17) comprise the nuclear lamina (1, 11, 16, 27, 28) of most mammalian somatic cells. Individual lamins are substrates for posttranslational modifications that include cell cycle-dependent phosphorylation (7, 15, 37, 42, 52), methyl-esterification (9), and covalent modification with a mevalonate-derived product (4, 6, 34, 47, 49, 50, 58). The transient depolymerization of lamins, an event that is associated with the hyperphosphorylation of lamins (7, 14, 15, 37, 42) during mitosis, has been suggested to play a key regulatory role in the concomitant dissociation and reformation of the nuclear envelope. Membrane association appears to be an important function of the mevalonate-derived modifications to lamins in light of the apparent nuclear membrane localization of mammalian lamin B (7, 15, 18, 31), chicken lamin B$_2$ (55), and the lamin A precursor polypeptide (22), all of which are modified by isoprenoids. The isoprenoid substituent of lamin B(11a), p21^{ras} (8, 46), and yeast polypeptide pheromones (3, 23, 25, 39, 45) has been identified as farnesyl.

Gerace et al. (17) have shown that in CHO cells, prelamin A becomes NP-40 insoluble within 5 min of synthesis, and that it is in this lamina-associated environment that processing to mature lamin A occurs. We have previously reported (4) that this 74-kD precursor form of lamin A is posttranslationally modified by a metabolic product derived from R-[2-^{14}C]mevalonate. Site-specific point mutation and deletion analyses have established that the carboxy-terminal -cysteine-aliphatic-aliphatic-methionine (-C-A-A-M)[1] motif, acting in concert with other signal sequences, is required for efficient targeting of human prelamin A (22) and *Xenopus* lamins L$_1$ and A (29) to the nuclear membrane. In mammalian (4, 17, 30, 42, 56) and avian (33, 55) cells, prelamin A to lamin A maturation is associated with a 2-kD decrease in molecular mass. We have demonstrated (4) that, after this maturation event, lamin A no longer contains the R-[2-^{14}C] mevalonate-derived moiety.

In this report, we examine the processing of the 74-kD prelamin A precursor polypeptide in human (HeLa) and hamster (CHO-K1) cells. The requirement for mevalonate, and hence, isoprenoids, for the conversion of prelamin A to lamin A is demonstrated both in cultured cells treated with mevinolin as well as by mevalonate starvation of mev-1, a CHO-K1 mevalonate auxotroph. The results presented in this report are consistent with the existence of an isoprenylation-dependent pathway for the formation of mature lamin A, and support our earlier proposal (4) that isoprenylation

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1. *Abbreviations used in this paper:* -C-A-A-M, cysteine-aliphatic-aliphatic-methionine; -C-A-A-X, cysteine-aliphatic-aliphatic-other; 2D-NEPHGE, two-dimensional nonequilibrium pH gradient electrophoresis.

is a required step in the processing of prelamin A and other isoprenylated proteins.

Materials and Methods

Materials

R-[2-^{14}C]mevalonic acid lactone (56.7 Ci/mmol, 2.10 GBq/mmol) was purchased from Amersham Corp. (Arlington Heights, IL); R,S-[5-^{3}H(N)]-mevalonolactone (24 Ci/mmol, 888.0 GBq/mmol) from New England Nuclear (Boston, MA); and [^{35}S]methionine (tran^{35}S-label, 1,005 Ci/mmol) from ICN Radiochemicals (Irvine, CA). Mevinolin (lovastatin) was a gift from A. W. Alberts of Merck, Sharp, and Dohme (Rahway, NJ). (3-R,S)-3-fluoromevalonate was a gift of Zoecon Corporation (Palo Alto, CA). Triton X-100 and SDS (surfact-amps) were from Pierce Chemical Company (Rockford, IL). Nitrocellulose membranes (trans-blot) and all reagents used for SDS-PAGE were from Bio-Rad Laboratories (Richmond, CA). Silver-stain "DPC" kit was from Integrated Separation Systems (Hyde Park, MA). Molecular mass marker standards were from Pharmacia Fine Chemicals (Uppsala, Sweden). Ampholytes (Iso-Dalt Grade) were from Serva Fine Biochemicals Inc. (Garden City Park, NJ). DNase I (Code:DP) and RNase A (Code:RASE) were from Organon Teknika-Cappel (Malvern, PA). Antilamin (A + C) human autoimmune serum (35) was a gift of Dr. F. McKeon (Harvard Medical School); normal human serum was from Rockland, Inc. (Gilbertsville, PA), and protein A-Sepharose CL-4B was from Sigma Chemical Co. (St. Louis, MO). Goat anti-human IgG (H + L chain)-alkaline phosphatase antibody conjugate and Immun-Blot Assay Kits were from Bio-Rad Laboratories. A CHO-K1 derivative, met-18b-2, was a gift from Dr. J. Faust (Tufts University, Boston, MA). All cell culture reagents were from Gibco Laboratories (Grand Island, NY). Fungizone (Amphotericin B) was obtained from E. R. Squibb (Princeton, NJ). All other chemicals were purchased from Sigma Chemical Co.

Cell Culture and Radioisotopic Labeling

The cell lines used were CHO-K1 (26); mev-1, a CHO-K1 3-hydroxy-3-methylglutaryl CoA synthase mutant (48, 51); met-18b-2, a CHO cell line that displays elevated mevalonate uptake and metabolism (12), and HeLa, a human adenocarcinoma cell line (24). Cells were grown in Ham's F12 medium (20), supplemented with FCS (5% [vol/vol] for CHO cell lines and 10% [vol/vol] for HeLa), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml Fungizone (medium I). Mev-1 cells were grown in medium I supplemented with 60 μg/ml mevalonate (medium II). Met-18b-2 cells were grown in medium I supplemented as described (12) with 8 μg/ml mevinolin and 37 μg/ml mevalonate (medium III).

For labeling with R,S-[5-^{3}H(N)]mevalonate or R-[2-^{14}C]mevalonate, cells were plated in the appropriate medium at one-half maximum density, and allowed to attach overnight. Before addition of mevalonate labeling medium, the cells were washed with PBS (130 mM NaCl, 2 mM KCl, 8 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.2). Mevalonate labeling medium consisted of F12, penicillin, streptomycin, Fungizone, and 5% (vol/vol) dialyzed (against PBS) FCS (medium IV), and was supplemented with radiolabeled mevalonate (either 2-10 μCi/ml of R-[2-^{14}C]mevalonic acid lactone or 10 μCi/ml R,S-[5-^{3}H(N)]mevalonolactone) with the appropriate concentrations of mevinolin (details are given in the figure legends). Mev-1 cells are auxotrophic for mevalonate, and do not require mevinolin treatment to block endogenous mevalonate synthesis. [^{35}S]Methionine labeling medium (medium V) consisted of F12 containing one-half the normal methionine concentration, penicillin, streptomycin, Fungizone, 5% (vol/vol) dialyzed FCS, and 100 μCi/ml [^{35}S]methionine.

Electrophoresis and Western Blotting

SDS-PAGE, two-dimensional nonequilibrium pH gel electrophoresis (2D-NEPHGE), and fluorography were performed as previously described (4). Electrophoretic transfer of proteins to nitrocellulose membranes was performed at 4°C for 12-14 h at 30 V (100 mA) using a Hoefer TE Series Transphor Electrophoresis Unit (Hoefer Scientific Products, San Francisco, CA) in the Tris-glycine-methanol buffer system of Towbin (54) containing 0.1% (wt/vol) SDS.

Immunoblot Analysis of Proteins Immobilized on Nitrocellulose

After electrophoretic transfer of proteins from the two-dimensional nonequi-

librium pH gradient electrophoresis (2D-NEPHGE) gel, the nitrocellulose membrane was rinsed in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) and blocked overnight at 4°C in TBS blocking buffer (TBS, 0.5% [vol/vol] Tween-20 and 5% [wt/vol] BSA [RIA grade]). The nitrocellulose membrane was then rinsed in TBS and placed in 5 ml of TBS blocking buffer containing a 1:1,000 dilution of LS1 anti-lamin (A + C) antiserum (35). After incubation for 6-12 h at 4°C with the primary antibody, the nitrocellulose membrane was washed twice in TBS with 0.05% Tween-20 for 10 min at 4°C. The nitrocellulose membrane was then reacted with goat anti-human IgG-alkaline phosphatase conjugate at a 1:1,000 dilution in TBS, 5% (wt/vol) BSA for 1-2 h at 25°C. The nitrocellulose membrane was then rinsed three times for 5 min in TBS. Color development of the alkaline phosphatase-conjugated second antibody was performed according to the manufacturer's directions.

Filter Binding Assay for [^{35}S]methionine and R-[2-^{14}C]mevalonate-labeled Proteins

A small fraction of the R-[2-^{14}C]mevalonate is incorporated into isopentenyl tRNA (47, 50), which must be hydrolyzed (100 μg/ml RNase A, 37°C, 30 min) before precipitation of proteins with TCA. Carrier protein and lipids were added in the form of FCS (20 μl/assay). The samples were then adjusted to 10% (wt/vol) TCA, and incubated on ice for at least 60 min. Samples were transferred to GF/A glass microfiber filters (Whatman Inc., Clifton, NJ) and washed sequentially with three 5-ml volumes each of acetone, followed by chloroform:methanol (2/1), and 95% ethanol. The filters were dried and radioactivity determined.

Results

Inhibition of Mevalonate Biosynthesis Prevents the Conversion of Prelamin A to Lamin A

We have demonstrated (4) that growth of mammalian cells in the presence of mevinolin and R-[2-^{14}C]mevalonate results in the labeling of three proteins present in nuclear lamina intermediate filament preparations. These R-[2-^{14}C]mevalonate-labeled proteins are present in a number of different cell lines (Beck, L. A., C. H. Campbell, J. Logel, and M. Sinensky, submitted for publication) including HeLa and CHO. Proteins visualized by silver-staining of a 2D-NEPHGE gel of nuclear lamina intermediate filament preparations from R-[2-^{14}C]mevalonate labeled CHO-K1 (Fig. 1 A) are lamin A (72 kD), lamin B (68 kD), lamin C (62 kD), a lamina-associated protein of 70 kD (*solid triangle*), vimentin (56 kD), and actin (43 kD). We have determined that the 70 kD isoprenylated protein is lamina-associated based on (a) its biochemical characteristics and (b) immunofluorescence localization and immunoblot identification by a chicken lamin B$_2$-specific antibody (Beck, L. A., C. H. Campbell, J. Logel, and M. Sinensky, submitted for publication). The fluorogram of this 2D-NEPHGE gel is presented in Fig. 1 C. The two R-[2-^{14}C]mevalonate-labeled polypeptides in the acidic region of the 2D-NEPHGE gel are lamin B and the 70-kD lamina-associated polypeptide (*solid triangle*). As we previously reported (5), the R-[2-^{14}C]mevalonate-labeled polypeptide in Fig. 1 C, with an apparent molecular mass of 74 kD but no visible silver-staining mass is the lamin A precursor (designated as lamin A$_o$ [17]).

The silver-stained 2D-NEPHGE gel of a nuclear lamina intermediate filament fraction from similarly treated HeLa cells contains a similar complement of proteins (Fig. 1 B). The 74 kD prelamin A polypeptide (A$_o$) and lamin B are labeled by R-[2-^{14}C]mevalonate. However, in HeLa cells, in contrast to CHO-K1, there are two R-[2-^{14}C]mevalonate-labeled lamina-associated polypeptides (D, *solid triangles*) that display lower molecular masses and more basic isoelec-
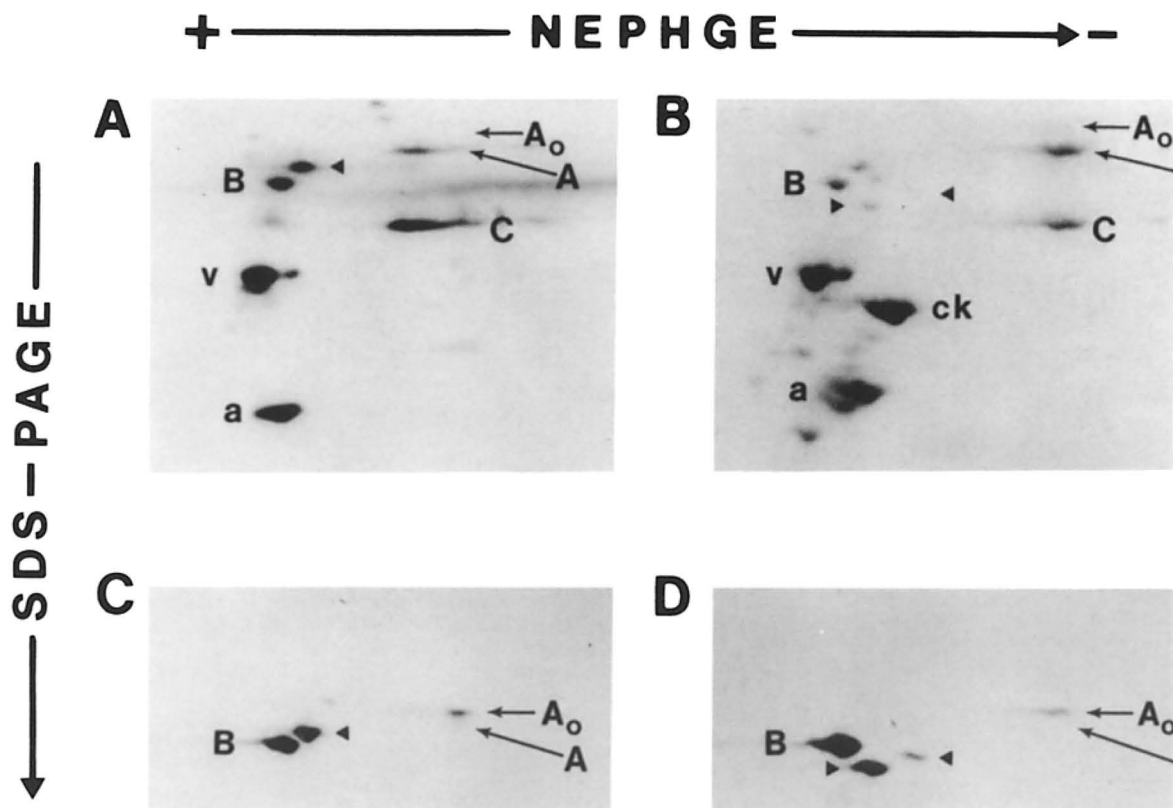
Figure 1. Identification of R-[2-^{14}C]mevalonate–labeled proteins in CHO-K1 and HeLa nuclear lamina-intermediate filament preparations by 2D-NEPHGE and fluorography. CHO-K1 and HeLa cells were labeled with 2 μCi/ml R-(2-^{14}C) mevalonate for 17 h in medium IV. Nuclear lamina intermediate filament fractions were analyzed by 2D-NEPHGE and fluorography as previously described (4). Proteins were visualized by silver staining. (*A*) Silver-stained CHO-K1 nuclear lamina intermediate filament preparation. (*B*) Silver-stained HeLa nuclear lamina intermediate filament preparation. (*C* and *D*) Fluorograms of the 2D-NEPHGE gels in *A* and *B*, respectively. Only the relevant portion of each fluorogram is shown. Fluorographic exposure was for 45 d. Indicated are lamins A (72 kD), B (68 kD), and C (62 kD); the 74-kD isoprenylated prelamin A polypeptide (A_o), the 70-kD lamina-associated isoprenylated polypeptide (*solid triangle*), vimentin (*v*), and actin (*a*). The arrows in *A* and *B* (as well as in subsequent figures) indicate the relative position of prelamin A (A_o) and lamin A in *C* and *D* as determined by superimposing each fluorogram on its silver-stained gel.

tric points than does the 70 kD lamina-associated CHO-K1 homologue (compare the solid triangles in the silver-stained gels [*A* and *B*] with the corresponding fluorograms [*C* and *D*]).

Immunological Identification of Prelamin A and Lamin A in Nuclear Lamina Intermediate Filament Preparations

We have shown (4) that prelamin A migrates on 2D-NEPHGE gels with an identical set of pH isovariants and at a slightly higher molecular mass than lamin A, is precipitated with antilamin (A + C) antiserum, and exhibits peptide mapping homology with lamin A. We now present immunological evidence identifying the 74-kD R-[2-^{14}C]mevalonate-labeled polypeptide as prelamin A. Nuclear lamina intermediate filament fractions were prepared from HeLa cells labeled with [^{35}S]methionine for 7 h and then shifted to medium containing mevinolin and [^{35}S]methionine for an additional 10 h. Visible in the autoradiogram, produced by the [^{35}S]methionine-labeled polypeptides transferred to nitrocellulose, (Fig. 2 *A*) is newly synthesized prelamin A (A_o), which accumulates under conditions of mevalonate starvation. Also present in these cells is mature lamin A that was synthesized and processed during the preliminary 7 h growth in the ab-

sence of mevinolin. In contrast, the autoradiogram from cells not treated with mevinolin (Fig. 2 *B*) illustrates that cells grown in the absence of mevinolin process prelamin A normally.

Positive identification of these polypeptides as prelamin A and lamin A was made by immunoblot analysis using an antilamin (A+C) serum. Both prelamin A and mature lamin A, (Fig. 2 *C*) are present in mevinolin-treated cells. In contrast, only lamin A is detectable in the immunoblots from untreated cells (*D*). Immunoblot analyses of nuclear lamina intermediate filament preparations from a number of similarly treated cell types yield identical results (Beck, L. A., C. H. Campbell, J. Logel, and M. Sinensky, submitted for publication). Both the 74 kD R-[2-^{14}C]mevalonate–labeled polypeptide and the 72 kD nonisoprenylated lamin A are recognized by an antilamin (A + C) antibody, consistent with their identification as lamin A and its precursor polypeptide, prelamin A. Only in mevinolin-treated cells, in the absence of any exogenously added mevalonate, does prelamin A accumulate to a detectable level (Fig. 2 *A*). Even the addition of trace amounts of mevalonate, present only as R-[2-^{14}C]mevalonate, results in a sufficient intracellular concentration of mevalonate to allow biosynthesis of isoprenoids, posttranslational modification, and processing of prelamin A
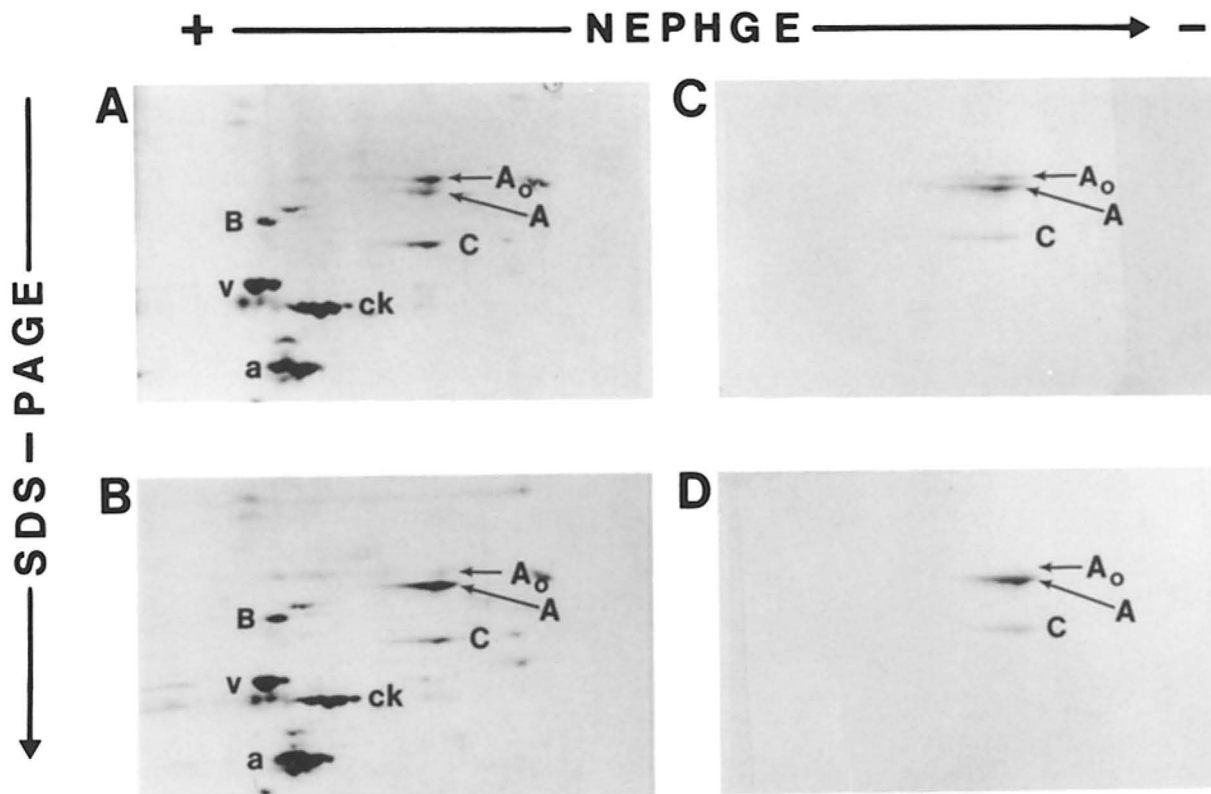
Figure 2. Immunoblot analysis of the accumulation of prelamin A in mevinolin-treated cells. HeLa cells were grown for 7 h in medium V containing 100 µCi/ml [^{35}S]methionine. The labeling medium was then changed to either medium V with 100 µCi/ml [^{35}S]methionine and 5 µg/ml mevinolin (*A*) or to medium V (no mevinolin) with 100 µCi/ml [^{35}S]methionine (*B*). After an additional 10 h of growth, nuclear lamina intermediate filament fractions were prepared, displayed on 2D-NEPHGE gels, and transferred to nitrocellulose membranes. [^{35}S]methionine-labeled polypeptides were visualized autoradiographically from the nitrocellulose blots with Kodak X-OMAT AR film. After autoradiography, immunoblot analysis was then performed. A 1:1,000 dilution of human autoimmune antilamin (A + C) serum was used as the primary antibody and a 1:2,000 dilution of alkaline phosphatase conjugated goat anti–human IgG was used as the second antibody. Indicated are prelamin A (A_o), lamins A, B, and C, vimentin (*v*), cytokeratin (*ck*), and actin (*a*). (*A*) Autoradiogram obtained from the nitrocellulose blot of nuclear lamina intermediate filament preparation from mevinolin-treated HeLa cells. (*B*) Autoradiogram obtained from the nitrocellulose blot of nuclear lamina intermediate filament preparation from the untreated HeLa cells. (*C*) Immunoblot from *A*. (*D*) Immunoblot from *B*.

(Fig. 1 *A* and *B*). The R-[2-^{14}C]mevalonate-labeled 74-kD pre-lamin A fluorographic signal (Fig. 1, *C* and *D*) is produced by those molecules of prelamin A that have been modified by the mevalonate-derived product but have not yet been processed.

Verification of the Isoprenoid Nature of the Mevalonate-derived Modification

(3-R,S)-3-Fluoromevalonate is a competitive inhibitor (41) of pyrophosphomevalonate decarboxylase. This enzyme catalyzes the reaction leading directly to isopentenyl pyrophosphate, the immediate precursor to all isoprenoids. Treatment of CHO met-18b-2 cells with (3-R,S)-3-fluoromevalonate inhibits the posttranslational modification of proteins with a metabolic product derived from R-[2-^{14}C]mevalonate in a dose-dependent manner (Fig. 3 *A, solid circles*). Inhibition by (3-R,S)-3-fluoromevalonate is specific for the isoprenylated proteins, since it produces only a slight effect on the incorporation of [^{35}S]methionine into total cellular protein (Fig. 3 *A, open circles*). Consistent with the postulated isoprenoid nature of this type of modification to the lamina proteins, (3-R,S)-3-fluoromevalonate blocks the R-[2-^{14}C]mevalonate-

derived labeling of proteins of nuclear lamina intermediate filament preparations (Fig. 3 *B, solid circles*) with little effect on the net synthesis of these proteins as measured by [^{35}S]methionine labeling (Fig. 3 *B, open circles*). These results are consistent with the premise that the mevalonate-derived modification to prelamin A, as well as to other mevalonate-labeled proteins, is isopentenyl pyrophosphate, or an isoprenoid moiety metabolically distal to isopentenyl pyrophosphate.

Inhibition of the Processing of Prelamin A to Lamin A Is Not Due to Nonspecific Mevinolin Effects

It was important to confirm that the block to prelamin A processing is a direct consequence of mevinolin depletion of isoprenoids, and is not due to nonspecific mevinolin effects. We compared the processing of prelamin A in mevinolin-treated CHO-K1 and HeLa cells to the processing of prelamin A in a cell line auxotrophic for mevalonate (mev-1), grown under conditions of mevalonate starvation. These studies demonstrate (Fig. 4) that only mature lamin A is present in control CHO-K1 or HeLa cells grown in the absence of mevinolin (Fig. 4, lanes *a* and *d*), or in Mev-1 cells supplemented with exogenous mevalonate (Fig. 4, lane *g*). Accumulation
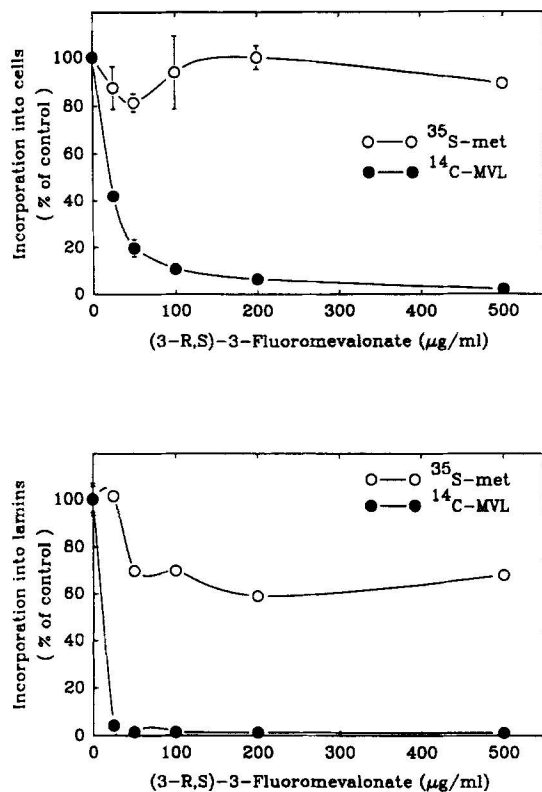
Figure 3. (3-R,S)-3-Fluoromevalonate inhibition of R-[2-^{14}C]mevalonate incorporation into isoprenylated proteins. CHO-K1 (met-18b-2) cells were preincubated in the absence of radioisotopes for 2 h (to deplete endogenous pools of mevalonate) in either medium IV (for R-[2-^{14}C]mevalonate labeling) or medium V (for [^{35}S]methionine labeling) containing 8 μg/ml mevinolin and 0–500 μg/ml (3-R,5)-3-fluoromevalonate. At 2 h, an equal volume of medium IV containing 5 μCi/ml R-[2-^{14}C]mevalonate or medium V containing 60 μCi/ml [^{35}S]methionine with the appropriate (3-R,S)-3-fluoromevalonate concentrations was added. Labeling was then allowed to proceed for an additional 17 h. Duplicate aliquots, containing equal numbers of cells, were analyzed for total [^{35}S]methionine or R-[2-^{14}C]mevalonate incorporation into protein using the filter binding assay described in Materials and Methods. (*A*) Dose-response curve for the (3-R,S)-3-fluoromevalonate inhibition of R-[2-^{14}C]mevalonate incorporation into cellular isoprenylated proteins (*solid circles*), and [^{35}S]methionine incorporation into cellular protein (*open circles*). (*B*) Dose response curve for the (3-R,S)-3-fluoromevalonate inhibition of R-[2-^{14}C]mevalonate incorporation into protein in nuclear lamina intermediate filament preparations (*solid circles*) and [^{35}S]methionine incorporation into proteins of nuclear lamina intermediate filament fractions (*open circles*).

of prelamin A can be manifested either by mevinolin treatment of CHO-K1 or HeLa cells (Fig. 4, lanes *b* and *e*) with mevinolin, or by growth of Mev-1 in the absence of exogenous mevalonate (Fig. 4, lane *h*). To test further that processing of prelamin A is mevalonate-dependent, cells were treated simultaneously with mevinolin and mevalonate. Under these growth conditions, cells contain only mature lamin A (Fig. 4, lanes *c*, *f*, and *i*). These results demonstrate that (*a*) mevalonate starvation, whether the consequence of mevinolin treatment or auxotrophy, blocks the processing of prelamin A; and (*b*) the presence of mevalonate, even during mevinolin treatment, restores isoprenoid synthesis and allows processing of prelamin A to lamin A.

Kinetics of Prelamin A Processing: Immunoprecipitation Analysis

We next examined the kinetics of the isoprenoid-dependent processing of prelamin A. Both HeLa and CHO-K1 cells were labeled for 17 h with [^{35}S]methionine plus mevinolin to accumulate newly synthesized ^{35}S-labeled prelamin A. Processing was initiated by removing the mevinolin and [^{35}S]methionine labeling medium and adding fresh medium containing 1 mM unlabeled mevalonate. Disappearance of the [^{35}S]methionine labeled 74-kD prelamin A precursor and the concomitant appearance of mature 72-kD lamin A was monitored by immunoprecipitation with antilamin (A + C) antiserum, SDS-PAGE, and fluorography. HeLa cells grown in the absence of mevinolin contain only the mature form of lamin A (Fig. 5 *A*, lane *a*), whereas cells treated with mevinolin accumulate [^{35}S]methionine-labeled prelamin A (Fig. 5 *A*, lane *b*). The mevalonate-induced chase of prelamin A
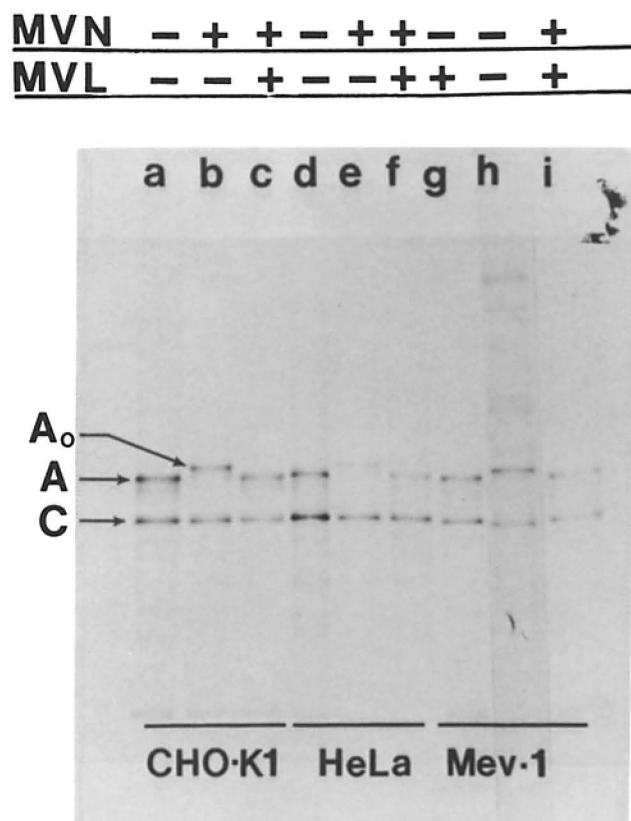

Figure 4. Immunoprecipitation analysis of prelamin A in mevinolin-treated cells. Immunoprecipitation analysis, SDS-PAGE, and fluorography of CHO-K1, HeLa, and mev-1 cells, labeled for 17 h with 50 μCi/ml [^{35}S]methionine were performed as previously described (4). (Lanes *a–c*) CHO-K1 cells grown in normal (medium V) medium (lane *a*), medium V with 5 μg/ml mevinolin (lane *b*), or medium V with 5 μg/ml mevinolin plus 1-mM mevalonate. (Lanes *d–f*) HeLa cells grown in normal (medium V) medium (lane *d*), medium V with 5 μg/ml mevinolin (lane *e*), or medium V with 5 μg/ml mevinolin plus 1 mM mevalonate. (Lanes *g–i*) Mev-1 cells grown in medium V supplemented with 1 mM mevalonate (lane *g*), or medium V only (lane *h*), or medium V with 5 μg/ml mevinolin plus 1 mM mevalonate (lane *i*). The presence (+) or absence (−) of mevinolin (*MVN*) or mevalonate (*MVL*) is indicated in the legend at the top of the figure. Arrows identify prelamin A (*A$_o$*), lamin A, and lamin C.
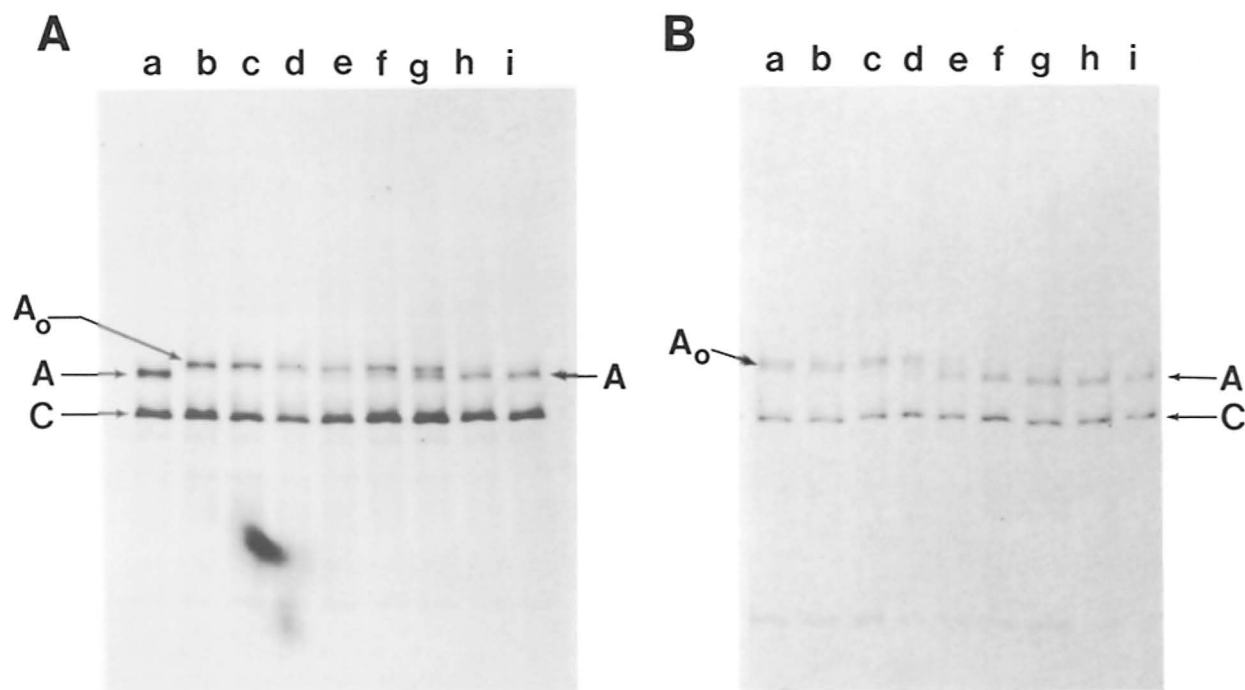
Figure 5. Time course analysis of the isoprenylation-dependent processing of prelamin A. HeLa cells were grown for 17 h in medium V containing 100 μCi/ml [^{35}S]methionine and 5 μg/ml mevinolin. Mev-1 cells were grown in the same medium without mevinolin. To initiate the isoprenylation-dependent conversion of the [^{35}S]methionine-labeled prelamin A to lamin A, labeling medium was replaced with medium I containing 1 mM unlabeled mevalonate. At appropriate intervals, cells were harvested in situ with solubilization buffer. Details of the immunoprecipitation procedure using antilamin A + C autoimmune serum, as well as SDS-PAGE and fluorography have been described (4). (*A*) SDS-PAGE/fluorographic analysis of the time course of the isoprenylation-dependent conversion of prelamin A to lamin A in HeLa cells. (Lane *a*) 17 h, no mevinolin treatment; (lane *b*) 17 h with mevinolin treatment; (lanes *c–i*) mevalonate chase of prelamin A to lamin A at 10 min (lane *c*); 35 min (lane *d*); 60 min (lane *e*); 80 min (lane *f*); 100 min (lane *g*); 180 min (lane *h*); and 240 min (lane *i*) after removal of mevinolin and the shift to mevalonate-containing growth medium. (*B*) SDS-PAGE/fluorographic analysis of the conversion of prelamin A to lamin A in mev-1 cells. (Lane *a*) 17 h growth under mevalonate starvation conditions; (lanes *b–i*) mevalonate induced chase of prelamin A to lamin A at 20 min (lane *b*); 35 min (lane *c*); 45 min (lane *d*); 105 min (lane *e*); 120 min (lane *f*); 180 min (lane *g*); 240 min (lane *h*); and 300 min (lane *i*) after removal of mevinolin and shift to mevalonate-containing growth medium. Indicated are the 74-kD prelamin A polypeptide (A_o), the 72-kD mature lamin A form (*A*), and lamin C (*C*).

into lamin A (Fig. 5 *A*, lane *c–i*) reveals a time-dependent processing of [^{35}S]methionine-labeled prelamin A. Lamin A processing is first detectable 60 min after the addition of exogenous mevalonate (Fig. 5 *A*, lane *e*). By 100 min (Fig. 5 *A*, lane *g*), approximately half of the prelamin A, that accumulated during the mevinolin block, has been converted to lamin A. By 240 min (lane *i*), all of the prelamin A has been converted to lamin A. Identical results were obtained using the mevalonate auxotroph Mev-1 (Fig. 5 *B*). Only prelamin A is present in Mev-1 cells starved for mevalonate (Fig. 5 *B*, lane *a*). Processing of prelamin A is first detectable ∼45 min (lane *d*) after mevalonate addition. By 180 min (lane *g*), all of the [^{35}S]methionine labeled prelamin A has been processed to lamin A.

Kinetics of Prelamin A Processing: 2D-NEPHGE Analysis

As an independent method of examining the kinetics of prelamin A processing, we used a 2D-NEPHGE/fluorographic analysis of nuclear lamina intermediate filament preparations from HeLa cells labeled with [^{35}S]methionine in the presence or absence of mevinolin. Consistent with the data obtained from the immunoprecipitation analysis of untreated HeLa cells (Fig. 5 *A*, lane *a*), we found that all of the

[^{35}S]methionine labeled lamin A in nuclear lamina intermediate filament preparations from untreated cells is present as the mature 72-kD form (Fig. 6 *A*; note the absence of prelamin A). In mevinolin-treated cells, processing is blocked and the lamin A precursor polypeptide accumulates (Fig. 6 *B*). The rate of conversion of [^{35}S]methionine-labeled prelamin A to lamin A from parallel cultures, that had been transferred to unlabeled chase medium containing 1 mM exogenous mevalonate (Fig. 6 *C–F*), was identical to the data presented in Fig. 5 *A*. By 120 min (Fig. 6 *D*), after release from the mevinolin block, most of the prelamin A has been converted to lamin A. From 120 to 250 min after the addition of mevalonate (Fig. 6 *D*, *E*, and *F*), there is a steady decrease in prelamin A accompanied by a concomitant increase in lamin A. At 250 min (*F*), newly synthesized prelamin A is no longer detectable.

Quantitation of the Precursor Product Relationship between Prelamin A and Lamin A

Radioactive spots corresponding to prelamin A, lamin A, and 13 other well-separated and clearly distinguishable [^{35}S]-methionine-labeled polypeptides were excised from a representative subset of 2D-NEPHGE gels obtained from this analysis and counted. The fractional change in the amount
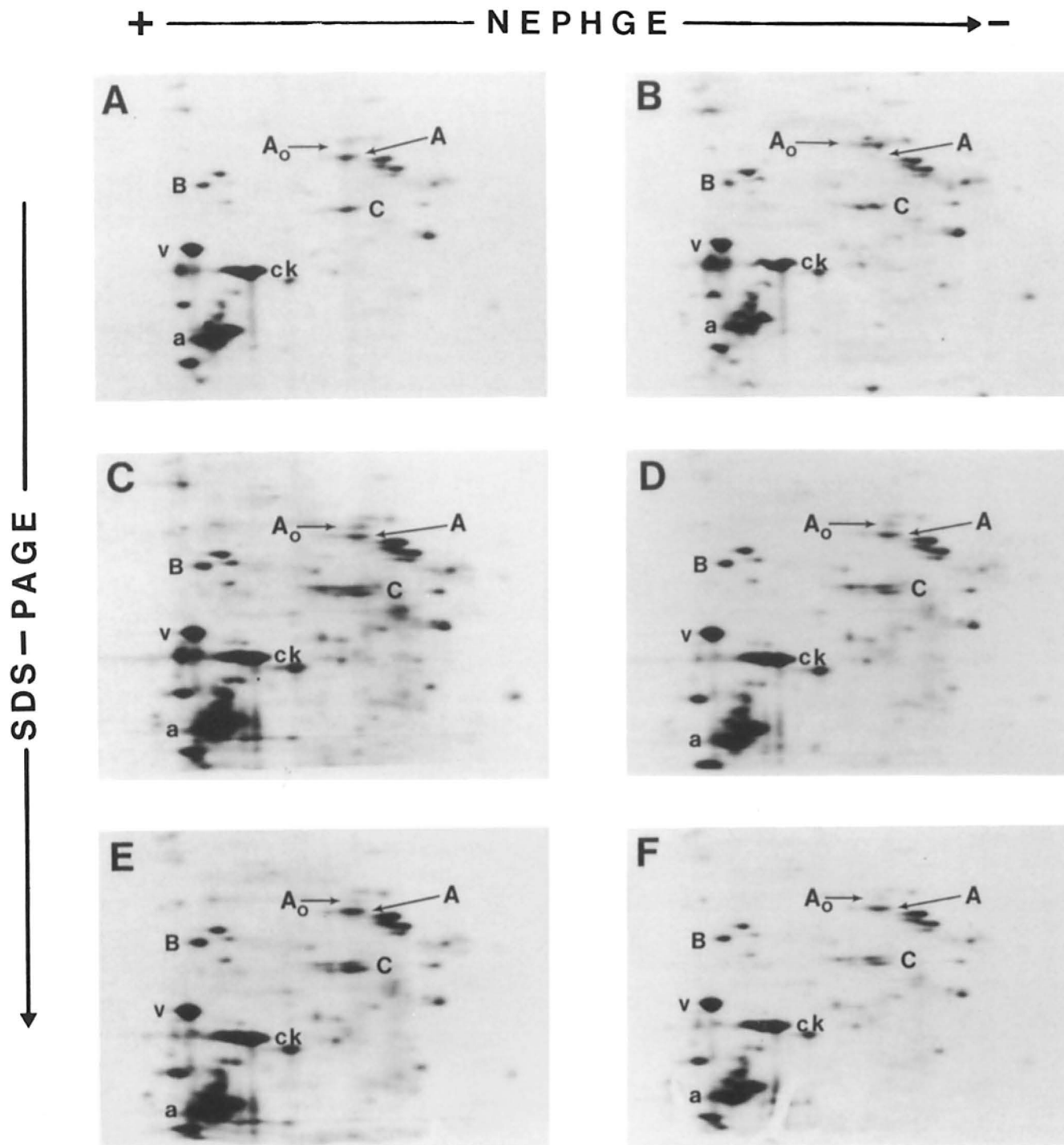
1494

Figure 6. 2D-NEPHGE analysis of the precursor product relationship between prelamin A and lamin A in HeLa. Experimental conditions are given in the legend to Fig. 5. At intervals after the addition of exogenous mevalonate, cells were collected and counted. For subsequent 2D-NEPHGE analysis, a micropreparation procedure for isolation of the nuclear lamina intermediate filament fraction was used. This method paralleled the large scale nuclear lamina intermediate filament isolation procedure previously described (4). The resulting nuclear lamina intermediate filament fractions were solubilized in 63 mM Tris-HCl (pH 7.5), 2% SDS, 5% β-mercaptoethanol, 8 M urea, and stored at $-80°C$. 2D-NEPHGE analysis and fluorography were performed as described (4). Fluorograms from a representative subset of these 2D-NEPHGE gels are presented in *A–F.* (*A*) Control, 17 h growth in ^{35}S-labeling medium without mevinolin. (*B*) 17 h growth with 5 $\mu g/ml$ mevinolin. Arrows for prelamin A (A_o) in *A* and for lamin A (*A*) in *B* are for orientation purposes. (*C–F*) Mevalonate-induced chase of prelamin A to lamin A after removal of mevinolin and shift to mevalonate-containing growth medium. (*C*) 90 min after release from mevinolin block. (*D*) 120 min; (*E*) 200 min; (*F*) 250 min. Indicated are lamins A, B, C, the 74-kD prelamin A polypeptide (A_o), vimentin (*v*), cytokeratin (*ck*), and actin (*a*). Electrophoresis in the NEPHGE dimension was for 3,000 V h.
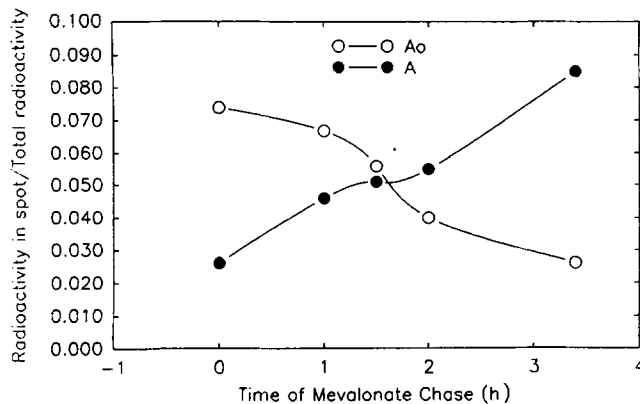
Figure 7. Quantitation of the precursor product relationship between prelamin A and lamin A. A subset of the fluorograms obtained from the 2D-NEPHGE/fluorographic analysis of the prelamin A to lamin A processing (see Fig. 6) were used as templates to carefully excise the [^{35}S]methionine-labeled prelamin A, lamin A, and 13 other well-separated and clearly identifiable polypeptides. The gel slices were dissolved in 30% hydrogen peroxide at 65°C, and the radioactivity was contained in each polypeptide determined by liquid scintillation counting. The fractional ratio of the radioactivity contained in each polypeptide spot, relative to the total radioactivity contained in all 15 spots, was then determined. The fractional change in prelamin A (A_o, *open circle*) and lamin A (*A*, *solid circle*) as a function of time after release from the mevinolin treatment is presented.

of radioactivity contained in the prelamin A and lamin A polypeptide spots, relative to the total radioactivity contained in all 15 spots, was then determined. The result of this examination (Fig. 7) demonstrates that (*a*) prelamin A accumulates as a consequence of a mevinolin-induced isoprenoid starvation; (*b*) prelamin A is converted to lamin A with a half-life of ~90-120 min after the removal of the mevinolin block; and (*c*) prelamin A and lamin A exhibit a precursor product relationship.

Kinetics of the Loss of the Isoprenoid Substituent from Prelamin A

We next compared the rate of loss of the isoprenoid moiety from prelamin A with the rate of conversion of prelamin A to lamin A. [^{3}H]isoprenylated lamins in Mev-1 cells were obtained by growth in medium IV with R,*S*-[5-^{3}H(*N*)]-mevalonate for 17 h. The labeling medium was removed and replaced with fresh medium containing 1 mM unlabeled mevalonate. At various times from 0 to 30 h, cells were harvested and nuclei prepared. The rate of loss of the tritium label ([^{3}H]isoprenylation) from the isoprenylated lamina proteins was analyzed by SDS-PAGE and fluorography. The resultant fluorogram (Fig. 8 *A*) was then analyzed by densitometry. The results of this analysis are presented in Fig. 8 *B*. The loss of the isoprenoid substituent from prelamin A is relatively rapid, displaying a half-life, determined graphically, of ~100-120 min. In contrast, lamin B and the 70-kD lamina-associated protein are characterized by a very stable form of isoprenylation. This rate of loss of the isoprenylated substituent from prelamin A is comparable to the rate of conversion of prelamin A to lamin A, as determined from the immunoprecipitation and 2D-NEPHGE/fluorographic analyses presented in Figs. 5, 6, and 7. These results are consis-

tent with the premise that the loss of the isoprenoid substituent is associated with the proteolytic processing mechanism during the conversion of prelamin A to lamin A.

Discussion

In earlier reports we have shown that prelamin A, lamin B, and a 70 kD lamina-associated polypeptide are posttranslationally modified with a metabolite derived from R-[2-^{14}C]-mevalonate (4). In this report we confirm the isoprenoid nature of this modification by demonstrating that (3-R,*S*)-3-fluoromevalonate inhibits the incorporation of R-[2-^{14}C]-mevalonate into these proteins (Fig. 3). We have identified the 74 kD isoprenylated protein as prelamin A on the basis of its subcellular distribution, migration on 2D-NEPHGE gels, and peptide maps (4). Furthermore, it is recognized on 2D-NEPHGE/immunoblots with antilamin (A + C) antibodies (Fig. 2). Mevalonate starvation and subsequent depletion of isoprenoid pools can be effected by mevinolin treatment or through auxotrophy (Fig. 4). The metabolic consequence of either treatment is inhibition of isoprenoid-dependent proteolytic processing, rapidly followed by an accumulation of prelamin A. Subsequent addition of mevalonate abolishes isoprenoid starvation and allows in vivo conversion of prelamin A to lamin A (Figs. 5 and 6). The rate of this conversion (Fig. 7) is similar to the loss of the isoprenoid moiety from prelamin A (Fig. 8 *B*).

The -C-A-A-X Carboxy Terminal Motif of Prelamin A Is Required for Its Maturation to Lamin A

Clarke et al. have catalogued a number of proteins that contain a -C-A-A-X carboxy terminal amino acid sequence (10). They have suggested that this consensus amino acid sequence may provide a general recognition site for posttranslational modifications that include lipidation and carboxymethylation. This sequence is present in yeast peptidyl pheromones (3, 5, 38, 46), yeast *ras*1 and *ras*2 (43, 46), and mammalian p21^{ras} (8, 10, 19, 21, 46, 57). Mammalian and avian lamins, characterized by a -C-A-A-M carboxy terminal motif (55), can be considered a subfamily of this class of proteins. The similarity between the -C-A-A-X motif of *ras* proteins, yeast peptidyl pheromones, and the -C-A-A-M motif of lamins, in addition to their posttranslational isoprenylation, suggests that the functional role of this carboxy terminal sequence may be similar. Moreover, *ras* proteins, yeast peptidyl pheromones, and B-type lamins undergo proteolytic processing and membrane localization subsequent to isoprenylation. We propose, as has Holtz et al. (22), that prelamin A is also a nuclear membrane protein and that nuclear membrane localization is required for its processing and assembly into the lamina. The isoprenylated form of prelamin A is hypothesized to be required for this membrane localization.

Isoprenylation Is Required for the Processing of Prelamin A to Lamin A

We have demonstrated that depletion of endogenous isoprenoid pools through mevinolin treatment can block the processing of prelamin A. This occurs in HeLa and CHO-K1 cells (Figs. 1, 2, and 4), as well as in a number of other human, hamster, and chicken cell lines (our unpublished data).
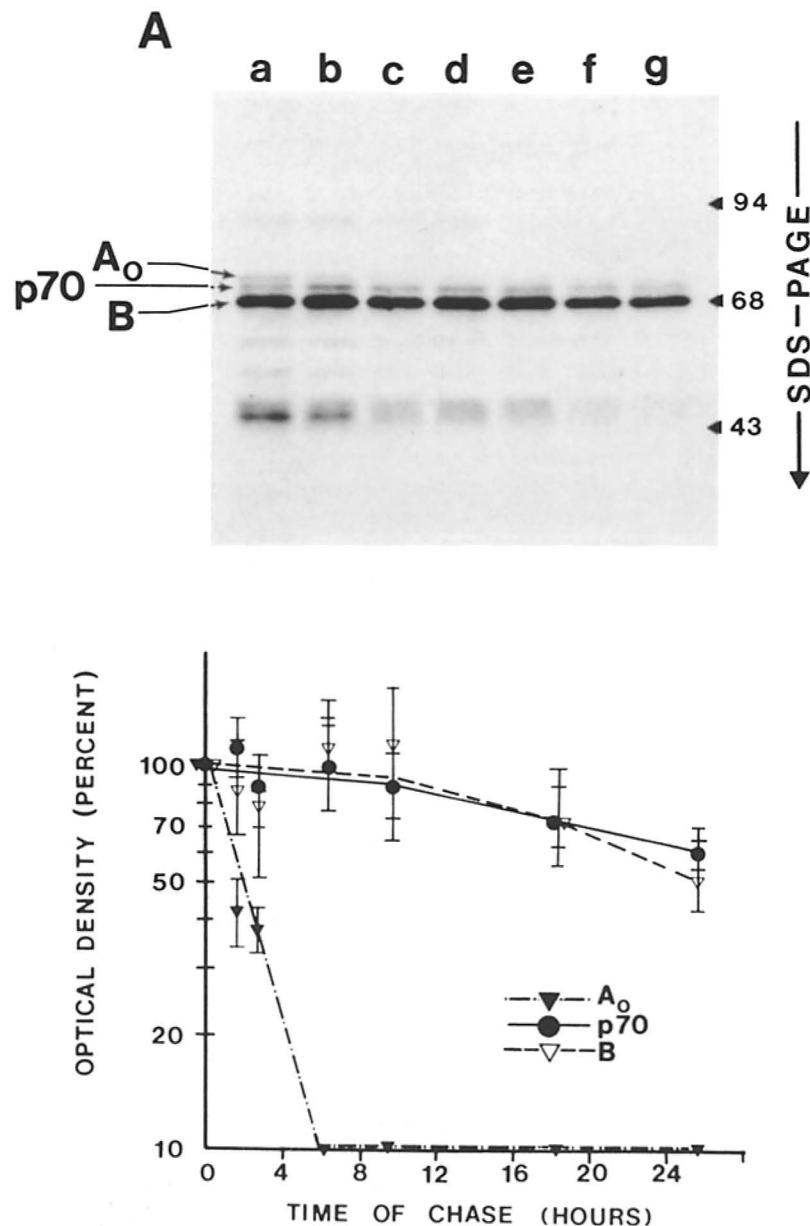
Figure 8. Analysis of the rate of turnover of [³H]isoprenylated prelamin A by SDS-PAGE and fluorography. Mev-1 cells were labeled in medium IV for 17 h with 10 μCi/ml of R,S-[5-³H(N)]mevalonate. To initiate the mevalonate-induced turnover of the [³H]mevalonate–labeled prelamin A, cells (in duplicate) were incubated with medium I containing 1 mM cold mevalonate. At the times indicated, nuclei were prepared (4) and examined by SDS-PAGE and fluorography. Densitometry of the fluorogram in *A* was used to quantitate the rate of loss of the [³H]isoprenylated moiety from the isoprenylated nuclear lamina proteins. (*A*) Fluorogram of the SDS-PAGE analysis of the loss of tritium label from the [³H]isoprenylated nuclear lamina intermediate filament proteins as a function of time of mevalonate chase. (Lane *a*) Control; (lanes *b–g*) time after initiation of mevalonate chase: 1.5 h (lane *b*); 2.5 h (lane *c*); 6.1 h (lane *d*); 9.6 h (lane *e*); 18.4 h (lane *f*); 25.1 h (lane *g*). Molecular mass standards (in kilodaltons) are presented at the right of the fluorogram. Fluorographic exposure was for 90 d. (*B*) Results of densitometric analysis of the fluorogram in *A*. Indicated are the 74-kD isoprenylated prelamin A polypeptide (*solid triangle*), the 70-kD lamina-associated polypeptide (p70, *solid circle*), and lamin B (*open triangle*).

Starvation of the mevalonate auxotroph Mev-1 (Fig. 4) verifies that the inhibition of prelamin A processing is a specific consequence of isoprenoid starvation and is not due to nonspecific mevinolin effects. Furthermore, the inhibition of processing is readily reversible. In mevinolin-treated cells, addition of exogenous mevalonate initiates protein isoprenylation, followed rapidly (Fig. 4) by the in vivo conversion of prelamin A (74 kD) to mature lamin A (72 kD). In vivo studies on the fate of recombinant prelamin A (22, 55), *Xenopus* lamins L_1 and A (29), and chicken lamin B_2 (55), modified by site-directed mutagenesis, have demonstrated the importance of the -C-A-A-M cysteine residue for proper nuclear envelope assembly and proteolytic maturation. Vorburger et al. have demonstrated that the in vitro isoprenylation and proteolytic processing of chicken lamin B_2 are blocked by a cysteine to alanine mutation in the -C-A-A-M motif (55). In addition, they have demonstrated that this processing pathway is mevalonate dependent. Similarly, a cys-

teine to methionine mutation in the -C-A-A-M motif of human prelamin A blocks nuclear envelope association and proteolytic processing (22). These mutational studies on the presumed isoprenylation site of these proteins are also consistent with our proposal that isoprenylation is required for the processing of prelamin A.

Processing of Prelamin A Involves the Loss of the Isoprenylated Moiety

Analysis of mature lamin A by peptide mapping revealed that the entire carboxy terminal -C-A-A-M motif of prelamin A is cleaved during maturation, whereas in lamin B_2 at most three amino acids are lost (55, 56). We have clearly demonstrated (Figs. 1 and 8, and reference 4) that, lamin B retains its isoprenylation, whereas mature lamin A does not. Lamin C, which is missing the carboxy terminal 93 amino acids that are present in lamin A (13, 36), is not isoprenylated (Fig. 1; reference 4), further supporting the premise that the -C-A-A-M

motif is the site of isoprenylation. Sequence analysis (56) of the carboxy-terminal 14-kD fragment of mature murine lamin A has revealed that mature lamin A lacks the carboxy terminal 18 amino acids predicted from the prelamin A cDNA sequence (13, 36), thus providing direct evidence that mammalian prelamin A maturation occurs by carboxy terminal proteolysis. It is reasonable, therefore, to presume that the polypeptide removed from prelamin A during the endoproteolytic processing event contains the isoprenylated moiety. In agreement with this assumption, is our demonstration that the rate of loss of isoprenylation from prelamin A (Fig. 8) is similar to the rate of conversion of prelamin A to lamin A (Figs. 5–7). These lines of evidence, combined with our in vivo demonstration of mevalonate dependency for the conversion of prelamin A to lamin A, are not consistent with the earlier suggestion (32) that the 72-kD lamin A polypeptide arises artifactually.

The data presented here support the hypothesis that the loss of isoprenylation from prelamin A during maturation is due to its localization (presumably the cysteine residue of the -C-A-A-M motif) at the carboxy terminus. Confirmation of this premise must await chemical identification of the derivatized amino acids and direct sequencing of the carboxy terminus of prelamin A.

Prelamin A Maturation: Similarity to Other Systems

The sequence of metabolic events implicated in the maturation of prelamin A is analogous to that described for yeast peptidyl pheromones and *ras* proteins. In *Tremella mesenterica*, the mating pheromone tremerogen A-10, is isoprenylated with a farnesyl substituent in a thioether linkage to the carboxy terminal cysteine residue of the -C-A-A-X motif (39, 40). After isoprenylation, the 26-kD precursor polypeptide becomes membrane bound where it is processed to a 24-kD polypeptide and the 2-kD mature pheromone (39, 40). In like manner, *Saccharomyces cerevisiae* a-mating factor is synthesized as a higher molecular mass precursor and, after isoprenylation of the -C-A-A-X cysteine residue with a farnesyl moiety, undergoes endoproteolytic as well as carboxy terminal processing (3, 46). Other studies have shown that both carboxy terminal processing and membrane localization of p21^{ras} are dependent on isoprenylation (3, 10, 19, 21, 46, 57).

Processing of Prelamin A: A Proposed Model

Our results, together with those presented by a number of other laboratories, enable us to propose the following model for the isoprenylation-dependent maturation of prelamin A. Prelamin A is transported rapidly into the nucleus after translation. We hypothesize that, in analogy to steps described above for the maturation of p21^{ras}, yeast peptidyl pheromones, and chicken lamin B_2, prelamin A is then isoprenylated, presumably at the cysteine of the carboxy terminal -C-A-A-M motif. This facilitates its association with the nuclear membrane, the site of the subsequent proteolytic removal of three C-terminal amino acid residues. This carboxypeptidase-type processing of prelamin A yields a prelamin A intermediate containing an isoprenylated carboxy terminal cysteine. The nature of the isoprenylation reaction is not yet understood, however, in preliminary studies we have found that nonisoprenylated prelamin A is transported

into the nucleus. In the final stage of the maturation pathway, a membrane-bound endopeptidase cleaves an additional polypeptide from the carboxy terminus of prelamin A. The loss of this polypeptide, which presumably contains the isoprenoid substituent, accounts for the 2-kD molecular mass shift observed during maturation of prelamin A. Whether other posttranslational modifications are also involved in the altered mobility observed during processing of prelamin A is still not known.

Additional mammalian proteins that undergo an isoprenylation-dependent proteolytic maturation most likely exist since mevinolin treatment has been shown to alter the electrophoretic mobility of a number of cytosolic, isoprenylated proteins in murine erythroleukemic cells (44). The variability in the fate of the isoprenylated moiety, and the proteins so modified, advances the possibility of different isoprenoid substituents, multiple prenyl-transferases, endopeptidases, and/or carboxy peptidases involved in this pathway for proteolytic maturation.

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