

# The CaaX Motif Is Required for Isoprenylation, Carboxyl Methylation, and Nuclear Membrane Association of Lamin B<sub>2</sub>

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**Abstract.** Recent evidence suggests that the conserved COOH-terminal CaaX motif of nuclear lamins may play a role in targeting newly synthesized proteins to the nuclear envelope. We have shown previously that in rabbit reticulocyte lysates the cysteine residue of the CaaX motif of chicken lamin B<sub>2</sub> is necessary for incorporation of a derivative of mevalonic acid, the precursor of isoprenoids. Here we have analyzed the properties of normal and mutated forms of chicken lamin B<sub>2</sub> stably expressed in mouse L cells. Mutation of the cysteine residue of the CaaX motif to alanine or introduction of a stop codon immediately after the

cysteine residue was found to abolish both isoprenylation and carboxyl methylation of transfected lamin B<sub>2</sub>. Concomitantly, although nuclear import of the mutant lamin B<sub>2</sub> proteins was preserved, their association with the inner nuclear membrane was severely impaired. From these results we conclude that the COOH-terminal CaaX motif is required for isoprenylation and carboxyl methylation of lamins *in vivo*, and that these modifications are important for association of B-type lamins with the nucleoplasmic surface of the inner nuclear membrane.

**T**HE nuclear lamina is a fibrous protein layer lining the nucleoplasmic surface of the inner nuclear membrane. It is believed to stabilize the nuclear envelope and to play a role in organizing the distribution of interphase chromatin (for reviews see Gerace and Burke, 1988; Nigg 1988, 1989; Burke 1990). Its major constituents, the nuclear lamins, are members of the intermediate filament protein family (McKeon et al., 1986; Fisher et al., 1986; for reviews see Franke, 1987; Stewart, 1990). Based on biochemical properties and structural comparisons, lamins can be classified as either A- or B-type (e.g., Burke and Gerace, 1986; Höger et al., 1988; Peter et al., 1989). During mitosis, when the nuclear envelope breaks down, A-type lamins disperse as soluble oligomers, whereas B-type lamins, although similarly depolymerized, remain associated with remnants of the nuclear membrane (Gerace and Blobel, 1980; Stick et al., 1988). The reversible mitotic depolymerization of the nuclear lamina has long been shown to correlate closely with transient hyperphosphorylation of the lamins (Gerace and Blobel, 1980), and recent results point to a direct role of the mitotically activated *cdc2* kinase in controlling this process (Peter et al., 1990; see also Ward and Kirschner, 1990; Heald and McKeon, 1990).

Analysis of the amino acid sequences of B-type lamins has not revealed any particularly hydrophobic domains that might explain their preferential membrane association (Krohne et al., 1987; Höger et al., 1988, 1990; Peter et al., 1989;

Vorburger et al., 1989a). It is possible, therefore, that B-type lamins may bind to integral protein components of the inner nuclear membrane (Worman et al., 1988; see also Senior and Gerace, 1988; Padan et al., 1990). Alternatively, membrane association of lamin proteins might be facilitated by posttranslational modifications with lipophilic substituents. Specifically, lamin proteins have been shown to be both isoprenylated (Beck et al., 1988; Wolda and Glomset, 1988; Vorburger et al., 1989b; Farnsworth et al., 1989; Pollard et al., 1990) and carboxyl methylated (Chelsky et al., 1987, 1989). Whereas both A- and B-type lamins are modified in this fashion shortly after synthesis, lamin A is subject to an additional proteolytic processing event (Laliberté et al., 1984; Gerace et al., 1984; Dagenais et al., 1985; Lehner et al., 1986a), which results in removal of the hydrophobically modified COOH-terminal cysteine residue (Beck et al., 1988; Vorburger et al., 1989b; Weber et al., 1989).

The interpretation of the posttranslational processing occurring in lamin proteins has been greatly facilitated by the realization that all known members of this protein family, with the exception of mammalian lamin C (McKeon et al., 1986; Fisher et al., 1986), contain a COOH-terminal motif commonly referred to as the "CaaX box" (C = cysteine, a = aliphatic, X = any amino acid). Such motifs are also conserved at the COOH termini of several other proteins, most notably members of the *ras* (proto-) oncogene family, small GTPbinding (G-) proteins, and yeast mating pheromones (reviewed in Clarke et al., 1988; Magee and Hanley, 1988). As shown conclusively for several of these proteins, the COOH-terminal CaaX tetrapeptide is the substrate for a series of

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posttranslational modifications, namely isoprenylation, followed by proteolytic removal of the three-COOH-terminal amino acids, and carboxyl methylation of the resulting COOH-terminal isoprenylated cysteine residue (for reviews see Glomset et al., 1990; Rine and Kim, 1990).

In experiments designed to determine if the CaaX motif of lamin proteins is isoprenylated, we showed previously that the cysteine of the CaaX motif of chicken lamin B<sub>2</sub> is essential for incorporation, in a rabbit reticulocyte lysate, of a moiety derived from mevalonic acid, the precursor of isoprenoids (Vorburger et al., 1989b; see also Pollard et al., 1990). The corresponding substituent in human lamin B was identified as farnesyl (Farnsworth et al., 1989). Evidence was also obtained to indicate that, subsequent to isoprenylation, lamin B<sub>2</sub> is subject to proteolytic removal of three COOH-terminal amino acids (Vorburger et al., 1989b). By analyzing stably transfected mouse cell lines expressing wild-type and mutant forms of chicken lamin B<sub>2</sub>, we now demonstrate that the cysteine residue of the COOH-terminal CaaX motif is required for both isoprenylation and carboxyl methylation *in vivo*. In addition, we extend recent findings from other laboratories (Krohne et al., 1989; Holtz et al., 1989) by demonstrating the importance of these modifications for correct targeting of B-type lamin proteins to the nuclear membrane.

## Materials and Methods

### Site-directed Mutagenesis and Plasmid Constructions

Mutations in the coding region of the chicken lamin B<sub>2</sub> cDNA (Vorburger et al., 1989a) were introduced by oligonucleotide-directed mutagenesis (Kunkel et al., 1987), using the BioRad Muta-Gene kit. Individual cysteine (Cys) TGC codons (for either Cys191 or Cys597, or both residues together) were changed to GCC codons specifying alanines (Fig. 1 A). To delete the last three amino acids at the COOH terminus (i.e., the three amino acids of the CaaX motif believed to be proteolytically cleaved from the lamin B<sub>2</sub> precursor; Vorburger et al., 1989b), a termination codon (TGA) was introduced immediately after Cys597 (Fig. 1 A). All mutations were verified by nucleotide sequence analysis. Moreover, mutant cDNAs were subcloned into pGEM vectors (Promega Biotec, Madison, WI) and successfully used for *in vitro* translation studies (Vorburger et al., 1989b; Peter M., G. T. Kitzen, and E. A. Nigg, unpublished results), indicating that none of the mutations adversely affected translation of the mutated lamin B<sub>2</sub> proteins. For the *in vivo* expression studies reported here, the constructs were subcloned into the pHTRcDEX vector (Fig. 1 B), obtained from Dr. L. Kühn (ISREC). This vector is a modification of pcD-TR-1 construction 10 described previously (Owen and Kühn, 1987): the Eco RV site at position +12 from the cap site and a BamHI site in the segment carrying the SV-40 late region polyadenylation signal were changed to EcoRI sites to facilitate direct cloning of Eco RI fragments. In this vector transcription initiation is directed by a 322-bp genomic segment derived from the human transferrin receptor promoter, and 3' processing of the transcript is directed by an SV-40 polyadenylation signal.

### Transfection of Mouse L tk<sup>-</sup> Cells

Thymidine-kinase-deficient (tk<sup>-</sup>) mouse L cells, kindly provided by Dr. L. Kühn (ISREC), were cultured in 6% CO<sub>2</sub> at 37°C in standard medium (alpha minimal essential medium without nucleosides, supplemented with 10% heat-inactivated FCS and 50 µg/ml gentamicin). 15 µg of pHTRcDEX plasmid containing either the wild-type chicken lamin B<sub>2</sub> cDNA construct or the various mutated versions of this cDNA was transfected into L tk<sup>-</sup> cells (2 × 10<sup>5</sup>/6 cm plate), using the calcium phosphate precipitation method (Graham and van der Eb, 1973). Each plasmid was cotransfected with a plasmid containing the thymidine kinase gene of herpes simplex type 1 virus (100 ng pTK-x1; Enquist et al., 1979). Control cells were transfected with the pTK-x1 plasmid alone. In initial transfections 10 µg of carrier L

tk<sup>-</sup> genomic DNA was also included, but was found to be unnecessary and omitted in subsequent experiments. Precipitates were allowed to remain on the cells for 16–18 h after which cells were rinsed twice with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2) and then refed standard medium for 24 h. Cells were then passaged at low density to 10-cm plates and fed HAT medium (standard medium containing 100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine). To assess the transient expression of lamin B<sub>2</sub>, an aliquot of cells was seeded onto coverslips, cultured an additional 24 h in standard medium and then processed for immunofluorescent staining (see below). After 10–14 d, with HAT medium changed every 3 d, several individual colonies were isolated and subcloned to purity. The remaining transfectants were pooled. After multiple passages in HAT medium, the transfected L tk<sup>-</sup> cell lines were transferred to standard medium. The level of expression of lamin B<sub>2</sub> in each transfectant was determined by immunoblotting experiments and compared to the level of expression of endogenous mouse lamin B<sub>2</sub>. DU249 chicken hepatoma cells (Langlois et al., 1974) were cultured as described elsewhere (Nakagawa et al., 1989).

### Biosynthetic Labeling and Immunoprecipitation

10-cm plates of subconfluent (70–80%) cells were pretreated for 1–2 h with 30 µM mevinolin in standard medium before labeling for 18–20 h in the same medium containing 3 µCi/ml R-(2-<sup>14</sup>C) mevalonate (56.7 µCi/mmol, CFA 600; Amersham Corp., Arlington Heights, IL). Duplicate cultures were pretreated for 1 h with methionine-free DMEM supplemented with 4% dialyzed FCS and 50 µg/ml gentamicin and then labeled for 18–20 h with 30 µCi/ml [<sup>35</sup>S]methionine (SJ 204; Amersham Corp.) in the presence or absence of mevinolin (30 µM). Mevinolin was a gift from Dr. A. W. Alberts (Merck, Sharp & Dohme, Rahway, NJ) and was prepared as described previously (Kita et al., 1980).

Chicken lamin B<sub>2</sub> was immunoprecipitated from transfected mouse L cells using a rabbit serum specific for chicken lamins (Lehner et al., 1986b) as described previously (Nakagawa et al., 1989), except that protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was used for isolation of immune complexes, and samples were precleared with protein A Sepharose for 30 min before the addition of the primary antibody. After immunoprecipitation, labeled proteins were separated on 8% SDS polyacrylamide gels and visualized by fluorography using EnHance (Dupont Corp., Wilmington, DE).

### Methylation Assays

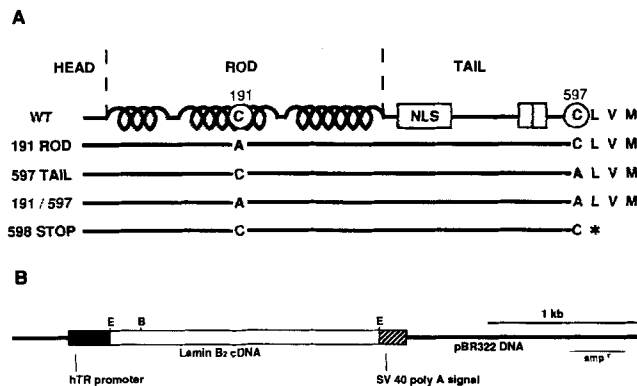
10-cm plates of subconfluent cells were pretreated for 1 h with methionine-free DMEM, as described above, and then labeled for 18–20 h in medium containing 10 µCi/ml L-[methyl-<sup>3</sup>H]methionine (15 Ci/mmol, TRK 209; Amersham Corp.). Lamin B<sub>2</sub> was immunoprecipitated, resolved by SDS-PAGE, and analyzed by fluorography. Bands corresponding to lamin B<sub>2</sub> were excised from the gels, which had been dried between two sheets of cellophane, and then treated for 16–20 h with 1.0 N NaOH to release esterified methyl groups as described elsewhere (Chelsky et al., 1984; Deschenes et al., 1989). The label released as [<sup>3</sup>H]methanol, representing methylation, and the label remaining in the proteins, representing [<sup>3</sup>H]methionine, were determined by scintillation counting. To determine non-specific background, several bands were cut out of lanes containing aliquots of labeled whole cell extracts.

### Detergent Solubilization Assays

Extracts of interphase and mitotic cells were prepared as described previously (Nakagawa et al., 1989). Briefly, cells grown to 70–80% confluency were allowed to either continue growing normally overnight or were treated with nocodazole (500 ng/ml; Janssen Pharmaceutica, Beerse, Belgium) to obtain cells in mitotic arrest. After harvesting and homogenizing the cells, the lysates were divided into two aliquots, one of which was treated with 1% Triton X-100. Each aliquot was then fractionated into a supernatant and a pellet by centrifugation (12,000 g, 15 min) and equivalent amounts of each fraction were separated by SDS-PAGE. Immunoblotting was carried out as described elsewhere (Borer et al., 1989) using a mouse mAb against chicken lamin B<sub>2</sub> (L-20; Lehner et al., 1986b), followed by <sup>125</sup>I-labeled sheep anti-mouse IgG antibody (IM131; Amersham Corp.).

### Immunofluorescent Localization of Lamin B<sub>2</sub>

Cells were seeded onto coverslips and after incubation for 18–24 h were



**Figure 1.** Lamin B<sub>2</sub> mutations and pHTRcDEX expression vector. (A) Diagrammatic representation of wild type (WT) and mutated forms of the chicken lamin B<sub>2</sub> protein. Cys (C) to Ala (A) mutations were obtained using site-directed mutagenesis of lamin B<sub>2</sub> cDNAs (see Materials and Methods). A stop codon (\*) immediately follows Cys597 in the 598 STOP mutant. The head, rod, and tail domains as well as the nuclear localization signal (NLS; Loe-winger and McKeon, 1988) are indicated. Note that the COOH-terminal four amino acid residues of wild-type lamin B<sub>2</sub>, Cys Leu Val Met, fit the criteria of a CaaX motif (C = Cys, a = aliphatic, X = any amino acid). (B) Schematic diagram of the pHTRcDEX vector. Transcription of a cDNA encompassing the entire open reading frame of lamin B<sub>2</sub> is directed by a 322-bp fragment of the human transferrin receptor (*hTR*) promoter and is terminated by an SV-40 polyadenylation signal. Relevant restriction sites are indicated: E, Eco RI; B, Bgl II.

fixed in 3% paraformaldehyde, 2% sucrose in PBS for 5 min. They were then rinsed with PBS, permeabilized for 5 min with cold (4°C) 0.5% Triton X-100 in PBS, rinsed again with PBS, and incubated with a 1:1,000 dilution of mAb L-20 ascites (see above). After several washes in PBS, the cells were treated with a 1:500 dilution of rhodamine-conjugated goat anti-mouse IgG antibody (Cappel Laboratories, Malvern, PA). Coverslips were mounted with 90% glycerol, 10% Tris-HCl (pH 9.0) and viewed at 100x magnification, using a Reichert-Jung Optivar fluorescence microscope.

## Results

### Expression of Wild-Type and Mutated Chicken Lamin B<sub>2</sub> in Mouse L Cells

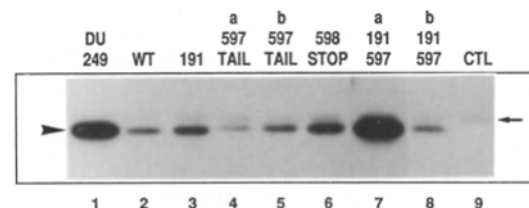
To assess the role of the CaaX motif in posttranslational modifications of lamin proteins *in vivo*, and the significance of these modifications for mediating the association of B-type lamins with the nuclear envelope, we transfected and stably expressed wild-type and mutated forms of chicken lamin B<sub>2</sub> in cultured mouse cells. These experiments were based on the premise that transfected chicken lamin B<sub>2</sub> would interact with the endogenous mouse nuclear lamina and membrane in a normal fashion. This assumption appeared justified in view of the high degree of structural similarity between murine and avian B-type lamin proteins (Höger et al., 1988, 1990; Peter et al., 1989; Vorburger et al., 1989a), and it was supported by the previous demonstration that chicken lamin B<sub>2</sub> localized correctly to the nuclear membrane when expressed in interspecies (chicken-mouse) heterokaryons (Borer et al., 1989).

Based on our previous *in vitro* data (Vorburger et al., 1989b) as well as evidence from other laboratories (Krohne et al., 1989; Holtz et al., 1989; Farnsworth et al., 1989), the

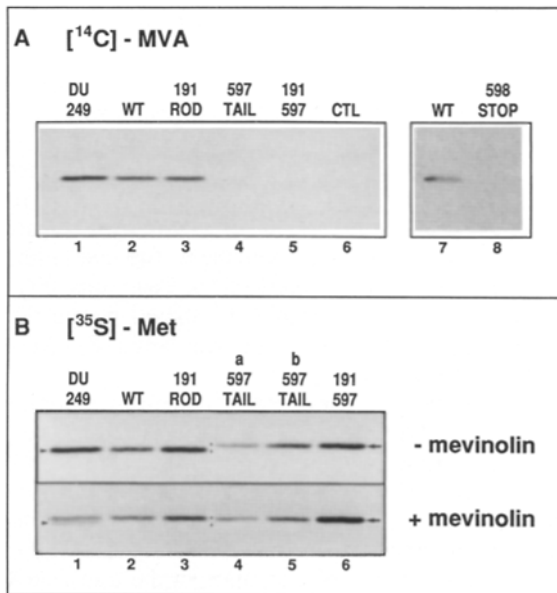
cysteine residue located four amino acids from the COOH terminus of lamin B<sub>2</sub> was expected to be the site of isoprenylation. Accordingly, we constructed a mammalian expression vector containing either the full-length wild-type chicken lamin B<sub>2</sub> coding sequence or lamin B<sub>2</sub> with mutations at either one, or both, of the two cysteine residues (Cys191 and Cys597) which occur normally in the native protein (Fig. 1, see also Vorburger et al., 1989a, b). An additional mutation was made by introducing a stop codon immediately after the cysteine residue of the CaaX box (Fig. 1). This truncated version of lamin B<sub>2</sub> should mimic the primary sequence of mature, processed lamin B<sub>2</sub> (Vorburger et al., 1989b).

From each transfection experiment, numerous colonies were obtained. Although some of the transfectants had noticeably different morphologies, doubling times or adhesion properties, no correlation could be made with a specific mutation, suggesting that these variations were most probably related to the amounts of lamin proteins expressed and/or to differences in the site(s) of integration of the transfected plasmids. For each mutation, several individual colonies were isolated and subcloned to establish homogeneous cell lines; these were named according to the type of transfected lamin B<sub>2</sub> they contained (Fig. 1). The remainder of the colonies were pooled. Results obtained from colony pools were in agreement with those obtained with subcloned cell lines (not shown). Where appropriate, control cells were obtained from a pool of mouse L cells transfected with the thymidine kinase selection plasmid alone.

The stably transfected cell lines used here were selected for detailed studies because, as judged by both Northern analyses (not shown) and immunoblotting experiments (Fig. 2), they expressed similar amounts of chicken lamin B<sub>2</sub>.



**Figure 2.** Immunoblot analysis of chicken lamin B<sub>2</sub> expression in transfected mouse L cell lines. Homogenates representing  $2 \times 10^5$  cells from each cell line were separated by SDS-PAGE and then transferred to nitrocellulose. After incubation with a mAb (E-3) that recognizes both chicken and mouse lamin B<sub>2</sub>, reactive proteins were visualized with <sup>125</sup>I-labeled secondary antibody. Chicken and mouse lamin B<sub>2</sub> proteins can be differentiated since they migrate with slightly different mobilities. The arrowhead marks the migration of chicken lamin B<sub>2</sub>, and the arrow marks the migration of the mouse homologue. Although clones expressing comparatively high amounts of chicken lamin B<sub>2</sub> were isolated (e.g., the 191/597a transfectant; lane 7), most of the transfected mouse cell lines used in the studies presented here expressed chicken lamin B<sub>2</sub> at levels lower than seen in chicken DU249 cells (lane 1). Also, note that endogenous mouse lamin B<sub>2</sub> is expressed at approximately the same levels in all transfectants, including the transfection control cells which do not express chicken lamin B<sub>2</sub> (arrow). For designation of the various lamin B<sub>2</sub> mutants see Fig. 1, a and b denote independently isolated transfectants. CTL: transfection control.



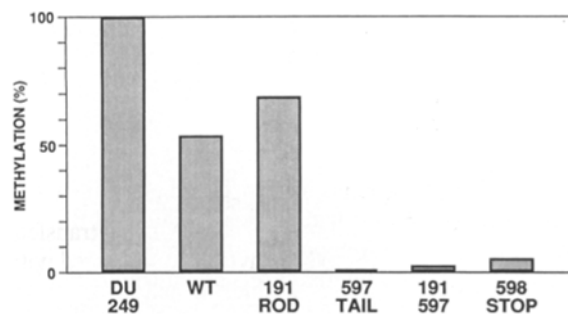
**Figure 3.** In vivo labeling of lamin B<sub>2</sub> by [<sup>14</sup>C]mevalonate and effect of isoprenylation on electrophoretic mobility of wild-type and mutant lamin B<sub>2</sub>. (A) In vivo incorporation of [<sup>14</sup>C]mevalonate into lamin B<sub>2</sub>. Wild-type (WT) and mutated forms of chicken lamin B<sub>2</sub> were immunoprecipitated from transfected mouse L cells that had been cultured in the presence of [<sup>14</sup>C]mevalonic acid (MVA) for 20 h. Samples were analyzed by SDS-PAGE and fluorography. Radiolabeled bands migrating to the same position as the native lamin B<sub>2</sub> immunoprecipitated from chicken DU249 cells (lane 1) were present in wild type (lanes 2 and 7) and 191 ROD (lane 3)-transfected mouse cells. Labeled lamin B<sub>2</sub> was not detected in 597 TAIL (lane 4), 191/597 (lane 5), or 598 STOP (lane 8) transfectants, nor in control (CTL) mouse L cells (lane 6). Results from two independent experiments are shown. Only the relevant parts of the gels are shown. (B) CaaX mutation or inhibition of isoprenoid biosynthesis with mevinolin blocks formation of mature (faster migrating) lamin B<sub>2</sub>. Duplicate cultures of those used in A were labeled with [<sup>35</sup>S]methionine in the presence or absence of 30 μM mevinolin for 20 h. Then lamin B<sub>2</sub> was immunoprecipitated and analyzed as above. Care was taken to run the gel under optimal conditions to visualize minor differences in electrophoretic mobilities. In the absence (-) of mevinolin (top), lamin B<sub>2</sub> from wild-type (WT; lane 2) and 191 ROD transfectants (lane 3) migrated with the same mobility as native, mature lamin B<sub>2</sub> from chicken DU249 cells (lane 1; arrowhead). In contrast, lamin B<sub>2</sub> isolated from 597 TAIL (lanes 4 and 5; two different cell clones) and 191/597 mutants (lane 6) migrated with a clearly slower mobility. Previous in vitro data indicated that this slower migrating band represents the nonisoprenylated precursor of lamin B<sub>2</sub> (Vorburger et al., 1989b). In the presence (+) of mevinolin (bottom), lamin B<sub>2</sub> from DU249, WT and 191 ROD cells also migrated with a reduced mobility (lanes 1-3; note a trace amount of mature lamin B<sub>2</sub> in lane 1; arrowhead), and thus comigrated precisely with the 597 TAIL and the 191/597 mutants (lanes 4-6). No [<sup>35</sup>S]methionine-labeled chicken lamin B<sub>2</sub> was immunoprecipitated from control mouse cells (not shown).

Most studies were done with cells lines expressing approximately equal levels of mutant and wild-type chicken lamin B<sub>2</sub> (Fig. 2, compare lane 2 with lanes 3-6 and 8), but results were very similar over quite a large range of expression levels (e.g., Fig. 2, lane 7). Also, we note that levels of chicken lamin B<sub>2</sub> in high-expressor mouse cell lines did not significantly exceed those observed in chicken cells,

where lamin B<sub>2</sub> is the predominant B-type lamin (Fig. 2, compare lanes 7 and 1). Finally, we emphasize that expression of chicken lamin B<sub>2</sub> in L cells did not detectably influence the levels of expression of endogenous mouse lamin B<sub>2</sub> (Fig. 2, see arrow). This information could be obtained by exploiting the observation that mouse and chicken lamin B<sub>2</sub> proteins differ slightly in their electrophoretic mobility, and by using, for this particular experiment, an mAb (E-3) which displays some cross-reactivity with mammalian lamin B<sub>2</sub> (Lehner et al., 1986b). In all other experiments shown in this study, chicken-specific mAbs were used for detection of ectopically expressed lamin B<sub>2</sub>.

### An Intact CaaX Motif Is Required for Both Isoprenylation and Carboxyl Methylation of Lamin B<sub>2</sub>

In earlier experiments, we found that Cys597 of chicken lamin B<sub>2</sub> is essential for in vitro modification by a mevalonic acid derivative, and that this modification produces a slight increase in the electrophoretic mobility of lamin B<sub>2</sub> (Vorburger et al., 1989b). Because an identical mobility shift occurs shortly after synthesis of lamin B<sub>2</sub> in vivo (Lehner et al., 1986a), it seemed reasonable to assume that conversion of the lamin B<sub>2</sub> precursor to a faster migrating form might be used to monitor isoprenylation of this protein in vivo (see below). To examine whether or not isoprenoid modification of lamin B<sub>2</sub> in vivo requires Cys597 within an intact CaaX motif, cell lines expressing wild-type and mutated forms of chicken lamin B<sub>2</sub> were cultured in the presence of [<sup>14</sup>C]mevalonic acid. Parallel cultures of the same cell lines were incubated with [<sup>35</sup>S]methionine, in the presence or absence of mevinolin, an inhibitor of isoprene biosynthesis. Lamin B<sub>2</sub> was then immunoprecipitated and incorporation of a mevalonic acid derivative was analyzed by autoradiography. The lamin B<sub>2</sub> proteins precipitated in par-



**Figure 4.** Methylation of wild type and mutated forms of lamin B<sub>2</sub>. Wild-type (WT) and mutated forms of chicken lamin B<sub>2</sub> were immunoprecipitated from transfected mouse L cell which had been cultured in the presence of [methyl-<sup>3</sup>H]methionine for 20 h. Radioactivity due to [<sup>3</sup>H] methyl groups attached via an ester linkage and that due to incorporation of methionine into protein was determined separately (see Materials and Methods). For calibration, methylation counts were standardized against protein counts and expressed here as the percentage of the corresponding value determined for chicken DU249 cells (100%). Lamin B<sub>2</sub> isolated from WT and 191 ROD transfectants was methylated, but not to as great an extent as from DU249 cells. Very low values were observed in lamin B<sub>2</sub> from 597 TAIL, 191/597, and 598 STOP transfectants; this residual radioactivity most likely represents background.

allele from the [<sup>35</sup>S]methionine labeled cells were used to monitor mobility shifts indicative of isoprenylation.

As shown in Fig. 3 A, lamin B<sub>2</sub> immunoprecipitated from chicken DU249 cells was readily labeled by [<sup>14</sup>C]mevalonic acid (lane 1), as were wild-type and 191 ROD chicken lamin B<sub>2</sub> proteins immunoprecipitated from transfected mouse cells (lanes 2, 3, and 7). Conversely, no incorporation of label was observed when lamin B<sub>2</sub> proteins were immunoprecipitated from the 597 TAIL and 191/597 transfectants (lanes 4 and 5), nor was there any label detected in immunoprecipitates from control (CTL) mouse L cells (lane 6). Likewise, no mevalonic acid derivative was incorporated into lamin B<sub>2</sub> isolated from the 598 STOP cell line (lane 8). Fig. 3 B summarizes the results of the immunoprecipitations from [<sup>35</sup>S]methionine-labeled cells. These data illustrate that lamin B<sub>2</sub> was immunoprecipitated with similar efficiencies from all cell lines. Moreover, they demonstrate that lamin B<sub>2</sub> proteins carrying CaaX mutations display a slower electrophoretic migration than wild-type lamin B<sub>2</sub> or lamin B<sub>2</sub> carrying the 191 ROD mutation (Fig. 3 B, top; compare lanes 4–6 with lanes 1–3). Treatment of cells with mevillin caused an identical retardation in electrophoretic mobility for all lamin B<sub>2</sub> proteins, due to inhibition of isoprene biosynthesis (Fig. 3 B, bottom; note the presence of a trace of faster migrating, mature lamin B<sub>2</sub> in lane 1). These results demonstrate that CaaX box mutations interfere with *in vivo* isoprenylation of this protein, and they confirm the notion that conversion of lamin B<sub>2</sub> to a faster migrating form reflects isoprenylation (Vorbürger et al., 1989b). Although no attempt was made in the present study to precisely determine the structure of the isoprenoid substituent on chicken lamin B<sub>2</sub>, we note that analysis of human lamin B by gas chromatography and mass spectrometry identified a farnesyl group attached to cysteine via a thioether linkage (Farnsworth et al., 1989).

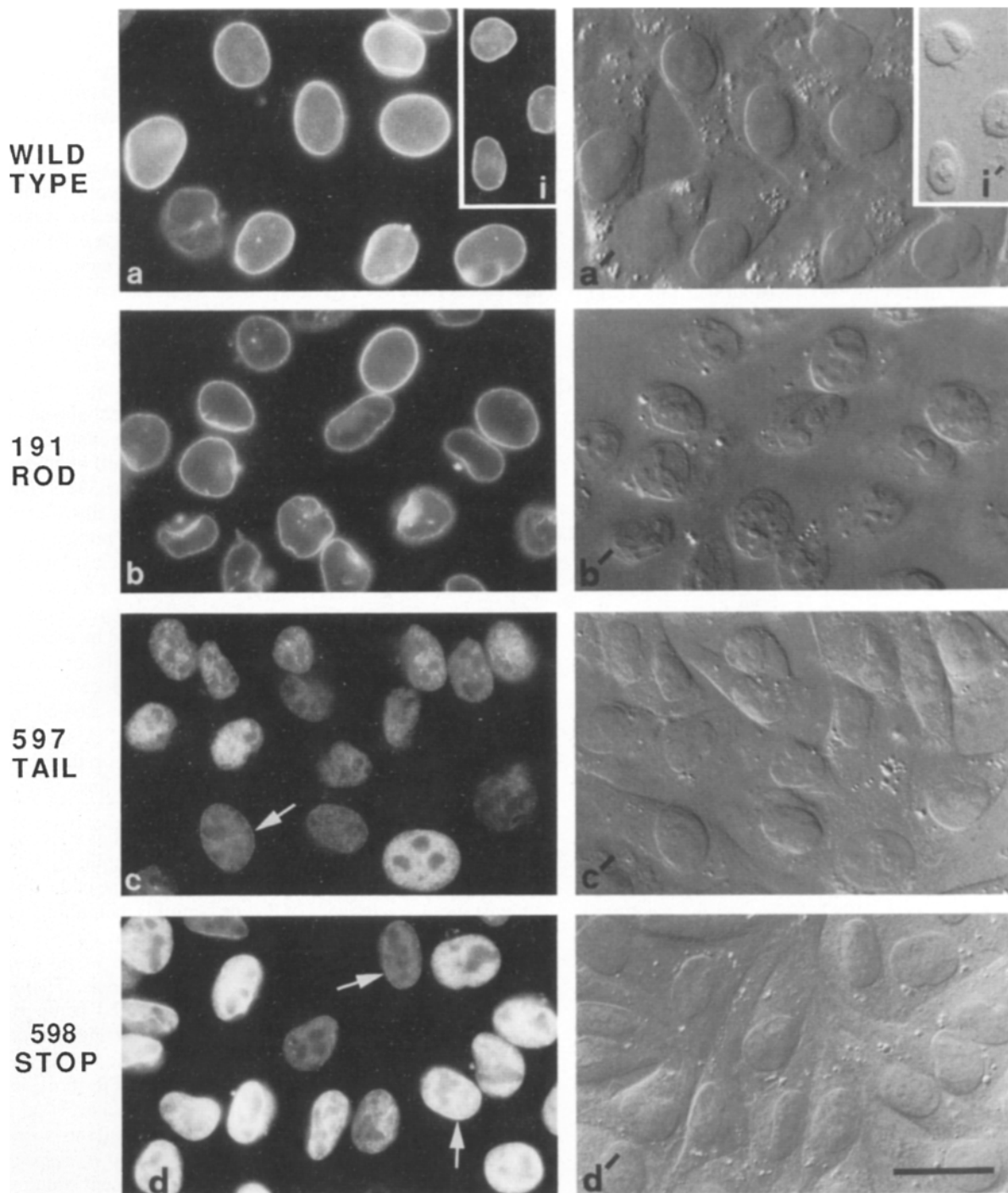
B-type lamin proteins have also been shown to be carboxyl methylated (Chelsky et al., 1987, 1989), but the identity of the modified residue(s) had not previously been determined. We therefore assayed the methylation state of wild-type and mutated forms of chicken lamin B<sub>2</sub>, employing the stably transfected cell lines described above. For these experiments cells were labeled with L-[methyl-<sup>3</sup>H]methionine, lamin B<sub>2</sub> proteins were immunoprecipitated, and the extent of carboxyl methylation determined by a vapor phase methyl release assay (Chelsky et al., 1984). As shown in Fig. 4, lamin B<sub>2</sub> proteins isolated from wild-type and 191 ROD transfectants were extensively carboxyl methylated. Compared with lamin B<sub>2</sub> isolated from DU249 chicken cells, the transfected lamin B<sub>2</sub> appeared to be modified to a somewhat lower degree (Fig. 4); the significance of this observation is presently unclear. However, and most importantly, mutant lamin B<sub>2</sub> proteins isolated from the 597 TAIL, 191/597, or 598 STOP transfectants were essentially unmethylated (Fig. 4). These results support the notion that both isoprenylation and carboxyl methylation of lamin B<sub>2</sub> occur on Cys597 of the CaaX motif. In addition, they indicate that the COOH-terminal cysteine is the only major site of methylation on lamin B<sub>2</sub>, and that a COOH-terminal cysteine is not a substrate for carboxyl methylation unless it has been isoprenylated. This latter conclusion is also supported by recent studies with a purified carboxyl methyltransferase (Stephenson and Clarke, 1990).

### Mutations of the CaaX Motif Perturb Lamin B<sub>2</sub> Localization

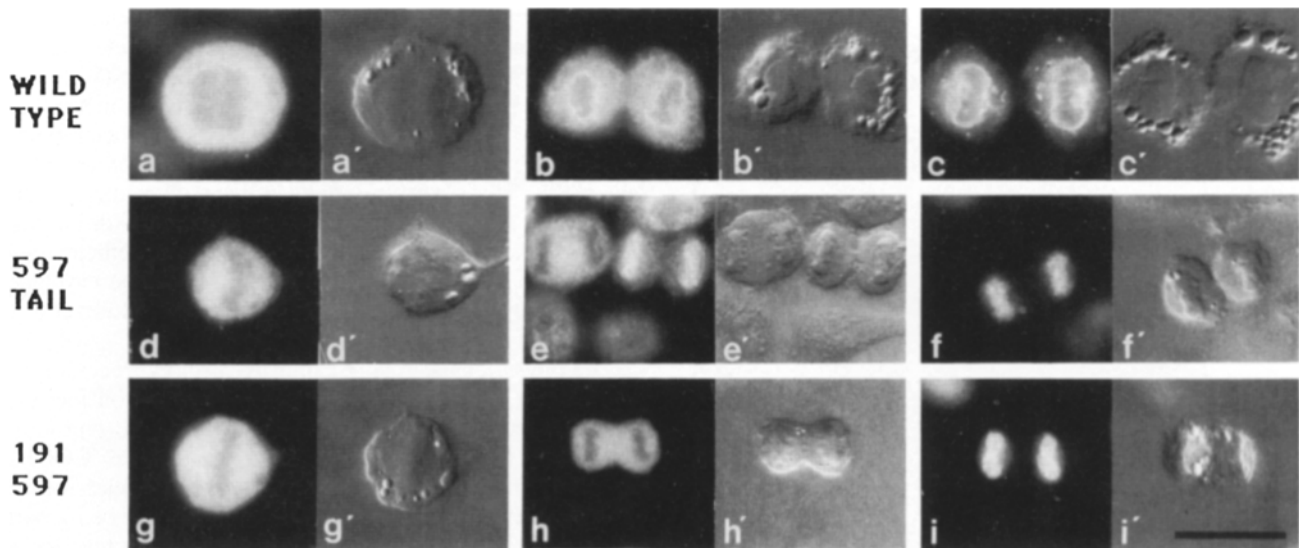
Fig. 5 illustrates the subcellular distribution, as analyzed by indirect immunofluorescence microscopy, of wild-type and mutated chicken lamin B<sub>2</sub> proteins expressed in mouse L cells. As shown in Fig. 5 a, expression of lamin B<sub>2</sub> in cells transfected by a wild-type cDNA resulted in a typical lamin staining pattern. At the level of resolution of these assays, the distribution of the exogenous lamin B<sub>2</sub> in mouse cells was indistinguishable from the one observed for the endogenous protein in chicken cells (Fig. 5 i, inset). A characteristic lamin staining was also observed with the 191 ROD mutant form of lamin B<sub>2</sub> (Fig. 5 b), suggesting that the cysteine residue in the rod domain of lamin B<sub>2</sub>, although conserved in B-type lamins (e.g., Höger et al., 1988, 1990; Vorbürger et al., 1989a; Peter et al., 1989), does not play an essential role in targeting the protein to the nuclear envelope. In contrast, in cells expressing mutants in which the CaaX motif had been altered, i.e., either the 597 TAIL (Fig. 5 c) or the 598 STOP mutant (Fig. 5 d), the bulk of lamin B<sub>2</sub> was distributed throughout the nucleoplasm. Only a fraction of mutant protein appeared to be properly localized to the nuclear lamina (Fig. 5, c and d, arrows), possibly due to polymer formation with endogenous mouse lamins. This residual lamina-associated lamin B<sub>2</sub> could more readily be visualized by extracting the nucleoplasmic lamin B<sub>2</sub> with detergent before fixation and immunofluorescent staining of the cells (not shown). Finally, to the extent that this could be studied by immunofluorescence microscopy using antibodies specific for mouse lamins, we did not obtain evidence for redistributions of endogenous lamins in any of the cell lines investigated (not shown).

The above observations suggested that mutations affecting the CaaX motif perturbed the interactions of lamin B<sub>2</sub> with the nuclear envelope of interphase cells. Since one of the distinguishing characteristics of B-type lamins is their ability to remain associated with membranes throughout mitosis, it was of interest to determine if these mutations would also affect the distribution of lamin B<sub>2</sub> during mitosis. Therefore, the distribution of the various transfected lamin B<sub>2</sub> proteins was examined in spontaneously occurring mitotic cells (Fig. 6). During metaphase and anaphase, wild-type lamin B<sub>2</sub> was distributed throughout the cell (Fig. 6 a), as is characteristic of lamin proteins (e.g., Lehner et al., 1986b). 597 TAIL and 191/597 lamin B<sub>2</sub> mutants were similarly dispersed (Fig. 6, d and e and g and h, respectively); at these early stages of mitosis, fluorescent staining was homogeneous and virtually identical in all other lines analyzed, including 191 ROD, 598 STOP and DU249 cells (not shown).

At the end of mitosis, lamin B<sub>2</sub> progressively reassociated with the reforming daughter cell nuclei in all of the cell lines. However, when compared with wild-type lamin B<sub>2</sub>, a noticeable difference was observed in the behavior of the mutant lamins in which the CaaX motif had been altered. In the wild-type transfectant (Fig. 6, b and c) a pronounced staining of the telophase chromosome surface became visible, presumably reflecting reformation of a nuclear lamina structure concomitant with reformation of the nuclear membranes. In contrast, although mutant lamin B<sub>2</sub> proteins accumulated within reforming nuclei in 597 TAIL and 191/597



**Figure 5.** Immunofluorescent localization of wild-type and mutated chicken lamin B<sub>2</sub> in mouse L cells. Stably transfected mouse L cells expressing approximately equivalent amounts of wild type or mutated forms of chicken lamin B<sub>2</sub> were cultured on coverslips for 18–24 h and then prepared for immunofluorescence microscopy (*left*) using a mouse mAb specific for chicken lamin B<sub>2</sub> (see Materials and Methods). The corresponding differential interference contrast (DIC) images are shown on the right. Control mouse L cells transfected with pTK-x1 plasmid alone were completely lacking fluorescence (not shown). Wild-type (*a*) and 191 ROD (*b*) lamin B<sub>2</sub> transfectants displayed a nuclear lamina ring staining pattern indistinguishable from the characteristic localization of native lamin B<sub>2</sub> observed in DU249 chicken cells (*i*, *inset*). In contrast, in both 597 TAIL and 598 STOP transfectants the majority of the fluorescently localized lamin B<sub>2</sub> appeared to be distributed throughout the nucleoplasm (*c* and *d*, respectively). Nucleoli were often apparent as dark, spherical structures, contrasted by the fluorescently labeled nucleoplasmic lamin proteins. Note the minor amount of peripheral nuclear lamina staining that persisted in the 597 TAIL and 598 STOP cells (*c* and *d*, *arrows*). Some variation in fluorescence intensity was routinely observed despite repeated subcloning of cell lines (see, for instance, 597 TAIL). The cause of this phenotypic variation is presently unknown but similar effects were recently seen in the course of expressing chicken vimentin in mouse cells (Ngai et al., 1990). Also, minor variations in the intensity and granular appearance of nucleoplasmic lamin B<sub>2</sub> could be seen when comparing various 597 TAIL and 598 STOP cell lines (as well as 191/597, not shown); the significance of these minor variations is not clear. Bar, 20  $\mu$ m.



**Figure 6.** Localization of chicken lamin B<sub>2</sub> in mouse cells during mitosis. The immunofluorescent distribution of chicken lamin B<sub>2</sub> in wild type (a–c), 597 TAIL (d–f) and 191/597 (g–i) transfected mouse L cells at various stages of mitosis was determined as described in the legend to Fig. 5. (a, d, and g) metaphase to early anaphase; (b, e, and h) late anaphase to telophase; (c, f, and i) late telophase; (a'–i') represent the corresponding DIC images. Lamin B<sub>2</sub> appeared to be equally dispersed throughout the cytosol of wild type, 597 TAIL and 191/597 cells during metaphase, anaphase and early telophase (a, b, d, e, g, and h). During late telophase, a decrease in cytosolic staining paralleled a concomitant increase in localization of lamin B<sub>2</sub> to the reforming nucleus. In transfectants expressing wild type lamin B<sub>2</sub>, a reassembled nuclear lamina ring pattern was observed (b and c), whereas in 597 TAIL and 191/597 cells lamin B<sub>2</sub> appeared to colocalize with condensed chromatin and staining of the nuclear lamina was not detectable (e, f, and i). Bar, 20 μm.

transfectants, no preferential staining of the chromosome surfaces could be detected (Fig. 6, e, f, and i; identical results were observed with the 598 STOP line; not shown). These results suggest that the lamin B<sub>2</sub> proteins containing CaaX mutations reassociate with daughter nuclei, but are unable to participate in the reformation of nuclear envelopes. In none of the stably transformed cell lines did we observe structures resembling the cytoplasmic inclusions described after transient expression of mutated human lamin A proteins in hamster cells (Holtz et al., 1989); however, we note that such aberrant distributions were occasionally seen in transiently transfected cells (data not shown).

#### ***Cys597 of the CaaX Motif Is Required for Association of Lamin B<sub>2</sub> with Nuclear Membranes***

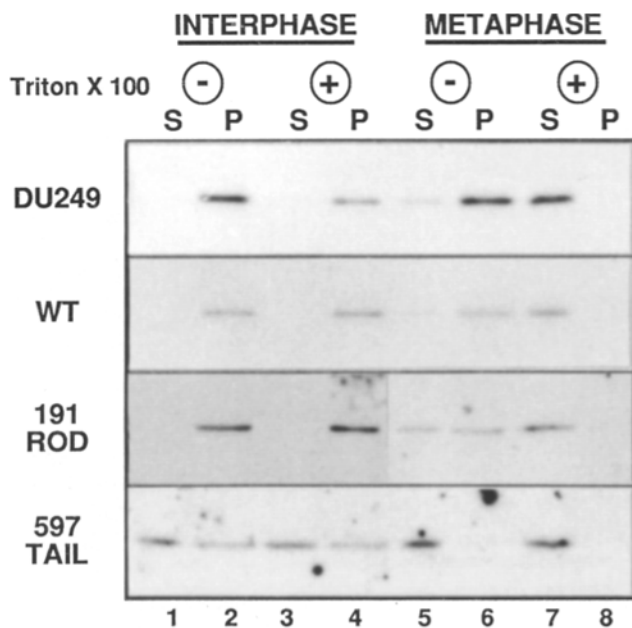
To extend the observations described above, the interactions of wild-type and mutated forms of lamin B<sub>2</sub> with the nuclear envelope were monitored using a detergent (Triton X-100) solubilization assay (see Materials and Methods). This assay allows one to distinguish between lamin proteins that are insoluble because of integration into a nuclear lamina from those that sediment because of nuclear membrane association. For comparison, the fractionation of wild-type lamin B<sub>2</sub> in chicken DU249 cells is illustrated in Fig. 7. As expected, lamin B<sub>2</sub> remained almost exclusively in the detergent-resistant fraction during interphase (Fig. 7, lanes 2 and 4), but was readily solubilized from metaphase cells when the nuclear membranes were dissolved by Triton X-100 (Fig. 7, lanes 7 and 8). Conversely, due to its persistent membrane association, lamin B<sub>2</sub> was pelletable when mitotic DU249 cells were fractionated in the absence of detergent (Fig. 7, lanes 5 and 6).

Analysis of mouse cell transfectants expressing either

wild-type or the 191 ROD mutant lamin B<sub>2</sub> protein showed a pattern of partitioning similar to the one seen with DU249 cells (Fig. 7). In contrast, the 597 TAIL transfectant displayed drastically different solubilization characteristics. Although some of the mutated lamin B<sub>2</sub> still fractionated with the pellets during interphase (Fig. 7, lanes 2 and 4), the majority of the protein was recovered in the supernatants, even in the absence of detergent (Fig. 7, lanes 1 and 2). This observation suggested that mutating the cysteine residue of the CaaX motif of lamin B<sub>2</sub> to an alanine was sufficient to render much of the protein soluble. The observation that solubilization was not complete most probably reflects a residual capacity of the mutated lamin B<sub>2</sub> to form polymers, either with itself, or with the endogenous mouse lamins. This interpretation is supported by the results of immunofluorescent staining described above (Fig. 5). Most importantly, in metaphase samples prepared from 597 TAIL transfectants, all of the lamin was in the supernatant fraction, irrespective of the presence or absence of detergent during fractionation (Fig. 7, lanes 5–8). Thus, during mitosis, when lamins are depolymerized, membrane association was completely abolished by mutation of the cysteine residue of the CaaX motif. This result strongly suggests that CaaX box mutations abolish the ability of lamin B<sub>2</sub> to interact with nuclear membranes, rather than interfering with lamina polymerization.

#### ***Discussion***

By stably expressing mutated chicken lamin B<sub>2</sub> proteins in mammalian cells, we have demonstrated that the cysteine residue of the COOH-terminal CaaX motif is required for isoprenylation and carboxyl methylation of lamin B<sub>2</sub> in



**Figure 7.** Detergent solubilization characteristics of wild type and mutated lamin B<sub>2</sub> in interphase and metaphase cells. Interphase cells or cells arrested in metaphase with nocodazole were harvested, homogenized, and then divided into two aliquots, one of which was treated (+) with 1% Triton X-100. Aliquots were subjected to centrifugation and an equivalent amount of each of the resulting supernatant (S) and pellet (P) fractions was analyzed by immunoblotting, using a mAb specific for chicken lamin B<sub>2</sub>. Control mouse cells were completely negative (not shown). The solubilization characteristics of wild type (WT) and 191 ROD molecules closely resembled those of the native lamin B<sub>2</sub> in chicken DU249 cells (cf. lanes 1–8 of each). A clearly different solubilization pattern was revealed for lamin B<sub>2</sub> molecules in the 597 TAIL transfectant. Lamin B<sub>2</sub> was present in the supernatant fractions of interphase and metaphase cells even in the absence of detergent treatment (lanes 1 and 5). Moreover, whereas some lamin B<sub>2</sub> was retained in the interphase pellet fractions (lanes 2 and 4), it was completely absent from metaphase pellets (lanes 6 and 8). Only the relevant part of the gel is shown.

vivo. Moreover, we show that mutations of the CaaX motif interfere with the correct targeting of newly synthesized B-type lamins to the inner surface of the nuclear membrane. Together with previous data (for references see introduction), these findings allow us to depict the following pathway for posttranslational modification of newly synthesized lamin proteins. Shortly after synthesis on free ribosomes, both A- and B-type lamins are modified at their CaaX termini. These modifications occur in three steps, namely, addition of an isoprene moiety to the CaaX box cysteine, followed by removal of three COOH-terminal amino acids, and carboxyl methylation of the resulting COOH-terminal isoprenylated cysteine. As discussed in more detail below, several lines of evidence suggest that these modifications are required for efficient association of newly synthesized lamins with the nuclear membrane.

#### **Lamin A-specific Processing Results in Removal of the Modified COOH-Terminus**

Concomitant with or shortly after incorporation into the lamina, lamin A is subject to an additional processing event

(Laliberté et al., 1984; Gerace et al., 1984; Dagenais et al., 1985; Lehner et al., 1986a), which results in cleavage of some 18 amino acids from the COOH terminus (Weber et al., 1989). While this proteolytic cleavage results in removal of the modified COOH-terminal cysteine, it only occurs subsequent to modification of the lamin A CaaX box (Beck et al., 1990). This observation falls in line with evidence indicating that the relevant protease is associated with the nuclear envelope (Lehner et al., 1986a), and that efficient association of the lamin A precursor with the nuclear envelope requires isoprenylation (Krohne et al., 1989; Holtz et al., 1989; Beck et al., 1990).

The physiological role of lamin A-specific processing is not entirely clear. However, it is interesting to consider the consequences of this event for the fate of A- and B-type lamins during mitosis. It would appear that mature, COOH-terminally truncated lamin A remains at the nuclear envelope of interphase nuclei primarily by virtue of being part of a polymeric structure. When the lamina depolymerizes at the onset of mitosis, lamin A would then be expected to be readily released from nuclear membranes, as is observed (Gerace and Blobel, 1980; Stick et al., 1988). Conversely, the fact that mature B-type lamins retain the hydrophobically modified COOH-terminal cysteine may contribute to explain the observed persistent association of these proteins with mitotic remnants of the nuclear membrane (Gerace and Blobel, 1980; Stick et al., 1988).

Considering the need for CaaX box modifications for correct subnuclear targeting of both A- and B-type lamins during interphase, the question arises how mature, COOH-terminally truncated lamin A can be reutilized at the end of mitosis. One plausible explanation derives from the observation that mature lamin A binds to the surface of telophase chromosomes before reformation of the nuclear envelope (Burke and Gerace, 1986; Benavente and Krohne, 1986; Glass and Gerace, 1990). Thus, the mechanism allowing correct subcellular localization of mitotic lamin A would seem to be different from the one operating during interphase for newly synthesized lamin A.

#### **What is the Molecular Function of CaaX Box Modification of Lamin Proteins?**

Although the importance of CaaX box modification for targeting newly synthesized lamins to the correct subnuclear location is well documented (Krohne et al., 1989; Holtz et al., 1989; this study), no molecular explanation for this observation has previously been provided. In principle, at least two different mechanisms could account for the defect in envelope association of mutant lamin B<sub>2</sub> proteins. First, mutations might lead to misfolding of the protein that in turn might interfere with lamina assembly. That such a process may lead to intranuclear accumulation of lamin proteins is well documented for the case of an A-type lamin carrying a mutation within the central rod domain (Holtz et al., 1989). Second, CaaX mutations could interfere directly with membrane binding of lamin B<sub>2</sub>. According to this scenario, lack of hydrophobic modification of the CaaX box by isoprenylation and carboxyl methylation would disturb interactions of the mutated lamin with either the lipid bilayer or an integral membrane protein receptor.

Based on the following arguments, we strongly favor the



view that CaaX box mutations disturb the interaction of lamin proteins with nuclear membranes, rather than affecting lamin polymerization per se. The most important argument stems from observing the behavior of wild-type and mutant lamins during mitosis. During this stage of the cell cycle, the lamina is depolymerized and the envelope association typical of wild-type lamin B<sub>2</sub> must be due primarily to direct interactions with the nuclear membrane. Thus, our finding that CaaX mutations abolish membrane association of lamin B<sub>2</sub> proteins during mitosis (see Fig. 7) strongly suggests that hydrophobic modifications of the CaaX box contribute to membrane binding of the wild-type lamin B<sub>2</sub>. Conversely, several lines of evidence argue against a major role of the CaaX box in lamin-lamin interactions: first, lamin B<sub>2</sub> proteins carrying CaaX mutations were at least partly capable to participate in lamina formation during interphase (see Figs. 5 and 7). Second, the 597 TAIL mutant could as readily be cross-linked by cupric *o*-phenanthroline catalyzed disulphide bridge formation as the wild-type lamin B<sub>2</sub> (Peter, M., and E. A. Nigg, unpublished results). Considering that this zero-length cross-linking (involving Cys 191 within the lamin rod domain) requires coiled-coil interactions between two parallel unstaggered lamin proteins (Krohne et al., 1987), its success indicates that mutation of Cys597 did not interfere with lamin dimer formation. Third, wild-type lamin B<sub>2</sub> proteins expressed in *Escherichia coli* readily assemble in vitro into dimers, filaments of ~10 nm width, and paracrystals, despite the fact that their CaaX boxes had not been modified by the prokaryotic host (Heitlinger et al., 1991). Fourth, mature lamin A as well as lamin C, two proteins lacking CaaX boxes and displaying completely different COOH-terminal amino acid sequences, readily assemble lamin filaments in vitro (Aebi et al., 1986; Moir et al., 1990). Taken together, these data would seem to argue against a direct role of the CaaX box in lamin polymer formation.

It remains to be determined to what extent isoprenylation and carboxyl methylation alone are sufficient to explain membrane association of B-type lamins. In the case of *ras* proteins, these modifications have been shown to be required for membrane association and biological activity (Hancock et al., 1989; Casey et al., 1989; Schafer et al., 1989; Jackson et al., 1990), but it appears that they are not sufficient to confer strong membrane association. Instead, additional palmitoylation of cysteine residues located in close proximity to the isoprenylated COOH terminus, or the presence of a stretch of basic residues, were found to be necessary for strong membrane binding of *ras* proteins (Hancock et al., 1989, 1990). In analogy to these findings, one might expect additional factors to contribute to the membrane association typical of B-type lamins. However, none of the presently known lamins is palmitoylated, and, with the exception of the nuclear localization signal, there are no conspicuous stretches of basic residues that might correspond functionally to those present in human p21<sup>K-ras(B)</sup> (Hancock et al., 1990).

When considering possible determinants conferring membrane affinity to B-type lamins, it is noteworthy that B- as well as A-type lamins readily form dimers, and that even in their mitotically disassembled state they may exist as dimers or tetramers (Gerace and Blobel, 1980; Havre and Evans, 1983; Benavente et al., 1985; Aebi et al., 1986; Krohne et

al., 1987; Dessev et al., 1990; Heitlinger et al., 1991). If this is the case, each lamin oligomer would be held at the membrane by at least two isoprene substituents, and overall avidity for the nuclear membrane would be substantially increased. An alternative explanation for the preferential membrane attachment of B-type lamin proteins is suggested by studies describing integral membrane proteins that may function as receptors for B-type lamins (Worman et al., 1988; see also Senior and Gerace, 1988; Padan et al., 1990). These candidate receptors could interact either with the lamin B polypeptide itself, or they could function as farnesyl receptors. In the latter case, their apparent specificity for B-type lamins would readily be explained by the lack of an isoprenoid substituent on mature lamin A.

While the role of the isoprene moiety is beginning to emerge, the significance of carboxyl methylation remains to be determined. Methyl esterification of a carboxyl group shields a negative charge at the COOH terminus of modified proteins and is expected to enhance the hydrophobic effect of isoprenylation. However, it is presently difficult to rationalize the reported mitosis-specific demethylation of B-type lamins (Chelsky et al., 1987).

#### What Enzymes Modify Lamin CaaX Boxes?

It will be important to determine when lamin CaaX box processing occurs relative to nuclear transport. Based on previous kinetic studies of lamina biogenesis (Lehner et al., 1986a), we favor the view that CaaX box processing of lamins is a cytoplasmic event preceding nuclear transport (see, however, Beck et al., 1990). If correct, this raises the possibility that nuclear lamins may be modified by the same enzymes that act on proteins that ultimately associate with the plasma membrane or various endomembrane systems. Consistent with this view, a purified 70–100-kD farnesyl protein transferase capable of farnesylating p21<sup>H-ras</sup> was recently found to be inhibited by peptides based on either *ras* or lamin sequences (Reiss et al., 1990). However, it would clearly be premature to exclude the possibility that lamin proteins might be modified after nuclear transport, in which case nuclear isoprene transferases, proteases and carboxyl methylases may await discovery.

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