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Cellular distribution of cholesterogenesis-linked, phospho-isoprenylated proteins in proliferating cells

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A set of isoprenylated proteins has been detected in rapidly proliferating, suspension-grown murine lymphoma cells. Our evidence indicates that all of these isoprenylated proteins are phosphorylated. Subsequent to a 24 h incubation with mevinolin to deplete the intracellular mevalonate (MVA) level, cells were incubated with [^{3}H]MVA and/or $^{32}P_i$ and both total cell and subcellular fraction proteins were resolved via 1- and 2-D gel electrophoresis, then assessed via subsequent autoradiography. The phospho-isoprenylated proteins comprise a set spanning a molecular mass range of 21–69 kDa and all dispay acidic p*I*. MVA-derivatized proteins of 21–24 kDa, which consist of multiple isoforms, are present in both cytosolic and nuclear fractions. Larger phospho-isoprenylated protein species (44–69 kDa) are specifically localized within the nucleus, where applicable extraction protocols indicate that they are part of or closely affiliated with the nuclear matrix-intermediate filament (NM-IF) components. The localization of the 69 kDa prenylated species within the NM-IF fraction, together with evidence of its phosphorylation, supports recent indications that this protein is the nuclear matrix component lamin B.

Isoprenylated protein; Mevinolin; Cell cycle; Cholesterogenesis

1. INTRODUCTION

A direct correlation has been shown between the rate of cell replication and that of carbon flux through the cholesterogenesis pathway [1–4]. The normal S-phase burst of DNA synthesis is specifically prevented upon inhibition, during mid-to-late G_1, of the rate controlling enzyme of cholesterogenesis, 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) with either oxygenated sterols [5] or with competitive inhibitors of the enzyme, compactin and mevinolin [6–12]. By inhibiting HMGR, cells become depleted of mevalonate. Many observations collectively indicate that mevalonate deprivation starves cells of additional factors apart from those isoprenoid intermediates that are employed in cholesterol synthesis and the more common prenyl metabolites that arise from branches off the main cholesterogenic pathway. Thus, while mevalonate serves not only as direct precursor for the synthesis of sterol and other well-known isoprenoid metabolites (e.g., dolichol, the prenyl side chain of ubiquinone, etc.), it has been established that, in addition, it is employed as a source from which a new class of terpenylated proteins is generated [13–16].

Soon after the discovery and preliminary characterization of these mevalonate-derived, isoprenylated proteins [13], Sinensky and Logel [14] convincingly demonstrated their unique importance with regard to DNA replication relative to other prenyl intermediates generated by the sterol synthesis pathway, such as isopentenyl t-RNA. Subsequently, Maltese and Sheridan [17] reported detecting a set of similarly derivatized proteins in a variety of transformed as well as normal cell lines

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Abbreviations: MVA, mevalonic acid; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; NEPHGE, non-equilibrium pH gel electrophoresis; ARG, autoradiogram; NM-IF, nuclear matrix-intermediate filament; TdR, thymidine

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in culture. They showed such proteins to have molecular masses ranging from about 17 to 66 kDa, and that they are differentially distributed within subcellular fractions, including the nucleus. Recently, Wolda and Glomset [18] reported that one of these isoprenylated proteins, affiliated with the nuclear matrix, is immunologically definable as lamin B.

We provide here further characterization of mevalonate-derived, isoprenylated proteins detected in a murine lymphoma cell line grown in suspension, and show that the larger of these protein species are preferentially localized within the nucleus where they appear to be associated predominantly with nuclear matrix material, confirming recent findings [18,19]. Furthermore, we find that every prenylated protein within this set of MVA-derivatized proteins is phosphorylated.

2. MATERIALS AND METHODS

2.1. *Reagents*

(RS) [5-^{3}H]Mevalonolactone, [^{32}P]orthophosphate, and EN^{3}HANCE were from Dupont-New England Nuclear (Boston, MA). Mevinolin was a kind gift from Dr A. Alberts (Merck & Co.) and was converted to the Na^+ salt before use as described in [19]. Nonidet P-40, leupeptin, pepstatin, DNase I, and RNase A were from Sigma (St. Louis, MO). All electrophoresis reagents were from RioRad (Rockville Centre, NY). Fetal bovine serum (FBS), RPMI 1640, penicillin/streptomycin and glutamine were from Gibco (Grand Island, NY). All other reagents and chemicals were from standard commercial suppliers and were used without further treatment.

2.2. *Cells*

The pre-B murine lymphoma cell line, 70Z/3, was grown in suspension in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 5 μM 2-mercaptoethanol, 0.05% pluronic acid and 50 U/ml each of penicillin and streptomycin (complete medium). Viability was determined via trypan blue dye exclusion cell counts in a hemocytometer, and all studies were initiated with cultures displaying viabilities >95%.

2.3. *Incubations with [^{3}H]mevalonate and ^{32}P*

Exponentially growing 70Z/3 cultures (2.5×10^5 cells/ml) were treated for 24 h with 30 μM mevinolin in complete medium. Viability after this 24 h incubation period still exceeded 86%. The mevinolin-treated cells were harvested by low-speed centrifugation and resuspended at 10^8 cell/ml in a labelling cocktail containing 200 μCi/ml [^{3}H]mevalonolactone in complete culture medium that contained 30 μM mevinolin. Cells were incubated at 37°C for 3 h in this radioactive mixture and harvested by low-speed centrifugation. The pellet was washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 10 mM Tris, pH 7.4, 100 mM KCl, 2 mM $MgCl_2$, 30 mM NaF, 1 mM dithiothreitol (DTT), 5 μg/ml each of leupeptin and pepstatin, and 50 μg/ml of DNase I and RNase A. After homogenizing the cells (Dounce), the homogenate was warmed to 27°C to permit nuclease activity for 15 min. Total cellular protein was precipitated with 5 vols of cold acetone, then dried under a stream of N_2, and resuspended in the appropriate gel buffer for electrophoresis. For in vivo phosphorylation with [^{32}P]orthophosphate, mevinolin-treated cells were preincubated for 1 h in phosphate-free RMPI medium. Thereafter, cells were labelled for 3 h with 400 μCi/ml $^{32}P_i$ in mevinolin-containing, phosphate-free medium, supplemented with either 400 μCi/ml of *(RS)* [5-^{3}H]MVA or 100 μM cold mevalonolactone. After the labelling period, cells were processed as above, except that all buffers contained phosphatase inhibitors (30 μM NaF, 440 μM EDTA).

2.4. *Gel electrophoresis*

For non-equilibrium pH, 2-D gel electrophoresis (NEPHGE) samples were dissolved in freshly prepared 9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, 2% ampholyte mixture (pH range 3–10), and both electrophoretic dimensions were run sequentially as described [20]. The pH profile in the first (isoelectric focusing) dimension was determined according to [21], and low molecular mass protein markers were co-electrophoresed in the second (12.5% SDS-PAGE) dimension. Gels were stained with Coomassie blue R-250, partially destained, impregnated with EN^{3}HANCE, dried, and exposed to Kodak X-Omat AR film at −80°C. 1-D SDS-PAGE was performed according to Laemmli [22].

2.5. *Cell fractionation*

Cells (10^9) were incubated for 24 h in the presence of mevinolin, then radiolabelled with [^{3}H]mevalonolactone for 3 h and harvested as described above. The pellet was resuspended in 0.25 M sucrose, 4 mM $MgCl_2$, 10 mM Tris, pH 7.4, and 5 μg/ml each of leupeptin and pepstatin, and homogenized on ice. Subcellular fractionation was performed via two different procedures. The first was according to Boland and Tweto [23], and nuclear matrix was retrieved as recently described [24]. An alternate procedure was also employed. After Dounce homogenization in PBS on ice and centrifugation ($650 \times g$, 5 min, 4°C), the supernatant proteins were precipitated with cold acetone, while the nuclear pellet was resuspended in 5% (w/v) Na-citrate. This suspension was overlayered onto a 30% sucrose cushion and centrifuged ($2000 \times g$, 5 min, 4°C). After washing the nuclear pellet with 50 mM NaCl/10 mM Na-acetate buffer, pH 5, it was resuspended in 0.5 ml of this buffer. For both cell fractionation procedures, each fraction was subjected to acetone precipitation and resuspended to the same protein concentration in SDS-gel electrophoresis sample buffer, then resolved on 12.5% gels. Autoradiograms of the gels were prepared after impregnation with EN^{3}HANCE and exposure to X-OMAT film for 1 month at −80°C. Nuclear fractionation of mevinolin-treated, [^{3}H]MVA-labelled 70Z/3 cells was performed as described for B lymphocytes [24].

3. RESULTS AND DISCUSSION

3.1. *Detection of mevalonate-derived, phospho-isoprenylated proteins*

The ability to detect proteins derivatized with

Table 1

Effect of mevinolin on sterol synthesis and murine lymphoma cell growth

		Control	Plus mevinolin
Sterol synthesis[a]	(3)[c] [U-^{14}C]acetate	94272 ± 9790 dpm (100%)	3806 ± 389 dpm (4%)
	(3) [2-^{14}C]pyruvate	1018 ± 20 dpm (100%)	25 ± 5 dpm (2.4%)
Cell growth parameters[b]	(22) Inhibition in total cell number (%)	–	43.5 ± 2.1
	(22) Viability (post 24 h)	>95%	>86%
	(12) ^{3}H-TdR incorp. into TCA-precip. counts	41542 ± 1655 dpm (100%)	10620 ± 738 dpm (25.6%)
	(22) Doubling time (h)	12.07 ± 0.41	22.13 ± 1.50

[a] ^{14}C incorporation into digitonin-precipitable sterol after a 3 h incubation in complete medium with 5 μCi/ml acetate or 7.5 μCi/ml pyruvate under incubation conditions given

[b] DNA synthesis assessed as TCA-precipitable counts after a 24 h incubation under conditions given, via a 30 min pulse with [CH_3-^{3}H]TdR (1 μCi/1.25 × 10^5 cells)

[c] Number of individual experiments are reported in parentheses next to the condition assessed

mevalonate metabolites requires prior depletion of the intracellular mevalonate pool in order to follow the fate of added [^{3}H]mevalonate. Incubation of cell cultures with 30 μM mevinolin for 24 h inhibits HMGR almost completely [13,18]. Under these conditions, incorporation of [U-^{14}C]acetate and [2-^{14}C]pyruvate into digitonin-precipitable sterols is reduced by 96% and 98%, respectively, compared to untreated cultures. In addition, cell growth is inhibited by ~44%, with a concomitant increase in the doubling time from 12 h to >20 h (table 1). After mevinolin treatment, 70Z/3 cells are arrested in late G_1 or at the G_1/S boundary. This was verified by comparing the kinetics of [^{3}H]TdR incorporation into trichloroacetic acid-precipitable counts for both mevinolin-treated cells that were re-fed MVA, and double thymidine block-synchronized cultures; the kinetic patterns and the total cpm incorporated are indistinguishable (fig.1).

Subsequent to mevinolin inhibition, cells were pulse labelled with [^{3}H]MVA and their proteins were then extracted and resolved via 2-D NEPHGE. Fig.2 indicates that such ^{3}H-labelled proteins constitute a set which spans a molecular mass range of 21 to about 69 kDa, and all the

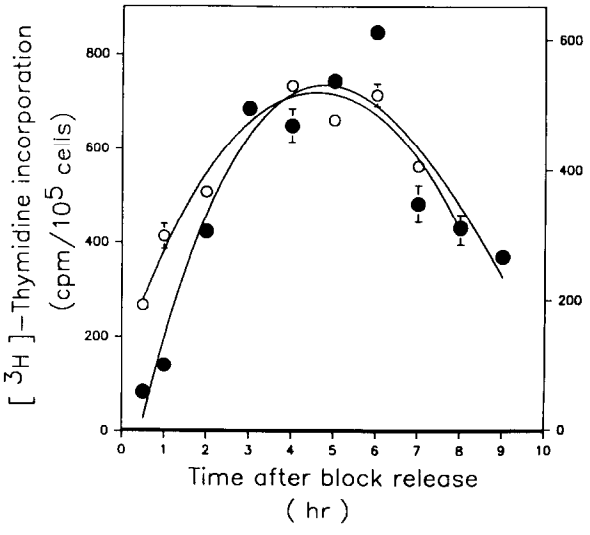

Fig.1. Kinetics of [^{3}H]TdR incorporation. 70Z/3 cells, incubated according to Rao and Coleman [25], were treated either with 30 μM mevinolin for 24 h (●) or synchronized by double thymidine blockade (○). At different times after release from the cell cycle block by resuspension in fresh complete medium, 1.25 × 10^5 viable cells/0.25 ml were pulsed with 1 μCi of [CH_3-^{3}H]TdR for 30 min. Cells were harvested by 10% TCA precipitation onto glass fiber filters, and the filters were washed, dried and counted via liquid scintillation. Each point represents the mean of 12 determinations.

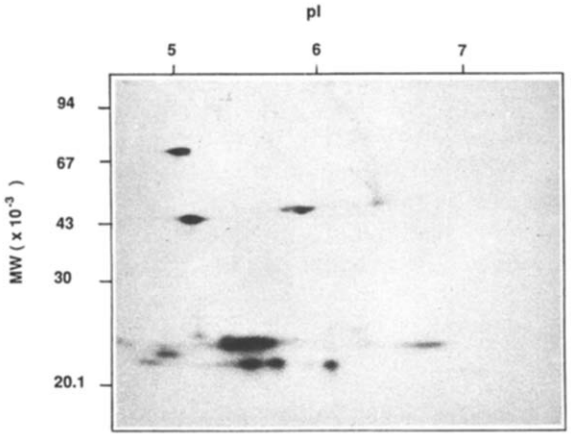

Fig.2. 2-D NEPHGE fluorographic X-ray pattern of [^{3}H]MVA incorporation into total proteins of mevinolin-treated lymphoma cells. Cells were treated for 24 h with 30 μM mevinolin, then incubated for an additional 3 h in the same mevinolin-containing complete medium plus 200 μCi/ml of *RS* [5-^{3}H]-mevalonolactone. Samples were prepared and 2-D gels were run and exposed to X-ray film as described in section 2.

members of this set exhibit acidic p*I* values. Another modified protein (56 kDa), due to its very acidic p*I*, did not resolve in the 2-D gel system, but is evident via 1-D SDS-PAGE (see below, fig.4). The existence of two distinct p*I* families of isoforms with molecular masses of about 21 and 24 kDa led to the suspicion that for both species, varying degrees of phosphorylation might have occurred. Mevalonate-depleted cells were therefore double labelled by incubation with both [^{3}H]-mevalonate together with [^{32}P]orthophosphate for 3 h, and the isolated proteins resolved via 2-D NEPHGE. The difference in β-particle emission energies and penetration levels of ^{3}H versus ^{32}P over a 48 h exposure permitted the specific detection of the ^{32}P pattern by blocking the X-ray film from ^{3}H emission with a sheet of paper interposed between gel and film (complete ^{3}H blockage was obtained via this procedure). Tritium and ^{32}P distribution were evaluated by excising individual Coomassie-stained spots from the dried gel, and upon digestion in Protosol (Dupont-NEN), assessing radioactivity by liquid scintillation. Superimposition of the ^{3}H pattern of fig.2 onto that of the ^{32}P ARG pattern of fig.3 (A or B) clearly indicates that all MVA-derivatized proteins are also phosphorylated, although to different extents. Fig.3A shows those spots containing both ^{32}P and ^{3}H (ar-

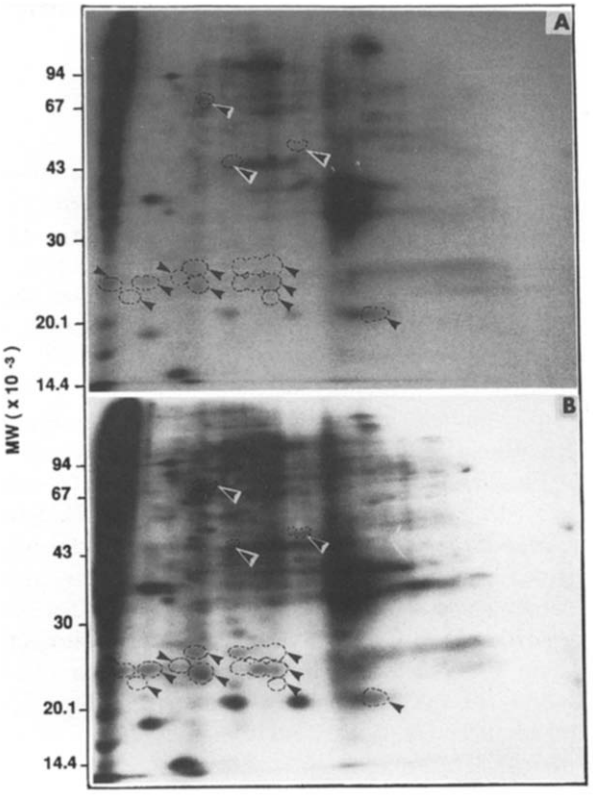

Fig.3. 2-D NEPHGE autoradiogram of ^{32}P incorporation into mevinolin-treated lymphoma cell proteins. Labelling incubations and sample preparation were identical with those for fig.2, except that all buffers were supplemented with phosphatase inhibitors (30 μM NaF and 440 μM EDTA), and labelling incubations for (A) were double labelled, containing *RS* [5-^{3}H]MVA (400 μCi/ml) as well as 400 μCi/ml [^{32}P]PO_4^{3-}, whereas for (B) they contained 100 μM cold MVA together with 400 μCi/ml [^{32}P]PO_4^{3-} in phosphate-free RPMI (otherwise complete) medium. 2-D NEPHGE slabs were Coomassie-stained, destained, dried, and exposed to Kodak X-OMAT AR film for 2 days at −80°C (without EN^{3}HANCE) with a heavy paper sheet interposed between gel and film. Arrows in B indicate those ^{32}P-labelled proteins that, via scintillation counting, also were found to contain the [^{3}H]MVA in A.

rows). A control experiment (fig.3B) in which cells were singly labelled with ^{32}P (plus cold mevalonate) demonstrates that the ^{32}P ARG pattern is identical to that obtained when the incubation contained both ^{3}H and ^{32}P labels.

Fig.3A and B provides strong evidence for the phosphorylation of all the prenylated proteins detected. It should be recognized that the current lack of antibodies against these species rendered the double-labelling experiments the only pro-

cedure by which the entire set could be unequivocally identified.

3.2. *Subcellular distribution of the MVA-derivatized proteins*

Regardless of which of the two cell fractionation procedures was employed, MVA-derivatized proteins of high molecular mass (44–69 kDa) were always localized within the nuclear fraction (fig.4, lane N), whereas the two families of low molecular mass isoforms (21–24 kDa) appeared to be concentrated in the cytosol (fig.4, lane S). None of these prenylated proteins was found within the mitochondrial, microsomal or plasma membrane fractions (fig.4, lanes D, M and P, respectively).

Further nuclear subfractionation was performed in order to generate nuclear matrix-intermediate filament (MN-IF) and chromatin fractions. It was found that the 69, 47 and 44 kDa isoprenylated proteins are affiliated with the NM-IF scaffold of the 70Z/3 cells. Thus, upon nuclease treatment, these MVA-derivatized proteins resisted extraction with Triton X-100 in a low ionic strength buffer (chromatin fraction), and remained insoluble in this buffer even when supplemented with 2 N NaCl (NM-IF fraction). In contrast, the small amount of residual 21–24 kDa derivatized proteins present within the nucleus was easily extracted in the chromatin fraction. Fig.5A shows the distribution among different cell fractions of [^{3}H]MVA-derived proteins resolved via 10% SDS-PAGE. The differential distribution of the large and small derivatized proteins in the NM-IF and chromatin fractions is clearly discernable. The NEPHGE autoradiogram of NM-IF proteins (fig.5B) confirms the absence of low molecular mass isoprenylated proteins in this fraction; only the 69, 47 and 44 kDa proteins are evident.

Recently, Wolda and Glomset [18] identified the 69 kDa prenylated protein as lamin B. This major laminar component (and the only lamin present in lymphoid cells [26]), is subject to a variety of post-translational modifications [27–30]. This report indicates for the first time that every one of the prenylated proteins in this set of cholesterogenically-linked, MVA-derivatized proteins, is phosphorylated, including the prenyl-modified lamin B. The known posttranslational modifica-

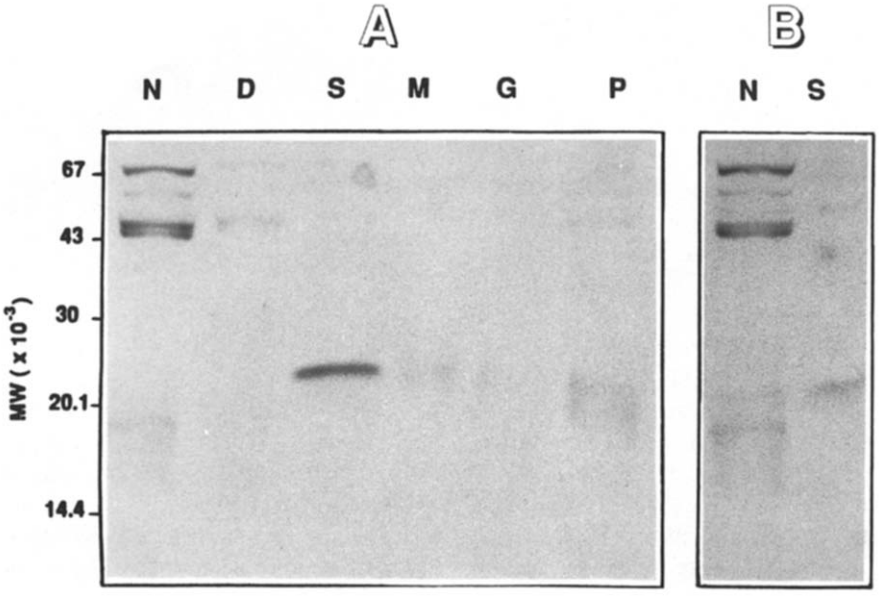

Fig.4. Cell fractionation of mevinolin-treated, [^{3}H]MVA-labelled cells. For A, cell homogenates obtained via the methods for fig.2 were fractionated according to [24], and proteins from the following fractions were analyuzed via 12.5% SDS-PAGE and subsequent fluorography. Lanes: N, nuclei; D, mitochondria; S, cytosol; M, microsomes; P and G, plasma membrane and the 30% sucrose gradient layer, respectively. For B, cells were homogenized in 5% citric acid, and the nuclear fraction separated and further purified via centrifugation as in section 2. Nuclear (N) and cytoplasmic (S) proteins were assessed as in A. A nuclear isoprenylated protein of 56 kDa, and an isoelectric point less than pH 4.5, is resolved via these 1-D SDS gels.

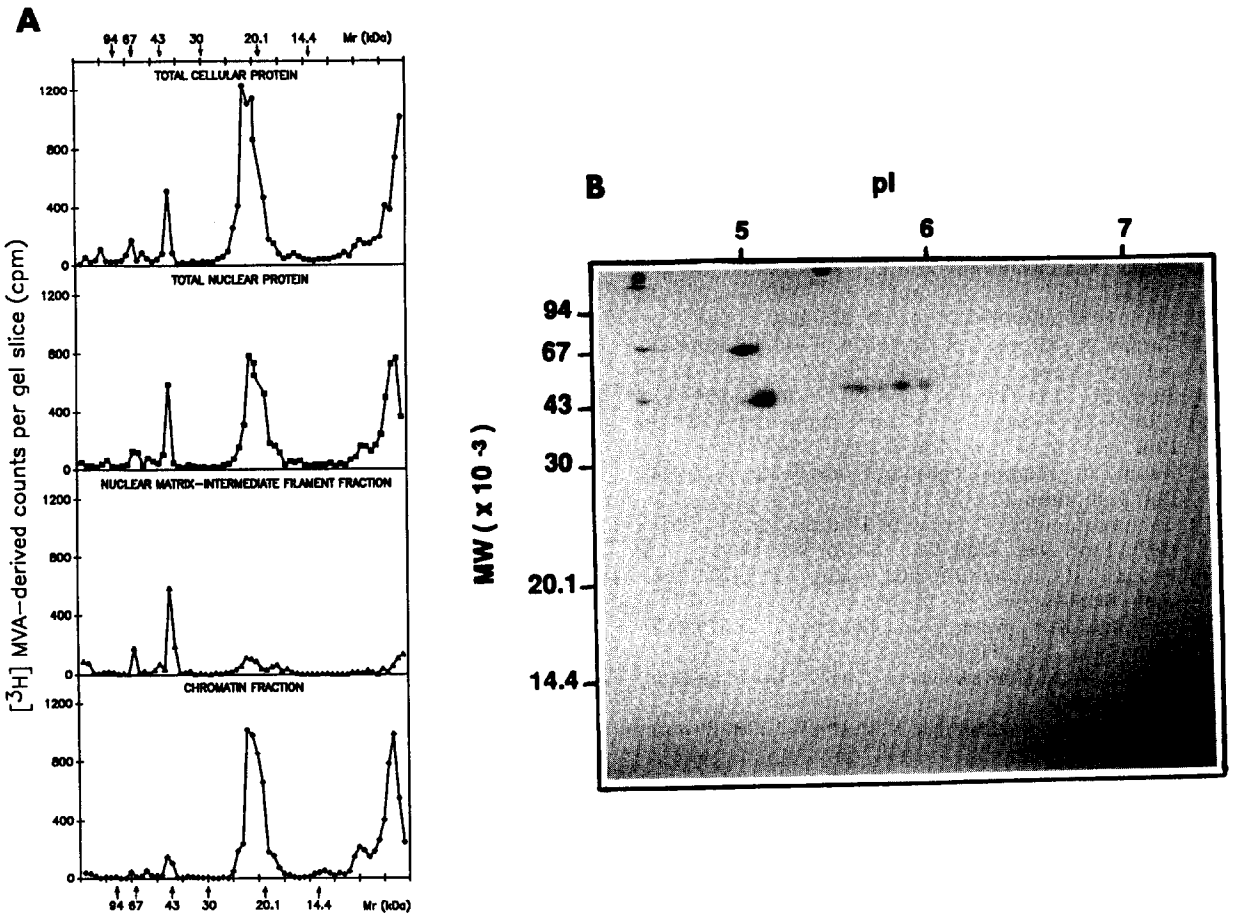

Fig.5. Nuclear fractionation of mevinolin-treated, [^{3}H]MVA-labelled cells. Matrix and chromatin fractions were obtained according to [24], and proteins from each fraction were extracted via acetone precipitation then assessed by 10% SDS-PAGE or 2-D NEPHGE. For A, the ^{3}H cpm profiles were derived from 10% SDS-PAGE slices of total cellular, total nuclear, nuclear matrix and chromatin fractions, as indicated. For B, the 2-D NEPHGE fluorogram of the [^{3}H]MVA-labelled, nuclear matrix-associated (IF) proteins is shown.

tions of lamin B affords room for additional speculation, since its methylation and phosphorylation have been shown to be cell cycle regulated; hypermethylation is manifest during interphase and a high degree of multiple site phosphorylations occurs during mitosis [27–30].

The predominant localization of this set of phospho-isoprenylated proteins within the nucleus prompts interesting questions concerning their roles during DNA synthesis. It is conceivable that the isoprenylation and phosphorylation serve as recognition signals for their translocation into the nucleus, thereby classifying these proteins among the 'amphoteric' species described by Burn [31]. It is also reasonable to propose that their covalent prenylation functions as a hydrophobic linker that helps both to anchor certain of them (e.g., lamin B) to the nuclear envelope as well as to particular chromatin domains in the nuclear matrix. The lamina itself provided not merely a substructure for nuclear envelope organization, but is also a major chromatin anchoring site during interphase [32]. Thus, the dependence of DNA replication on the synthesis of mevalonate, and thereby on the creation of this set of mevalonate-derived, phospho-isoprenylated proteins, may provide a clue regarding the interdependence of an active cholesterogenesis pathway and cell proliferation.

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