Identification of Distinct Messenger RNAs for Nuclear Lamin C and a Putative Precursor of Nuclear Lamin A

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ABSTRACT The lamins are the major components of the nuclear matrix and are known as lamins A, B, and C with M_r 72,000, 68,000, and 62,000 when analysed by SDS PAGE. These three polypeptides are very similar, as determined by polypeptide mapping and immunological reactivity. Lamins A and C are so homologous that a precursor-product relationship has been proposed. Using an antiserum against nuclear matrix proteins that specifically immunoprecipitates the three lamins, we examined their synthesis in the rabbit reticulocytes lysate. Four bands of M_r 62,000, 68,000, 70,000, and 74,000 were specifically immunoprecipitated when polysomes or polyadenylated RNA were translated in vitro. By two-dimensional gel electrophoresis, the 68,000- and the 62,000-mol-wt proteins were identified as lamins B and C, respectively, and the 74,000-mol-wt polypeptide had properties of a precursor of lamin A. The mRNAs of lamin C and of the putative precursor of lamin A were completely separated by gel electrophoresis under denaturing conditions, and their respective sizes were determined. These results suggest that lamin A is not a precursor of lamin C.

The structure and functions of nuclear proteins, particularly those that are part of the insoluble matrix, are still obscure. The nuclear matrix is the residual structure remaining after extensive extraction of the nucleus with high concentration of salts and detergent (1, 2). Examination by electron microscopy reveals that it is composed of a lamina with nuclear pores, a residual nucleolus, and an internal fibrogranular network (1–4). Biochemical analysis indicates that proteins form the bulk of the nuclear matrix along with a small proportion of nucleic acids apparently enriched in newly synthesized molecules. Prompted by this finding, a number of studies have been done to characterize the residual RNA and DNA molecules and to evaluate the role of the matrix in replication and transcription (for a review, see reference 5).

Of the nuclear matrix proteins, the most abundant in the rat liver are three polypeptides of apparent molecular weight (M_r) in SDS gels of 72,000–62,000, designated lamins A, B, and C (6). Polypeptides of similar size were found in the nuclear envelope of a variety of organisms (7–11), and the nuclear pore complex-lamina, described by Aaronson and Blobel (7), is a subfraction of the nuclear envelope consisting almost exclusively of the lamins (6). However, their exact organization within that structure and their relation with other nuclear components is still controversial (6, 10, 12–14). Analyses of the lamins by two-dimensional gel electrophoresis and partial proteolysis have established that lamins A and C are structurally related (4, 8). The two proteins are also immunologically related (6, 13, 15). In view of this homology, the observation that mild proteolysis of lamin A could yield polypeptides of the size of lamin C in vitro (16) has led to the suggestion that there is a precursor-product relationship between the two polypeptides (8). This hypothesis predicts that only one mRNA codes for both and that only lamin A should be synthesized in vitro in the reticulocyte lysate.

In the present study, we have examined the in vitro synthesis of the nuclear matrix proteins and found that lamins A and C are synthesized from different messenger RNAs. In addition, lamin A mRNA is apparently translated into a precursor of the polypeptide found in the nuclear matrix.

MATERIALS AND METHODS

Nuclear Matrix and Antibodies: Nuclear matrices were prepared from BHK 21 cells by a modification (Dagenais, A., V. Bibor-Hardy, and R.

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Simard, manuscript submitted for publication) of the technique described by Berezney and Coffey (2) for rat liver. Exponentially growing BHK cells were trypsinized and washed with phosphate-buffered saline (PBS). Cell pellets were resuspended in 20 vol of TECK buffer (10 mM Tris-HCl pH 7.8, 1 mM EDTA, 3 mM $CaCl_2$, 10 mM KCl) (17) containing 0.5% Triton X-100, kept on ice for 10 min, and homogenized for 20 s with an Ultra-Turrax (Janke & Kunkel, Staufen, W. Germany). Nuclei were obtained after centrifugation at 400 *g* and cleaned, as judged by light and electron microscopy. The nuclear pellet was resuspended in 10 volumes of low-magnesium buffer (Tris-HCl pH 7.4, 0.2 mM $MgCl_2$), kept for 10 min on ice, and centrifuged for 10 min at 400 *g*. After a second low-magnesium buffer treatment, the pellet was washed with 5 vol of low-magnesium buffer and digested with 10 μg/ml of DNase during 10 min at room temperature. After centrifugation, the pellet was resuspended in 5 vol of low-magnesium buffer and brought to 2 M NaCl by slow addition of a 4 M NaCl solution. After an incubation of 10 min on ice, the extracted nuclei were centrifuged for 20 min at 400 *g*. The pellet was extracted a second time with 2 M NaCl and washed once more in low-magnesium buffer before analysis. Antibodies against whole nuclear matrices were obtained after injection into rabbits and IgGs were purified by chromatography through protein A–Sepharose (18).

Indirect Immunofluorescence Localization: Baby hamster kidney (BHK)[1] cells grown on glass cover glasses were fixed twice with methanol at −20°C during 5 min and permeabilized with 0.4% Triton X-100 in PBS during 4 min. Cells were then incubated for 30 min either with the antiserum against nuclear matrix proteins (dilution 1:10) or with a preimmune serum. After extensive washing with PBS containing 0.1% casein, both preparations were incubated for 60 min with fluorescein isothiocyanate–conjugated protein A (Pharmacia Fine Chemicals, Piscataway, NJ) diluted 1:10. Washed cell samples were mounted in 2% n-propyl gallate in Tris-HCl, pH 8.4, and were photographed under fluorescent light.

Preparation of Polysomes and Polyadenylated RNA: To minimize the possibility of RNase contamination, all glassware was treated with 0.5% diethylpyrocarbonate and, whenever possible, disposable plasticware was used. All buffers were filtered and autoclaved before use.

For in vitro translation, a fraction rich in polysomes was prepared by the method of Ramsey and Steele (19) except that 1% Nonidet P-40 and 0.5% sodium deoxycholate were added during homogenization. The polysome pellet was resuspended in 10 mM Tris-HCl, pH 7.6, 20 mM KCl, 2 mM magnesium acetate, and 20% (vol/vol) glycerol, at 200 A_{260} U/ml.

For isolation of polyadenylated RNA, polysomes were obtained by streptomycin sulfate precipitation (20, 21) in the presence of Vanadyl Ribonucleoside Complex (Bethesda Research Laboratories, Gaithersburg, MD) in all solutions. After digestion with proteinase K, polyadenylated RNA was purified by two passages through oligo (dT)-cellulose (22).

In Vitro Translation: Protein synthesis was carried out at 29°C for 60 min (23) in a solution containing the following components: 100 μl of nuclease-treated rabbit reticulocytes lysate, 10 mM HEPES, pH 7.5, 0.2 mM GTP, 1 mM ATP, 2.5 mg/ml creatine phosphate, 0.5 mg/ml phosphocreatine kinase, 20 μM amino acids in a mix minus methionine, 1 mCi/ml [^{35}S]methionine (New England Nuclear, Boston, MA) 10 μg/ml hemin, 2.6 A_{260} U/ml yeast t-RNA, 0.5 mM spermidine, 1.5 mM magnesium acetate, 80 mM KCl, and either 25 μg/ml polyadenylated RNA or 20 A_{260} U/ml of polysomes in a final volume of 200 μl.

Immunoprecipitations: The translation mixture or labeled cells resuspended in a small volume of PBS were made 500 mM NaCl, 20 mM Tris-HCl, pH 7.6, 20 mM methionine, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS and 1 mM phenylmethylsulfonyl fluoride in a final volume of 600 μl and centrifuged at 35,000 rpm (SW 50.1 [Beckman Instruments, Palo Alto, CA]) for 45 min. After addition of 10 μl of nonimmune IgGs and 10 μl of 100 mM phenylmethylsulfonyl fluoride, the mixture was incubated for 2 h at 37°C and overnight at 4°C. 50 μl of IgGSorb (Enzyme Center, Boston, MA) were added and the mixture was incubated at room temperature for 1 h, and then centrifuged at 12,000 *g* for 2 min. The procedure was repeated after addition of 10 μl of immune IgGs to the supernatant. The two pellets were washed five times with 1 ml of 500 mM NaCl, 20 mM Tris-HCl pH 7.6, 20 mM methionine, 0.5% sodium deoxycholate, 1% Nonidet P-40 and 0.1% SDS, and once with 1 ml of PBS containing 20 mM methionine. (For further information on immunoprecipitations, see reference 24.)

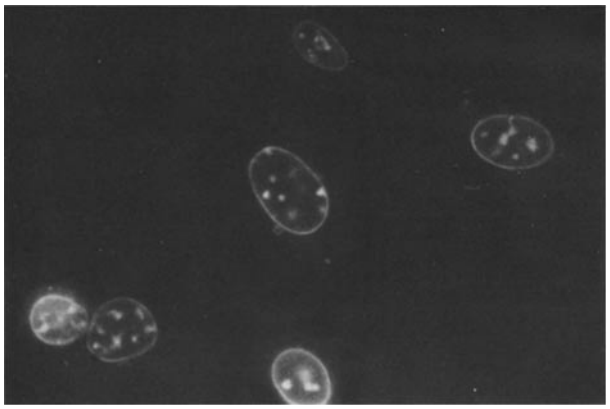
Protein Electrophoresis: Electrophoresis in 5–12.5% gradient polyacrylamide SDS gels was carried out as described (25). The gels were treated with En^{3}Hance (New England Nuclear), dried, and exposed to pre-sensitized radioautographic films (Kodak RP-X-Omat) (26). Two-dimensional electrophoresis was carried out according to O'Farrell (27) using Biolyte 3-10 (Bio-Rad Laboratories, Richmond, CA).

Fractionation of Polyadenylated RNA on Methyl Mercury Agarose Gel: Methyl mercury agarose gels were run as described by Bailey and Davidson (28). 100 μg of polyadenylated RNA were loaded onto a horizontal 1.2% gel made from low-gelling-temperature agarose and fractionated by electrophoresis at 30 V for 18–19 h at room temperature. After the electrophoresis, the gel was soaked in 20 mM β-mercaptoethanol for 30 min and cut into 2-mm slices. The RNA in each slice was extracted according to Weislander (29) and precipitated by addition of ethanol.

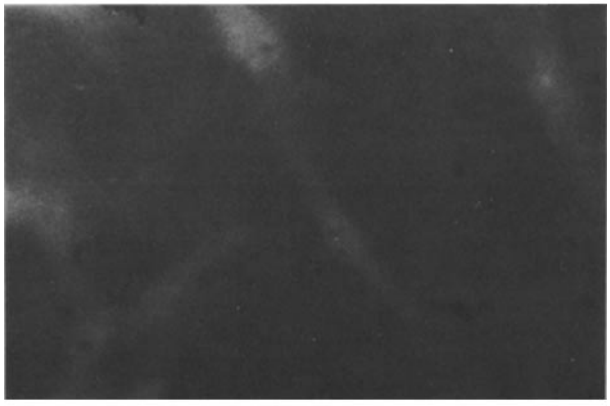
RESULTS

Immunoprecipitation of Nuclear Matrix Proteins from BHK Cells

Analysis of BHK-21 nuclear matrix by SDS PAGE reveals five major proteins of apparent molecular weight 72,000, 70,000, 68,000, 62,000, and 45,000 (see Fig. 2). An antiserum against whole nuclear matrix proteins was raised in rabbits using such preparation. Indirect immunofluorescence was performed on BHK cells. The antiserum labeled the nuclear envelope and possibly an intranuclear structure (Fig. 1 *a*). In the control experiment with a preimmune serum, the immunofluorescence gave a diffuse pattern of low intensity (Fig. 1 *b*). To further determine the specificity of this antiserum, IgGs were purified from immune and nonimmune sera by adsorption on protein A–Sepharose and immunoprecipita-



A



B

FIGURE 1 Indirect immunofluorescence localization with antiserum against nuclear matrix proteins. BHK cells were fixed and then incubated with (*A*) antiserum against nuclear matrix proteins or with (*B*) preimmune serum. Fluorescence staining was obtained by incubation with fluorescein isothiocyanate-conjugated protein A. × 400.

[1] *Abbreviations used in this paper:* BHK, baby hamster kidney; PBS, phosphate-buffered saline.

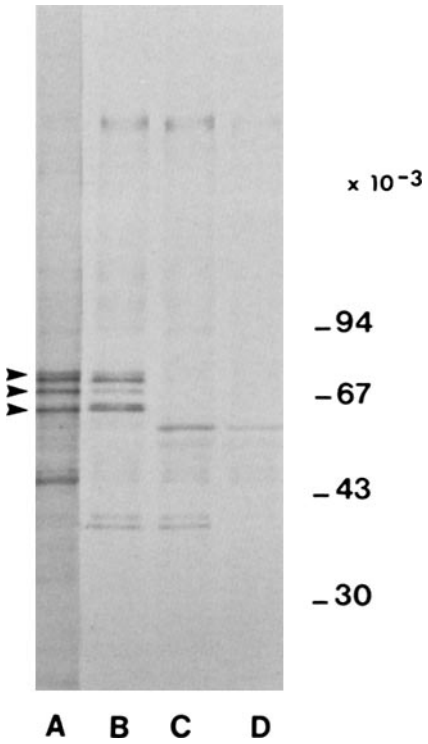


FIGURE 2 Immunoprecipitation of nuclear matrix proteins from [^{35}S]methionine-labeled BHK cells. 3 × 10^7 BHK cells were incubated for 16 h in 7 ml of modified α-medium containing 3 mM methionine and 100 μCi of [^{35}S]methionine. They were then washed with PBS, scraped of the plastic dish, lysed, and incubated with IgGs. The immunoprecipitates were electrophoresed in a 5–12.5% polyacrylamide SDS gel. (*A*) nuclear matrix proteins; (*B*) proteins immunoprecipitated with antinuclear matrix IgGs from cell lysates; (*C*) same as lane *B* except that 1 μg of unlabelled nuclear matrix proteins was present during the immunoprecipitation; (*D*) proteins immunoprecipitated from cell lysates with nonimmune IgGs. The molecular weights were calculated from the mobilities of standard protein markers (phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000 and carbonic anhydrase, 30,000). Lane *A* was exposed for 3 d while lanes *B*, *C*, and *D* were exposed for 1 d only.

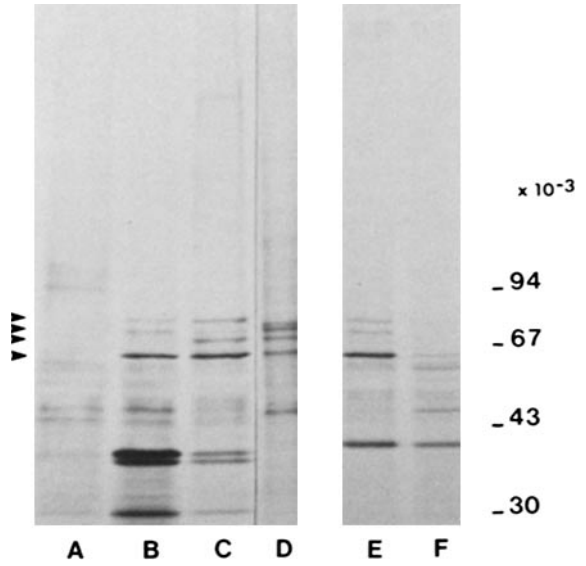


FIGURE 3 Immunoprecipitation of nuclear matrix proteins synthesized in vitro. Translation products from polyadenylated RNA or from polysomes were incubated with IgGs, and the immunoprecipitates were electrophoresed in a 5–12.5% polyacrylamide SDS gel. (*A*) polyadenylated RNA translation products immunoprecipitated with nonimmune IgGs; (*B*) same as lane *A* except that immunoprecipitation was performed with antinuclear matrix IgGs; (*C*) polysomes translation products immunoprecipitated with antinuclear matrix IgGs; (*D*) nuclear matrix proteins labelled in vivo; (*E*) polyadenylated RNA translation products immunoprecipitated with antinuclear matrix IgGs; (*F*) same as lane *E* except that 1 μg of unlabeled nuclear matrix proteins was present during the immunoprecipitation. Arrows indicate the positions of proteins specifically immunoprecipitated by the antinuclear matrix IgGs.

tions were performed on homogenates of whole cells labeled in culture for 16 h with [^{35}S]methionine (Fig. 2, *b* and *d*). The results indicate a reaction mainly with five major polypeptides of M_r 60,000–72,000, of which four co-migrate with the major components of the nuclear matrix. These 72,000-, 70,000-, 68,000-, and 62,000-mol-wt polypeptides are related to the nuclear matrix proteins since they were not precipitated when unlabeled nuclear matrix proteins were added in the reaction (Fig. 2*c*). A doublet of M_r 38,000–40,000 (Fig. 2*b*) was not displaced by nuclear matrix proteins (Fig. 2*c*) and therefore these two polypeptides were not considered in this study.

In Vitro Synthesis of Nuclear Matrix Proteins

The lamins are the main proteins of the nuclear matrix; they have a low turnover and represent 0.1% of cellular proteins (2, 4, 6, 15). Therefore their mRNAs are not expected to be abundant. For each immunoprecipitation, polyadenylated RNA purified from 6 × 10^7 BHK-21 cells growing exponentially was translated in vitro. Three polypeptides of M_r 62,000, 70,000, and 74,000 accounting for ~0.05% of the radioactivity incorporated into proteins were specifically immunoprecipitated (Fig. 3*b*) as judged by the absence of reaction in the presence of unlabeled nuclear matrix proteins (Fig. 3, *e* and *f*). Sometimes a faint band of 68,000-mol-wt was detectable (Figs. 3*b*; and see 5*a*). All except the 74,000-mol-wt polypeptide comigrate with major nuclear matrix components.

When this experiment was repeated with free and membrane-bound polysomes (Fig. 3*c*) the same four bands were immunoprecipitated, the difference being that the 68,000-mol-wt was clearly visible while the 70,000-mol-wt was almost undetectable. This difference in the abundance of the translation products of polyadenylated RNA and polysomes probably reflects the difference between the state of the messenger RNA added to the translation mixture. In polysomes, the mRNA is already engaged in translation, which could facilitate the synthesis of certain proteins, and both polyadenylated and nonpolyadenylated RNAs are present. In addition, some components may be present in the polysome fraction that may have some effects on the stability of certain translation products (30). Further studies are now underway to explain this difference.

The conclusion of these experiments is that nuclear matrix proteins and a 74,000-mol-wt polypeptide immunologically related to the nuclear matrix can be synthesized in vitro from polyadenylated RNA and polysomes.

Characterization of the Immunoprecipitated Polypeptides

The four proteins identified as the major components of the nuclear matrix (Figs. 2*a* and 3*d*) have properties which

indicate that they could be lamins (15). First, in addition to being found in the nuclear matrix, they are the main components of the nuclear pore complex-lamina fraction (Dagenais, A., V. Bibor-Hardy, and R. Simard, manuscript submitted for publication). Secondly, they have molecular weights very similar to those of the mammalian and avian lamins (4, 8). Finally, when they were examined on two-dimensional gels, the 72,000- and the 62,000-mol-wt polypeptides were resolved into at least four isovariants of pI 6.5–7.4 while the 68,000-mol-wt polypeptide appeared as one major spot of pI 5.6 (Fig. 4*a*). These properties are characteristic of lamins A, C, and B, respectively, as described for rat liver (4), chicken liver, and chicken erythrocytes (8). The remaining protein of M_r 70,000 gave a spot of pI 6.1 (Fig. 4*a*). In this gel, it is noticeable that the spot identified as the 70,000-mol-wt polypeptide is less intense, relative to the other components, than what would be expected from Fig. 2*a*. This is probably due to incomplete entry of the proteins in the isofocussing gel since the spot of pI 6.1 co-migrated with the intense 70,000-mol-wt band when the two were run in the same gel (result not shown).

When immunoprecipitates of in vitro translated polysomes products were analysed on a two-dimensional gel (Fig. 4*b*), the 70,000-mol-wt polypeptide was not observed as expected from the experiment described above (Fig. 3*c*). In contrast, the 68,000-mol-wt polypeptide was resolved into a number of isovariants with an acidic component around pI 4.3, several spots around pI 6.5–7.4, and a major double spot at pI 5.7–5.9 that could be lamin B (Fig. 4*b*). However, definitive identification of lamin B will require further studies. The 62,000- and 74,000-mol-wt polypeptides synthesized in vitro from polysomes were each resolved into a cluster of spots of pI 6.5–7.4 (Fig. 4*b*) that identify the 62,000-mol-wt polypeptide as lamin C and suggest that the 74,000-mol-wt polypeptide is a precursor of lamin A.

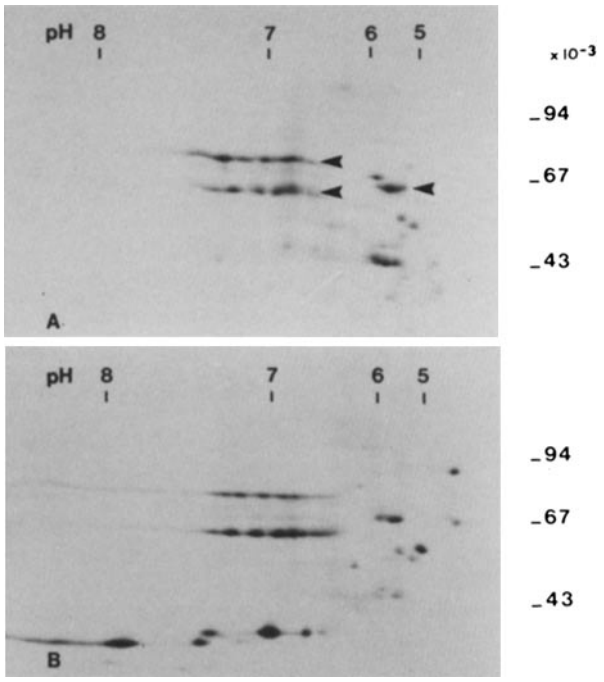


FIGURE 4 Two-dimensional gel electrophoresis of nuclear matrix proteins. Nuclear matrix proteins were subjected to equilibrium isoelectric focusing followed by 5–12.5% polyacrylamide SDS gel electrophoresis. (*A*) nuclear matrix proteins labelled in vivo; (*B*) nuclear matrix proteins immunoprecipitated from the products of in vitro translation of polysomes. Arrows indicate the positions of lamins A, B, and C.

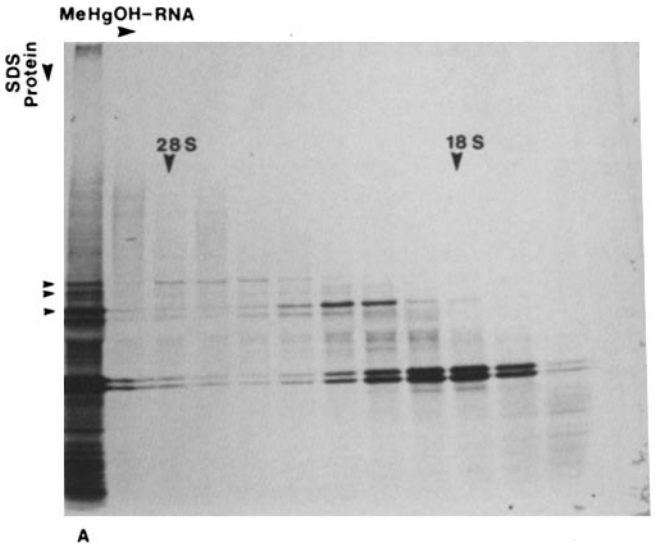


FIGURE 5 Size determination of lamin messenger RNAs. Polyadenylated RNA was fractionated in a methyl mercury agarose gel, extracted, and translated in vitro. Translation products were incubated with antinuclear matrix IgGs and the immunoprecipitates were electrophoresed in a 5–12.5% polyacrylamide SDS gel. Each lane represents immunoprecipitated translation products of the RNA present in a 2-mm slice of the agarose gel except for lane *A* which represents immunoprecipitated translation products of unfractionated polyadenylated RNA. Arrowheads indicate the positions of proteins specifically immunoprecipitated by the antinuclear matrix IgGs.

Identification of Two Lamin mRNAs

That the 74,000- and 62,000-mol-wt lamins are synthesized in vitro should imply that two mRNAs, possibly of different size, are involved since the rabbit reticulocytes lysate has a limited capacity to process or glycosylate proteins (23, 31, 32). This possibility was verified by fractionating polysomal polyadenylated RNA in a methyl mercury agarose gel. The gel was sliced, and the RNA was extracted and translated in vitro. The translation products were precipitated by the antinuclear matrix antibodies and were analysed by gel electrophoresis (Fig. 5). Examination of the fluorogram indicates that the 62,000-mol-wt polypeptide is associated with RNAs of 1.9 to 2.3 kilobases while the 74,000-mol-wt polypeptide is found in fractions containing 2.5 to 3.1 kilobases RNAs. The size of these mRNAs is slightly larger than needed to accommodate the sequences coding for proteins of M_r 62,000 and 74,000, respectively, as has been found with many other mRNAs.

DISCUSSION

The antiserum used in this study was raised against purified nuclear matrix from BHK cells, and lamins were the main components of the antigen. This identification is based on the properties of the proteins found in the nuclear matrix preparation. First, they are found in an insoluble nuclear fraction which includes the lamina (2). Secondly, they have molecular weights that are very close to the value reported for the lamins (6–11). Finally, when analysed by two-dimensional gel elec-

trophoresis they gave rise to species of isoelectric points identical to those of the lamins (4, 8). These criteria constitute the definition of the nuclear lamins (4, 6). Not surprisingly, the antiserum labeled the nuclear envelope and immunoprecipitated polypeptides having molecular weights and isoelectric points identical with those of the lamins. Nonimmune serum did not react with these polypeptides, and their specific immunoprecipitation was blocked by nuclear matrix proteins.

Our results with RNA and polysomes show that lamins are synthesized in vitro. This is based on the molecular weights and isoelectric points of the proteins specifically immunoprecipitated by the antiserum. The 62,000-mol-wt polypeptide has the characteristic pattern of lamin C on two-dimensional gel. The 68,000-mol-wt polypeptide behaves like lamin B, but definitive proof that it is indeed lamin B will require further studies. In addition, we found a protein of M_r 74,000 that is specifically immunoprecipitated by the antinuclear matrix antiserum and has ionic properties similar to those of lamin A in two-dimensional gel electrophoresis. Indeed, its immunoprecipitation was inhibited when unlabeled antigen was added, showing that it shares some homology with the nuclear matrix proteins and, in two-dimensional gels, the 74,000 mol wt was resolved in four to five spots of pI identical to those found for the 72,000- and the 62,000-mol-wt lamins, indicating again the resemblance between the three proteins. On the basis of these observations and considering that no polypeptide with the molecular weight of lamin A was immunoprecipitated after synthesis in vitro, we identify the 74,000-mol-wt polypeptide as a putative precursor of lamin A. Another explanation is that the 72,000-mol-wt protein seen in the nuclear matrix is a degradation product of a 74,000-mol-wt lamin A. However, this is unlikely since we find no evidence of a 74,000-mol-wt polypeptide in immunoprecipitates of whole cell lysates although the conditions used were chosen to minimize protease activities and were very similar to those used with the in vitro synthesized products.

All nuclear proteins studied so far contain in their mature form the information necessary for their entry and their accumulation in the nucleus (33–36). This seems to be the case for lamin C, since the in vitro synthesized form has the same mobility on SDS PAGE as the one isolated from the nuclear matrix. In contrast, lamin A seems to have a precursor with approximately 20 amino acids more. This finding raises the question of the function of this precursor. By analogy with proteins that have to interact with membranes for their biological activity or to reach their site of function, it is tempting to postulate the existence of a signal sequence (37, 38), but the precise localization of this additional peptide in the precursor remains to be established. Another possibility is the use of this extra sequence to allow the protein to reach its final destination where cleavage of the precursor would make the process irreversible. Finally, the precursor could be involved in the folding of lamin A. It will be interesting to know why lamin A has a precursor while lamin C does not, even though they are associated with the same nuclear structure (10, 13, 14, 15) and share common sequences (4, 6, 8, 13, 15).

We found that lamin C and the putative precursor of lamin A are coded for by different messenger RNAs. This was first suggested by the synthesis of both polypeptides in vitro in the rabbit reticulocytes lysate, which is not expected to be efficient in protein processing (23, 31) and glycosylation (32), and confirmed by the separation of their messenger RNAs by agarose gel electrophoresis. The homologies between lamins A and C are well documented (4, 6, 8, 13, 15) and, on this basis, the hypothesis that lamin C is produced in vivo by proteolytic cleavage of lamin A has been put forward (8, 16). We propose instead that the homologies between lamins A and C and the identification of different mRNAs for the two proteins imply the existence of two closely related genes or of a single gene transcribed into an RNA which is subsequently differentially spliced.

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