Lamins A and C Appear during Retinoic Acid-induced Differentiation of Mouse Embryonal Carcinoma Cells

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Abstract. The lamin complement of nuclear matrix isolated from F9 embryonal carcinoma cells was studied during retinoic acid-induced differentiation in culture. Differentiation of the original cells into parietal endoderm-like cells was accompanied by the gradual appearance of lamins A and C while lamin B was present throughout all stages. Lamins were identified by their molecular masses, isoelectric points, recognition by a monoclonal antibody and a polyclonal antiserum, and by peptide mapping. The increase in the amounts of lamins A and C found in the matrix was due to de novo synthesis as no extranuclear pools of these lamins were detected in the undifferentiated cells. These results provide biochemical evidence that, as in amphibian embryogenesis, there are variations in nuclear lamina composition during mammalian development.

THE peripheral lamina is a structural component of the nuclear envelope that interacts with peripheral chromatin and with the nucleoplasmic face of the inner nuclear membrane (for recent reviews see references 9, 10, 15). During mitosis, the lamina plays an important role in the process of nuclear envelope breakdown and reformation which is controlled by a phosphorylation-dephosphorylation cycle of its constituent proteins called lamins (9, 10). During interphase, the lamins associate to form the insoluble lamina in a manner similar to the interaction of intermediate filament (IF)[1] proteins: cDNA sequencing studies on lamins A and C (7, 25) and epitope mapping studies using mAbs on lamin B (18; Raymond, Y., and G. Gagnon, manuscript submitted for publication) have shown that lamins share important structural homologies with IF proteins.

The parallel between lamins and IF proteins can be further extended to include differential expression during embryogenesis and differentiation. The lamins appear to constitute a family of proteins with major and minor species (19), the expression of which varies among cell types (reviewed in 15). In amphibians, the type and number of lamins vary during embryogenesis, gametogenesis, and in some adult somatic cells (15). In early *Xenopus* embryos, regulation of lamin expression seems to be at the level of translation (32). In mammalian cells, immunofluorescence studies have shown variations in the number and distribution of lamins during gametogenesis (24) and embryogenesis (28), but these results have not been confirmed by biochemical studies due to the lack of sufficient material. In all cases, changes in the composition of the lamina were correlated with changes in nuclear morphology.

The requirements for more than one lamin type and the specific role of each species are not yet fully understood. In vertebrate cells, lamins A and C appear responsible for interacting with chromatin (5) while lamin B may provide the anchor to the inner nuclear membrane during both interphase and mitosis (10, 17). These rules are, however, tentative as preliminary results indicate the possibility of membrane interaction for lamins A and C (our unpublished results) and chromatin interaction for lamin B (4). Depletion of all three lamins by microinjection of an antibody prevented normal chromatin dispersion and nucleolus reformation after mitosis (2), which indicates an important role for lamins A, B, and C in these processes.

To biochemically study the expression of lamins in mammalian cells, we chose to use mouse embryonal carcinoma (F9EC) cells. These cells are stem cells that can be induced in culture to differentiate into parietal or visceral endoderm-like cells as defined in the post-implantation embryo (11, 12, 30, 31). The differentiation process is accompanied by morphological and biochemical changes (1, 12, 13, 20) including the synthesis of novel IF proteins (16, 26, 27).

Using a mAb reactive against lamins A, B, and C (6), we show that lamins A and C appear during retinoic acid-induced differentiation of F9EC cells while lamin B is present throughout all stages. This change in composition of the lamina results from de novo synthesis of lamins A and C and not from mobilization from an extranuclear pool of lamins as in amphibian oocytes (8, 22). This report constitutes the first biochemical demonstration of differential expression of lamins in mammalian cells.

Materials and Methods

Cell Cultures

The F9EC cell line (1) and the F9-AC cl9 clone (13) were obtained from Dr.

1. *Abbreviation used in this paper*: IF, intermediate filament.

Daniel Skup of this institute. F9-21 cells, a subclone of F9, were cultivated in DME containing 15% heat-inactivated (1 h, 56°C) FBS in gelatin-coated (0.1%) culture dishes. Cells were passaged every 2 or 3 d using 2.5 mM EDTA in PBS in the presence of 2% FBS. F9-AC c19 cells, a clonal line selected from retinoic acid-induced differentiated cells, were cultivated in DME containing 10% FBS and harvested with trypsin-EDTA. F9-21 cells were treated with 10^{-6} M retinoic acid (30, 31) in the medium described above and seeded at a density of 3×10^6 cells per 150-mm dish for the 24-h culture, and 2.5×10^6 cells per dish for the 48-h culture.

Isolation of Nuclear Matrix

All solutions contained 1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. Mouse liver nuclear matrix was isolated as described previously for rat liver (17). Embryonal carcinoma cells were incubated for 15 min at 37°C in 5 ml of PBS (containing calcium and magnesium). Cells were scraped from petri dishes using a rubber policeman and centrifuged at 180 *g* for 5 min. The following steps were performed at 4°C. Cells were washed twice with PBS, resuspended in 5 ml of 10 mM Tris-HCl, pH 7.4/1 mM $MgCl_2$, and incubated for 25 min. Swollen cells were disrupted with an Ultraturrax homogenizer (Janke-Runkel, Staufen, Federal Republic of Germany) set at 120 V and speed 60, until intact nuclei appeared free of cytoplasmic contamination, which took from 10 to 35 s according to cell type. The suspension was spun for 10 min at 1,000 *g*, the pellet washed three times, and the nuclei were resuspended at a concentration of 10^8/ml. Nuclei were digested with 250 μg/ml RNase A and 500 μg/ml DNase 1 for 60 min with occasional shaking and then centrifuged for 15 min at 1,600 *g*. To minimize proteolytic degradation, samples of RNase had been boiled for 10 min and samples of DNase had been treated with soybean trypsin inhibitor (Raymond, Y., and G. Gagnon, manuscript submitted for publication). The nuclear pellet was resuspended in 0.2 vol of 10 mM Tris-HCl, pH 7.4/0.2 mM $MgSO_4$, and 0.8 vol of the same solution containing 2 M NaCl was slowly added. After a 15-min incubation, nuclei were centrifuged for 15 min at 5,900 *g*, extracted a second time with high salt as above, and the final pellet of nuclear matrix was solubilized, reduced, and alkylated as previously described (17). Total cell lysates were prepared by direct solubilization of washed cell pellets in lysis buffer (containing 9.5 M urea) for two-dimensional gel electrophoresis. Protein was estimated by the Lowry method (23) using BSA as a standard.

Electrophoresis and Immunoblotting

One- and two-dimensional electrophoresis and staining of gels (17), electrophoretic transfer of proteins onto nitrocellulose sheets, and subsequent immunodetection (18) were as previously described. An mAb (IgM) that recognizes lamins A, B, and C (6) was kindly provided by Dr. Brian Burke (European Molecular Biology Laboratory, Heidelberg, FRG) in the form of ascites fluid which was used throughout this study at a dilution of 1:1,000. The second antibody used was ^{125}I-labeled goat anti-mouse IgM (10 μCi per transfer, 3 h at room temperature) and the nitrocellulose transfers were radioautographed at room temperature for the indicated periods of time.

Peptide Mapping

Samples of nuclear matrix proteins were labeled with [^{3}H]dansyl chloride: proteins were dissolved at a concentration of 1–2 mg/ml in a 0.1 M sodium carbonate–bicarbonate buffer (pH 9.8) containing 2% SDS. To 60 μl of this solution we added 60 μCi of [^{3}H]dansyl chloride in 25 μl of acetone and the solution was incubated for 30 min at 37°C. Proteins were precipitated in 12.5% TCA, the pellet was washed with acetone, and the final pellet dissolved in electrophoresis buffer. Incorporation varied from 2×10^4–1.5×10^5 dpm/μg of protein.

Lamins A, B, and C were individually purified by excising the appropriate spots from two-dimensional gels after fluorography, rehydrated, and cleaved at tryptophan residues using *N*-chlorosuccinimide (21) with the following modifications: reaction time was 60 min instead of 30 min and the final equilibration before electrophoresis was for 30 min in 0.125 M Tris-HCl, pH 6.8, 0.5% SDS, and 10% glycerol. The resulting one-dimensional gel containing the fragments was fluorographed at −70°C.

Results

Lamins A, B, and C from vertebrate cells are characterized by their molecular masses and their isoelectric points. A typical two-dimensional polypeptide profile from mouse liver nuclear matrix is shown in Fig. 1 *a*. The major component of nuclear matrix prepared using this protocol (i.e., in the absence of reducing or alkylating agents) was the peripheral lamina (cf. references 14 and 17) and very few internal matrix

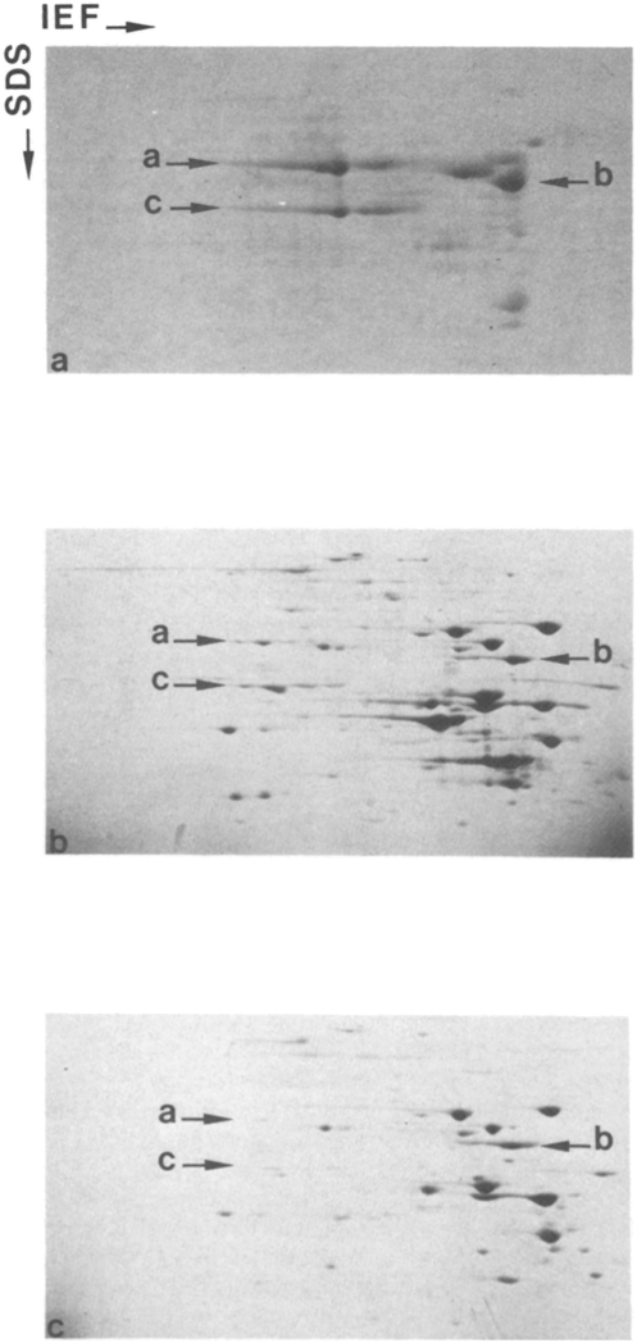

Figure 1. Protein profiles of nuclear matrix from mouse liver and embryonal carcinoma cells. Samples of nuclear matrix (100 μg protein) from mouse liver (*a*), differentiated c19 cells (*b*), and undifferentiated F9 cells (*c*) were separated by isoelectric focusing (*IEF*) in the first dimension and SDS-PAGE (*SDS*, 8% polyacrylamide) in the second dimension, and stained with Coomassie Blue. Positions of lamins A (*a*), B (*b*), and C (*c*) are indicated. Only the central portion of the gels are shown where the majority of proteins migrated.

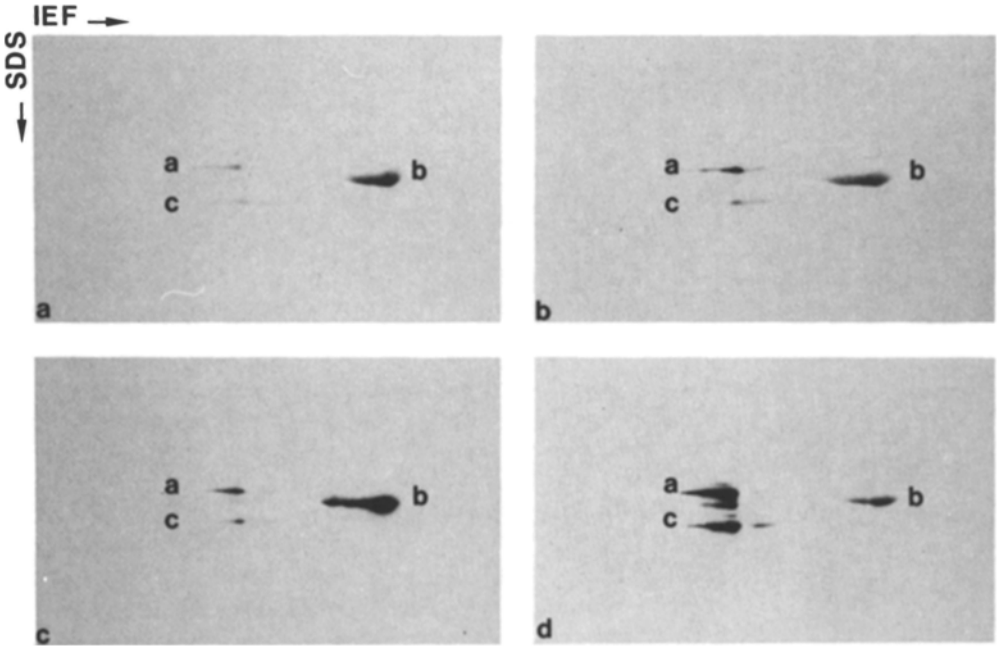

Figure 2. Immunoblotting of nuclear matrix samples from embryonal carcinoma cells. Samples of nuclear matrix (200 μg protein) from undifferentiated F9 cells (*a*), F9 cells treated for 24 (*b*) or 48 h (*c*) with retinoic acid, and from differentiated cl9 cells (*d*) were separated by two-dimensional gel electrophoresis, transferred onto nitrocellulose membranes, and probed with the anti-lamin mAb followed by ^{125}I-labeled anti-mouse IgM as described under Materials and Methods. Positions of lamins A (*a*), B (*b*), and C (*c*) are indicated. The same central portion of the gels as shown in Fig. 1 is presented. Exposure time was 3 d (*a–c*) or 20 h (*d*). Control experiments using as first antibody an ascites fluid prepared from myeloma cells followed by the ^{125}I-labeled antibody gave no reaction over similar samples (not shown).

proteins were present. Lamins are known to be exclusively localized in the peripheral lamina in interphase cells (10). Lamins A (69 kD) and C (62 kD) both showed a number of isoelectric point variants ranging from 6.8 to 7.2 while lamin B (67 kD) was present as a single species (isoelectric point of 5.7). The three lamins were present in roughly equal amounts.

The polypeptide profile of nuclear matrix prepared from differentiated embryonal carcinoma cells (F9-AC cl 9; referred to hereafter as cl9) is shown in Fig. 1 *b*. Lamin B was readily identified on this Coomassie-stained gel where it appeared to be present in a slightly larger amount than lamins A and C (Fig. 1 *b*). These cl9 cells represent one of the end products of retinoic acid–induced differentiation of the F9 cell line and resemble the parietal endoderm of the post-implantation embryo (30, 31). Nuclear matrix isolated from undifferentiated F9 cells is illustrated in Fig. 1 *c*. Lamin B was easily seen while very little stainable material migrating at the positions of lamins A and C could be detected (Fig. 1 *c*). It appears therefore that, based on results of Coomassie staining of gels, the polypeptide composition of the lamina was changing during differentiation of the F9EC cells into parietal endoderm: the latter resembled mouse liver lamina, with a full complement of lamins A, B, and C, while the former appeared to be composed almost exclusively of lamin B.

A number of polypeptides other than lamins were seen in nuclear matrix prepared from F9 (Fig. 1 *c*) and cl9 (Fig. 1 *b*) cells in contrast with the mouse liver matrix preparation (Fig. 1 *a*). This result is often seen when nuclear matrix is prepared from cultured cells and it appears to be due to the copurification of cytoskeletal elements that remain physically attached to the isolated nuclei (29). Definitive identification of the lamins in nuclear matrix prepared from F9EC cells was therefore sought by using a mAb reactive against rat liver lamins A, B, and C.

This mAb, prepared by Dr. Brian Burke (6), did not cross-react with cytoskeletal elements (see Fig. 1 of reference 6) and it recognized the lamina in a large number of mammalian species. When it was used to probe nuclear matrix prepared from cl9 cells (Fig. 2 *d*), the mAb reacted against all three lamins exclusively confirming the assignment of the stained spots in Fig. 1 *b*. Reactive material migrating between the positions of lamins A and C (Fig. 2 *d*) probably represented degradation products of lamin A.

Using the mAb, we sought to determine at which point, during the in vitro differentiation process, embryonal carcinoma cells acquired lamins A and C in their lamina. F9 cells were treated with retinoic acid and nuclear matrix prepared after 24 and 48 h of treatment, separated by two-dimensional gel electrophoresis, and probed on nitrocellulose transfers with the mAb. The results are shown in Fig. 2. A strong signal was observed over lamin B from F9 cells (Fig. 2 *a*) while only a very faint signal was seen at the positions of lamins A and C. The signal over lamin B did not change appreciably at the 24-h (Fig. 2 *b*) and 48-h (Fig. 2 *c*) time points and it was comparable to that of the differentiated cells (Fig. 2 *d*; exposed for a shorter time). Identical amounts of lamin B were detected by Coomassie staining of matrix samples from 24- and 48-h–treated cells (data not shown). In contrast to lamin B, the amount of lamins A and C detected by the mAb increased during the differentiation process and this was seen as early as 24 h (cf. Fig. 2 *b* and 2 *a*) after addition of retinoic acid. The levels of lamins A and C at the 48-h (Fig. 2 *c*) time point were similar to those of the 24-h–treated cells (Fig. 2 *b*) and they had not reached the high level seen in the fully differentiated cells (Fig. 2 *d*).

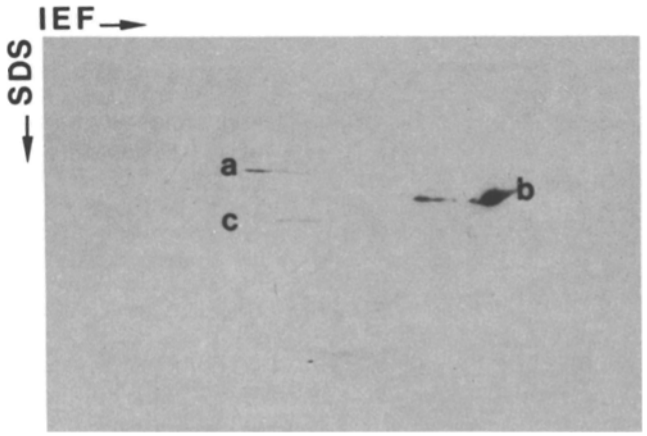

Figure 3. Lamins in a total cell lysate of F9 cells. A total cell lysate was prepared from the same number of F9 cells used to prepare the nuclear matrix sample shown in Fig. 2 *a*. After two-dimensional gel electrophoresis, the proteins were transferred onto a nitrocellulose membrane and probed with the mAb as described in the legend to Fig. 2. Positions of lamins A (*a*), B (*b*), and C (*c*) are indicated. Exposure time was 3 d.

The presence of a small but detectable amount of lamins A and C in the F9 nuclear matrix sample (Fig. 2 *a*) may be due to the presence of a small number of spontaneously differentiating cells which possess a full complement of all three lamins: studies using immunodetection of keratin as a marker for differentiation (27) have estimated that 3% of the original cell population had already acquired keratin filaments before the addition of the differentiating agent.

Taken together, the results of Figs. 1 and 2 show that embryonal carcinoma cells acquired lamins A and C during retinoic acid–induced differentiation (from F9 to cl9 cells) while levels of lamin B remained relatively unchanged. The same results were obtained using a rabbit autoimmune serum reacting against lamins A, B, and C (data not shown): this confirmed that the mAb was detecting an increase in the amounts of lamins A and C and not merely a progressive unmasking of its epitope on these antigens.

Amphibian oocytes possess an extranuclear pool of lamins from which they can mobilize material for the formation of a new lamina around daughter nuclei or around exogeneously added DNA (8, 22). To verify whether F9 cells also possessed extranuclear pools of lamins, we probed with the mAb a total cell lysate corresponding to the same number of cells used to generate the nuclear matrix sample seen in Fig. 2 *a*, and these results are shown in Fig. 3. The cell lysate contained the same amount of lamins A and C relative to lamin B (Fig. 3) as seen in the nuclear matrix preparation from the same F9 cells (Fig. 2 *a*). We can therefore exclude the possibility of an extranuclear pool of lamins and conclude that lamins A and C arose from de novo synthesis during differentiation.

Further confirmation of the identity of lamins was obtained by peptide mapping studies. Nuclear matrix preparations were labeled with [^{3}H]dansyl chloride and the spots corresponding to lamins A, B, and C were excised from two-dimensional fluorograms and hydrolyzed at tryptophan residues with *N*-chlorosuccinimide (21). The use of this reagent was dictated by its ability to generate identical fragments from a defined polypeptide over a wide range of protein concentration (21) and by the knowledge that lamins A and C (7, 25) as well as B (Raymond, Y., and G. Gagnon, manuscript submitted for publication) have only few tryptophan residues. A majority of the fragments generated by the hydrolysis of lamins A and C were expected to be of identical sizes since the amino acid sequence of lamin C is identical to lamin A except for the last six amino acids of the carboxy-terminal of lamin C (7, 25). These results are shown in Fig. 4.

The peptide pattern generated from lamins A and C of mouse liver (Fig. 4, lanes *5* and *6*, respectively) was identical to that of differentiated (cl9) cells (Fig. 4, lanes *3* and *4*). Lamins A and C from cells treated for 48 h with retinoic acid (Fig. 4, lanes *1* and *2*) gave only weak signals due to their low abundance in the nuclear matrix but the major peptides detectable (indicated by bars in Fig. 4) had the same apparent mobilities as those of the two previous samples. Lamin B from 48-h–treated cells (Fig. 4, lane *7*), differentiated cells (Fig. 4, lane *8*), and mouse liver (Fig. 4, lane *9*) gave rise to identical fragments, different from those of lamins A and C as previously seen with rat liver lamins (Raymond, Y., and G. Gagnon, manuscript submitted for publication). Hydrolysis of a spot tentatively identified as vimentin (Fig. 4, lane *10*) showed a profile different from those of the lamins indicating the specificity of the *N*-chlorosuccinimide reaction.

Discussion

In summary, we have shown that undifferentiated F9EC cells possessed a peripheral lamina where only lamin B was present and that they acquired, by de novo synthesis, lamins A and C during retinoic acid–induced differentiation into parietal endoderm–like cells (cl9 cells). Lamins have been identified by four criteria: (*a*) their presence in the insoluble nuclear matrix; i.e., the lamina; (*b*) their molecular masses and isoelectric points; (*c*) their recognition by an mAb and a polyclonal serum; and (*d*) their peptide maps as compared with mouse liver lamins.

The increase in the amount of lamins A and C was detectable as early as 24 h after the addition of retinoic acid. After this relatively early start, however, the process of accumulation of lamins A and C was slow as illustrated by the higher level of these two lamins in fully differentiated cells (cl9) than in 48-h–treated cells. A similar situation occurred during changes in the composition of the cytoskeleton where a number of days were required for the differentiating F9 cells to acquire the full complement of IF proteins seen in cl9 cells (16, 26, 27). On the other hand, the possible presence of undifferentiated cells in the 48-h–treated population may have led to an underestimation of the amount of lamins A and C in these cells.

Differentiation of F9 cells in the presence of retinoic acid is accompanied by a reduction in their rate of multiplication and their rate of DNA and protein synthesis, and the differentiated cells loose the malignancy associated with the original population (20). Interestingly, in amphibians, gametes and early developing embryos have only one type of lamin, while somatic cells have two types of lamin, and highly differentiated cells (myocytes, neurons) have three types (15). These results are suggestive of a correlation between the rate of proliferation of the cells, their differentiation state, and their lamin complement.

One important test for the relationship between prolifera-

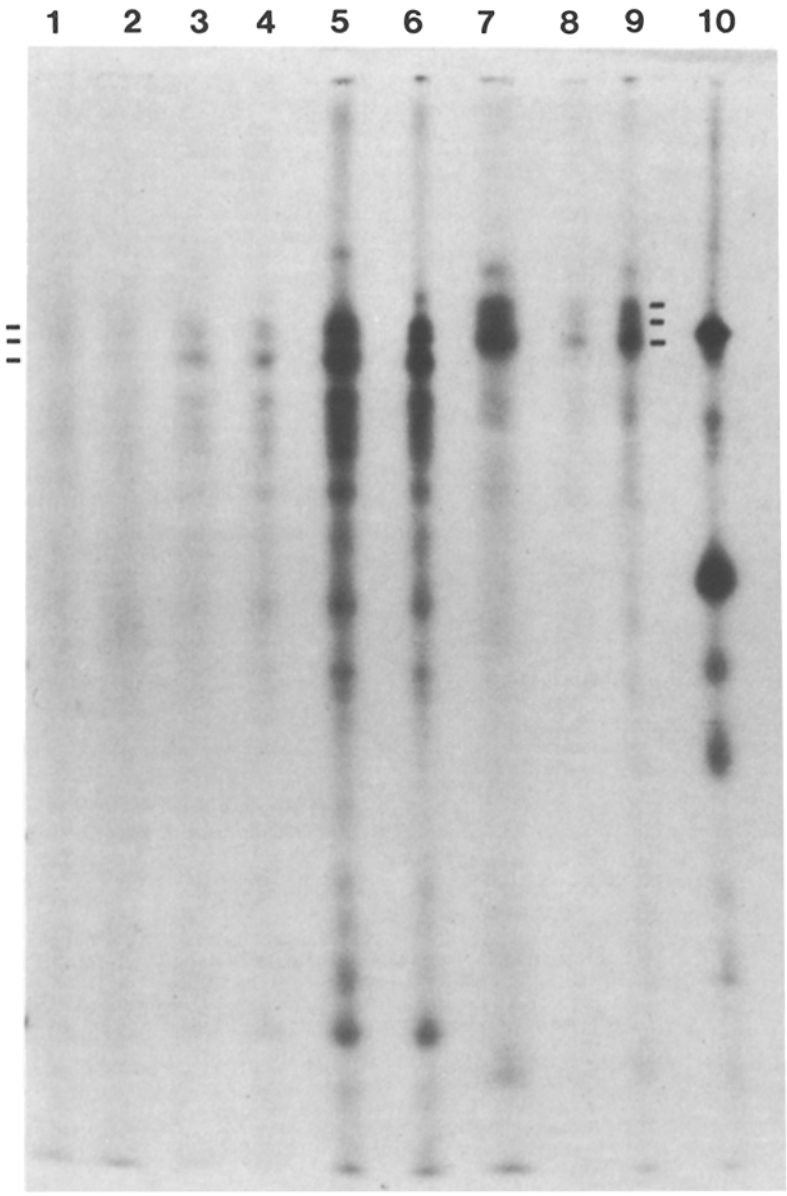

Figure 4. Peptide mapping analysis of lamins from mouse liver and embryonal carcinoma cells. [^{3}H]Dansyl chloride–labeled lamins were hydrolized with *N*-chlorosuccinimide and the fragments were subjected to electrophoresis as described in Materials and Methods. (Lanes *1* and *2*) Lamins A and C, respectively, from 48-h–treated cells; (lanes *3* and *4*) lamins A and C from cl9 cells; (lanes *5* and *6*) lamins A and C from mouse liver; (lane *7*) lamin B from 48-h–treated cells; (lane *8*) lamin B from cl9 cells; (lanes *9*) lamin B from mouse liver; (lane *10*) a spot tentatively identified as vimentin from cl9 cells. Exposure time was 30 d. Bars along lanes *1* and *9* indicate positions of major fragments generated from lamins A and B, respectively.

tion and lamina composition would be to see whether it is reversed when differentiated somatic cells undergo changes from a quiescent state to a rapidly dividing state. Although such comparisons between the polypeptide composition of nuclear matrix (of which the peripheral lamina was the major component) of rapidly dividing hepatoma cells and quiescent adult liver cells have been performed (3), these studies lacked two-dimensional gel analysis and immunological characterization of the lamins and are therefore not conclusive from this point of view. No differences were observed in the overall polypeptide composition of nuclear matrix from regenerating and normal rat liver (3).

Lamins A and C appear to be involved in the interaction between lamina and chromatin as evidenced by recent studies of their reassociation characteristics in vitro: nuclear envelope reassembly around the chromosomes was strongly inhibited by immunological depletion of lamins A and C from a cell-free extract (5). Whether lamins A and C are absolutely required in the process of envelope reformation in mammalian cells is questioned by the results of this study and by immunofluorescence studies (28; these still need biochemical confirmation) on early mouse embryos where cells appear to proliferate rapidly while their lamina are composed exclusively of lamin B. Whether lamins A and C are directly involved in the organization of chromatin or whether other polypeptides, present in embryonal carcinoma cells and early embryos, play such a role is still unresolved.

Quantitatively minor lamin species have been identified recently (19) that are recognized by some but not all anti-lamin antibodies and these minor components could be playing an essential role in the lamina. The possibility exists that the polyclonal antiserum used to deplete lamins from cell-free extracts (5) and the antibodies microinjected into mitotic cells (2) were reacting with minor as well as major lamin components: in the case of the latter, a reaction of the antibodies can be seen on one of the minor lamin components from rat liver lamina (see Fig. 1 *a* of reference 2). The conclusions drawn from the above studies (2, 5) on the role of

lamins A, B, and C in chromatin rearrangements after mitosis will have to be confirmed using antibodies reacting exclusively with the minor lamin components. In the present study, the mAb used recognized only the major lamin species in embryonal carcinoma cells but this does not exclude the possible presence of minor species as this mAb does not cross-react with minor lamin spots in rat liver lamina (our unpublished results).

Changes in lamin number and distribution are often associated with changes in nuclear morphology and, possibly, chromatin rearrangements. What correlation exists between changes in chromatin organization, rate of cellular proliferation, and lamin complement is still a question to be investigated. We think that embryonal carcinoma cells may prove to be a useful model for the study of these aspects of lamina structure and physiology.

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