Differential Expression of Nuclear Lamin Proteins during Chicken Development

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Abstract. By immunocytochemistry, quantitative immunoblotting, and two-dimensional gel electrophoresis, we have analyzed the distribution of nuclear lamin proteins during chicken embryonic development. Whereas no qualitative differences in the patterns of expression of lamins A, B_1, and B_2 were observed during gametogenesis in either the female or the male germ line, profound changes in the composition of the nuclear lamina occurred during the development of somatic tissues. Most unexpectedly, early chicken embryos were found to contain little if any lamin A, although they contained substantial amounts of lamins B_1 and B_2. During embryonic development, lamin A became increasingly prominent, whereas the amounts of lamin B_1 decreased in many tissues. Interestingly, the extent and the developmental timing of these changes displayed pronounced tissue-specific variations. Lamin B_2 was expressed in fairly constant amounts in all cell types investigated (except for pachytene-stage germ cells). These results have implications for the purported functional specializations of individual lamin proteins. In addition, they suggest that alterations in the composition of the nuclear lamina may be important for the establishment of cell- or tissue-specific differences in nuclear architecture.

THE nuclear lamina is a filamentous meshwork closely apposed to the nucleoplasmic surface of the inner nuclear membrane (1; for review see reference 15). It is thought to serve a nucleoskeletal role important for nuclear envelope integrity (8, 12, 28) and interphase chromatin organization (4, 14, 17, 22); this organization in turn may be important for DNA replication and differential gene expression (for references see 6, 9, 17). Lamin proteins have been found in a wide variety of organisms, including insects, amphibia, birds, and mammals (for review see reference 19). The three major mammalian lamins have been designated as lamins A, B, and C (12). Lamin B is implicated in anchoring the lamina to the nuclear membrane, whereas lamins A and C are thought to interact with chromatin (8, 13, 15). Based on cDNA sequence data (11, 27) as well as direct structural information obtained from electron microscopy (1), members of the lamin protein family were recently shown to constitute a novel class of (nuclear) intermediate filament proteins.

With the aid of monoclonal antibodies we have begun to study the composition and assembly of the nuclear lamina in chicken (24, 25). In particular, we have recently characterized three structurally distinct chicken proteins that we have termed lamins A, B_1, and B_2 (25). In chicken embryo fibroblasts lamins A and B_2 predominate, whereas lamin B_1 represents a quantitatively minor component (25). By immunological criteria, chicken lamin B_1 is related to mammalian lamin B, whereas chicken lamin B_2 is related to lamin A as well as to a quantitatively minor mammalian protein that had not previously been considered as a member of the lamin protein family (25). At present there is no definitive information about functional homologies between individual avian and mammalian lamin proteins.

In view of the purported involvement of the nuclear envelope in determining the three-dimensional architecture of the interphase nucleus, it is of great interest that lamin proteins are differentially expressed during embryogenesis (5, 19, 36) and gametogenesis (3, 19, 20, 35) of *Xenopus laevis*. Studies on *Drosophila* (33), mouse (26, 32), and sea urchin (32) have also been taken to suggest that reorganizations of the lamina might accompany important developmental transitions, but proof for changes in the lamin protein composition in these latter organisms is lacking. Here, we have studied the distribution of lamin proteins in different tissues during chicken development. Our studies unequivocally demonstrate that chicken lamins A, B_1, and B_2 are expressed according to a developmentally controlled and tissue-specific pattern. These results are discussed with respect to the developmental regulation of lamin protein expression in amphibia, and the purported functional specialization of lamin proteins in mammals.

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Materials and Methods

Cell Culture

Hepatocytes were isolated from livers of 17–18-d[1] chicken embryos according to Giger and Meyer (16) and cultured in Leibovitz L15 medium supplemented with 20% FCS, 10% tryptose phosphate broth, glutamin, and antibiotics (penicillin/streptomycin, 100 U/ml).

Spinal cord cell cultures were prepared by trypsinization of spinal cord tissue dissected from 8–10-d chicken embryos. After filtration through a sterile nylon filter (10 μm), cells were plated on poly-L-lysine (Sigma P-1274)-coated culture dishes and grown in DME containing 5% horse serum, 4% FCS, 1% chicken serum, 2% embryo extract, glutamin, and antibiotics.

Antibodies

The production and characterization of anti-lamin antibodies has previously been described (23, 25). Briefly, these antibodies react with the following chicken lamin proteins: the rabbit serum recognizes both lamins A and B_2. The mouse mAbs O-1 and L3-4B4 react with lamin A, the mAb L-5 recognizes lamin B_1, and the mAbs E-3 and L3-5D10 are specific for lamin B_2 (23, 25; Stick, R., unpublished data). The two mAbs specific for lamin A recognize two distinct epitopes; they were used as a mixture for staining of cryosections as well as for immunoblotting experiments. This minimizes the possibility that absence of immunoreactivity might arise from modification or inaccessibility of epitopes.

mAbs recognizing a gangliosid specific for the oligodendrocytic cell lineage (34) and the 200-kD neurofilament subunit, respectively, were kindly provided by Drs. P. Caroni and M. Schwab (University of Zurich).

Isolation of Nuclei

Nuclei from 3–7-d chicken embryos were isolated according to the method of Blobel and Potter (7) with the following slight modifications (23).

All steps were carried out on ice. Embryos were removed from the eggs and washed once in 0.25 M sucrose in TKM (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 3 mM β-mercaptoethanol). After resuspending the embryos (or parts) in an equal volume of 0.25 M sucrose in TKM, they were homogenized in a Potter-Elvehjem homogenizer. Cell disruption was routinely controlled by phase-contrast microscopy. The homogenate was filtered through three layers of nylon mesh (110 μm) before the sucrose concentration was raised by addition of 3 vol of 2.3 M sucrose in TKM. 20 ml of the mixture were then layered on a cushion of 15 ml 2.1 M sucrose in TKM and nuclei were pelleted in an SW-27 rotor (Beckman Instruments, Inc., Palo Alto, CA) during 60 min at 25,000 rpm. Finally, nuclei were washed once in TKM.

For the preparation of liver nuclei, livers were dissected from 10-, 14-, or 18-d embryos or from adult chicken and further processed as described above for the preparation of nuclei from total embryos. The same procedure was also applicable for the isolation of heart nuclei, except that homogenization of the tissue was more vigorous. Brain nuclei could not be pelleted through the 2.1 M sucrose/TKM cushion. Therefore a modified cushion was prepared by overlaying 5 ml 2.0 M sucrose in TKM with 10 ml 1.9 M sucrose in TKM. During the relatively mild homogenization of embryos or liver and brain tissues, erythrocytes remained intact and were not pelleted by subsequent centrifugation. Contamination with erythrocyte nuclei was therefore very low in these preparations. In the case of adult heart nuclei, however, contamination by erythrocytes was significant.

To isolate erythrocyte nuclei, blood from 4-, 5.5-, 7-, and 18-d chicken embryos or from adult animals was collected into citrate buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 15 mM sodium citrate). To bleed young embryos (4, 5.5, and 7 d), they were removed as fast as possible from the eggs, put in citrate buffer, decapitated, and cut in several parts. To remove the embryos after bleeding, the suspension was filtered through a nylon mesh (110 μm). Erythrocytes were sedimented at 1,000 *g* for 10 min and washed twice in citrate buffer. The buffy coat was carefully removed after each sedimentation. After a 10-min incubation in lysis buffer (10 mM Tris-HCl, pH 7.2, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 3 mM β-mercaptoethanol), erythrocytes were lysed by dounce homogenization; nuclei were pelleted for 10 min at 2,000 *g* and washed three times in lysis buffer.

1. *Abbreviations used in this paper*: d, days of incubation after egg laying.

Preparation of Pore-Complex Lamina Fractions

Pore-complex lamina fractions were prepared according to Dwyer and Blobel (10), with the following slight modifications: nuclei (2–5 mg protein) were washed with TKM and resuspended on a vortex by dropwise addition of 300 μl ice cold 0.1 mM $MgCl_2$. Then, 1.5 μl of DNase 1 and RNase A stock solutions (each 1 mg/ml in 10 mM Tris-HCl, pH 7.5, 0.1 mM $MgCl_2$, 10% sucrose) were added, followed rapidly by the addition of 1.2 ml of digestion buffer 1 (10 mM Tris-HCl, pH 8.5, 0.1 mM $MgCl_2$, 10% sucrose, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 3 mM β-mercaptoethanol). After digestion for 15 min at room temperature, the nuclear residues were pelleted (2,000 *g*, 10 min) and resuspended in 100 μl digestion buffer 2 (10 mM Tris-HCl, pH 7.5, 0.1 mM $MgCl_2$, 10% sucrose, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 3 mM β-mercaptoethanol). 1.5 μl of DNase 1 and RNase A stock solutions were added and digestion was again for 15 min at room temperature. The digested nuclei were pelleted (8,000 *g*, 10 min) and resuspended in 90 μl digestion buffer 2. After addition of 10 μl Triton X-100 (10% wt/vol) extraction was carried out on ice for 10 min. The unsoluble structures were pelleted (13,000 *g*, 10 min) and resuspended in 70 μl digestion buffer 2. Finally, 30 μl of high salt buffer (100 mM Tris-HCl, pH 7.5, 2 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 3 mM β-mercaptoethanol) were added and extraction was for 10 min on ice. The residual structures (i.e., the pore-complex lamina fraction) were sedimented (13,000 *g*, 10 min) and washed once in distilled water.

Gel Electrophoresis and Quantitative Immunoblotting

One-dimensional SDS-PAGE was carried out according to Laemmli (21). Two-dimensional analyses involving nonequilibrium pH gradient electrophoresis (NEPHGE) in the first and SDS-PAGE in the second dimension were performed as described previously (25, 30). The solubilization of pore-complex lamina fractions for two-dimensional gel electrophoresis has also been described (25, 31).

For immunoblotting, proteins were resolved by SDS-PAGE and transferred to nitrocellulose paper (23, 39). After overnight incubation in blocking buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 3% BSA, 0.1% Triton X-100), nitrocellulose filters were incubated for 4 h at room temperature with ascites fluids diluted 2,000-fold in the same buffer. Excess antibody was removed by two quick washes with blocking buffer containing only 1% BSA, followed by three 10-min incubations in the same buffer. Secondary antibodies were (^{125}I)iodinated sheep anti-mouse antibodies (Amersham Corp., Arlington Heights, IL) diluted to 0.1 μCi/ml in blocking buffer. After two washes in blocking buffer containing only 1% BSA, nitrocellulose papers were thrice incubated for 10 min in blocking buffer containing 0.5 M NaCl and only 0.1% BSA. Nitrocellulose papers were then rinsed quickly with 10 mM Tris-HCl, pH 7.5, dried, and autoradiographed using intensifying screens.

Densitometric quantitation of the lamin proteins (Table I) was carried out as follows. The signal intensities ($S_{\text{lamin x}}$) on the autoradiographs of the immunoblots were scanned using a Desaga Chromatogramm Densitometer CD50/Shimadzu CS930. In parallel, the amounts of core histones (S_{histone}) in the corresponding nuclear samples were quantified densitometrically by scanning Coomassie Blue-stained gels. The relative lamin contents ($S_{\text{lamin x}}/S_{\text{histone}}$) were then calculated as the ratios between $S_{\text{lamin x}}$ and S_{histone}. To ascertain linearity of the assay, serially diluted nuclear samples isolated from 18-d embryos and adult chicken were analyzed. All other determinations were subsequently carried out under conditions where linearity had been established. Finally, to standardize the values determined for different tissues, the amount of lamin A in the pore-complex lamina fraction prepared from liver nuclei of 18-d chicken embryos was arbitrarily set to 100%. The contents of lamins B_1 and B_2 in the same preparation were then determined by scanning Coomassie Blue-stained gels; relative to lamin A, they were found to be 27% for lamin B_1 and 100% for lamin B_2. Values obtained for other tissues were then expressed relative to these standards.

Indirect Immunofluorescence

For cryosections, 3 × 3 × 3-mm pieces of tissue were fixed during 2 h at room temperature in PBS containing 3% formaldehyde and 2% sucrose. The fixed tissue blocks were then washed three times for 5 min in PBS and incubated overnight in PBS containing 2.3 M sucrose. After freezing in liquid nitrogen, cryosections (0.8 μm) were cut using a Reichert-Jung ultramicrotome. Before labeling with antibodies, sections were permeabilized with PBS containing 0.5% Triton X-100 and washed three times in PBS. In the case of cryosections through ovaries, the isolated tissue was immediately

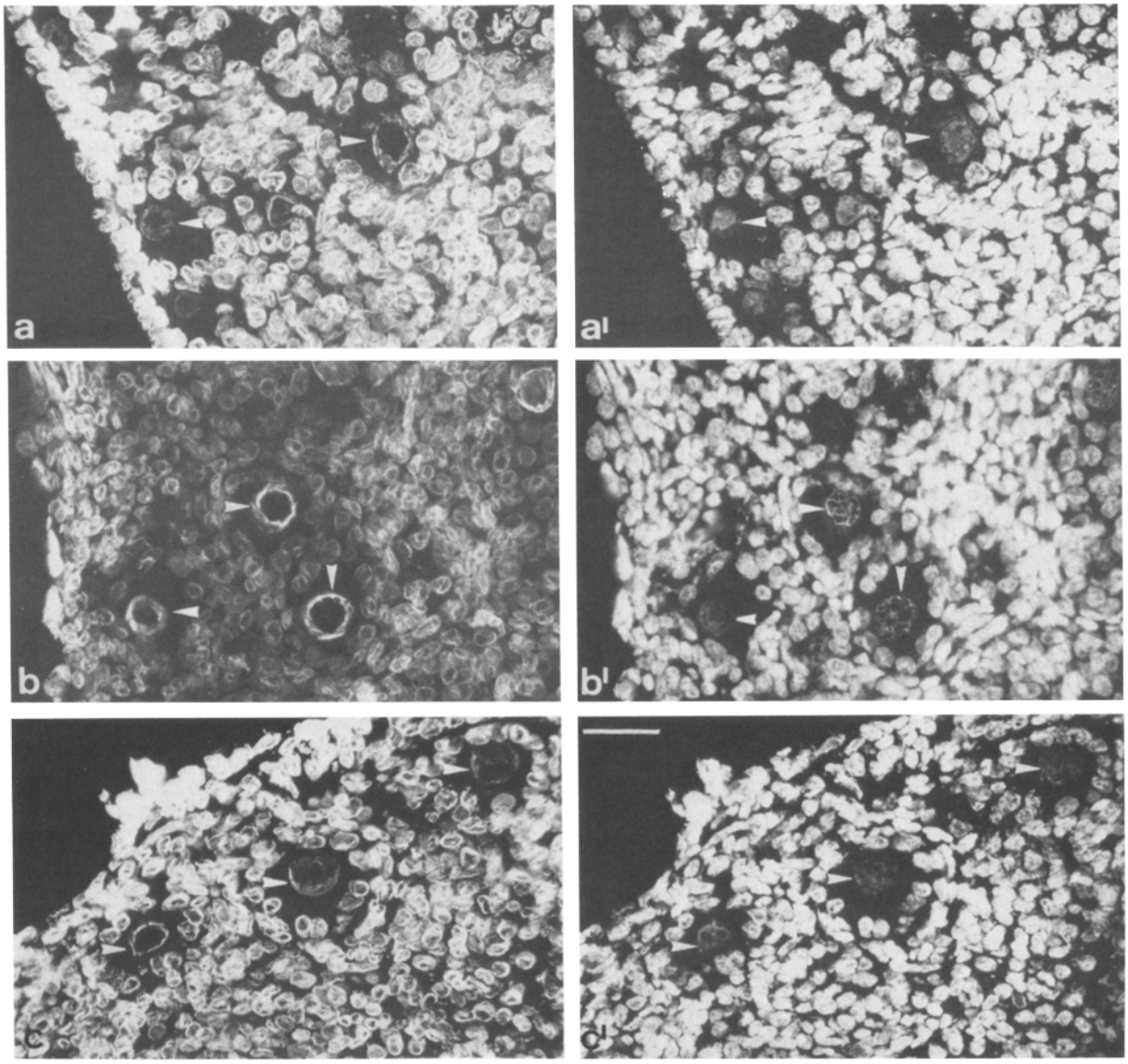

Figure 1. Immunofluorescent staining of lamins A, B_1, and B_2 in diplotene oocytes. Cryosections (6 μm) through ovaries of a 12-d-old chicken were stained by indirect immunofluorescence (*a–c*) and counterstained with diamidinophenylindole (*a'–c'*). The antibodies used were (*a*) mAb L3-4B4, anti-lamin A; (*b*) mAb L-5, anti-lamin B_1; (*c*) mAb L3-5D10, anti-lamin B_2. Arrowheads point to diplotene oocyte nuclei. Bar, 25 μm.

frozen in *N*-methylbutane cooled with liquid nitrogen. Fixation was then carried out after sectioning (6 μm) by immersion in acetone (−20°C) for 10 min. Incubation with primary antibodies was for 10 min at room temperature. All sections were routinely counterstained using either diamidinophenylindole (DAPI) or the rabbit serum against lamins A and B_2 (which was directly diluted 1:300 into hybridoma supernatants). Before applying the secondary antibodies, sections were washed three times for 5 min in PBS. Secondary antibodies were rhodamine-conjugated sheep anti-mouse IgG (Cappel Laboratories, Cochranville, PA) and fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories) both diluted 1:300 in PBS. After a 10-min incubation at room temperature the cryosections were again washed three times for 5 min in PBS, and mounted on a drop of 90% glycerol, 10% 1 M Tris-HCl (pH 9.0).

Cells grown in culture dishes were fixed for 5 min in 3% formaldehyde, 2% sucrose in PBS (23, 29). After washing three times in PBS, the cells were permeabilized for 5 min using 0.5% Triton X-100 in PBS, and washed again three times in PBS. Incubations with primary and secondary antibodies were as described above for cryosections. To perform immunofluorescence experiments on isolated nuclei, these were centrifuged onto glass slides using a Cytospin centrifuge. In this case fixation was with methanol (−20°C) for 5 min, followed by acetone (−20°C) for 20 s. Labeling with antibodies was as described above.

For immunofluorescence microscopy a Zeiss Standard Model 18 microscope equipped with a Zeiss Planapo ×63 oil immersion objective was used.

Results

Immunocytochemical Analysis of Lamin Protein Distribution in Chicken Germ Cells

To analyze the lamin protein distribution in female germ cells, cryosections (6 μm) were cut through developing chicken ovaries and stained with mAbs specific for either lamin A, lamin B_1, or lamin B_2. Confirming and extending earlier results (35, 38), no lamin proteins could be detected in pachytene-stage oocytes (not shown), but all three lamins

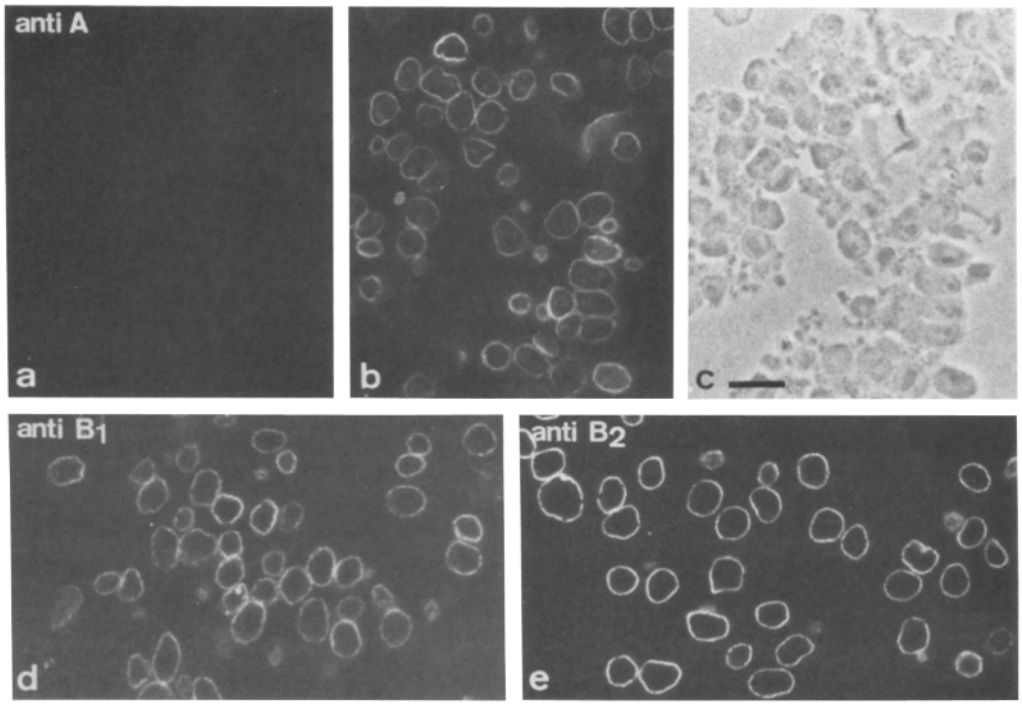

Figure 2. Immunofluorescent staining of lamins A, B_1, and B_2 in early embryos. Cryosections (0.8 μm) through the head region of a 3-d chicken embryo were stained by indirect immunofluorescence (*a*, *b*, *d*, and *e*) using the following antibodies: (*a*) mAbs L3-4B4 and O-1 (mixed), anti-lamin A; (*b*) rabbit serum anti-lamins A and B_2 (same field as in *a*); (*d*) mAb L-5, anti-lamin B_1; (*e*) mAb E-3, anti-lamin B_2. The phase-contrast micrograph (*c*) corresponds to the field shown in *a* and *b*. Bar, 10 μm.

were readily stained in diplotene oocytes (arrowheads in Fig. 1, *a–c*). As judged by the intensity of the fluorescent labeling, lamin B_1 appeared to be more abundant in oocytes than in the surrounding follicle cells (Fig. 1 *b*), whereas lamins A and B_2 seemed to be present in comparable amounts in both cell types (Fig. 1, *a* and *c*).

By immunofluorescent staining of cryosections through testes, none of the three lamin proteins could be detected in male germ cells after the pachytene stage of meiosis (not shown). The absence of lamins A, B_1, and B_2 from purified sperm was confirmed by immunoblotting (not shown). Thus, to the extent that gametogenesis has been studied, there are no major qualitative differences in the expression of lamins A, B_1, and B_2 (35, 37, 38; this study).

Immunocytochemical Analysis of Lamin Expression during Chicken Embryogenesis

Fig. 2 shows cryosections through the head region of a 3-d chicken embryo stained with antibodies against the different lamin proteins. Surprisingly, no significant labeling was produced by mAbs directed against lamin A (Fig. 2 *a*), even though nuclear envelopes on the same section were readily labeled by a polyclonal rabbit serum recognizing both lamins A and B_2 (Fig. 2 *b*). Strong staining was produced also by mAbs specific for either lamin B_1 (Fig. 2 *d*) or lamin B_2 (Fig. 2 *e*). Cryosections through the body region of a 3-d chicken embryo revealed a similar distribution of lamin proteins: only very few cells were positive for lamin A (not shown). In all likelihood, these cells were of the erythroid lineage; indeed, maturing erythroid cells were found to contain relatively high amounts of lamin A already in very early stages of embryogenesis (see below).

Fig. 3 summarizes the results obtained when labeling cryosections through tissues of 10-d embryos: lamin A was virtually undetectable in 10-d embryonic brain (Fig. 3 *a*), but was readily stained on cryosections through 10-d embryonic liver (Fig. 3 *b*), muscle, or heart tissue (not shown). Conversely, lamin B_1 was brightly stained in brain (Fig. 3 *c*), but only weakly in liver (Fig. 3 *d*). Intense and uniform labeling of nuclear envelopes of either tissue was produced by mAbs against lamin B_2 (Fig. 3, *e* and *f*). We emphasize that all cryosections were routinely counterstained using either diamidinophenylindole or the rabbit serum recognizing both lamins A and B_2. This control serum invariably produced strong and uniform labeling of all nuclear envelopes even on sections where staining for lamin A was virtually undetectable (i.e., in brain tissues) or staining for lamin B_1 was very weak (i.e., most cells in liver tissues). Results very similar to those shown in Fig. 3 were obtained when analyzing brain and liver tissues from 18-d embryos (not shown).

In adult tissues, most brain cells did contain lamin A, but labeling was variable and many nuclei were labeled to a very low extent (not shown). The majority of cells in adult liver were brightly stained by antibodies against lamin A, but it is noteworthy that a few rare cells remained unstained (arrowhead in Fig. 4 *a*). Conversely, lamin B_1 was readily stained in brain cells (not shown), but only low levels of labeling were observed in adult liver (Fig. 4 *b*). Again it is remarkable that in liver rare cells (around 5%) were strongly labeled by anti-lamin B_1 antibodies (arrowheads in Fig. 4 *b*).

Lamin Expression in Cultured Cells

To determine the distribution of individual lamin proteins with respect to defined cell types, analyses were extended to tissue culture cells. For further analysis of lamin A expression, cultures were prepared from spinal cord tissue of 8-d chicken embryos. Neurons were identified by morphological criteria (aggregation, axon outgrowth), by positive immunolabeling with a mAb specific for neurofilament protein (Fig. 5 *a*), and by the absence of reaction with an mAb (34)

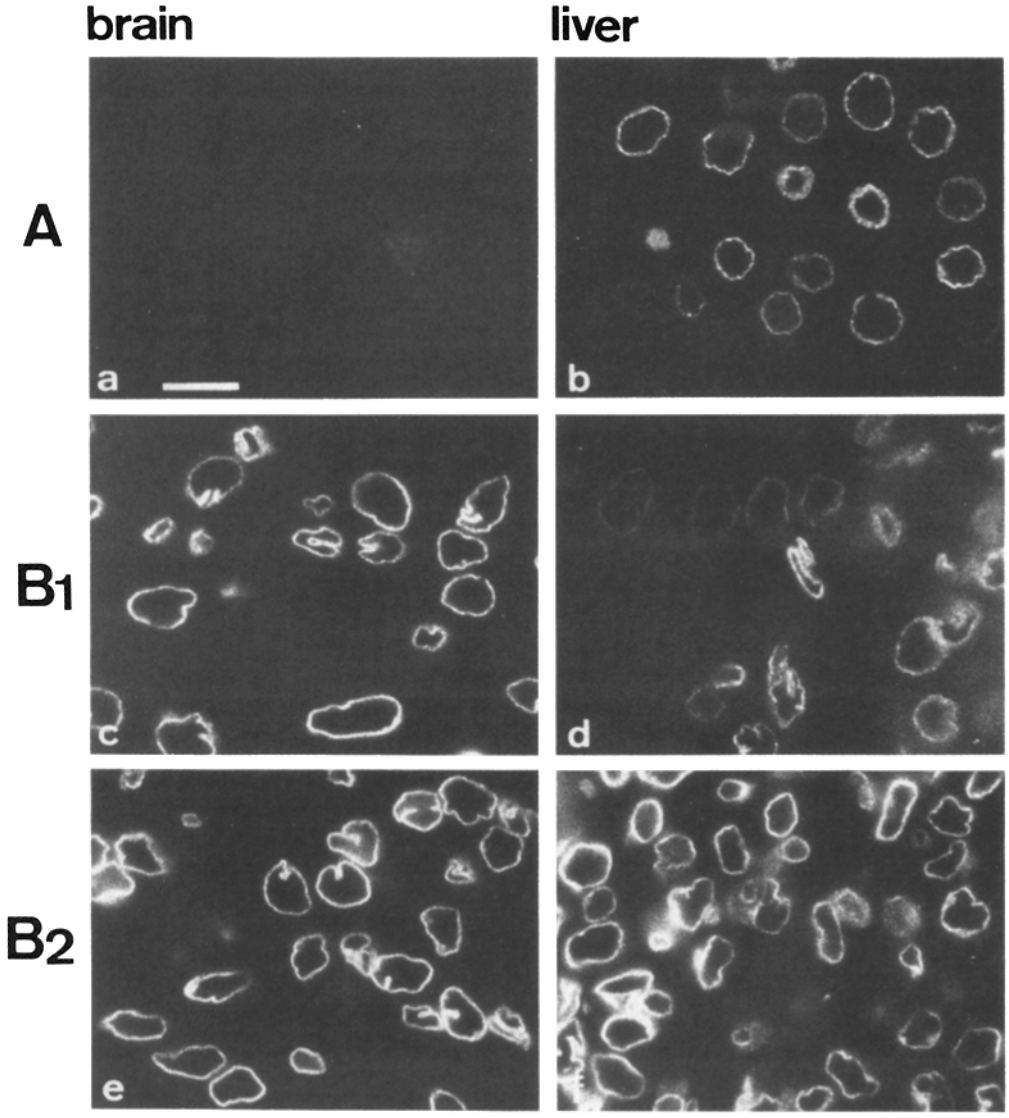

Figure 3. Immunofluorescent staining of lamins A, B_1, and B_2 in 10-d embryonic brain and liver. Cryosections (0.8 μm) through brain (*a*, *c*, and *e*) or liver (*b*, *d*, and *f*) of a 10-d chicken embryo were stained by indirect immunofluorescence using the following antibodies: (*a* and *b*) mAbs L3-4B4 and O-1 (mixed), anti-lamin A; (*c* and *d*) mAb L-5, anti-lamin B_1; (*e* and *f*) mAb E-3, anti-lamin B_2. Bar, 10 μm.

directed against a surface glycolipid specific for cells of the oligodendrocyte lineage (Fig. 5 *b*). Vice versa, cells of the glial lineage did not contain neurofilaments, but many reacted readily with the antibody against the oligodendrocyte marker (not shown). Consistent with the results reported above, lamin A was almost undetectable in the nuclear envelopes of either neurons (Fig. 5 *c*) or glial cells (not shown). In contrast, the very rare fibroblasts contaminating the cultures were very brightly stained by the anti-lamin A antibodies (not shown). mAbs against lamins B_1 and B_2 readily stained both neurons (Fig. 5, *d* and *e*) and glial cells (not shown).

To confirm the low level of expression of lamin B_1 in liver cells, hepatocytes were prepared from livers of 18-d chicken embryos. When these cultures were labeled with an antibody specific for lamin B_1, hepatocytes reacted very weakly (Fig. 5 *f*), whereas the rare contaminating fibroblasts were strongly stained (arrowheads in Fig. 5 *f*). As shown by double-immunofluorescence microscopy, virtually identical staining of hepatocytes and fibroblasts was produced by the polyclonal rabbit serum recognizing both lamins A and B_2 (Fig. 5 *g*). Hepatocytes were also readily labeled by mAbs specific for either lamins A (Fig. 5 *i*) or B_2 (Fig. 5 *k*).

Thus, immunofluorescent staining of cultured embryonic spinal cord cells and hepatocytes revealed the same lamin protein distribution as found by the analysis of sections through the corresponding tissues: neurons and glial cells appeared to contain little if any lamin A, whereas hepatocytes seemingly contained only low amounts of lamin B_1.

Analysis of Lamin Protein Distribution by Quantitative Immunoblotting

To confirm the results of the immunofluorescence experiments by immunoblotting, nuclei were prepared from embryonic tissues of different developmental stages. Whereas 3-d chicken embryos were directly used for the preparation of nuclei. 5-d embryos were first divided into a head region and a body region. In the case of 7-d embryos, the brains were separated from the rest of the embryos and both parts

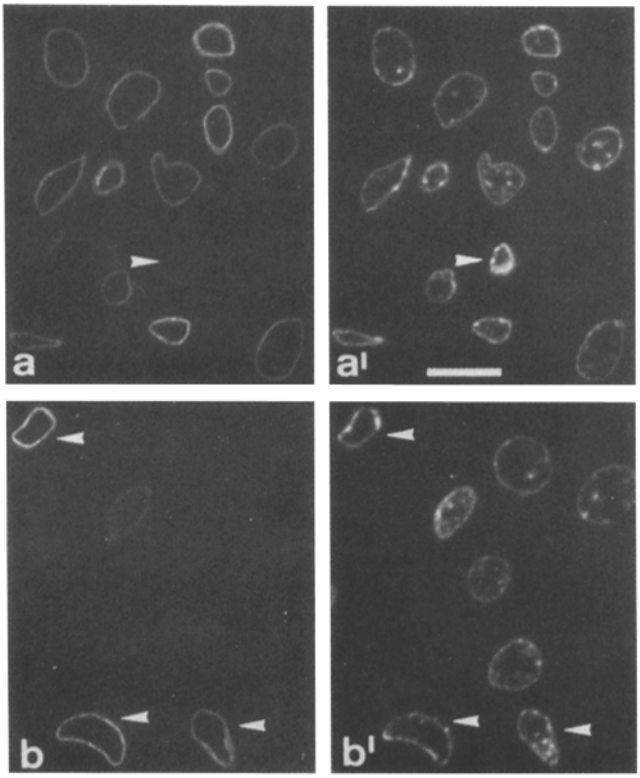

Figure 4. Heterogeneity of lamin protein distribution in liver cells. Cryosections through adult liver tissue were stained by indirect immunofluorescence (*a* and *b*) using either the mAb L3-4B4 recognizing lamin A (*a*) or the mAb L-5 recognizing lamin B_1 (*b*). The sections were counterstained with diamidinophenylindole (*a'* and *b'*). Arrowheads in *a* and *a'* point to a nucleus devoid of lamin A staining; in *b* and *b'* the arrowheads point to a few nuclei that are intensely labeled by the mAb L-5 (anti-lamin B_1). Bar, 10 μm.

were subsequently used for preparation of nuclei. In the case of later (10-, 14-, and 18-d) embryos and adult chicken, nuclei were isolated from brain as well as from liver. The proteins of the individual preparations were separated by SDS-PAGE and either stained with Coomassie Blue (Fig. 6 *A*) or processed for immunoblotting with antibodies directed against lamin A (Fig. 6 *B*), lamin B_1 (Fig. 6 *C*), or lamin B_2 (Fig. 6 *D*). Consistent with the results of the immunofluorescence experiments, lamin A could not be detected in 3-d embryos (Fig. 6 *B*, lane *1*). In 5–7-d embryos, lamin A was detectable in the body region (Fig. 6 *B*, lanes *3* and *5*), but not in the head or brain (Fig. 6 *B*, lanes *2* and *4*) and only very low amounts of lamin A were detected in brains from 10-, 14-, and 18-d embryos (Fig. 6 *B*, lanes *6*, *8*, and *10*). Even in adult brain nuclei (Fig. 6 *B*, lane *12*) the amounts of lamin A were still markedly lower than those detectable in liver nuclei from 10-, 14-, and 18-d embryos (Fig. 6 *B*, lanes *7*, *9*, and *11*) or from adult chicken (Fig. 6 *B*, lane *13*). In contrast to lamin A, lamin B_1 was detectable in all samples, but the amounts of lamin B_1 were somewhat lower in liver nuclei than in brain nuclei (Fig. 6 *C*, compare lanes *7*, *9*, *11*, and *13* with lanes *6*, *8*, *10*, and *12*, respectively). No major changes were noted in the case of lamin B_2 expression (Fig. 6 *D*).

Consistent with the impression gained in the course of the immunocytochemical experiments, erythrocytes were found to contain lamin A already in early embryos (Fig. 7). Crude nuclei from erythrocytes were prepared using blood from 4-d (Fig. 7, *A–D*, lanes *1*), 5.5-d (Fig. 7, *A–D*, lanes *2*), or 7-d (Fig. 7, *A–D*, lanes *3*) embryos. Immunoblotting experiments using the appropriate antibodies revealed an increase of lamin A (Fig. 7 *B*), a decrease of lamin B_1 (Fig. 7 *C*), and relatively constant amounts of lamin B_2 (Fig. 7 *D*) during terminal differentiation of primitive red blood cells.

To analyze the immunoblotting experiments in more quantitative terms, lamin protein contents were determined by densitometric scanning. For calibration, lamin contents were calculated relative to the amounts of core histones present in individual nuclear preparations (for details see Materials and Methods). The results summarized in Table I clearly show that early embryonic cells contain high amounts of lamin B_1 and lamin B_2 but little or no lamin A. Later in embryonic development, a large raise in lamin A content occurs in most embryonic tissues. This increase in lamin A is roughly paralleled by a decrease in lamin B_1. Among the tissues studied here, the first cells to change their lamin protein composition are the red blood cells during primitive erythropoiesis. Somewhat later, the change of the lamin protein composition is detectable in liver. As judged by immunofluorescence microscopy, a similar transition occurs at about the same time in heart and muscle (not shown). Surprisingly, very little change in the lamin protein composition occurs during embryonic brain development, and even adult brain, when compared to liver, heart, or erythrocytes, clearly contains low amounts of lamin A (Table I).

The molar ratios between lamins A and B_2 as well as between lamins B_1 and B_2 are also summarized in Table I. By immunofluorescent staining of isolated nuclei from samples that were later to be used for quantitative immunoblotting experiments, we have tried to estimate the variability of the lamin protein content among individual nuclei in a given preparation (not shown). The preparations from erythrocytes, livers, and early (3-d) embryos showed low variabilities in labeling intensities when stained with antibodies against either one of the three lamin proteins. Thus, for these preparations the determined molar ratios may (approximately) reflect the stoichiometries of the lamin proteins in individual cells. In contrast, brain nuclei and embryonic nuclei from 5-d or 7-d embryos were found to be very heterogeneous with respect to size and intensity of immunofluorescent labeling by antibodies against lamins A or B_1. For these cell populations, the estimated molar ratios therefore represent only average values.

Biochemical Confirmation of Immunochemical Results

The interpretation of immunochemical data may in principle be complicated by alterations or inaccessibilities of epitopes, and absence of immunoreactivity may not a priori be taken to imply the absence of a corresponding antigen. To eliminate these potential ambiguities and to confirm our results by antibody-independent techniques, we have analyzed pore-complex lamina preparations from all relevant tissues by two-dimensional gel electrophoresis. As shown by the representative results summarized in Fig. 8, the lamin protein composition in such preparations can readily be visualized by Coomassie Blue staining. In agreement with our immunochemical data, no lamin A was found in preparations from

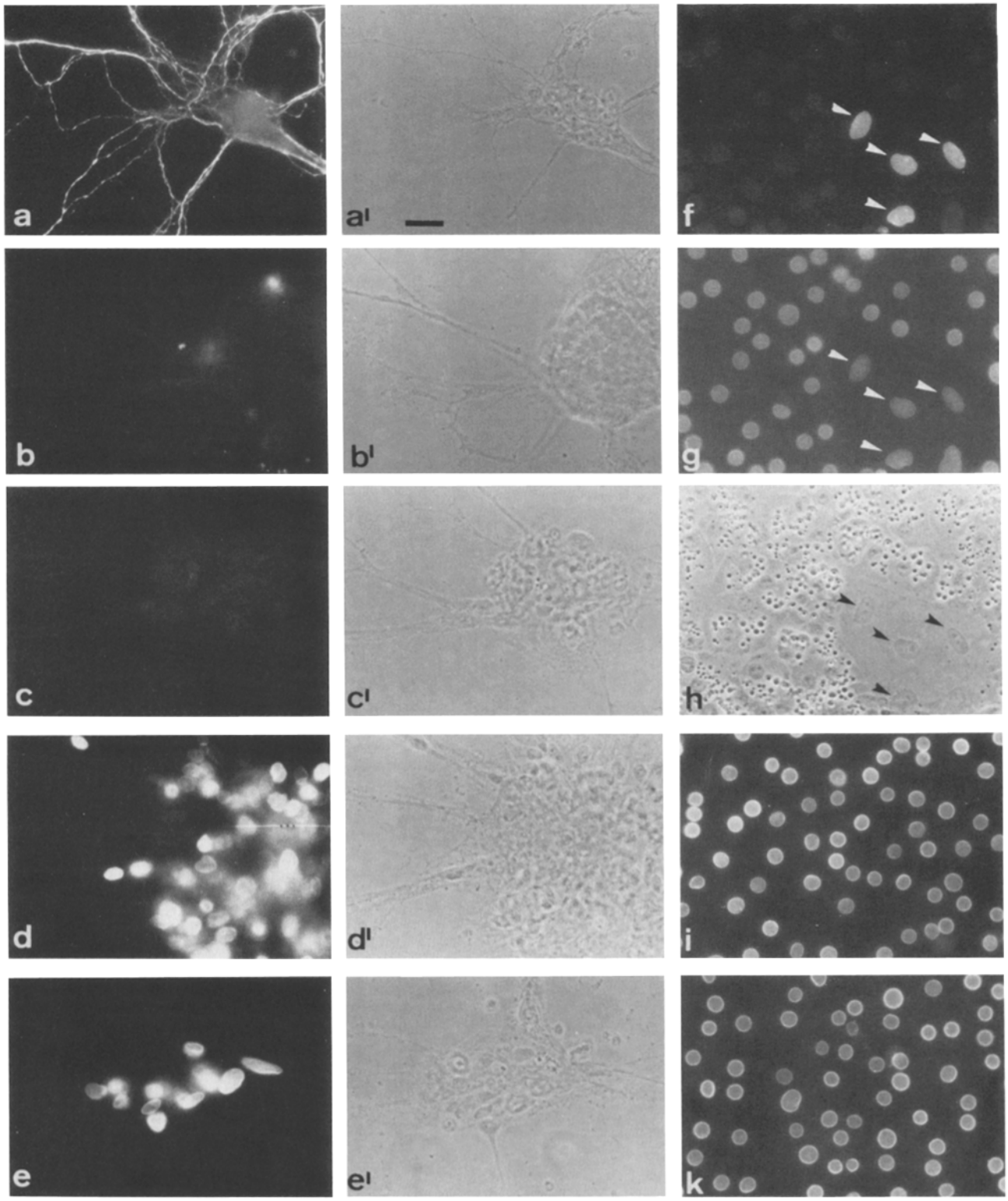

Figure 5. Immunofluorescent analysis of lamin protein distribution in cultured cells. (*a*,*a'*–*e*,*e'*) Cell cultures were prepared from spinal cord tissue of 8-d chicken embryos. After 7 d in culture, cells were fixed with formaldehyde and permeabilized with Triton X-100 (except for the cells shown in *b*,*b'* which were used unpermeabilized). Indirect immunofluorescent labeling was carried out using the following antibodies: (*a*) mAb recognizing the 200-kD subunit of neurofilaments; (*b*) mAb recognizing a gangliosid specific for the oligodendrocytic lineage (34); (*c*) mAb O-1, anti-lamin A; (*d*) mAb L-5, anti-lamin B_1; (*e*) mAb E-3, anti-lamin B_2. The phase-contrast micrographs *a'*–*e'* correspond to frames *a*–*e*. Bar, 20 μm. (*f*–*k*) Cultures were prepared from liver tissue of 18-d chicken embryos, incubated for 2 d, and then processed for immunofluorescent staining as described above. Antibodies used were (*f*) mAb L-5, anti-lamin B_1; (*g*) rabbit serum anti-lamins A and B_2 (same field as in *f*); (*i*) mAb 0-1, anti-lamin A; (*k*) mAb E-3, anti-lamin B_2. The phase-contrast micrograph (*h*) corresponds to the field shown in *f* and *g*. The arrowheads in *f*–*h* point to strongly lamin B_1-positive nuclei of fibroblasts which contaminate the hepatocyte cultures.

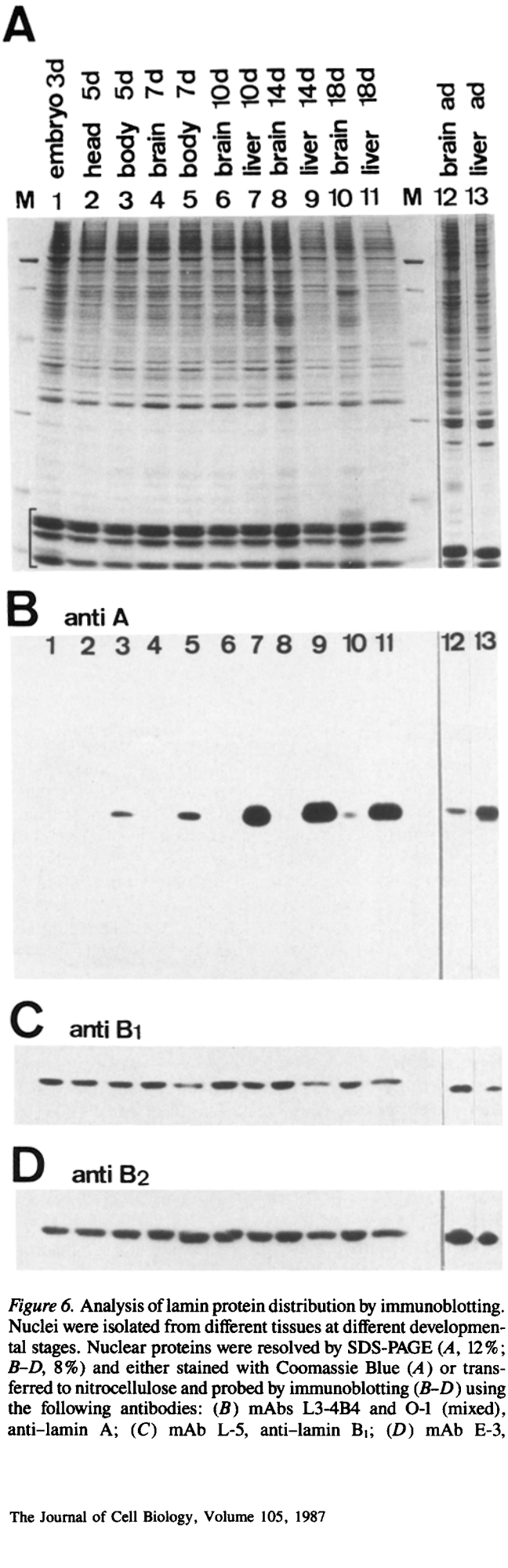

Figure 6. Analysis of lamin protein distribution by immunoblotting. Nuclei were isolated from different tissues at different developmental stages. Nuclear proteins were resolved by SDS-PAGE (*A*, 12%; *B–D*, 8%) and either stained with Coomassie Blue (*A*) or transferred to nitrocellulose and probed by immunoblotting (*B–D*) using the following antibodies: (*B*) mAbs L3-4B4 and O-1 (mixed), anti-lamin A; (*C*) mAb L-5, anti-lamin B_1; (*D*) mAb E-3, anti-lamin B_2. Nuclei were prepared from (lanes *1*) 3-d embryos; (lanes *2*) 5-d embryos, head region; (lanes *3*) 5-d embryos, body region; (lanes *4*) 7-d embryos, brain; (lanes *5*) 7-d embryos, body region; (lanes *6*) 10-d embryos, brain; (lanes *7*) 10-d embryos, liver; (lanes *8*) 14-d embryos, brain; (lanes *9*) 14-d embryos, liver; (lanes *10*) 18-d embryos, brain; (lanes *11*) 18-d embryos, liver; (lanes *12*) adult chicken, brain; (lanes *13*) adult chicken, liver. The lanes denoted by *M* contain marker proteins, from top to bottom: phosphorylase B (92,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (31,000), and soybean trypsin inhibitor (21,000). Core histones are indicated by a bracket next to lane *1*. Only the relevant parts of the immunoblots are shown in *C* and *D*.

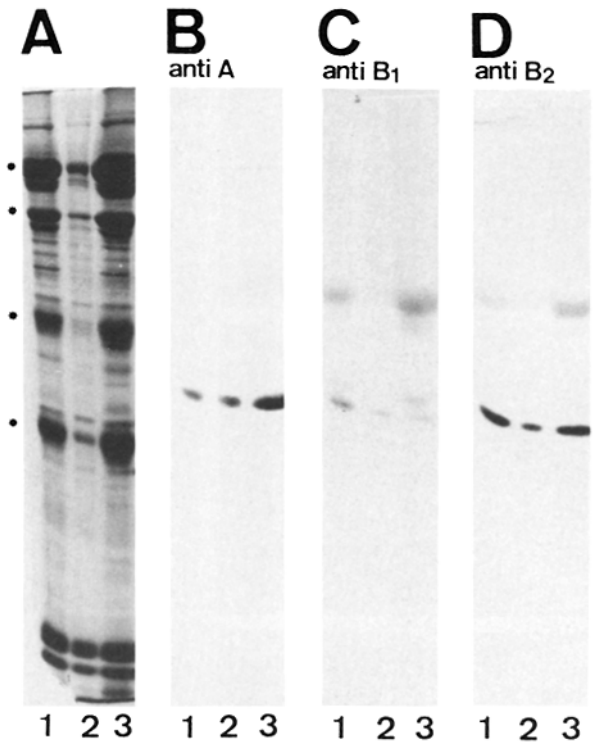

Figure 7. Immunoblotting analysis of lamin protein distribution in terminally differentiating red blood cells. Erythrocyte nuclei were prepared using blood of 4-d (lanes *1*), 5.5-d (lanes *2*), or 7-d chicken embryos (lanes *3*). Nuclear proteins were resolved by SDS-PAGE (*A*, 12%; *B–D*, 8%) and either stained with Coomassie Blue (*A*) or transferred to nitrocellulose and probed by immunoblotting (*B–D*) using the following antibodies: (*B*) mAbs L3-4B4 and O-1 (mixed), anti-lamin A; (*C*) mAb L-5, anti-lamin B_1; (*D*) mAb E-3, anti-lamin B_2. Asterisks denote yolk proteins contaminating different nuclear preparations to a variable extent; these yolk proteins gave rise to nonspecific reactions in the immunoblotting experiments (pronounced in *C* and *D*).

either early embryos (Fig. 8 *a*) or 18-d embryonic brain (Fig. 8 *b*), while considerable amounts of lamin A were present in preparations from 18-d embryonic livers (Fig. 8 *c*). In adult brain, lamin A could be detected (Fig. 8 *d*), but the relative amount was clearly lower than in adult liver (Fig. 8 *e*). Lamin B_1 was found in relatively high amounts in pore-complex lamina fractions from either early embryos (Fig. 8 *a*) or embryonic brain (Fig. 8 *b*), but was present in comparatively minor amounts in preparations from liver (Fig. 8, *c* and *e*) or adult brain (Fig. 8 *d*). Thus, the results of biochemical analyses of pore-complex lamina preparations fully support our immunochemical findings.

Table I. Quantitation of Lamins A, B_1, and B_2 during Chicken Embryogenesis

Origin of nuclear preparation		Relative lamin content*			Molar ratios	
					Lamin A	Lamin B_1
Age	Organ	Lamin A	Lamin B_1	Lamin B_2	Lamin B_2	Lamin B_2
		%	%	%		
3 d	Embryo	<0.6	65	65	<0.01	1
5 d	Head	<0.6	66	84	<0.01	0.79
7 d	Brain	<0.6	70	112	<0.01	0.62
10 d	Brain	<0.6	91	128	<0.01	0.71
14 d	Brain	3.2	75	118	0.03	0.64
18 d	Brain	8.4	76	147	0.06	0.54
Adult	Brain	31	53	130	0.23	0.38
5 d	Body	8	63	118	0.07	0.54
7 d	Body	12.3	33	125	0.1	0.27
10 d	Liver	92	70	122	0.75	0.58
14 d	Liver	100	48	100	1	0.48
18 d	Liver	100	27	100	1	0.27
Adult	Liver	148	35	100	1.48	0.35
18 d	Heart	56	26	96	0.58	0.27
Adult	Heart	114	35	82	1.39	0.18
4 d	Erythrocytes	14	11	31	0.45	0.34
5.5 d	Erythrocytes	14	5.4	29	0.48	0.18
7 d	Erythrocytes	47	4.7	34	1.38	0.13
18 d	Erythrocytes	32	4.8	28	1.14	0.17
Adult	Erythrocytes	42	5.2	22	1.9	0.23

* The determination of the relative lamin contents ($S_{\text{lamin x}}/S_{\text{histone}}$) is described in detail in Materials and Methods.

Discussion

Using both immunochemical and biochemical techniques, we have studied the tissue distribution of nuclear lamin proteins during chicken embryonic development. Based on immunofluorescent staining of tissue sections and cultured cells, quantitative immunoblotting, and two-dimensional gel electrophoretic analyses of pore-complex lamina preparations, we demonstrate that chicken embryogenesis is accompanied by profound changes in the composition of the nuclear lamina.

Lamin B_2 Is a Constant Element of the Chicken Nuclear Lamina

Whereas lamins A and B_1 were barely or not at all detectable in certain cell types, all chicken cells (except pachytene-stage germ cells which are known to lack a lamina structure [35, 37, 38]) were found to contain relatively high amounts of lamin B_2. Indeed, in all immunofluorescence experiments with antibodies against lamin B_2, all nuclei displayed indistinguishable labeling intensities, suggesting that the amounts of lamin B_2 may actually be proportional to the nuclear surface area. Some support for this notion stems from correlating the relative amounts of lamin B_2 with the estimated nuclear surface areas from different nuclear preparations (Table I): lamin B_2 contents are lower for the small nuclei of erythrocytes, and higher for the relatively large nuclei of brain. On the other hand, the results of our quantitative immunoblotting assay suggest that there may be some increase of lamin B_2 during early embryonic development (Table I).

Lamin A and Lamin B_1 Display a Variable Expression

In contrast to the comparatively constant expression of lamin B_2, the relative amounts of lamin A and, to a minor extent, of lamin B_1, showed striking variations. Thus, most if not all embryonic tissues were characterized by a low content of lamin A and a high content of lamin B_1. Conversely, all adult tissues investigated (except for brain) were found to contain high amounts of lamin A, but only low amounts of lamin B_1. It is particularly remarkable that, depending on the cell type, the transitions from an "embryonic" to an "adult" lamin protein composition occurred at different stages of development. These transitions were observed at a very early time (around day 5) in the primitive red blood cells, somewhat later (around day 7) in liver, muscle, and heart tissue, and still later in brain. It is of interest also, that rare cells in liver (and other tissues) seemingly retained a lamin protein composition characteristic of early embryos (i.e., comparatively high amounts of lamin B_1, but little if any lamin A). Work is currently in progress to identify the nature of these cells. Preliminary results indicate that lamin A–negative cells include lymphocyte and macrophage precursors and resting lymphocytes (Stick, R., and H. Beug, unpublished results); moreover, differentiation of macrophage precursor cells is accompanied by an increase in lamin A (Stick, R., and H. Beug, unpublished results). These results extend the observations reported here for the case of synchronously differentiating (18) primitive red blood cells.

Lamin Protein Composition in the Chicken Germ Line

None of the three known chicken lamin proteins could be detected in male or female germ cells during pachytene stages

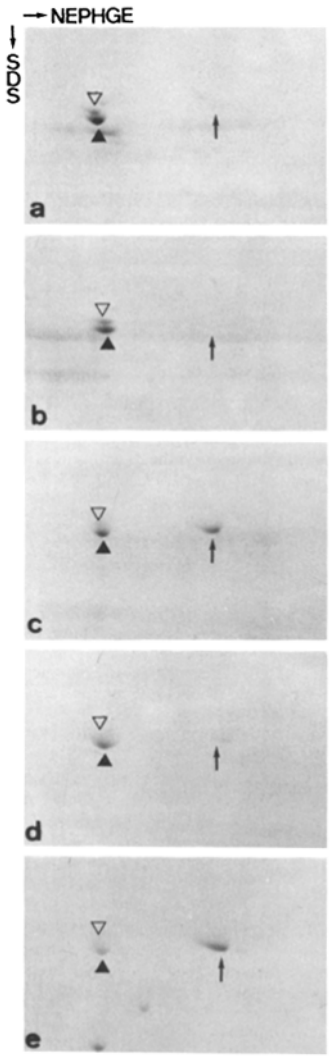

Figure 8. Two-dimensional gel electrophoretic analysis of pore-complex lamina preparations. Proteins (7 μg) from pore-complex lamina fractions were resolved using non-equilibrium pH gradient electrophoresis (NEPHGE) in the first and SDS-PAGE (8%) in the second dimension and stained with Coomassie Blue. Arrows denote the position of lamin A, filled triangles that of lamin B_2, and open triangles that of lamin B_1. The pore-complex lamina fractions were prepared from (*a*) 7-d embryos; (*b*) 18-d embryos, brain; (*c*) 18-d embryos, liver; (*d*) adult chicken, brain; (*e*) adult chicken, liver.

of meiosis. This result confirms and extends previous work of Stick and Schwarz (37, 38). These authors showed that during this stage of gametogenesis no lamina structure could be detected by ultrastructural analysis, and no lamina staining was produced by antibodies recognizing both lamins A and B_2. These previous studies had also revealed positive immunostaining of chicken diplotene oocytes, indicating that either lamins A and/or B_2 are expressed at these later stages of meiosis. Here, using mAbs specific for individual lamin proteins, we show that in fact both lamins A and B_2 appear to be present in diplotene oocytes (see also reference 35). In addition, we provide evidence that lamin B_1 is expressed in these cells and that it indeed appears to be present in relatively large amounts. One possible caveat in the interpretation of these results is that they are based on immunofluorescence experiments only. A biochemical confirmation has not been feasible due to the difficulties of obtaining sufficient quantities of purified oocytes. With respect to the male germ line, immunocytochemical and immunoblotting analyses indicate that none of the chicken lamin proteins identified so far are present after the pachytene stage of meiosis. It remains an open question, however, whether or not sperm-specific chicken lamin proteins exist as is the case in *Xenopus laevis* (3).

Comparison of Lamin Protein Expression in Chicken and Xenopus laevis

It is interesting to compare our present results with recent studies on the developmental regulation of lamin protein expression during early embryonic development of *Xenopus laevis*. In this organism, a single major lamin protein, L_{III}, is present in diplotene oocytes (19, 20), while a protein designated as L_{IV} has been reported to be specific for the male germ line (3). L_{III} is the only major lamina constituent of the early developing embryo (5, 19, 36), until expression of the major somatic lamin proteins, L_I and L_{II}, starts around midblastula transition and gastrulation, respectively. L_{III} then gradually disappears from most tissues of the developing organism, but, curiously, it reappears later in certain somatic cell types; in particular, together with L_I and L_{II}, L_{III} is reexpressed in neurons and in Sertoli, retina, and muscle cells from adult animals (5, 19). No expression of L_{III} was found in adult hepatocytes, erythrocytes, fibroblasts, and endothelial cells (5, 19).

It is remarkable that the distribution of lamin B_1 reported here bears some resemblence to the distribution of lamin L_{III}. High levels of lamin B_1 have been found in diplotene oocytes, early embryos, Sertoli cells (our unpublished observation), glial cells and neurons, whereas only minor amounts have been detected in hepatocytes, erythrocytes, and fibroblasts. However, although this distribution is clearly reminiscent of the expression of lamin L_{III}, it should be stressed that low amounts of lamin B_1 were found in all chicken tissues investigated, whereas the *Xenopus* lamin L_{III} was undetectable in most somatic tissues (5, 19). Moreover, none of the presently available antibodies against lamin B_1 cross-reacts with lamin L_{III} (our unpublished result).

Functional Considerations

Finally, it is of interest to consider our results with respect to functional specializations as they have been proposed for mammalian lamin proteins (8, 13, 15). Based on a series of elegant experiments, Gerace and coworkers have provided strong evidence to indicate that mammalian lamin B may function primarily in connecting the nuclear lamina to the membrane, whereas mammalian lamins A and C may be involved more directly in mediating interactions between the lamina and chromatin (8, 13, 15). Thus, according to a simplistic model, one might expect that lamina-mediated envelope-chromatin interactions in mammals would require the coexpression of lamins A/C and B (2). Indeed, as far as mammalian cells have been analyzed biochemically, lamins A, B, and C were found to occur in roughly equimolar stoichiometries. Our present findings suggest that lamin B_2 is a constant element of the chicken lamina, whereas the incorporation of variable amounts of lamins A and/or B_1 may lead to significant modifications of lamina structure during development. The constant expression of lamin B_2 in amounts roughly parallel to nuclear surface area might be taken to imply that lamin B_2 is closely associated with the inner nuclear membrane. Indeed, preliminary analyses of mitotic cells indicate that lamin B_2 remains associated with membrane vesicles, whereas lamin A becomes soluble (Stick, R., unpublished results). Hence it is possible that chicken lamin B_2 might be functionally homologous to mammalian lamin B, although the extent of structural homology is far from being clear (25). At present we have no information about the

fate of chicken lamin B_1 in mitotic cells, and it would be premature to speculate on the role of this protein. However, based on the properties of lamin A and its variable amounts of expression, in particular its large abundance in nuclei of mature red blood cells, we consider it possible that the extent of apposition of lamin A onto a membrane-bound layer formed by lamin B_2 might relate to the degree of chromatin compaction in a given cell type. If this interpretation were correct, alterations in the composition of the nuclear lamina might profoundly affect the differential organization of nuclei in a developing organism.

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