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, B1, and B2 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 B1 and B2. During embryonic development, lamin A became increasingly prominent, whereas the amounts of lamin B1 decreased in many tissues. Interestingly, the extent and the developmental timing of these changes displayed pronounced tissue-specific variations. Lamin B2 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, amphibians, birds, and mammals (for review see reference 19). The three major mammalian lamins have been designated as lamins A, B1, 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, B1, and B2 (25). In chicken embryos fibroblasts lamins A and B2 predominate, whereas lamin B1 represents a quantitatively minor component (25). By immunological criteria, chicken lamin B1 is related to mammalian lamin B, whereas chicken lamin B2 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, B1, and B2 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 amphibians, and the purported functional specialization of lamin proteins in mammals.
Materials and Methods

Cell Culture

Hepatocytes were isolated from livers of 17-18-d' chicken embryos according to Giger and Meyer (16) and cultured in Leibovitz L15 medium supplemented with 20% FCS, 10% tryptophan 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. The mouse mAbs O-1 and L3-4B4 react with lamin A, and the mAbs L-5 recognizes lamin B1, and the mAbs E-3 and L3-SD10 are specific for lamin B2 (23, 25, Stiek, Z., 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.

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 MgCl2. Then, 1.5 μl of DNase I and RNase A stock solutions (each 1 mg/ml in 10 mM Tris–HCl, pH 7.5, 0.1 mM MgCl2, 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 MgCl2, 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 rain) and resuspended in 100 μl digestion buffer 2 (10 mM Tris–HCl, pH 7.5, 0.1 mM MgCl2, 10% sucrose, 1 mM phenylmethylsulfonyl fluoride, 1% Trasylol, 3 mM β-mercaptoethanol). 1.5 μl of DNase I 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 8 μl Triton X-100 (10% wt/vol) extraction was carried out on ice for 10 min. The insoluble 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 1% BSA, followed by three 10-min incubations in the same buffer. Secondary antibodies were (125I)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 then rinsed quickly with 10 mM Tris–HCl, pH 7.5, dried, and autoradiographed using intensifying screens.

Densitometric quantitation of the lamin protein contents (Table I) was carried out in two ways. The signal intensities (Sintens) on the autoradiographs of the immunoblots were scanned using a Desaga Chromatogrammen Densitometer CD50/ Shimadzu CS930. In parallel, the amounts of the proteins (Sim) in the corresponding nuclear samples were quantified densitometrically by scanning Coomassie Blue–stained gels. The relative lamin contents (Sim/Sintens) were then calculated as the ratios between Sim and Sintens. 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%.

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 cryosectioned 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, B₁, and B₂ 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 L3-41M, anti-lamin A; (b) mAb L-5, anti-lamin B₁; (c) mAb L3-5D10, anti-lamin B₂. 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 B₂ (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 B₁, or lamin B₂. Confirming and extending earlier results (35, 38), no lamin proteins could be detected in pachytene-stage oocytes (not shown), but all three lamins
were readily stained in diplotene oocytes (arrowheads in Fig.
1, a–c). As judged by the intensity of the fluorescent labeling, lamins A and B2 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, B1, and B2 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, B1, and B2 (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 B2 (Fig. 2 b). Strong staining was produced also by mAbs specific for either lamin B1 (Fig. 2 d) or lamin B2 (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 B1 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 B1 (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 B2. 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 B1 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 B1 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 B1 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)
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 B1 and B2 readily stained both neurons (Fig. 5, d and e) and glial cells (not shown).

To confirm the low level of expression of lamin B1 in liver cells, hepatocytes were prepared from livers of 18-d chicken embryos. When these cultures were labeled with an antibody specific for lamin B1, 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 B2 (Fig. 5 g). Hepatocytes were also readily labeled by mAbs specific for either lamins A (Fig. 5 i) or B2 (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 B1.

**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
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 B1 (Fig. 6 C), or lamin B2 (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 B1 was detectable in all samples, but the amounts of lamin B1 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 B2 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 B1 (Fig. 7 C), and relatively constant amounts of lamin B2 (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 B1 and lamin B2, 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 B1. 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 B1 as well as between lamins B1 and B2 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 B1. For these cell populations, the estimated molar ratios therefore represent only average values.

**Biochemical Confirmation of Immunocytochemical 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-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 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 B1; (e) mAb E-3, anti-lamin B2. 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 B1; (g) rabbit serum anti-lamins A and B2 (same field as in f); (h) mAb O-1, anti-lamin A; (i) mAb E-3, anti-lamin B2. The phase-contrast micrograph (h) corresponds to the field shown in f and g. The arrowheads in f-h point to strongly lamin B1-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 B1; (D) mAb E-3, anti-lamin B2. 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 B1; (D) mAb E-3, anti-lamin B2. 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 B1 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.

Figure 8. Immunoblotting analysis of lamin protein distribution. 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 B1; (D) mAb E-3, anti-lamin B2. 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.
Table I. Quantitation of Lamins A, B1, and B2 during Chicken Embryogenesis

| Age | Organ | Relative lamin content* | Molar ratios |
|-----|-------|-------------------------|-------------|
|     |       | Lamin A | Lamin B1 | Lamin B2 | Lamin A | Lamin B1 | Lamin B2 |
| 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 ($S_{rel}$/$S_{nucleus}$) 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 B2 Is a Constant Element of the Chicken Nuclear Lamina**

Whereas lamins A and B1 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 B2. Indeed, in all immunofluorescence experiments with antibodies against lamin B2, all nuclei displayed indistinguishable labeling intensities, suggesting that the amounts of lamin B2 may actually be proportional to the nuclear surface area. Some support for this notion stems from correlating the relative amounts of lamin B2 with the estimated nuclear surface areas from different nuclear preparations (Table I): lamin B2 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 B2 during early embryonic development (Table I).

**Lamin A and Lamin B1 Display a Variable Expression**

In contrast to the comparatively constant expression of lamin B2, the relative amounts of lamin A and, to a minor extent, of lamin B1, 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 B1. Conversely, all adult tissues investigated (except for brain) were found to contain high amounts of lamin A, but only low amounts of lamin B1. 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 B1, 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.

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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 B2. These previous studies had also revealed positive immunostaining of chicken diplotene oocytes, indicating that either lamins A and/or B2 are expressed at these later stages of meiosis. Here, using mAbs specific for individual lamin proteins, we show that both lamins A and B2 appear to be present in diplotene oocytes (see also reference 35). In addition, we provide evidence that lamin B1 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, Lm, is present in diplotene oocytes (19, 20), while a protein designated as Ltv has been reported to be specific for the male germ line (3). Ltv is the only major lamina constituent of the early developing embryo (5, 19, 36), until expression of the major somatic lamin proteins, L4 and LH, starts around midblastula transition and gastrulation, respectively. Ltv then gradually disappears from most tissues of the developing organism, but, curiously, it reappears later in certain somatic cell types; in particular, together with L4 and Ltv, Ltv is reexpressed in neurons and in Sertoli, retina, and muscle cells from adult animals (5, 19). No expression of Ltv was found in adult hepatocytes, erythrocytes, fibroblasts, and endothelial cells (5, 19).

It is remarkable that the distribution of lamin B1 reported here bears some resemblance to the distribution of lamin Lm. High levels of lamin B1 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 Ltv, it should be stressed that low amounts of lamin B1 were found in all chicken tissues investigated, whereas the Xenopus lamin Ltv was undetectable in most somatic tissues (5, 19). Moreover, none of the presently available antibodies against lamin B1 cross-reacts with lamin Ltv (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 B2 is a constant element of the chicken lamina, whereas the incorporation of variable amounts of lamins A and/or B2 may lead to significant modifications of lamina structure during development. The constant expression of lamin B2 in amounts roughly parallel to nuclear surface area might be taken to imply that lamin B2 is closely associated with the inner nuclear membrane. Indeed, preliminary analyses of mitotic cells indicate that lamin B2 remains associated with membrane vesicles, whereas lamin A becomes soluble (Stick, R., unpublished results). Hence it is possible that chicken lamin B2 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 B1 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 expression, in particular its large abundance in nuclei of postmitotic reorganization of chromatin as demonstrated by microinjection of lamin antibodies. J. Cell Biol. 103:1847-1854.

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