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Original Article

The oocyte lamin persists as a single major component of the nuclear lamina during embryonic development of the surf clam

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ABSTRACT Nuclei and nuclear lamina-enriched fractions, isolated from 1 to 5-day-old embryos of the surf clam, *Spisula solidissima*, contain only one major lamin protein, which appears to be identical to the oocyte lamin (L67), as judged by 2D IEF/SDS PAGE, reactivity with a polyclonal antibody directed against L67 and ^{125}I tryptic peptide mapping. The same protein is also present in liver, muscle, nerve and testis from adult animals. No proteins – recognized by several poly- and monoclonal antibodies, specific for somatic lamins from different vertebrate species or the oocyte lamin L_{III} of *Xenopus* – have been detected in nuclei or NL-enriched preparations, isolated from embryos or adult tissues. Synthesis of L67 is detectable in embryos 2h after fertilization; it reaches a maximum in 6h-old embryos and gradually declines thereafter. These results argue that the composition of the NL bears no obvious relationship to the structural and functional changes that take place during the embryonic developoment of this invertebrate.

KEY WORDS: *nuclear lamina, lamins, embryonic development, surf clam*

Introduction

The nuclear lamina (NL) is a polymeric protein structure located between the double nuclear membrane and chromatin (Franke *et al.*, 1981; Gerace and Blobel, 1982; Gerace, 1986). It is composed of a small number of slightly acidic proteins with Mr between 60 and 75 kDa, termed lamins (Aaronson and Blobel, 1974; Krohne and Benavente, 1986), and appears to be associated with the nuclear pore complexes, inner nuclear membrane (Franke *et al.*, 1981) and chromatin (Hancock and Hughes, 1982; Lebkowski and Laemmli, 1982; Krachmarov *et al.*, 1986b; Galcheva-Gargova and Dessev, 1987). It has been suggested that the interaction between the NL and specific chromatin domains (Jones, 1970; Pardue and Gall, 1970; Hochstrasser *et al.*, 1986) may be important in the functional organization of the nucleus (Hancock, 1982; Blobel, 1985; Benavente and Krohne, 1986).

In a number of vertebrate species, the protein composition of the NL has been found to change during development and differentiation. In *Xenopus laevis* the oocyte lamin L_{III} is gradually replaced by two somatic lamins, L_I and L_{II}, beginning at midblastula stage (Benavente *et al.*, 1985; Stick and Hausen, 1985; Krohne and Benavente, 1986). Still another lamin, L_{IV}, has been found in sperm nuclei of the same organism (Benavente and Krohne, 1985). Changes in the NL composition have also been observed during the development of other species. Thus, early chick embryos contain predominantly lamins B_1 and B_2 (Lehner *et al.*, 1987), while increasing amounts of lamin A appear at later stages (Lehner *et al.*, 1987; Lourim and Lin, 1989). Similar changes have been described during mouse embryogenesis (Schatten *et al.*, 1985; Stewart and Burke, 1987; Houliston *et al.*, 1988; Rober *et al.*, 1989), and during differentiation of a number of mammalian cell types (Guilly *et al.*, 1987; Lebel *et al.*, 1987; Stewart and Burke, 1987; Paulin-Levasseur *et al.*, 1988). These findings have suggested the possibility that the changes in the NL composition may be related to the reorganization of chromatin architecture and, as such, may be part of the mechanisms that control the pattern of gene expression during development and differentiation. At present, however, there is little direct evidence either for or against this hypothesis.

A different situation has been encountered in *Drosophila*, where the two lamin polypeptides initially described (Smith and Fisher, 1984) turned out to be generated by post-translational processing

Abbreviations used in this paper: NL, nuclear lamina; L67, 67 kDa clam oocyte lamin; IEF, isoelectric focussing; SDS PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; MFSW, millipore filtered sea water; PMSF, phenylmethylsulfonyl fluoride; PIPES, piperazine-N, N'-bis (ethanesulfonic acid); EDTA, ethylenediamine tetracetic acid, disodium salt.

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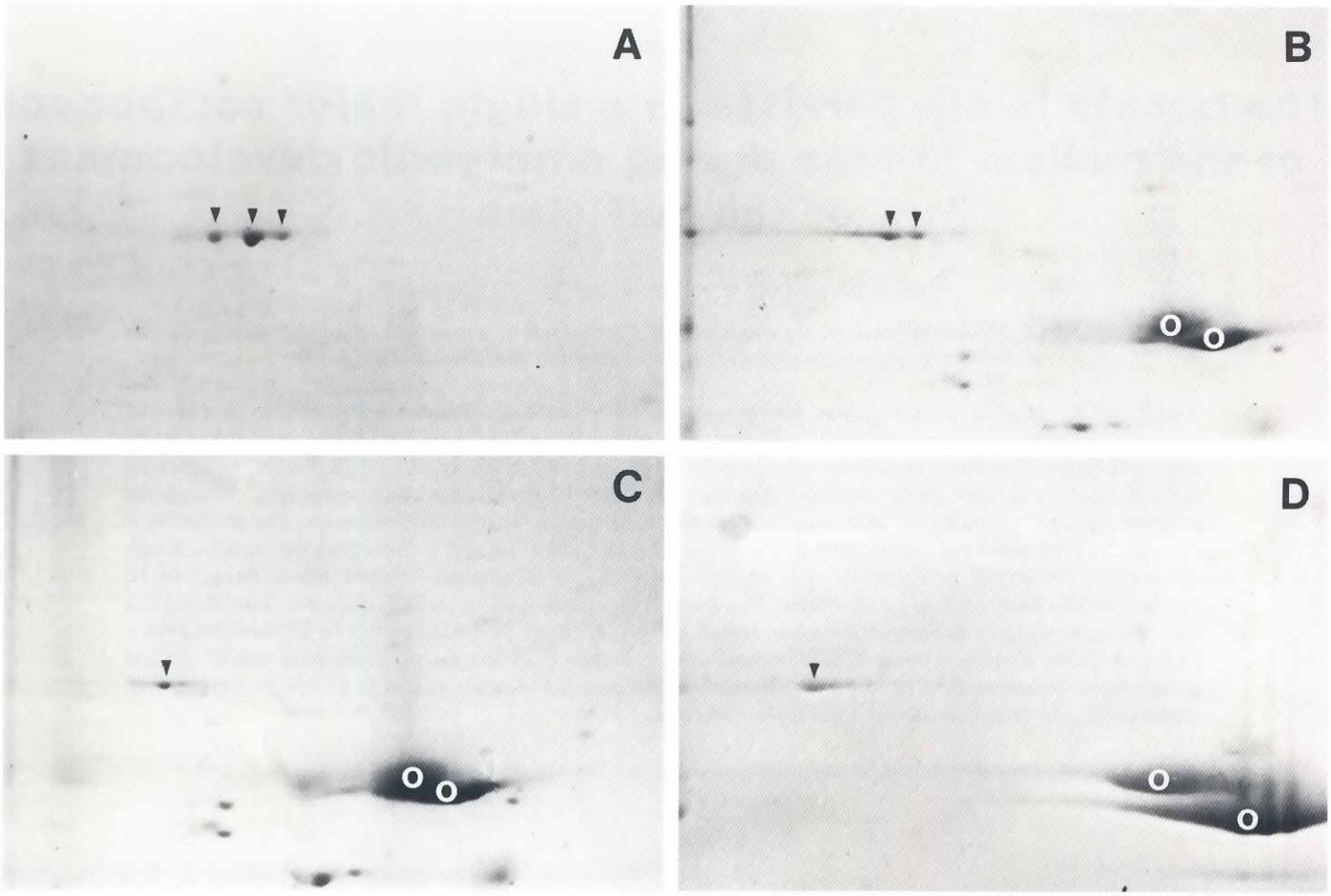

Fig. 1. NL-enriched fractions isolated from *Spisula* oocytes (A) *and embryos of different ages: 24h-old* **(B)**; *48h-old* **(C)** *and 72h-old* **(D)**, *analyzed by 2D IEF/SDS PAGE and stained with Coomassie Blue. The isoelectric variants of L67 used in the tryptic peptide mapping (Fig. 4) are indicated by arrowheads. The open circles show two abundant, probably cytoskeletal, proteins with Mr's around 56 kDa, which comigrate with vimentin from bovine lens.*

of a precursor, encoded by a single gene (Smith *et al.*, 1987; Gruenbaum *et al.*, 1988; Smith and Fisher, 1989). These results suggest that the pattern of lamin expression during development of invertebrate organisms may be different from that observed in higher vertebrate species, which express multiple stage- and tissue-specific lamins.

To obtain further information relevant to this problem we studied lamin expression during the development of another invertebrate, the surf clam *Spisula solidissima*, whose estimated evolutionary age is about one billion years (Wallace *et al.*, 1981). The embryonic development of the surf clam is rapid and accompanied by extensive morphological and functional changes (Costello and Henley, 1971 and the references therein). The 4h-old embryo is a non-swimming blastula. Eight hours after fertilization a ciliated swimming larva is formed. By 48h the larva has mouth and anal openings and clearly defined esophagus, stomach, intestine, liver, muscle and shell glands, as well as a bivalved shell. After 4-5 days the process of differentiation and morphogenesis is nearly complete and the young clam, which now displays almost all of the morphological and functional characteristics of the adult animal, continues to grow at a relatively slow rate. With these features, *Spisula* appears to be an excellent model for studying eventual developmental changes in the expression of the nuclear lamins.

The nuclear envelope of *Spisula* oocytes contains a major 67 kDa component (L67) which has been characterized as the single lamin of these cells by a number of criteria, including molecular mass, isoelectric point, insolubility in non-ionic detergents and high salt solutions (Maul *et al.*, 1984; Dessev and Goldman, 1988; Dessev *et al.*, 1989). Another similarity between the vertebrate lamins and L67 is that both are extensively phosphorylated during NEBD (Gerace and Blobel, 1980; Dessev and Goldman, 1988; Dessev *et al.*, 1989). In this paper we show that L67 is the only major lamin protein expressed and incorporated into the NL during the embryonic development of *Spisula*. L67 is also present in NL-enriched fractions of adult liver, muscle, nerve and testis. The nuclei or NL-enriched preparations from either embryos or adult tissues contain no proteins, recognized by a number of antibodies, specific for lamins from vertebrate species.

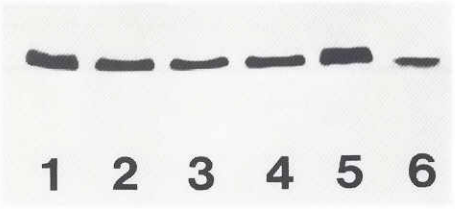

Fig. 2. An immunoblot of NL-enriched fractions *isolated from oocytes* (lane 1), *and embryos of different ages: 6h* (lane 2), *24h* (lane 3), *48h* (lane 4), *72h* (lane 5) *and 120h* (lane 6). *The blots were reacted with Pab 227 at a dilution of 1:1000 and were developed with a peroxidase-conjugated goat anti-rabbit second antibody at a dilution of 1:1000. The position of the marker, bovine serum albumin, is shown.*

Results

Spisula embryonic nuclei contain only one lamin, identical to the oocyte L67

Fig. 1 shows 2D IEF/SDS PAGE of NL-enriched preparations, isolated from *Spisula* oocytes and embryos of different ages. All samples contain a single major fraction in the region of 60-75 kDa with Mr of 67 kDa, as revealed by staining with Coomassie blue. This protein appears to be represented by a different number of isoelectric variants in oocytes and embryos of different ages (Fig. 1). On immunoblots of the same samples, this fraction reacted strongly with Pab 227 (Fig. 2). The same result was obtained with whole embryonic nuclei (not shown). The 67 kDa proteins in oocytes and embryos exhibited an identical electrophoretic behaviour in 2D IEF/SDS polyacrylamide gels, as demonstrated by mixed immunoblotting experiments (Fig. 3). Here the differences in the number and abundance of isoelectric variants are not obvious, perhaps due to the higher sensitivity of the detection method. Further evidence for structural similarity between these proteins was obtained by ^{125}I tryptic peptide mapping. Fig. 4 shows that the peptide maps of L67 isolated from oocytes and from embryos of different ages are very similar. In indirect immunofluorescence experiments, Pab 227 stained mainly the periphery of the embryonic nuclei (Fig. 5), suggesting that the antigen was located at the NL, in agreement with its insolubilty in 0.5% NP-40 and 1.5 M NaCl (see Materials and Methods). None of the antibodies against lamins from vertebrate species used in these experiments (see Materials and Methods) showed a positive reaction with oocyte or embryonic L67 by either immunoblotting (Fig. 6) and immunofluorescence (not shown). These results strongly suggest that the oocyte lamin is not only conserved, but remains the single major lamin species expressed during the embryonic development of *Spisula*.

L67 is synthesized during embryonic development

To find out whether there was a synthesis of L67 in the embryonic nuclei, we carried out a series of experiments in which embryos of different ages received a 30 min pulse with ^{35}S Translabel, followed by immunoprecipitation of L67 with Pab 227. Control immunoblotting experiments demonstrated that the immunoprecipitation efficiency was similar for L67 from embryos of different ages (not shown). Radioactive L67 could be detected as early as 2h after fertilization; its labeling reached a maximum in 6h-old embryos and later gradually decreased (Fig. 7). This time course probably reflects exhaustion of the maternal mRNA and an increase in the length of the cell cycle. Direct evidence for the presence of maternal mRNA for L67 in both oocytes and embryos has recently been obtained in this laboratory (Anne Goldman, unpublished data).

L67 is expressed in adult hepatopancreas, muscle, testis and nerve tissues, but not in sperm cells

A similar analysis for the presence of L67 was performed with adult *Spisula* tissues. However, unlike the embryonic nuclei, SDS gels of NL-enriched preparations from adult tissues contained a number of protein fractions in the region between 60 and 75 kDa, presumably of intranuclear and cytoskeletal origin, and it was not possible to see the less abundant L67 by Coomassie blue staining. Immunoblotting experiments demonstrated that the detergent-insoluble fractions from these tissues contained a single protein species, recognized by Pab 227 (Fig. 8). This protein was indistinguishable from oocyte L67 on 2D gels (results identical to those in Fig. 3, not shown). No evidence was obtained for the presence of the protein in sperm nuclei (Fig. 8, lane 6) in agreement with Stick and Schwarz (1982).

In an attempt to detect the presence of lamins other than L67 in the adult tissues, we used the same set of antibodies specific for vertebrate lamins, which were employed in the experiment shown in Fig. 6. None of these antibodies recognized proteins in the crude nuclear fractions from adult *Spisula* hepatopancreas, muscle, testis, ganglia or sperm cells using immunoblotting (results identical to those in Fig. 6, not shown).

Discussion

In this study we show that the single lamin of *Spisula* oocytes remains the only major lamin component of the NL of embryonic nuclei over a period of 5 days, in which the embryos complete most of their developmental program, including formation of a number of different cell types with specialized functions, and extensive mor-

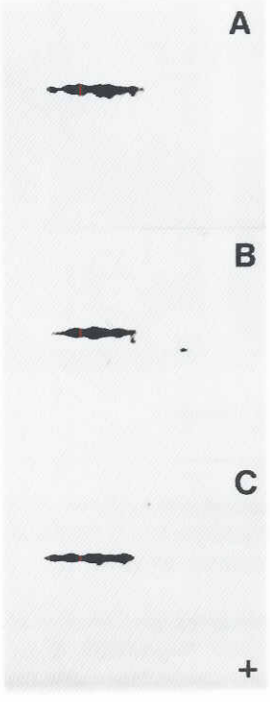

Fig. 3. Immunoblots of NL-enriched fractions *from oocytes* **(A)**, *72h-old embryos* **(C)** *and a mixture of the two* **(B)**. *The samples are separated by 2D IEF/SDS PAGE, transfered to nitrocellulose and reacted with Pab 227 (1:1000), followed by a peroxidase-conjugated goat anti-rabbit second antibody (1:1000).*

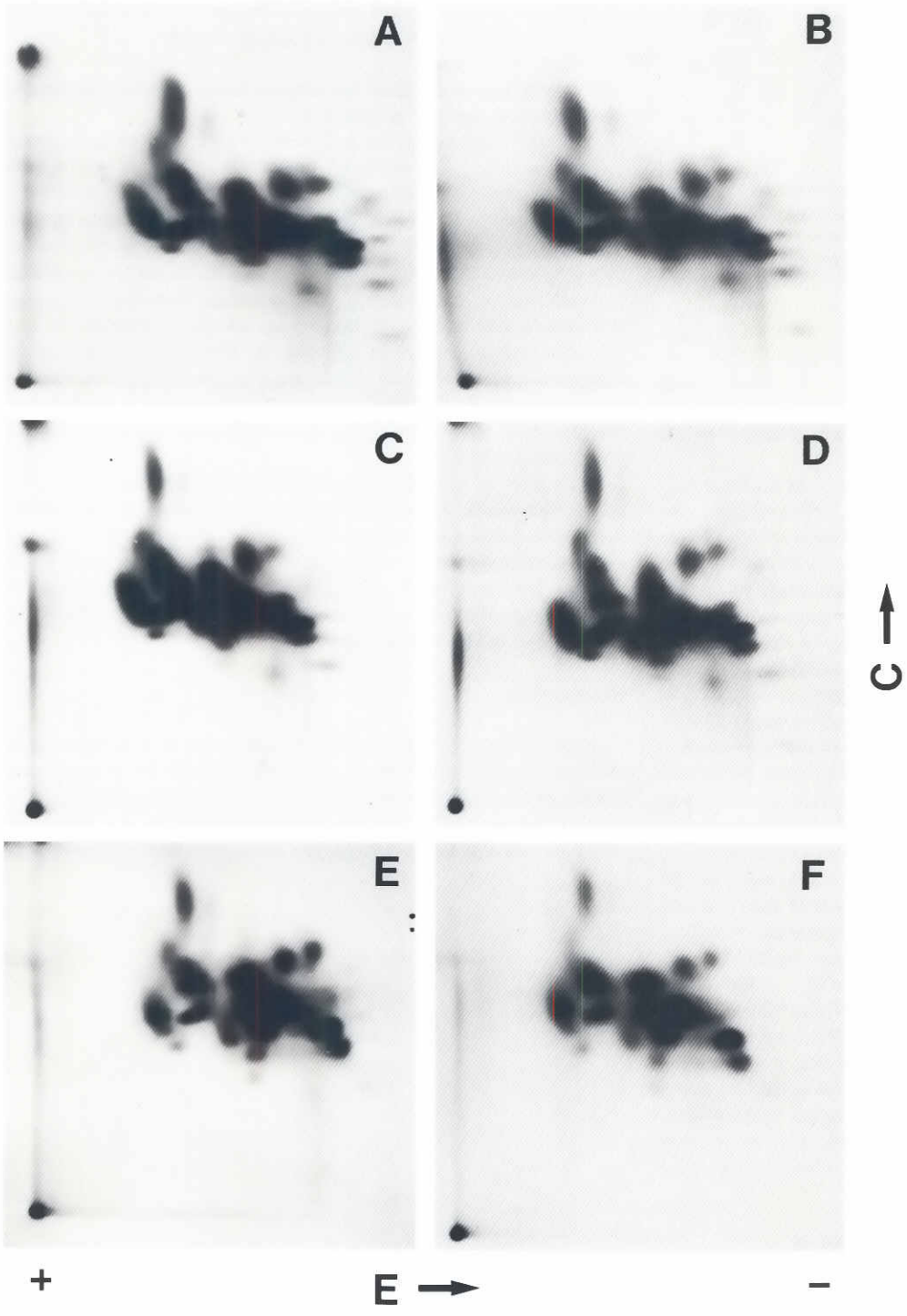

Fig. 4. 2D tryptic peptide maps of ^{125}I-labeled L67 *from oocytes* **(A)**, *24h-old embryos* **(B)**, *48h-old embryos* **(C)**, *a mixture of oocyte L67 and L67 from 48h old embryos* **(D)**, *72h-old embryos* **(E)** *and a mixture of oocyte L67 and L67 from 72h-old embryos* **(F)**. *NL-preparations from oocytes, embryos or mixtures of the two, each containing approximately equal amounts of protein, were separated by IEF/SDS PAGE, single isoelectric variants were cut out and processed for peptide mapping. The individual isoelectric variants of both oocyte and embryonic L67, indicated in Fig. 1, gave identical peptide maps.*

phogenesis. These results suggest that alterations in the NL composition are not necessarily involved in the structural and functional changes in the nucleus during the embryonic developmlent of *Spisula*.

Our findings are similar to the observations on *Drosophila*, (Smith and Fisher, 1984; Smith *et al.*, 1987; Gruenbaum *et al.*, 1988; Smith and Fisher, 1989) and are compatible with the possbility that a single nuclear lamin and a lack of developmental changes in NL composition may be characteristic for the lower eukaryotes. However, since two proteins cross-reacting with antibodies against turkey lamins A and B have been detected in yeast (Georgatos *et al.*, 1989), more data on lamin expression in other primitive eukaryotes of different evolutionary ages is needed to elucidate this question.

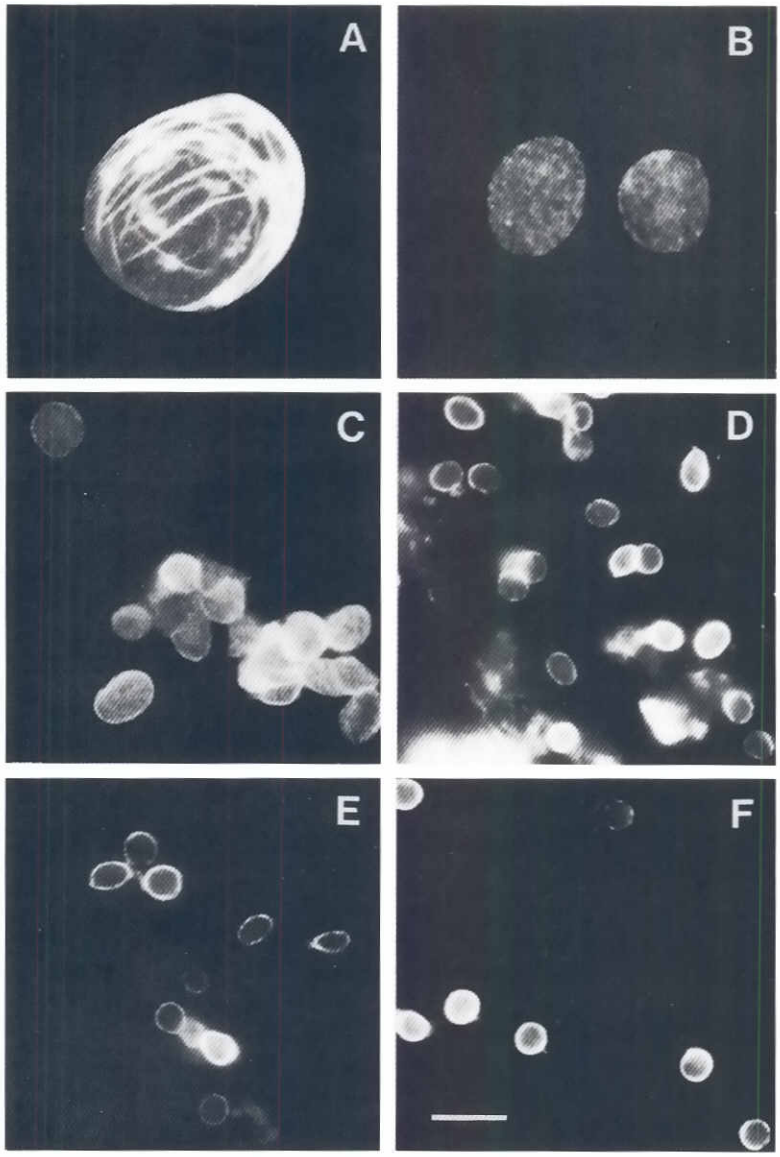

Fig. 5. Nuclei isolated from oocytes (A) *and embryos of different ages: 2h-old* **(B)**, *8h-old* **(c)**, *24h-old* **(D)**, *48h-old* **(E)**, *and 72h-old* **(F)**, *stained by indirect immunofluorescence using Pab 227 at a dilution of 1:50, followed by fluorescein-conjugated goat anti-rabbit IgG (1:50) as a second antibody. Bar, 10 μm.*

We have also shown that L67 is expressed in a number of adult clam tissues. However, while L67 may remain the only "adult" lamin in *Spisula*, our present results do not prove this, since other lamin species, unrecognized by the antibodies available to us, may be present in these tissues. We have been unable to detect L67 in sperm cells. Earlier, Stick and Schwarz (1982) found that NL is absent from chicken spermatides and spermatocytes. However, the presence of a sperm-specific lamin (L_{IV}) has been demonstrated in *Xenopus* (Benavente and Krohne, 1985). The meaning of these differences is not obvious; apparently, the presence of a NL structure is not critical for the organization of the sperm nucleus.

There is strong evidence that the control over gene expression during development operates at the level of large blocks of structural genes, rather than at the single gene level (for example see Gehring, 1985) and that different genetic blocks are activated as a whole at specific developmental stages. If these blocks contained genes encoding different functionally equivalent lamin proteins, this would result in changes in lamin expression during development (Benavente *et al.*, 1985; Stick and Hausen, 1985; Krohne and Benavente, 1986) which may not necessarily have functional significance. In a similar way one may explain the changes in lamin expression observed during differentiation of certain cell types (Lebel *et al.*, 1987; Stewart and Burke, 1987; Paulin-Levasseur *et al.*, 1988). Further, since individual development (ontogenesis) is known to recapitulate the evolutionary development of the species (phylogenesis), it is conceivable that a "single lamin" stage, perhaps characteristic for evolutionarily old organisms such as *Drosophila* and *Spisula*, may appear transiently during the oogenesis and early development of some more recently evolved vertebrate species (Krohne and Benavente, 1986; however, see Lehner *et al.*, 1987).

Using a number of monoclonal and polyclonal antibodies under

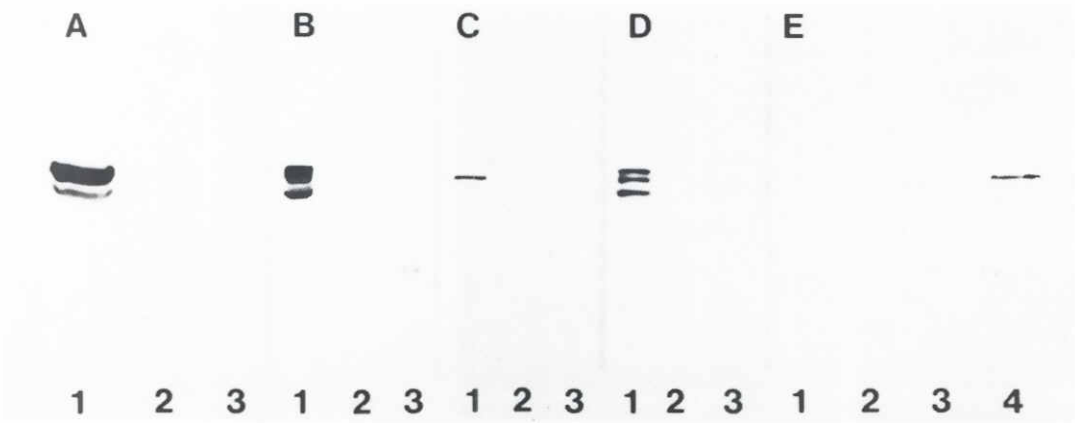

Fig. 6. NL-enriched fractions, *isolated from HeLa cells (lane 1), oocytes (lane 2), 72h-old embryos (lane 3), and cytosolic fraction (200,000xg supernatant) from Xenopus eggs (lane 4), were fractionated by SDS/PAGE, transfered to nitrocellulose, and reacted with different antibodies: a human auto-antibody specific for lamins A and C (LS-1)* (Panel A); *a chicken polyclonal antibody specific for lamins A and C (rat A/C)* (Panel B); *a chicken polyclonal antibody specific for lamin B (rat B)* (Panel C); *a monoclonal antibody against* Xenopus *somatic lamins (PKB8)* (Panel D) *and a monoclonal antibody, specific for* Xenopus *oocyte lamin* L_{III} *(Lo46F7)* (Panel E). *The antibodies were used at a dilution of 1:1000, except Lo46F7, which was used at a dilution of 1:100. The blots were developed by second peroxidase-conjugated antibodies (Southern Biotech) at a dilution of 1:1000 (goat anti-human (A), goat anti-chicken (B,C), and goat anti-mouse (D,E).*

our experimental conditions we have detected no immunological cross-reactivity between L67 and some vertebrate lamins. Other authors using a different set of antibodies have obtained evidence for epitopes shared between rat lamins and L67 (Maul *et al.*, 1984). The members of the lamin family exhibit a strong structural and

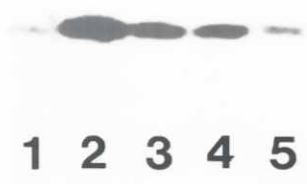

Fig. 7. Autoradiogram of L67 from embryos *2h-old* (lane 1) *6h-old* (lane 2), *18h-old* (lane 3), *29h-old* (lane 4) *and 48h-old* (lane 5), *labeled with [^{35}S] Translabel for 30 min, immunoprecipitated with Pab 227 and analyzed by SDS/PAGE.*

immunological relatedness. Determination of amino acid sequences has shown extensive homology in primary and secondary structure of several vertebrate lamins (Fisher *et al.*, 1986; McKeon *et al.*, 1986; Krohne *et al.*, 1987; Wolin *et al.*, 1987; Gruenbaum *et al.*, 1988; Steinert and Roop, 1988; Stick, 1988) as well as between

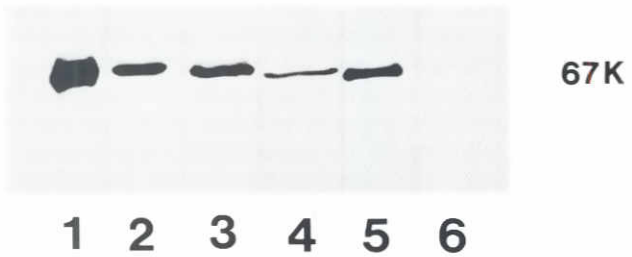

Fig. 8. Immunoblots of NL-enriched fractions, *isolated from oocytes* (lane 1), *hepatopancreas* (lane 2), *muscle* (lane 3), *ganglia* (lane 4), *testis* (lane 5) *and sperm* (lane 6). *The blots were reacted with Pab 227 at a dilution of 1:1000 and were developed with a peroxidase-conjugated goat anti-rabbit second antibody at a dilution of 1:1000. The position of the marker, bovine serum albumin, is shown.*

lamins and intermediate filament proteins (Fisher *et al.*, 1986; McKeon *et al.*, 1986). A number of antibodies recognizing the two main lamin types, A/C and B, have shown inter-species cross-reactivity (for example see Burke *et al.*, 1983; McKeon *et al.*, 1983; Benavente *et al.*, 1985; Stick, 1988; this paper) and cross-reactivity with intermediate filament proteins (Goldman *et al.*, 1986). The monoclonal antibody PKB8 (Benavente *et al.*, 1985) recognizes all three lamins of HeLa cells (Fig.6). Under these circumstances, one would expect to find immunological cross-reactivity between L67 and other lamins. Whether our negative results reflect more extensive structural differences between the vertebrate lamins and L67 remains to be elucidated. We feel that determination of its amino acid sequence, as well as those of other lower eukaryote lamins, will contribute towards understanding the evolution of the lamin family as well as the functions of the NL.

Materials and Methods

Surf clams were supplied by the Department of Marine Resources at the Marine Biological Laboratory, Woods Hole, Massachusetts, and kept in cold running sea water. The oocytes were obtained by dissection of the ovaries, washed four times with millipore-filtered sea water (MFSW) and fertilized according to Allen (1953). The embryos were then washed with MFSW three more times to remove the unattached sperm cells and were grown at 18°C with stirring at 60 r.p.m. in the presence of 50 U/ml penicillin and 50 μg/ml streptomycin.

Labeling of the embryos

Embryos of various ages were labeled in 10% suspension (v/v) in MFSW containing 1 mCi/ml of [^{35}S] Translabel (a mixture of labeled methionine and cysteine, ICN) for 30 min at 18°C with gentle rocking. The embryos were recovered by low speed centrifugation, washed once with MFSW and then either lysed directly by boiling in SDS-sample buffer or processed for isolation of nuclei as described below.

Isolation of nuclei from Spisula embryos

The embryos were resuspended in a solution containing 1.4 M glycerol, 15 mM phosphate buffer, pH 8.0, and 50 mM KCl. In this solution the

unfertilized oocytes are activated and lose their nuclei in about ten minutes (Dessev and Goldman, 1988). The same treatment renders the vitelline envelopes of both oocytes and early embryos soluble in NP-40 (Rebhun and Sharpless, 1964; Dessev and Goldman, 1988). After incubation at 20°C for 15 min, the embryos were sedimented and the pellet was resuspended in 20 volumes of lysis buffer (0.25 M sucrose, 0.5% NP-40, 10 mM PIPES pH 7.2, 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM PMSF and 1% Trasylol) at 0°C and repeatedly vortexed. The lysis was nearly complete in 2-3 min. The suspension was centrifuged at 800 r.p.m. in a clinical centrifuge for 2 min to sediment the unlysed cells, shells and large aggregates and the supernatant was layered over 0.4 M sucrose, 10 mM PIPES pH 7.2, 5 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM PMSF (sucrose cushion). The nuclei were recovered by centrifugation at 2000 r.p.m. for 5 min in a TJ-6 Beckman centrifuge. The nuclear pellet was resuspended in lysis buffer and sedimented once more through a sucrose cushion.

Preparation of crude nuclear fractions from hepatopancreas, muscle, testis, nerve and sperm cells

The hepatopancreas was removed from male animals, cut in 3-4 mm pieces, extensively washed in a large volume of MFSW at 0°C for 1 h and then homogenized in 100 volumes of lysis buffer in a tightly fitting Dounce homogenizer, using 5 strokes by hand. The homogenate was filtered through cheese cloth, layered over an equal volume of sucrose cushion and centrifuged for 10 min at 1700 r.p.m. in a TJ-6 Beckman centrifuge. The supernatant was removed and the walls of the tubes were wiped with paper tissues. The resulting nuclear pellet was resuspended in sucrose cushion solution and frozen in small aliquots at -80°C.

Preparation of crude nuclear fractions from testis, muscle and ganglia was carried out in the same way using 20 volumes of lysis buffer. The nuclear sediment was washed two more times by resuspension in lysis buffer and sedimentation through sucrose cushion. A large number of sperm heads were present in the nuclear fraction from testis.

To isolate sperm nuclei, the semen was resuspended in MFSW and freed of non-sperm cells and small pieces of tissue by centrifugation at 2,000 r.p.m. in a TJ-6 Beckman centrifuge. The sperm cells were collected from the supernatant at 10,000 r.p.m. for 10 min in a J-21 Beckman centrifuge. The pellet was homogenized in 20 volumes of lysis buffer and sedimented again at the same speed.

Isolation of NL-enriched fraction

The crude nuclear fractions were resuspended in 10 mM PIPES, pH 7.2, 1 mM $MgCl_2$, 1 mM KCl and 1% Trasylol and incubated for 20 min at 20°C in the presence of DNAse I (Sigma) and RNAse (Sigma) (50 μg/ml each). An equal volume of 3 M NaCl, 20 mM PIPES.NaOH, pH 7.2, 2 mM EDTA was added at 0°C and the samples were centrifuged for 10 min at 12,000 r.p.m. in a Beckman J-21 centrifuge. The pellet was dissolved in SDS-sample buffer by boiling. In the case of sperm nuclei the samples were sonicated to reduce viscosity.

Other methods

The protocols for immunoprecipitation, immunofluorescence and immunoblotting, one dimensional SDS PAGE and two dimensional equilibrium IEF/SDS PAGE were as previously described (Dessev and Goldman, 1988). Tryptic peptide mapping was carried out after labeling the proteins with ^{125}I in the gel, as described by Elder *et al.* (1977).

Antibodies

The polyclonal antibody against the oocyte L67 (Pab 227) was described previously (Dessev and Goldman, 1988; Dessev *et al.*, 1989). We also used the following antibodies specific for lamins from vertebrate species: LS-1, human auto-antibody recognizing mammalian lamins A and C (McKeon *et al.*,1983); Lo46F7 and PKB8, two monoclonal antibodies, specific for the oocyte lamin L_{III} and somatic lamins L_I/L_{II} of *Xenopus laevis*, respectively (Benavente *et al.*, 1985), and two polyclonal antibodies generated in chickens against rat liver lamins A/C and B (Kaufmann, 1989) (designated in the text as rat A/C and rat B, respectively), kindly supplied by Dr. Scott Kaufmann. The activity of these antibodies was tested using an NL preparation, isolated from HeLa cells according to Krachmarov *et al.* (1986a), or a cytosolic extract from *Xenopus* eggs in the case of Lo46F7. The results are shown in Fig. 6.

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