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 125I 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 LIII of Xenopus – have been detected in nuclei or NL-enriched preparations, isolated from embryos or adult tissues. Synthesis of L67 is detectable in embryos 2 h after fertilization; it reaches a maximum in 6 h 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 development 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 Gail, 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 LIII is gradually replaced by two somatic lamins, Lα and Lβ, beginning at midblastula stage (Benavente et al., 1985; Stick and Hausen, 1985; Krohne and Benavente, 1986). Still another lamin, Lγ, 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 B1 and B2 (Lehner et al., 1987), while increasing amounts of lamins 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; Robey 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.

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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 lamin.

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.
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 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 insolubility 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 35S 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 labelling 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-
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 development 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 possibility 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.
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 (Ls) 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
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 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) as well as between 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 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.

**Labeling of the embryos**

Embryos of various ages were labeled in 10% suspension (v/v) in MFSW containing 1 μCi/ml of [35S]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 (Dess et al. and Goldman, 1988). The same treatment renders the vitelline envelopes of both oocytes and early embryos soluble in NP-40 (Rebun and Sharpless, 1964; Dess et al. 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 MgCl₂, 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 MgCl₂, 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 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 50 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.

To isolate sperm nuclei, the semen was resuspended in MFSW and freed of non-sperm cells and small pieces of tissue by centrifugation at 2000 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 MgCl₂, 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 and the samples were centrifuged for 1 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 (Dess et al. and Goldman, 1988). Tryptic peptide mapping was carried out after labeling the proteins with [35S]methionine, as described by Elder et al. (1977).

**Antibodies**

The polyclonal antibody against the oocyte L67 (Pab 227) was described previously (Dess et al. and Goldman, 1988; Dess et al., 1989). We also used the following antibodies specific for laminas from vertebrate species: LS-1, a human auto-antibody recognizing mammalian laminas A and C (McKeon et al., 1983); Lo46F7 and PK88, two monoclonal antibodies, specific for the oocyte lamin L₀ and somatic laminas L₁/L₄ of Xenopus laevis, respectively (Benavente et al., 1985), and two polyclonal antibodies generated in chickens against rat liver laminas 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 isolation 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.

**References**


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