Conservation of the gene structure and membrane-targeting signals of germ cell-specific lamin LIII in amphibians and fish

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Targeting of nuclear lamins to the inner nuclear membrane requires CaaX motif-dependent posttranslational isoprenylation and carboxyl methylation. We previously have shown that two variants of lamin LIII (i.e., LIIIa and LIIIb) in amphibian oocytes are generated by alternative splicing and differ greatly in their membrane association. An extra cysteine residue (as a potential palmitoylation site) and a basic cluster in conjunction with the CaaX motif function as secondary targeting signals responsible for stable membrane association of lamin LIIIb. cDNA sequencing and genomic analysis of the zebrafish Danio rerio lamin LIII uncovers a remarkable conservation of the genomic organization and of the two secondary membrane-targeting signals in amphibians and fish. The expression pattern of lamin LIII genes is also conserved between amphibians and fish. Danio lamin LIII is expressed in diplotene oocytes. It is absent from male germ cells but is expressed in Sertoli cells of the testis. In addition, we provide sequence information of the entire coding sequence of zebrafish lamin A, which allows comparison of all major lamins from representatives of the four classes of vertebrates.

Introduction

The nuclear lamina is a filamentous meshwork associated with the nucleoplasmic surface of the inner nuclear membrane. In amphibian oocytes the organization of the lamina is particularly regular: 10-nm filaments form a near-tetragonal lattice, which harbors the nuclear pore complexes (Aebi et al., 1986). Lamins, members of the multigene family of intermediate filament (IF) proteins (Fuchs and Weber, 1994), are the major constituents of the lamina. They provide mechanical stability to the nuclear envelope and are involved in chromatin organization, DNA replication, and anchoring of nuclear pore complexes (Wilson et al., 2001). The importance of lamin function is highlighted by a growing number of mutants of the human gene encoding lamin A/C that give rise to a variety of dominant congenital diseases (Hegele, 2000; Hutchison et al., 2001), by the targeted disruption of the lamin A/C in mice (Sullivan et al., 1999), the study of a Drosophila mutant with reduced lamin Dmo activity (Lenz-Böhme et al., 1997), and by RNAi experiments with Caenorhabditis (Liu et al., 2000).

Vertebrates express a variety of lamins that are encoded by separate genes or are generated by differential RNA splicing (Döring and Stick, 1990; Fisher et al., 1986; Furukawa and Hotta, 1993; Furukawa et al., 1994; Machiels et al., 1996). Two types of lamins, A- and B-type, can be distinguished based on their domain structure. The larger A lamins possess about 100 additional amino acid residues in their tail domain that are encoded by an extra exon (Lin and Worman, 1993; Stick, 1992). It has been suggested that A-type lamins arose from a B-type lamin ancestor by insertion of this additional exon during vertebrate evolution (Stick, 1992). A-type lamins have been isolated so far from mammals, birds and amphibians. They seem to be restricted to vertebrates since all molecularly characterized invertebrate lamins are of the B-type (Riemer et al., 2000). The lamin B1 and B2 genes are generally constitutively expressed in somatic cells of mammals and birds, while the synthesis of lamin A is developmentally regulated (Lehner et al., 1987; Röber et al., 1989; Stewart and Burke, 1987; Wolin et al., 1987). Amphibians synthesize a fourth lamin in addition to lamin A, B1, and B2, which has been named lamin LIII or lamin B3 (Benavente et al., 1985; Stick and Hausen, 1985; Stick and Krohne, 1982). The latter name was originally chosen to emphasize its molecular structure, which is of the B-type (Stick, 1982).
Lamin LIII is the quantitatively major lamin of amphibian oocytes, eggs, and cleavage embryos (Benavente et al., 1985; Stick and Hauser, 1985; Stick and Krohne, 1982). In contrast to birds, which synthesize lamin A, B1, and B2 and diploptene oocytes (Lehner et al., 1987), B1 and B2 are very minor components in amphibian oocytes and lamin A is completely absent from these cells (Lourim et al., 1996; Wolin et al., 1987).

Lamins are posttranslationally farnesylated and carboxyl-methylated. These modifications depend on the presence of a C-terminal CaaX motif and are necessary for the association of lamins with the inner nuclear membrane (Holtz et al., 1989; Kitten and Nigg, 1991; Krohne et al., 1989; Loewinger and McKeon, 1988; Nigg et al., 1992). Lamin B1 and B2 are permanently isoprenylated and remain membrane associated after mitotic lamina disassembly (Gerace and Blobel, 1980; Hennekes and Nigg, 1994; Stick et al., 1988). Lamin A is proteolytically processed after incorporation into the lamina. This second maturation event removes the farnesylated and carboxyl-methylated cysteine residue of the processed CaaX motif (Beck et al., 1990; Kilic et al., 1999; Weber et al., 1989). This might explain why lamin A is selectively solubilized and dispersed during mitosis. Lamin LIII follows the rules of other B-type lamins. It is permanently isoprenylated and carboxymethylated (Firmbach-Kraft and Stick, 1993). In contrast to lamins B1 and B2, the majority of lamin LIII becomes soluble in the course of nuclear envelope breakdown and only a minor fraction remains membrane associated (Lourim and Krohne, 1993). This phenomenon has been explained by the existence of two lamin LIII variants, a and b (Hofmeister et al., 2000), resulting from alternative splicing of lamin LIII transcripts and differing in their CaaX-encoding exons (Döring and Stick, 1990). While both proteins are farnesylated and carboxymethylated (Firmbach-Kraft and Stick, 1993), the quantitatively minor variant LIIib carries an extra cysteine residue, as a potential palmitoylation site, and a cluster of six basic amino acid residues in proximity of the CaaX box. Both motifs function in conjunction with CaaX modifications as secondary signals in membrane association (Hancock et al., 1990; Hofmeister et al., 2000)

The specific expression of the lamin LIII gene in amphibian oocytes and early embryos has been interpreted as an adaptation to the particular type of oogenesis and early development found in these organisms. Amphibian oocytes stockpile large amounts of maternal RNA and proteins during the long phase of oogenesis (Hausen et al., 1985). The volume of a Xenopus oocyte nucleus is about 100 000 times that of a somatic cell nucleus (Hausen and Riebesell, 1991). The lamin present in the oocyte nuclear lamina serves as a pool for the generation of embryonic nuclei during the rapid cleavage divisions up to the 5000–10 000 cell stage (Stick and Hauser, 1985). During this early phase of development cells are transcriptionally silent, protein synthesis solely depends on the recruitment of maternal mRNA, and the cell cycle is reduced to a S- and M-phase (Newport and Kirschner, 1982a).

Mammals and birds do not show such a rapid cleavage phase in their early development. Correspondingly, a lamin LIII homologue has not been found in these two classes of vertebrates, and databank search of the human genome did not provide evidence for an additional lamin gene (Hesse et al., 2001). In contrast to mammals and birds, fish resemble amphibians in many respects of their oogenesis and early development (Kane, 1999). We therefore have searched for a homologue of the amphibian lamin LIII in the zebrasish Danio rerio, a model system of today’s developmental biology.

Here we describe the sequence of lamin LIII of Danio rerio as well as its localization in the oocyte lamina by immunohistochemistry. Genomic analysis uncovers a remarkable conservation between the amphibian and the fish gene, suggesting that also in the fish two variants of lamin LIII are generated by alternative RNA splicing. Moreover, the secondary signals that are responsible for membrane association of Xenopus lamin LIIib are perfectly conserved between amphibians and fish. In addition, we complete the sequence information for the major vertebrate lamins by providing the entire coding sequence of Danio rerio lamin A, thus allowing comparison of all major lamins from representatives of the four classes of vertebrates.

Materials and methods

DNA techniques

EST clones AW153707 and AW 421801 were obtained from Incyte Genomics (St. Louis, MO). Plasmids were purified using a plasmid purification kit (Qiagen, Hilden, Germany). Double stranded sequencing was done with internal primers using a Prism Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 392 DNA Sequencer. Since the Danio lamin A EST clones were not available at that time a DNA fragment covering the entire open reading frame was amplified by PCR. A cDNA library constructed from RNA of three-days-old Danio rerio embryos was used as template. This library was kindly provided by Holger Knaut (Max Planck Institute for Developmental Biology, Tübingen, Germany). PCR was carried out with sense primer 5'CAGGACAACCATGGAGACTC- CAGG3' and antisense primer 5'CAGGTCGACTTCGT-GAACGTGAC3' at 0.2 mM in a 100-ul reaction using TaqGold polymerase (Perkin Elmer-Cetus, Norwalk, CT) with the supplied buffer. Final concentration of MgCl2 was 10 mM. Cycling parameters were: initial denaturation (5 minutes, 95° C) followed by 35 cycles (45 seconds, 95° C; 30 seconds, 50° C; 2 minutes, 72° C) and a final polymerization step (10 minutes, 72° C). The PCR product was separated on an agarose gel, purified using a gel extraction kit (Qiagen), and then directly sequenced.

Antibodies

Peptides corresponding to amino acids 2–16 (N-term: AMVTSTPATPVPFR) and to amino acids 560–577 (C-term: PVRSRDHMRQGQTLHDC) of Danio rerio lamin LIII were synthesized by t-boc chemistry (Schnöller et al., 1992) and coupled via a carboxyl-terminal cysteine residue to maleimide-activated keyhole limpet hemocyanin (KLH; 1 mg peptide/1 mg KLH), using the Imject Activated Immunogen Conjugation kit (Pierce, Rockford, IL). Guinea pigs (two animals for each peptide) were immunized four times subcutaneously with 70–100 μg protein per injection. For the first injection the KLH-coupled peptides were emulsified with an equal volume of complete Freund’s adjuvant and, for booster injections at days 28, 49, and 70, with incomplete adjuvant. Blood was taken at day 86. Affinity purification of immunoglobulins was carried out with immobilized peptides. Peptides were coupled to SulfoLink (Pierce) with 1 mg peptide per ml matrix. Coupling was carried out by Peptide Specialty Laboratories (Heidelberg, Germany). Five ml peptide-resin was used per ml of serum. The peptide-resin was first equilibrated with five volumes of PBS (pH 7.4) and then mixed for 1 hour at room temperature with one ml of serum diluted 1:5 in PBS. The flow through was collected, and the resin was washed with 20 volumes of PBS. Antibodies were
eluted in fractions of 0.5 ml with 0.2 M glycine (pH 2.5) and immediately neutralized with 25–30 pl 1 M Tris-HCl (pH 9.5). Purified antibodies, supplemented with an equal volume of glycerol, containing BSA at a final concentration of 0.3% and 0.025% NaN₃, were stored at −20 °C.

**Animals and histological procedures**

Wild-type zebrafish (*Danio rerio*) was raised and kept under standard laboratory conditions at about 28 °C (Westerfield, 1995). Oocytes were staged as described (Selman et al., 1993). Tissues were frozen in isopentane cooled with liquid nitrogen to −140 °C, and then stored at −70 °C. Frozen tissues were embedded in Tissue-Tek (OCT 4583 embedding compound; Miles, Elkhart, IN) and frozen as described above. Tissues were sectioned (4–5 μm), fixed with acetone for 10 minutes at −20 °C, and then air-dried. Sections were blocked for 15 minutes with 1% BSA in PBS and incubated with appropriately diluted primary antibodies for 30 minutes, washed two times for 5 minutes, and incubated with Cy3-conjugated goat anti-guinea pig IgG (Dianova, Hamburg, Germany) for 30 minutes. After two washes for 5 minutes with PBS, sections were air-dried and mounted with Fluoromount-G (Southern Biotechnology Association, Birmingham, AL). Immuno-fluorescence was documented with an Axiophot photomicroscope (Zeiss, Jena, Germany) on T-max film (Kodak, Stuttgart, Germany). Films were scanned with a Coolscan 4000 scanner (Nikon, Tokyo, Japan) using SilverFast imaging software (LaserSoft Imaging, Kiel, Germany).

**Protein extraction, SDS-PAGE and immunoblotting**

A crude cytoskeletal preparation of zebrafish oocytes was prepared either from individual full-grown oocytes or small pieces of ovary. The tissue was homogenized using a tight-fitting, hand-driven homogenizer in an excess of extraction buffer (1 M NaCl, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.5). The cytoskeletal fraction was recovered by centrifugation for 10 minutes at 10000 g. It was washed twice in extraction buffer and twice in distilled water, dissolved in SDS-sample buffer, and treated with 0.5 U/μl Benzonase (Merck, Darmstadt, Germany) before boiling for 5 minutes. Samples were separated by 10% SDS-PAGE. Material equivalent to about 5 mg original tissue (wet weight) was loaded per lane. After electrophoresis polypeptides were transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The transferred proteins were stained with Coomassie Blue. Immunodetection was as described (Huttenlauch et al., 1998).

**Results**

**Characterization of a homologue of the amphibian lamin LIII in fish**

Databanks were searched for a homologue of the amphibian lamin LIII. The entire protein sequence of *Xenopus* lamin LIII (Stick et al., 1988) (accession number CA31567) was used as query in searches against the translated EST sequences of *Danio rerio* using the tBLASTN algorithm (Altschul et al., 1997). Among a number of hits with high scores to other vertebrate lamins, two sequences showed significant similarity to the N-terminal region of the query sequence. The inserts of these two clones (accession numbers AW 153707 and AW421801) were sequenced on both strands. Both cDNAs contained the complete open reading frame encoding *Danio rerio* lamin LIII. The two sequences differ in 12 nucleotide positions resulting in four amino acid exchanges. It is therefore reasonable that the two clones represent two different alleles of the same gene.

The conceptual translation product (Fig. 1) shows all features diagnostic for a vertebrate lamin protein (Fuchs and Weber, 1994), notably the long-coil 1b version in the central rod domain typical for lamins and IF proteins from protostomic phyla. Two p34cdc2 protein kinase phosphorylation consensus sites (amino acids 31 and 401) flank the rod domain. These sequences are

Fig. 1. Amino acid sequence of *Danio rerio* lamin LIII as deduced from the nucleotide sequence of EST clone GenBank accession number AW153707. The subdomains of the central rod (coils 1a, 1b, and 2) are marked by arrows. The two p34cdc2 protein kinase sites, that flank the rod domain, are marked by asterisks. The nuclear localization signal is underlined by a broken line. The 105-box within the tail domain is shown in brackets. The two peptides that were chosen for the generation of anti-peptide antibodies are underlined. The nucleotide sequence data are available from GenBank under the accession number AF397015.
involved in the reversible dissociation of lamin filaments during cell division (Heald and McKeon, 1990; Peter et al., 1990). A canonical NLS is present within the first part of the tail domain (amino acids 415–419), as well as a conserved region of about 105 amino acid residues, the 105-box, within the tail domain (amino acids 441–539) (Riemer et al., 2000), and a CaaX motif at the C-terminal. Danio LIII shows the greatest sequence drift among all vertebrate lamins and even in regions that are highly conserved between other lamins, as for example the 105-box within the tail domain, the Danio LIII sequences show significant sequence deviation. Danio lamin LIII lacks the extension of the tail domain that is characteristic of A-type lamins and can therefore be classified as a B-type lamin (Stick, 1992). Moreover, sequence comparison with mammalian, avian, amphibian, and fish lamins A, B1, B2 and with amphibian lamin LIII has revealed the closest sequence similarity to the latter, indicating that Danio lamin LIII is the homologue of Xenopus lamin LIII (see Fig. 6).

The amphibian lamin LIII is the major lamin of oocytes and early embryos (Stick and Hausen, 1985; Stick and Krohne, 1982). In other tissues this lamin occurs only in very few cells, as for example in Sertoli cells of the testis and in certain cells of neuronal and cardiac tissue (Benavente et al., 1985). To see whether the Danio lamin LIII gene is expressed in a similar pattern we have raised peptide antibodies that specifically recognize this lamin. Peptides for immunization were chosen from the N-terminus and the C-terminus, respectively, since these regions differ in sequence from all other three Danio lamins and are probably accessible to antibodies in the context of the entire protein. Two guinea pigs were immunized with each of the two peptides. All of the resulting four sera reacted strongly with the oocyte nuclear envelope on cryosections. In addition, these sera showed various but significant reactions with the vitelline envelope surrounding the oocytes. To clarify whether this was due to cross-reaction with the lamins, LIII-containing membranes (Hofemeister et al., 2000).

To clarify whether this feature of LIII is conserved between amphibians and fish we have amplified the genomic region of the Danio LIII gene that corresponds to intron 11 and the flanking exon regions of the Xenopus LIII gene. A genomic fragment of about 1850–1900 nucleotides was amplified by PCR that corresponded to about 1700–1750 nucleotides of intron sequence. The fragment was sub-cloned and sequenced from both ends. We obtained sequence information of 813 nucleotides from the 5′-end and 1032 nucleotides from the 3′-end that covered nearly the entire fragment (Fig. 4B). Since sequencing reactions from both ends terminated at the same positions under a variety of reaction conditions, we were unable to obtain an overlap of the two sequences. The 1032 nucleotides of the 3′-end, however, contained relevant information. It contained the sequences corresponding to the cDNA sequence of clone AW153707 as well as a short exon (exon 11b in Fig. 4A) encoding an amino acid sequence that closely resembled that of exon 11b of the LIII gene of Xenopus. The intron/exon structure of this region was identical to that of the homologue Xenopus gene, while, as expected, the actual sequences within the introns were not conserved (Fig. 4B) (Döring and Stick, 1990). Significantly, Danio exon 11b encodes a polybasic cluster flanked by an extra cysteine residue and a CaaX box. The sequence of the basic cluster is perfectly conserved between Danio and Xenopus and the CaaX sequence deviates only in one of the three variable residues (Fig. 4C). Taken together, this conservation strongly suggests that in Danio, like in Xenopus, two lamin LIII variants are generated by alternative splicing. This finding points to a specialized function that this lamin variant might fulfill during oogenesis and/or early development in amphibians and fish.

**Sequence of lamin A of Danio rerio**

Sequence information for the three lamin sub-types, A, B1, and B2 is available from at least one representative of the four classes of vertebrates, with the exception of lamin A from fish. Since it is widely assumed that A-type lamins evolved during vertebrate evolution we have been particularly interested to see whether lamin A is present in fish.

We therefore have searched the Danio EST data bank with either the Xenopus lamin A sequence (Wolin et al., 1987) or the lamin LIII sequence (Stick et al., 1988) as query. Several EST sequences were found that encode Danio lamin A. The ESTs contain sequences either from the 5′- or the 3′-end of the coding region (accession numbers BE201026, BE557685). Since the corresponding cDNA clones have not been available at that time we have amplified the entire coding region by PCR, using
as template oligo-dT-primed cDNA from 3-days-old embryos, anticipating that expression of *Danio* lamin A begins only at later stages of development, similar to the situation in other vertebrates. After gel purification the PCR product was directly sequenced in its entire length using internal primers. The conceptual translation product showed all features diagnostic for vertebrate lamins as explained above for lamin LIII (Fig. 5). It contained an extra domain of 71 amino acid residues (position 578–648) in its tail domain, the hallmark of A-type lamins (Fisher et al., 1986; Fuchs and Weber, 1994) (Fig. 5). *Danio* lamin A showed the largest sequence divergence of all A-type lamins. Interestingly, pairwise sequence alignment has revealed, that the LIII lamins are closer to A-type than to the other B-type lamins.

The intron positions of several lamin genes have been mapped (Düring and Stick, 1990; Lin and Worman, 1993, 1995; Zewe et al., 1991). They are highly conserved. In the course of our EST search for *Danio* lamin A we have also detected a sequence tag (accession number BE016225) containing an 82 nucleotides long intron. Comparison with other *Danio* ESTs and with genes encoding the *Xenopus* and the human lamin A genes have revealed that this intron is the homologue to intron 8 in the amphibian and human gene (Lin and Worman, 1993; Stick, 1992). Its position is perfectly conserved in the *Danio* gene.

![Fig. 2. Immunolocalization of lamin LIII in zebrafish ovary and testis.](image)

Cryosections (4–5 μm) of *Danio rerio* ovary (A–I) and testis (J, K) were fixed with cold acetone and stained by indirect immunofluorescence with affinity-selected anti-peptide antibodies directed against an N-terminal peptide of *Danio* lamin LIII (N-term: A), a C-terminal peptide (C-term: D, J), or with the flow through fraction of the affinity purification of an antiserum directed against the C-terminal peptide (Control: G). Sections were counterstained with diamidinophenylindole to visualize the position of nuclei (DAPI: B, E, H). Resolution of follicle cell nuclei is somewhat limited since acetone fixation, which was chosen to optimally maintain immuno-reactivity, does not fully preserve nucleic acids. Note that the concentration of DNA in the giant oocyte nuclei is too low to be detected under the conditions used here. Arrows in (G, H, and I) indicate the position of the nuclear envelope. The phase-contrast micrographs in (C, F, I, and K) correspond to frames (A/B, D/E, G/H, and J, respectively).

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Discussion

We have identified lamin A and a homologue of the amphibian lamin LIII in the zebrafish, *Danio rerio*, by searching the *Danio rerio* EST data bank. By providing the complete sequences of the two fish lamin polypeptides we complete the catalog of vertebrate lamin sequences. Classification of the two fish sequences was based on overall sequence similarity to the other known vertebrate lamins. The presence of an extra domain within the tail of *Danio* lamin A is diagnostic for A-type lamins and allowed an unambiguous identification of this protein as an A-type lamin (Fuchs and Weber, 1994; Lin and Worman, 1993; Stick, 1992). A-type lamins form a relatively homogenous group of lamins that seems to be restricted to vertebrates. The presence of lamin A in fish strengthens the view that A-type lamins have been acquired at the base of the vertebrate lineage. Their expression is developmentally controlled, and they are absent from undifferentiated cells (Lehner et al., 1987; Röber et al., 1989; Stewart and Burke, 1987). In mammals, the lamin A gene gives rise to several variants by differential splicing which points to a role in cell differentiation (Fisher et al., 1986; Furukawa et al., 1994; Machiels et al., 1996).

The sequence of lamin LIII from a second vertebrate class now allows positioning of this type of lamin within the lamin family: *Danio* lamin LIII shows the highest sequence similarity to *Xenopus* LIII but similarity values to A-type lamins are also high. However, sequence similarity between *Xenopus* LIII and several A-type lamins is even higher than between lamin LIII of *Xenopus* and fish. While our study was underway, Nahagama and colleagues cloned the goldfish lamin LIII (B3) (Yamaguchi et al., 2001), and identified the major polypeptide of the germinal vesicle lamina as lamin LIII by peptide sequence analysis and by cDNA sequencing.

Sequence identity between zebrafish and the goldfish sequences is about 70% while identity values drop to about 50% between the fish and the *Xenopus* sequences. Homologues of lamin LIII have not yet been identified in birds and mammals, and data bank search of the human genome has not provided evidence for an additional lamin gene (Hesse et al., 2001). Despite the large sequence drift and together with the evidence presented below we conclude that fish and amphibians contain and express an additional lamin gene, LIII, representing a distinct type (Fig. 6). Obviously, LIII lamins display the general features of B-type lamins, but on the other hand show the highest sequence similarity to A-type lamins. The high sequence drift between the three known LIII sequences is remarkable and suggests that these lamins represent an ancient form of (vertebrate) lamin serving functions common to fish and amphibians.

LIII lamin genes show a common expression pattern in fish and amphibians

Expression of the lamin LIII gene has been studied in particular detail in *Xenopus*, where it is the major lamin of oocytes and early embryos. In the ovary, it is expressed in diplotene oocytes. It is absent from male germ cells but is expressed in Sertoli cells of the testis as well as in a few cells in somatic tissues (Benavente et al., 1985; Stick and Hausen, 1985; Stick and Krohne, 1982). Moreover, in the present study the synthesis of *Danio* lamin LIII has been examined with antibodies that recognize peptide sequences unique to *Danio* lamin LIII. *Danio* lamin LIII is exclusively expressed in diplotene oocytes in the ovary and in Sertoli cells of the testis. In both tissues the expression pattern matches exactly that described for amphibian lamin LIII. Moreover, in *Xenopus* as well as in goldfish, LIII is the quantitatively major lamin of oocytes (Lourim et al., 1996; Stick and Krohne, 1982; Yamaguchi et al., 2001). These observations give further support to the view that these lamins are homologous. In contrast, birds synthesize all three lamin sub-types, B1, B2, and lamin A in diplotene oocytes (Lehner et al., 1987). We therefore assume that LIII serves a specialized function in fish and amphibians that is absent in other vertebrate classes.

Conservation of lamin LIII splice variants

The most compelling evidence for a common function of lamin LIII in amphibians and fish is the striking conservation of their CaaX motif-encoding exons. Two variants of lamin LIII are expressed in *Xenopus* (Döring and Stick, 1990), generated by alternative splicing of two C-terminal CaaX motif-encoding exons. The two variants differ greatly in their behavior during meiotic nuclear envelope breakdown: LIIIb remains selectively associated with membranes upon lamina disassembly (Hofemeister et al., 2000). Mutational analysis has shown that a cluster of six basic amino acids and a cysteine as a potential palmitoylation site is necessary for stable association of LIIIb with membranes. In contrast, lamin LIIIA, that lacks these secondary signals, becomes soluble during nuclear envelope breakdown. The genomic organization of the two alternative exons within the LIII gene is perfectly conserved between *Xenopus* and *Danio*. Even more strikingly, the actual sequence
of the LIIIb-specific exon, exon 11b, is highly conserved. It differs in only one of the two variable positions within the CaaX motif (Fig. 4C). In contrast, sequence conservation of the LIIIa-specific exon is limited to only one residue outside the CaaX motif. This suggests that also in the fish two variants of lamin LIII are expressed. A direct proof that the two proteins are indeed expressed in *Danio* would require variant-specific antibodies. Generation of lamin LIIIb-specific antibodies, however, poses a major problem. It is hampered by the fact that the processed forms of the two proteins differ only by eight amino acid residues. One of the eight residues of LIIIb, the extra cysteine residue, will be modified in vivo and will therefore have altered antigenic properties. Another six residues form a cluster of basic amino acids that is similar or...

Fig. 4. Genomic organization and nucleotide sequence of the 3'-end of the coding region of the *Danio rerio* lamin LIII gene. (A) Schematic representation of the exon/intron structure of the zebrafish lamin LIII gene (A). Numbering of exons and introns is according to the homolog gene of *Xenopus* (Drough and Stick, 1990). Splicing of introns 11a and 11b, giving rise to an mRNA encoding lamin variant LIIIb, is indicated by dotted lines and is inferred from analysis of the *Xenopus* lamin LIII gene. The positions of primers that were used for the amplification of the genomic fragment are shown by arrows. (B) Nucleotide sequence and predicted amino acid sequence of the genomic region covering the 3'-end of the coding region of the *Danio rerio* lamin LIII gene. Stop codons are marked by asterisks, the splice consensus sequences at the ends of the introns are underlined, the primer sequences used for amplifications are indicated by arrows. (C) Comparison of the amino acid sequences encoded by the two alternatively spliced exons of the *Xenopus* lamin LIII gene with the corresponding sequences of the *Danio* gene. Note the high conservation between the two LIIIb-specific sequences. The CaaX motifs are underlined, the extra cysteine residues, as potential palmitoylation sites, are boxed, and the polybasic clusters are underlined by broken lines.
even identical to the basic clusters constituting the classical nuclear localization signals. We have previously demonstrated that the basic cluster of *Xenopus* lamin LIIIb, which is identical to that of the zebrafish, can indeed function as an NLS (Hofemeister et al., 2000). Antibodies raised against the lamin LIIIb-specific peptide sequence, if obtained at all, would almost certainly recognize a wide variety of NLS-containing proteins making these antibodies useless as LIII-specific reagents.

The strict conservation of the sequence of the six basic residues in lamin LIII is also of interest in another context. Studies with K-ras have shown that a polybasic stretch of six amino acid residues, although with a different order of lysine and arginine residues, functions as a secondary signal to target localization signal is underlined by a *broken line*. The 105-box within the tail domain is shown in *brackets*. The boundaries of the lamin A-specific domain (amino acid residues 578–648) are marked by *arrowheads*. The nucleotide sequence data are available from GenBank under the accession number AF397016.

![Fig. 5. Amino acid sequence of *Danio rerio* lamin A as deduced from the nucleotide sequence of a cDNA clone obtained by PCR amplification using cDNA from three-days-old embryos (for details see Materials and methods). The subdomains of the central rod (coils 1a, 1b, and 2) are marked by *arrows*. The two p34*cdc2* protein kinase sites, that flank the rod domain, are marked by an *asterisk*. The nuclear localisation signal is underlined by a *broken line*. The 105-box within the tail domain is shown in *brackets*. The boundaries of the lamin A-specific domain (amino acid residues 578–648) are marked by *arrowheads*. The nucleotide sequence data are available from GenBank under the accession number AF397016.](image)

![Fig. 6. Phylogenetic relationship of vertebrate lamins. The rod domains of vertebrate lamins were aligned and submitted to phylogenetic analysis using the UPGMA (unweighted pair group with arithmetic mean method) method with the PAUP program version 40b2a (Swofford, 1998). Sequences are from man (Hs B1, P20700; Hs B2, the complete amino acid sequence of human lamin B2 was assembled from the partial cDNA sequence M94362 and the EST clone BE295831; Hs A, P02545), from chicken (Gg B1, P14731; Gg B2, P14732; Gg A, P13648), from *Xenopus* (*Xl B1, P09010; Xl B2, P21910; Xl A, P11048; Xl LIII, P10999), from zebrafish (Dr B1, AJ250201; Dr B2, AJ005936; Dr A, this study AF397016; Dr LIII, this study AF397015), and from *Caenorhabditis* (Ce B, S42257).](image)
K-ras to the plasma membrane (Hancock et al., 1990; Roy et al., 2000).

The giant nuclei of amphibian and fish oocytes are characterized by several peculiarities. Their chromatin is not closely associated with the nuclear lamina as it is in somatic cells but is organized in lampbrush chromosomes located in the center of the nucleolus. These oocytes also stockpile large amounts of maternal RNA and protein. Their chromosomes are in a post-replication state and are highly engaged in transcription. Early cleavage nuclei, in contrast, are rapidly replicating but are transcriptionally silent (Newport and Kirschner, 1982b). With the exception of the newly replicated DNA these nuclei are assembled entirely of maternal components. It is thus suggestive to speculate that this dramatic change of nuclear function needs nuclear components with special functional adaptations. The specific synthesis of two variants of a particular lamin subtype that differ in their association with nuclear membranes might be one of these adaptations. Early development of mammals and birds proceeds with normal cell cycles. This might explain why lamin LIII is restricted to fish and amphibians. Future studies will focus on the functional analysis of the lamin LIII variants in oocyte and early cleavage nuclei.

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