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cDNA cloning of the developmentally regulated lamin L_{III} of *Xenopus laevis*

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Lamins are nucleoskeletal proteins which form intermediate type filaments in close association with the inner nuclear envelope membrane. Based on molecular and biochemical properties the lamins were grouped as type-A and type-B lamins, respectively. I have cloned the cDNA encoding lamin L_{III} of *Xenopus* which is the lamin protein present in oocyte nuclei and in cleavage nuclei. The data presented here indicate that a pool of maternal lamin L_{III} RNA is synthesized very early in oogenesis and that it continues to be present until gastrulation when the vast majority of the L_{III} RNA is degraded. Despite the similarities shared by all lamin proteins, the lamin L_{III} sequence neither possesses the features diagnostic for either type-A or type-B lamins nor does it show greater sequence similarity to one of the lamin types than to the other and thus it may represent a third type of lamin protein which may reflect its special function in oogenesis and early development.

Key words: intermediate filaments/lamins/maternal RNA/nuclear lamina/*Xenopus*

Introduction

The nuclear lamina is part of the nuclear envelope which separates the cytoplasm from the nucleoplasm in eukaryotic cells (for review see Newport and Forbes, 1987). It forms a fibrous layer (usually 10-100 nm thick) in close apposition to the nucleoplasmic side of the inner nuclear membrane (Fawcett, 1966, 1981) and possibly serves nucleoskeletal functions which are important for nuclear envelope integrity (Gerace and Blobel, 1982; for review see Newport and Forbes, 1987) and for interphase chromatin organization (Lebkowski and Laemmli, 1982; Benavente and Krohne, 1986). This organization might be important for DNA replication and differential gene expression.

Lamin proteins have been identified in a wide variety of organisms (for review see Krohne and Benavente, 1986). In vertebrates several lamin subtypes have been characterized (Gerace *et al.*, 1978; Krohne *et al.*, 1981; Lehner *et al.*, 1986; Wolin *et al.*, 1987). They form a group of related proteins sharing common antigenic determinants (Krohne *et al.*, 1984), and sequence analysis of different lamins (McKeon *et al.*, 1986; Fisher *et al.*, 1986; Krohne *et al.*, 1987; Wolin *et al.*, 1987; Gruenbaum *et al.*, 1988), as well as structural analysis (Aebi *et al.*, 1986) has classified them as nuclear intermediate filament proteins. Characterization of lamin subtypes has been done most extensively in mammals, birds and amphibia. In mammals three major lamins, termed A, B and C, as well as at least one minor lamin have been described (Gerace *et al.*, 1978; Lehner *et al.*, 1986). Human lamin C is identical to human lamin A except for its C-terminus, and probably arises from alternate splicing of RNA (McKeon *et al.*, 1986; Fisher *et al.*, 1986). In chicken three lamins termed lamins A, B_1 and B_2 have been detected by immunological methods (Lehner *et al.*, 1986). Chicken lamins B_1 and B_2 resemble mammalian lamin B with respect to their subcellular distributions during mitosis, i.e. they remain associated with membrane vesicles when the nuclear lamina structure disassembles during mitosis. In contrast, the lamin A- and C-type proteins become freely soluble in the cytoplasm during lamina disassembly (Gerace and Blobel, 1980; Stick *et al.*, 1988).

Lamin proteins are differentially expressed during development and differentiation. The B-type lamins of mammals and chickens are present in all nuclei [except pachytene oocytes which lack a nuclear lamina structure (Stick and Schwarz, 1982, 1983)] and they possibly represent a constitutive element of the nuclear envelope. In contrast, the expression of lamins A and lamin C is under developmental control, i.e. they are expressed at low levels during early development and they increase in the course of cell differentiation (Guilly *et al.*, 1987; Lebel *et al.*, 1987; Lehner *et al.*, 1987; Stewart and Burke, 1987). The pattern of lamins is more complex in *Xenopus* than in mammals and birds. Five different lamins have been described, namely L_I, L_{II}, L_{III}, L_{IV} and L_A (for review see Krohne and Benavente, 1986; Wolin *et al.*, 1987). Lamins L_I, L_{II} and L_A are the major somatic lamins (Krohne *et al.*, 1981; Wolin *et al.*, 1987). Expression of lamin L_I and lamin L_{II} begins at midblastula and gastrula respectively (Stick and Hausen, 1985) while lamin L_A seems to be expressed in all adult somatic cells except for erythrocytes (Wolin *et al.*, 1987). $Lamin_{IV}$ seems to be restricted to male germ cells only, and it forms an atypical lamina structure in these cells (Benavente and Krohne, 1985). The lamin L_{III} is the only lamin protein in oocyte nuclei and cleavage nuclei (Benavente *et al.*, 1985; Stick and Hausen, 1985), i.e. the same protein forms the lamina structure in structurally and functionally diverse nuclei. While, for example, the oocyte nuclei are very actively involved in transcription, the cleavage nuclei are engaged in rapid DNA replication but they are transcriptionally inactive. Lamin L_{III} serves as a maternal lamin protein pool for the assembly of cleavage nuclei up to the mid-blastula stage (Stick and Hausen, 1985). It is expressed at mid-blastula (Stick and Hausen, 1985) and in a few specialized cell types of adult tissue (Benavente *et al.*, 1985). Based on biochemical properties *Xenopus* lamin L_{III} has been tentatively classified as embryonic type-C lamin (Wolin *et al.*, 1987). I have isolated and characterized a cDNA clone encoding lamin L_{III}. My study reveals that lamin L_{III} neither

possesses the features of type-A or type-B lamins nor does it show greater sequence similarity to one lamin type than to the other. This lamin is synthesized very early in oogenesis and is present until gastrulation when the vast majority of the maternal pool of L_{III} RNA becomes degraded.

Results

Cloning of a lamin L_{III} encoding cDNA clone

Four different monoclonal antibodies raised against *Xenopus* lamins were used to screen a λgt11 cDNA expression library constructed from *Xenopus* poly(A)$^+$ RNA (kindly provided by Dr L.Etkin, Houston, USA). The specificity of the monoclonal antibodies was shown by immunoblotting. Monoclonal antibody L6-8A7 recognizes a common epitope on lamins L_I, L_{II}, L_{III} (Figure 1b, lanes 1 and 2). L6-5D5 reacts with an epitope common to lamin L_{II} and L_{III} (Figure 1c, lanes 1 and 2). L7-4A2 exclusively reacts with lamin L_I (Figure 1e, lane 1) and L7-8C6 reacts with lamin L_{II} (Figure 1d, lane 1). Initial screening was done with a mixture of these four antibodies. Approximately 1.2×10^6 plaques were screened and seven independent clones were isolated. Five of these clones expressed a fusion protein reacting with the monoclonal antibody L6-8A7 and with a lamin specific polyclonal rabbit serum described earlier (Stick and Hausen, 1980). In addition, one of these clones reacted with the monoclonal antibody L6-5D5. This clone (D13) was further characterized. The cDNA is 4600 bp long and contains the whole coding region for lamin L_{III} coding for 583 amino acids (Figure 7), ~2900 bp of the 3′ non-coding region and 9 bp of the untranslated 5′ region. When total RNA from oocytes or embryos was probed with this cDNA it hybridized to an RNA of ~5200 bp (Figure 6a and c). Therefore the isolated cDNA lacks ~600 bp of the full length mRNA.

Identification of the lamin encoded by clone D13

To identify the lamin encoded by the recombinant λgt11D13 clone fusion protein was produced in *Escherichia coli* Y1089 lysogenized with λgt11D13. Cell lysates of infected and uninfected *E.coli* (Figure 1, lanes 3 and 4) together with lamins isolated from erythrocytes and oocytes (Figure 1, lanes 1 and 2) were probed by immunoblotting with the monoclonal antibodies described above. Only the antibodies recognizing a common epitope on lamins L_I, L_{II} + L_{III} (Figure 1b) and L_{II} + $_{III}$ (Figure 1c) bind to the fusion protein while antibodies specific for L_I (Figure 1e) or L_{II} (Figure 1d) failed to react with the fusion protein. I reproducibly detected three fusion protein bands with apparent mol. wts of 187, 172, and 148 kd. The largest protein most probably contains the entire lamin polypeptide fused to β-galactosidase by a short stretch of three amino acids translated in-frame from the 5′ region proximal of the lamin coding region. The lower bands may represent proteolytic products of the former one. Proteolysis of foreign proteins of this size is often observed in *E.coli*.

I have subcloned the cDNA into bluescribe M13+, prepared synthetic mRNA by transcription with T7 RNA polymerase, translated the RNA *in vitro* and immunoprecipitated the translation product with the monoclonal antibodies described above. Similar to the results obtained with SDS denatured polypeptides on immunoblots, the polypeptide synthesized *in vitro* was immunoprecipitated with antibodies reacting with L_I, L_{II} + L_{III} (Figure 2, lane 2) and L_{II} + L_{III} (Figure 2, lane 3) but not with antibodies specific for L_{II} (Figure 2, lane 5) and L_I (Figure 2, lane 4). In addition, an antibody that had been previously

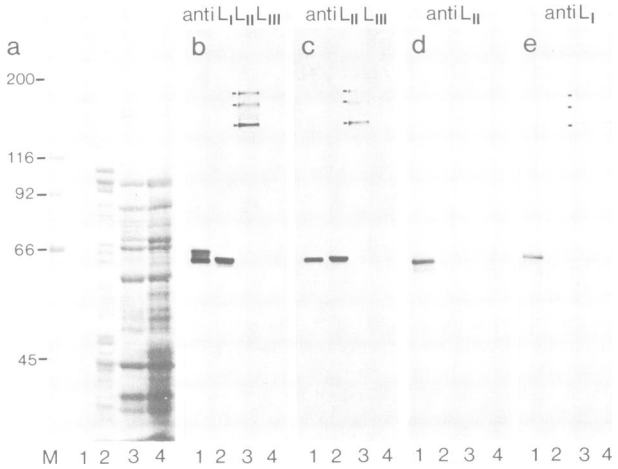

Fig. 1. Characterization of the fusion protein encoded by λgt11D13. Nuclear envelope fractions from erythrocytes (**lanes 1**), 20 oocyte nuclei each (**lanes 2**), cell lysates of *E.coli* Y1089 lysogenic for λgt11D13 (**lanes 3**), or λgt11 as controls (**lanes 4**) were separated on 7.5% SDS−PAGE gels and blots were either stained with Ponceau Red (**panel a**) or probed with monoclonal antibodies L6-8A7 (**panel b**), L6-5D5 (**lane c**), L7-8C6 (**panel d**) or L7-4A2 (**panel e**). Fusion protein bands are marked by short lines in (**b**), (**c**) and (**e**). The faint bands seen in panel (**e**), **lanes 3** and **4**, are due to a non-specific reaction of antibody L7-4A2 with *E.coli* protein; they do not correspond to a fusion protein band and are present also in lysates from cells infected with λgt11. Lamin L_{II} and L_{III} migrate at nearly identical positions in the gel system used here [7.5% SDS−polyacrylamide gel according to Laemmli (1970)]. Mol. wts of reference proteins are given in kd (**lane M**).

Fig. 2. Immunoprecipitation of *in vitro* synthesized polypeptides encoded by cDNA clone D13. Proteins were synthesized in a coupled transcription−translation system using cDNA D13 subcloned into bluescribe M13+ and immunoprecipitated with monoclonal antibodies L6-8A7 (**lane 2**), L6-5D5 (**lane 3**), L7-4A2 (**lane 4**), L7-8C6 (**lane 5**), $L_0$46F7 (Benavente *et al.*, 1985) (**lane 6**), and with goat anti-mouse IgG−Sepharose omitting a primary antibody (**lane 7**). Polypeptides were separated on10% SDS−polyacrylamide gels according to Laemmli (1970). Mol. wts of reference proteins are given in kd.

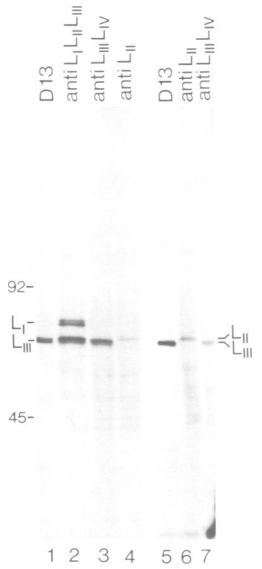

Fig. 3. Comparison of lamin L_{III} encoded by cDNA clone D13 with lamins translated from oocyte RNA *in vitro*. RNA was isolated from *Xenopus* stage II oocytes or transcribed *in vitro* from cDNA clone D13 using T7 RNA polymerase. RNA was translated in a rabbit reticulocyte lysate. Total translation products from synthetic lamin L_{III} RNA are shown in **lanes 1** and **5**. Translation products of oocyte RNA were subjected to immunoprecipitation using monoclonal antibodies L6-8A7 (**lane 2**), $L_0$46F7 (**lane 3** and **7**), and L7-8C6 (**lanes 4** and **6**). Polypeptides were separated on 12% SDS–polyacrylamide gels prepared according to Thomas and Kornberg (1974). Results of two separate experiments (lanes 1–4 and 5–7) are shown. Mol. wts of reference proteins are given in kd.

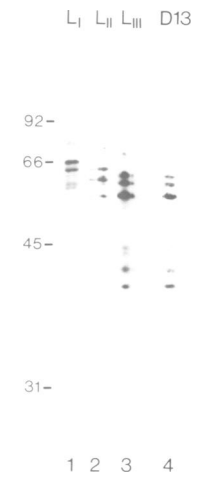

Fig. 4. Peptide map comparison of lamins L_I, L_{II} and L_{III} with the polypeptide encoded by cDNA clone D13. Lamin preparations from *Xenopus* erythrocyte nuclei or *Xenopus* oocyte nuclei and lamin L_{III} synthesized *in vitro* by coupled transcription–translation from cDNA clone D13 using [^{35}S]methionine were separated on SDS–polyacrylamide gels. Corresponding bands were excised and subjected to chemical cleavage with *N*-chlorosuccinimide. Cleavage products were separated on a 12% SDS–polyacrylamide gel (Laemmli, 1970). **Lanes 1, 2** and **3** show cleavage fragments of lamin L_I, L_{II} and L_{III}, respectively, visualized by indirect immunostaining with monoclonal antibody L6-8A7 (for details see Material and methods). **Lane 4** shows a fluorogram of cleavage products of the polypeptide synthesized *in vitro*. The lower mol. wt bands present in both lamin L_{II} and lamin L_{III} digests may reflect homologue positions of tryptophanyl residues in both polypeptides as discussed in the text. Mol. wts of reference proteins are given in kd.

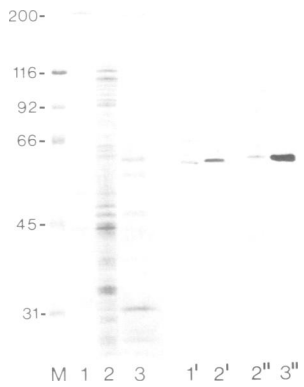

Fig. 5. Size comparison of lamin L_{III} from oocyte nuclei and L_{III} synthesized *in vitro* from cDNA clone D13. Lamin L_{III} was synthesized *in vitro* from cDNA clone D13 using a coupled transcription–translation system and separated on 10% SDS–polyacrylamide gels (Laemmli, 1970) (**lanes 3** and **3″**) adjacent to the lamin fraction of *Xenopus* erythrocyte nuclei (**lanes 1** and **1′**) and mixed with oocyte nuclei (**lanes 2, 2′** and **2″**). A protein blot of this gel was stained with Ponceau Red (**lanes M, 1, 2** and **3**) and the filter was processed for indirect immunostaining to visualize non-radiolabelled lamins (lanes 1′ and 2′) followed by fluorography (lanes 2″ and 3″) to detect the radiolabelled *in vitro* synthesis products. Mixture of the *in vitro* synthesized lamin L_{III} with the oocyte lamina fraction allows an accurate size comparison (lanes 2′ and 2″). Note the slightly lower mobility of lamin L_{III} synthesized *in vitro*. Mol. wts of reference markers (M) are given in kd.

demonstrated to recognize *Xenopus* lamins L_{III} + L_{IV} ($L_0$46F7) (Benavente *et al.*, 1985), immunoprecipitated the polypeptide encoded by clone D13 (Figure 2, lane 6). From these experiments it can be concluded that D13 most probably codes for lamin L_{III}. However, this conclusion is based on the fact that lamin L_I and L_{II} specific antibodies fail to react with the polypeptide encoded by clone D13. Furthermore, the specificity of antibody $L_0$46F7 is somewhat restricted as it also recognized lamin L_{II} in immunoblots under the conditions used here (not shown). Therefore, the next experiments were designed to distinguish between lamin L_{II} and L_{III}. Total RNA was isolated from stage II oocytes, the RNA was translated *in vitro*, lamins were immunoprecipitated and analysed by SDS–PAGE together with lamin encoded by cDNA clone D13 synthesized *in vitro* (Figure 3). Examples of two preparations run on separate gels are shown in Figure 3 (lanes 1–4 and 5–7). Both antibodies L6-8A7 (reacting with lamins L_I, L_{II} + L_{III}) (Figure 3, lane 2) and $L_0$46F7 (reacting with lamins L_{III} + L_{IV}) (Figure 3, lanes 3 and 7) precipitated a polypeptide co-migrating with the lamin polypeptide encoded by cDNA clone D13. L6-8A7 also precipitated a polypeptide of slower mobility which could be identified as lamin L_I. In contrast lamin L_{II} specifically precipitated by antibody L7-8C6 (Figure 3, lanes 4 and 6) migrated slightly slower in the gel system used (for details see Materials and methods). Since the RNA synthesized *in vitro* from clone D13 and the RNA isolated from oocytes was translated under identical conditions this indicates that the cDNA clone D13 encodes lamin L_{III}.

All evidence presented so far is based on the specificity of antibodies. To get independent evidence for the identity of the D13 encoded lamin, I subjected individual lamins, gel-purified either from erythrocyte (lamin L_I, L_{II}) or oocyte (lamin L_{III}) lamins, to chemical cleavage at

tryptophanyl residues with *N*-chlorosuccinimide (Lischwe and Ochs, 1982). The resulting peptide maps were compared with the cleavage pattern of the *in vitro* translated polypeptide encoded by cDNA clone D13. The map of the polypeptide encoded by cDNA clone D13 (Figure 4, lane 4) matches exactly that of lamin L_{III} isolated from oocyte nuclei (Figure 4, lane 3) and is significantly different from those of lamin L_{II} (Figure 4, lane 2) and lamin L_I (Figure 4, lane 1). Therefore one can conclude that clone D13 encodes *Xenopus* lamin L_{III}. I have also compared the size of lamin L_{III} synthesized from cDNA clone D13 (Figure 5, lanes 2″ and 3″) to that of lamin L_{III} isolated from oocyte nuclei (Figure 5, lane 2′). I found that the *in vitro* synthesized polypeptide is slightly larger than the protein isolated from oocytes.

Expression of lamin L_{III} RNA during oogenesis and early development

From previous reports we received evidence that the lamins are expressed from maternal RNA in early development (Stick and Hausen, 1985). I therefore investigated the expression of lamin L_{III} transcripts in oogenesis and early development by Northern blot analysis. To compare the amount of lamin L_{III} transcripts in oogenesis and early development I analysed total RNA from either a single oocyte or a single embryo on Northern blots (Figure 6a). The amount of lamin L_{III} RNA increases between stage I and II of oogenesis. It remains constant during later stages of oogenesis and in early development up to stage 9 (late blastula) and then falls to very low levels during gastrulation (stage 11–12). The amount of lamin L_{III} RNA slightly increases during tail bud stages from stage 38 onwards (Figure 6a, and results not shown). To check the quality of the RNA preparations Northern blots were reprobed with a cytoskeletal actin probe (see Materials and methods) (Figure 6b). The pattern of expression demonstrated reproducible recovery of RNA in our preparations. The level of lamin L_{III} RNA expression in stage I oocytes was somewhat variable (Figure 6a). In some samples I failed to detect significant amounts of lamin L_{III} RNA although skeletal actin RNA was present in normal amounts in all of these samples.

Our previous experiments (Stick and Hausen, 1985) had shown that translation of lamin L_{III} is turned on at mid-blastula and that this synthesis is directed by maternal RNA. To find out whether embryonic transcripts contribute significantly to the L_{III} RNA pool after the mid-blastula transition, I analysed lamin L_{III} RNA levels in embryos blocked in RNA polymerase II activity. Embryos were injected with α-amanitin at the one-cell stage and total RNA, isolated from injected and uninjected embryos, was analysed (Figure 6c). No significant difference in the amount of lamin L_{III} RNA could be detected in treated and untreated embryos between stage 6 and 10. Therefore one can conclude that the lamin L_{III} RNA present in early embryos is maternal in origin.

Amino acid sequence features of lamin L_{III}

The cDNA clone D13 encoding lamin L_{III} codes for a protein with a predicted mol. wt of 67 315 daltons (Figure 7). This is larger than would be expected from its electrophoretic mobility. The discrepancy may reflect some unusual sequence features of L_{III} such as a region exceptionally rich in negatively charged amino acids (11 out

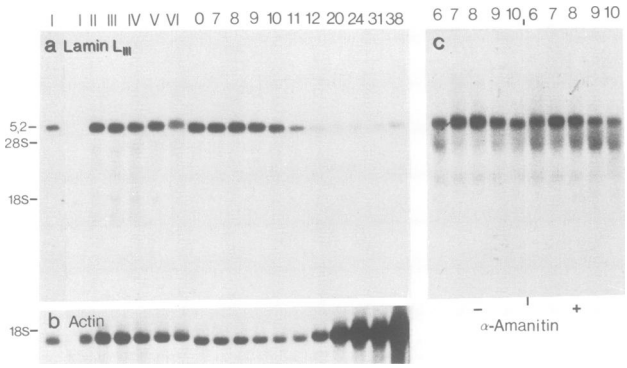

Fig. 6. Expression of *Xenopus* lamin L_{III} RNA during oogenesis and early development. Total RNA was isolated from oocytes or embryos of indicated stages. Oocyte stages (I–VI) according to Dumont (1972), embryonic stages (6–38) according to Nieuwkoop and Faber (1967), 0: unfertilized egg. Total RNA equivalent to one oocyte or one embryo was loaded in each lane of a 1% formaldehyde agarose gel. Northern blots were probed either with ^{32}P-labelled antisense RNA transcribed *in vitro* from cDNA clone D13 subcloned into bluescribe M13+ vector (**a** and **c**) or with a skeletal actin DNA cloned into pUC19 and ^{32}P-labelled by nick-translation (**b**). To inhibit endogenous transcription of RNA polymerase II embryos were injected with α-amanitin at the one-cell stage (c) (for details see Materials and methods). The sizes of RNAs are given in kd; positions of 28S and 18S RNA are indicated.

of 16) in the tail (position 550–565) (Figure 7) and/or incomplete unfolding of the L_{III} polypeptide in SDS-containing solutions. Incomplete denaturation would explain the different mobilities of lamin L_{III} relative to mol. wt standards that have been observed with changes in polyacrylamide gel concentrations (R.Stick, unpublished).

Lamin L_{III} displays the molecular organization typical for intermediate filament proteins (Geisler and Weber, 1982; McKeon *et al.*, 1986; Fisher *et al.*, 1986) with a 355 amino acid long central rod domain subdivided into three α-helical domains characterized by a heptade repeat of hydrophobic amino acids allowing the formation of interchain coiled coils. The rod is flanked by a 32 amino acid long head and a 196 amino acid long C-terminal domain (Figure 7). The sequence of lamin L_{III} shares a number of features with the sequences of other lamins.

An amino acid sequence comparison with *Xenopus* lamins L_I (Krohne *et al.*, 1987), and L_A (Wolin *et al.*, 1987) as well as with human lamins A and C (McKeon *et al.*, 1986; Fisher *et al.*, 1986) is shown in Figure 8. The complete rod domains of all lamins as well as a long region of the C-terminal domain from amino acids 430 to 560 (in L_{III}) can be aligned without introducing gaps (with a single exception in lamin L_I, which lacks a single amino acid between positions 475 and 476). The degree of similarity of the four lamins in these regions is remarkable. This becomes evident when, in addition to identical amino acids, conservative amino acid exchanges are also taken into account. The four lamins exhibit between 60 and 80% similarity of amino acids in these regions. Lamin L_{III} appears to be equally distant from lamin L_I and the type-A lamins.

The C-terminal region contains four tryptophanyl residues conserved in all lamins. The similarities in the lower mol. wt range of the peptide maps of lamins L_{II} and L_{III} observed after the tryptophanyl specific cleavage with *N*-chloro-succinimide (Figure 4, lanes 2 and 3) indicate that these tryptophanyl residues are also present in lamin L_{II}. Between

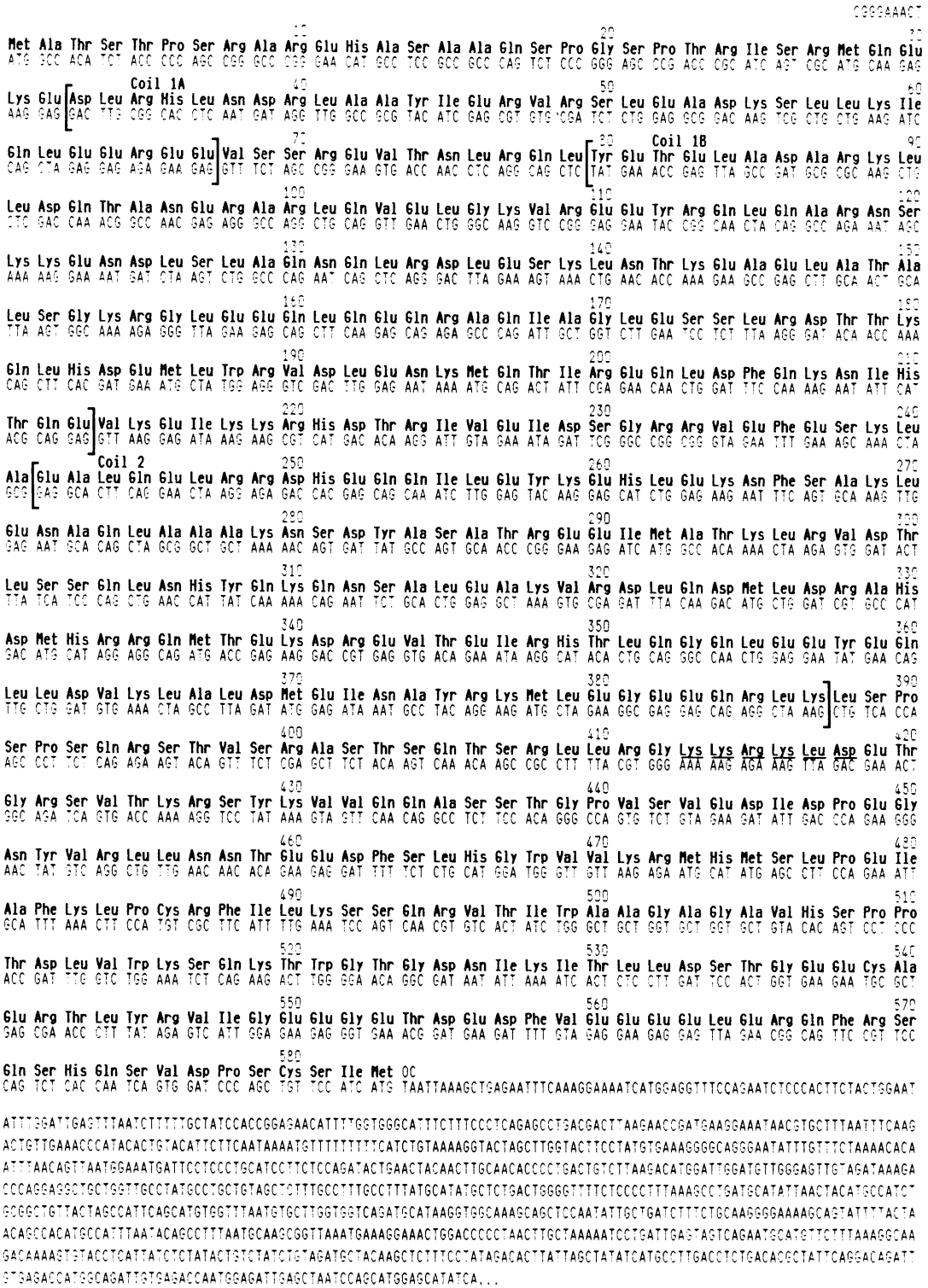

Fig. 7. cDNA sequence and deduced amino acid sequence of *Xenopus* lamin L_{III}. The 5′-terminal half of cDNA clone D13 containing the whole coding region of lamin L_{III} is shown. α-Helical domains (Coils 1A, 1B and 2) are shown by brackets. A putative nuclear migration signal (amino acid positions 413−418) is underlined.

the end of coil 2 and amino acid position 430 (in lamin L_{III}) several gaps must be introduced for optimal alignment between the four lamin sequences (Figure 8). Interestingly, in the region of amino acids 393−419 the similarity of lamin L_{III} to lamin L_I is much greater than to the other lamins. This region contains a putative nuclear localization signal in all four lamins with a consensus of four basic amino acids followed by a hydrophobic and an acidic amino acid residue (Figure 8). Most strikingly, the sequence similarity between lamin L_{III} and the other lamins drops drastically near the C-terminus beyond amino acid position 560 (in lamin L_{III}). And finally all lamins, with the exception of human lamin C (not shown), share a common C-terminus of the consensus S/NCS/AIM.

Fig. 8. Comparison of amino acid sequence of *X.laevis* lamin L_{III} with *Xenopus* lamins L_I (Krohne *et al.*, 1987), and L_A (Wolin *et al.*, 1987) and human lamin A (HL_A) (Fischer *et al.*, 1986). Identical amino acids are marked by bold letters. Coils 1A, 1B and 2 are indicated. Amino acid positions of lamin L_{III} are marked continuously. The arrowheads mark areas where gaps have to be introduced for alignment of all four sequences. The arrow denotes the amino acid at which the human lamin A sequence diverges from that of human lamin C.

Discussion

I have isolated a cDNA clone (D13) encoding *Xenopus* lamin L_{III} by screening a cDNA expression library constructed from *Xenopus* ovary poly(A)$^+$ RNA. The library was screened with a set of lamin specific monoclonal antibodies. The cDNA clone was shown by several criteria to encode lamin L_{III}. First, three monoclonal antibodies specific to lamins: (i) L_I, L_{II} + L_{III}, (ii) lamins L_{II} + L_{III} and (iii) lamins L_{III} + L_{IV} react with the fusion protein synthesized in *E.coli* lysogenic for λgt11D13 whereas antibodies specific for lamin L_I or lamin L_{II} do not react with the fusion protein.

Second, D13 encoded polypeptide synthesized *in vitro* by a coupled transcription−translation system co-migrates in SDS−polyacrylamide gels with lamin L_{III} but not with L_{II}.

Lastly, the peptide map of chemically cleaved protein encoded by clone D13 resembles that of lamin L_{III} isolated from oocytes and can be distinguished from the peptide maps of lamin L_I and L_{II} isolated from erythrocytes. The polypeptide synthesized *in vitro* has a slightly lower mobility in SDS−PAGE when compared to lamin L_{III} isolated from oocytes. This slight size difference must be due to post-transcriptional processing or a polypeptide modification event that occurs *in vivo*, since such a size difference was not observed when lamin L_{III} RNA isolated from oocytes and clone D13 encoded RNA were both translated in the same *in vitro* translation system.

Developmental expression of lamin L_{III} RNA

De novo synthesis of lamin L_{III} in early development begins at the mid-blastula, and from inhibitor experiments using α-amanitin it had previously been concluded that synthesis of lamin L_{III} in early development is achieved by activation of maternally stored RNA. Here I show the pattern of expression of lamin L_{III} RNA. The amount of lamin transcripts present in oocytes exceeds the amount expected to be necessary to direct lamin L_{III} protein synthesis for the growth of the oocyte nuclear lamina. The vast majority of these transcripts almost certainly represents a pool of maternal RNA stockpiled for the lamin synthesis starting at mid-blastula. The accumulation of this pool begins in stage I of oogenesis and the final size of the store is already reached at stage II. The maternal store of lamin L_{III} RNA is degraded during gastrulation and only a very low amount of RNA is found during the following development. The amount of lamin L_{III} protein decreases relative to the amounts of lamins L_I and L_{II} in these stages of development and it might be speculated that lamin L_{III} is successively replaced by the other lamins in most cell types. In later stages (~stage 36−38) the amount of lamin L_{III} RNA increases again. But the RNA does not reach the levels detected in early development. This latter observation correlates well with the findings of Benavente *et al.* (1985). These authors found expresion of lamin L_{III} in only a few specialized cell types from the tail bud stages onward. It has previously been shown that like lamin L_{III}, synthesis of lamin L_I and L_{II} is

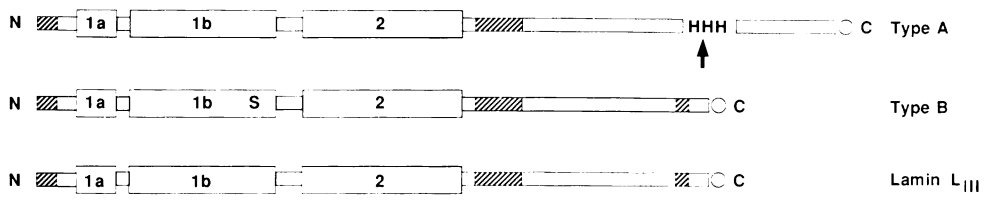

Fig. 9. Schematic representation of the three types of lamin proteins. α-Helical domains are indicated as 1a, 1b and 2, respectively. The areas where gaps have to be introduced for optimal alignment of the sequences are shown as hatched boxes. The oligohistidine block (HHH) in type-A and the cysteine residue (S) in coil 1b of type-B lamins are indicated. The arrow denotes the putative splice site in human lamins A and C. The presence of the C-terminal consensus sequence S/NCS/AIM is indicated by circles.

directed by maternal RNA in early development (Stick and Hausen, 1985; Wolin *et al.*, 1987). Lamin L_I as well as lamin L_{II} can be translated *in vitro* from RNA isolated from oocytes as early as stage II. Therefore, the time course of accumulation of their respective RNAs might be similar to that of lamin L_{III} described here.

Comparison of lamin L_{III} with type-A and type-B lamins

The family of lamins has been grouped into type-A and type-B lamins (Krohne and Benavente, 1986; Lehner *et al.*, 1987; Wolin *et al.*, 1987). A comparison of lamin L_{III} with other lamins on the basis of amino acid sequence and biochemical properties however shows that lamin L_{III} cannot be assigned easily to one of these groups. A schematic representation of lamin L_{III} and the type-A and type-B lamins with their diagnostic features is given in Figure 9. The amino acid sequence of lamin L_{III} displays the features diagnostic for lamins (McKeon *et al.*, 1986; Fisher *et al.*, 1986) including those features that characterize them as members of the intermediate filament protein family (Geisler and Weber, 1982). When all five vertebrate lamins sequenced so far are compared, gaps have to be introduced into the sequences to achieve optimal alignment. The gaps are clustered in three regions. These are the 14–18 amino acids at the extreme N-terminus, a region of ~40 amino acids following coil 2 and the C-terminal region beyond residue 560 (in lamin L_{III}) (Figure 9). The recently published sequence of a *Drosophila* lamin (Dm_0) (Gruenbaum *et al.*, 1988) fits into this general scheme. Interestingly, the *Drosophila* lamin possesses an extra 23 amino acids (compared to L_{III}) at its extreme N-terminus, the sequence homology with the other lamins drops beyond amino acid position 550 (in lamin L_{III}) and its C-terminal domain is slightly larger than that of lamins L_I and L_{III}.

In the region next to coil 2 and to a lesser extent at the N-terminus, lamin L_{III} shows greater similarity to lamin L_I than to the A-type lamins. However, the overall similarity between lamin L_{III} and L_I is similar to that between lamin L_{III} and *Xenopus* lamin A. Furthermore, the similarity to *Xenopus* lamin A is not higher than to human lamins A/C. Moreover, lamin L_{III} does not contain certain features that appear to be diagnostic for type-A or type-B lamins (Krohne *et al.*, 1987; Wolin *et al.*, 1987). Lamin L_{III} lacks an oligohistidine block found in the tail region of lamins A and lamin C and it also does not possess the extension of the tail domain characteristic for human and *Xenopus* lamins A (Fisher *et al.*, 1986; Wolin *et al.*, 1987). The presence of the C-terminal consensus sequence S/NCS/AIM present in all lamins with the exception of human lamin C argues against a recently proposed classification of *Xenopus* lamin L_{III} as a type-C lamin (Wolin *et al.*, 1987). Lamin L_{III} shares some sequence features with lamin L_I (see above) and both lamin L_{III} (Stick and Hausen, 1985) and the B-type lamins in mammals (Stewart and Burke, 1987) and chicken (Lehner *et al.*, 1987) seem to be the only lamin components present in nuclei of early embryos. However, lamin L_{III} lacks a cysteine in the rod domain which is found in *Xenopus* lamin L_I. The presence of this cysteine is thought to be a characteristic difference between type-B and type-A lamins (Krohne *et al.*, 1987).

Furthermore, based on cell fractionation experiments lamin L_{III} can clearly be distinguished from B-type lamins. Lamin L_{III} becomes freely soluble on nuclear envelope disassembly during meiotic maturation and mitosis (Benavente *et al.*, 1985; Stick and Hausen, 1985; Stick, 1987), in contrast to mammalian and chicken B-type lamins which remain associated with membrane cisternae during mitosis (Gerace and Blobel, 1980; Burke and Gerace, 1986; Stick *et al.*, 1988). However, the mechanism by which this membrane association is brought about is not clear. Other proteins might be involved in this process.

The amino acid sequence similarities found in the amino acids 393–419 of lamin L_{III} and lamin L_I could be explained either by exon shuffling or by functional constraints. The proportion of identical and similar amino acids between the lamins decreases drastically beyond residue 563 (in lamin L_{III}). It is in this region where the putative alternate splice sites of human lamins A and C would be expected (McKeon *et al.*, 1986; Fisher *et al.*, 1986). Furthermore, sequence similarity between a C-terminal region of human lamin A and a region of the N-terminal head domain of human type II keratin has been observed (Fisher *et al.*, 1986). Therefore, exon shuffling might have played an important role in lamin evolution. The striking similarity of the extreme C-terminus (consensus sequence S/NCS/AIM) of four lamins may also have arisen by recombination events. Alternatively, if the conservation of this sequence is due to functional constraints its absence in human lamin C has to be explained.

Materials and methods

Cloning and sequencing of Xenopus lamin L_{III}

A λgt11 expression library of *Xenopus* ovary cDNA was kindly provided by Dr L.Etkin (Houston, TX, USA). Plaques were probed with a mixture of four monoclonal antibodies reacting with epitopes on *Xenopus* lamins: L_I, L_{II}, L_{III} (L6-8A7), L_{II}, L_{III} (L6-5D5), L_I (L7-4A2), L_{II} (L7-8C6). Filters were blocked for 40 min in 1% BSA in 137 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4, pH 7.1 (PBS). They were then incubated for 2 h in primary antibody (mouse ascites fluid) 1:1000 diluted in 0.3% hemoglobin, 0.1% Tween-20 in PBS, washed for 30 min in several changes of 0.3% hemoglobin, 0.1% Tween-20 in PBS and incubated for 2 h in secondary antibody (alkaline phosphatase conjugated goat anti-mouse

IgG (Jackson Immunoresearch Laboratories, PA, USA) diluted 1:2000 in the above buffer). After two washes in 0.3% hemoglobin, 0.1% Tween-20 in PBS, one wash in 0.1% Tween-20 in PBS and one wash in 0.1 M Na_2CO_3, pH 10.2 the colour reaction was carried out in 0.2 mg/ml bromo-chloro-indolylphosphate, 0.33 mg/ml nitroblue tetrazolium in 0.1 M Na_2CO_3, pH 10.2 for appropriate times. Phages were purified from positive plaques and the probed with individual antibodies. Inserts were subcloned according to standard procedures (Maniatis *et al.*, 1982) into plasmids pEMBL8 (Dente *et al.*, 1983) and M13mp18 and 19 (Yanisch-Perron *et al.*, 1985) for sequencing, and into bluescribe M13+ (Stratagene, La Jolla, CA, USA) for *in vitro* synthesis of RNA. Sequencing by the didexoy chain termination method was done according to Sanger *et al.* (1977).

Fusion protein and nuclear skeletal fractions

E.coli Y1089 was lysogenized with λgt11 and λgt11D13 and fusion protein was prepared according to Huynh *et al.* (1985). A karyoskeletal fraction from *Xenopus* erythrocytes was prepared essentially as described by Krohne *et al.* (1981), a karyoskeletal fraction from *Xenopus* oocytes was prepared as described by Stick and Krohne (1982).

Gel electrophoresis and immunoblotting

SDS−PAGE was carried out either according to Laemmli (1970) or according to Thomas and Kornberg (1974). Proteins were electrophoretically transferred to nitrocellulose sheets. Proteins were visualized by brief staining of the sheets with Ponceau Red (Sigma, Munich, FRG). Nitrocellulose sheets were blocked for 30 min in 0.1% Tween-20 in PBS and 30 min in 5% non-fat dry milk, 0.1% Tween-20 in PBS. Primary antibodies (mouse ascites fluids) were diluted 1:1000 in 0.1% BSA, 0.1% Tween-20 in PBS and incubated for 2 h at room temperature, washed for 30 min in 0.1% Tween-20 in PBS, incubated for 2 h in secondary antibody (alkaline phosphatase conjugated goat anti-mouse IgG diluted 1:2000 in 0.1% BSA, 0.1% Tween-20 in PBS) and washed as above. The colour reaction was carried out as described above. In some experiments nitroblue tetrazolium was omitted to reduce non-specific background staining. ^{35}S-labelled proteins were detected by fluorography. The dried nitrocellulose sheets were dipped into 27% diphenyloxazol (PPO) in toluene for 30 s, air dried and exposed on pre-flashed film.

Peptide mapping

Lamins were separated by SDS−PAGE and stained with Coomassie blue. Individual bands were cut from the gel and subjected to chemical cleavage with *N*-chlorosuccinimide (Sigma, Munich, FRG) for 30 min essentially as described by Lischwe and Ochs (1982). When radiolabelled protein was analysed, gels were first processed for fluorography with EN^{3}HANCE (NEN, England) dried and exposed on pre-flashed film. Dried gel slices were rehydrated and processed as outlined above. The cleavage products were separated on 12% SDS−polyacrylamide gels, proteins were transferred to nitrocellulose sheets and lamin peptides were detected by immunoreaction using monoclonal antibody L6-8A7. Finally, nitrocellulose sheets were processed for fluorography. For details see under gel electrophoresis and immunoblotting.

RNA techniques

To prepare synthetic *Xenopus* lamin L_{III} mRNA, the cDNA insert D13 was cloned into bluescribe M13+ vector (Stratagene, La Jolla, CA, USA) and linearized with *Kpn*I. Transcription with T7 polymerase (Genofit, Geneva, Switzerland) was done according to manufacturer's instructions. The RNA was capped *in vitro* by including 0.5 mM m7G(5')pppG (CAP analog, Pharmacia, Uppsala, Sweden) in the reaction.

Total RNA was extracted from *Xenopus* oocytes and embryos [staged according to Dumont (1972) and Nieuwkoop and Faber (1967), respectively] as described by Krieg and Melton (1984). RNA from eggs and embryos was preciptiated with 2 M LiCl (final concentration) for 2 h at −20°C prior to ethanol precipitation. RNA of one oocyte or one embryo was separated on denaturating formaldehyde agarose gels, RNA was transferred to nylon membrane (Gene Screen, NEN, England) and probed either with ^{32}P-labelled antisense RNA (clone D13) or with ^{32}P-nick-translated plasmid DNA (pUC22 *Eco*RI−*Hin*dIII, encoding a genomic fragment of *Xenopus* non-muscle actin). This clone was kindly provided by G.Spohr, Geneva, Switzerland. For the hybridization I followed the protocol of Khandjian (1986). Hybridization of the antisense RNA was at 53°C, 50% formamide; hybridization of nick-translated DNA was at 42°C, 50% formamide. Filters were washed twice for 10 min at room temperature in 2 × SSC, 2% SDS and twice for 30 min at 68°C in 0.1 × SSC, 0.1% SDS. In experiments where α-amanitin was used embryos were injected at one-cell stage with 25 nl of α-amanitin (40 μg/ml) in 0.088 M NaCl, 0.015 M Tris−HCl, pH 7.5.

In vitro translation and immunoprecipitation

Total RNA, equivalent to 2.5 oocytes [stage II, according to Dumont (1972)] was used for translation *in vitro* using a rabbit reticulocyte lysate (Promega Biotec, Madison, WI, USA) and [^{35}S]methionine (Amersham, England) as label. Synthetic *Xenopus* lamin L_{III} RNA was translated *in vitro* without further purification in a coupled transcription−translation system (Hurt *et al.*, 1984). For immunoprecipitation goat anti-mouse IgG−Sepharose (kindly provided by Dr H.Schwarz, MPI für Biologie, Tübingen, FRG) was pre-washed in IPB [1% Triton-X100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Tris−HCl, pH 7.2, 150 mM NaCl, 2 mM EDTA, 20 mM methionione, 0.1 mM PMSF, 0.5% Trasylol (Bayer, Leverkusen, FRG)], incubated with 5 μl ascites fluid for 30 min at room temperature and then washed three times with IPB. Translation reactions (50 μl) were diluted to 400 μl with IPB and pre-incubated with Sepharose beads to remove non-specifically binding proteins. After 15 min the beads were sedimented and the supernatants were incubated with antibody-loaded beads for 2 h at 4°C. Beads were extensively washed with IPB (7−8 times), and washed once with 50 mM Tris−HCl, pH 7.5. Beads were suspended in SDS−gel sample buffer, boiled for 5 min, spun and the supernatants were analysed on 12% SDS−polyacrylamide gels (Thomas and Kornberg, 1974). After electrophoresis gels were processed for fluorography by impregnation with EN^{3}HANCE (NEN, England), dried and exposed on pre-flashed films.

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