INTRODUCTION

The nuclear envelope of eucaryotic cells is composed of three separate membrane domains: (i) the outer nuclear membrane is in continuity with the endoplasmic reticulum; (ii) the membranous wall of the nuclear pore complex is located where the outer and inner nuclear membranes fuse to form the pore channel; (iii) the inner nuclear membrane is associated with a meshwork of proteins, the nuclear lamina.

The inner nuclear membrane is structurally and functionally distinct from the other two membrane domains. It contains specific integral membrane proteins that link the membrane to the underlying lamina and the peripheral chromatin (for review see Georgatos et al., 1994; Gerace and Foisner, 1994; Ye et al., 1998).

Nuclear intermediate filament proteins, the lamins, are the major structural elements of the lamina. B-type lamins are constitutively expressed and remain attached to membranes during mitosis. In contrast, A-type lamins are expressed late during embryogenesis and form soluble oligomers in mitotic cells (for review see Gerace and Burke, 1988; Moir et al., 1995; Gant and Wilson, 1997). Lamins provide mechanical stability to the nuclear envelope (Newport et al., 1990; Lenz-Böhme et al., 1997; for review see Krohne, 1998), and are involved in the postmitotic reorganization of the nucleus, including chromatin decondensation and replication (Benavente and Krohne, 1986; Newport et al., 1990; Goldberg et al., 1995; Spann et al., 1997; Yang et al., 1997; for review see Benavente, 1991; Hutchison et al., 1994; Gant and Wilson, 1997).

More recently integral membrane proteins of the inner nuclear membrane have also been implicated in the structural organization of the nucleus by their binding to lamins and chromatin (for review see Ye et al., 1998). These are the lamin B receptor/p58 (LBR; Worman et al., 1990), the lamina-associated polypeptides 1A-C (LAP1A-C; Senior and Gerace, 1988; Martin et al., 1995) and 2 (LAP2 is now LAP2β, see Dechat et al., 1998; Foisner and Gerace, 1993; Furukawa et al., 1995; for details on the nuclear membrane proteins otefin and emerin see Ashery-Padan et al., 1997; Bione et al., 1994; Nagano et al., 1996).
One of the best characterized proteins of the inner nuclear membrane is LAP2β. LAP2β is the rat homologue to the human TPβ, a member of the thymopoietin (TP) protein family (Harris et al., 1994, 1995). The primary transcript of the TP/LAP2 gene is alternatively spliced giving rise to at least three different proteins: TP/LAP2 α, β, and γ (Harris et al., 1994; for nomenclature see also Dechat et al., 1998). All three proteins are identical for the amino-terminal 187 amino acids. LAP2α lacks the membrane spanning domain that is contained in LAP2β and γ and is found in association with chromatin (Dechat et al., 1998). LAP2γ differs from LAP2β by a 109 amino acid long deletion in the nucleoplasmic domain of LAP2β/TPβ (Harris et al., 1994, 1995). LAP2β binds in vitro to chromosomes and lamin B1, whereas its phosphorylation by mitotic cytosol inhibited both interactions (Foissner and Gerace, 1993). In the 409 amino acids long nucleoplasmic domain of the rat LAP2β, the chromatin binding domain resides near the amino terminus while the lamina/lamin binding domain resides between amino acids 298 and 373 (Furukawa et al., 1995, 1998; Furukawa and Kondo, 1998). Microinjection of a peptide comprising the lamina/lamin B1 binding region of LAP2β into cultured cells demonstrated that interaction between this LAP2β sequence and the lamina are required for the increase of nuclear volume at the end of mitosis and the progression into S-phase (Yang et al., 1997; Furukawa and Kondo, 1998).

The expression and localization of LAP2/TPs during rat spermiogenesis indicated that these proteins are apparently regulated during differentiation processes where the nuclear envelope is remodelled and the chromatin is reorganized and transcriptionally inactivated (Alsheimer et al., 1998).

Here we report on the developmentally regulated expression of XLAP2, an amphibian homologue to the mammalian LAP2β. In addition, we describe the identification of one additional member of the Xenopus LAP2/TP protein family that is expressed during oogenesis and early embryogenesis but not in somatic cells of adult animals.

MATERIALS AND METHODS

Cells, tissues, and embryos

The following cell lines were used for protein analysis and immunofluorescence microscopy: HeLa (human epithelial carcinoma cells), and A6 (kidney epithelial cells of Xenopus laevis). Cell lines were cultured according to standard procedures. Small pieces of Xenopus liver and spleen were directly homogenized and boiled in lysis buffer for analysis by SDS-PAGE.

To obtain stages of early Xenopus embryos, eggs were in vitro fertilized in 5% MMR (100% MMR: 100 mM NaCl, 2 mM KCl, 1 mM MgSO4, 0.1 mM EDTA, 5 mM Hepes (pH 7.8) as described (Wolf and Hedrick, 1971; Benavente et al., 1985) and cultured at 22-24°C in 5% MMR. Embryonic stages were classified according to the method of Nieuwkoop and Faber (1994). To arrest embryonic cells in interphase, embryos were transferred into 5% MMR containing 0.4 mg/ml cycloheximide for 1 hour. Embryos were then dejellied with 1.5% cysteine (pH 7.8), washed 4 times in 5% MMR, and either immediately processed for immunofluorescence microscopy (squash preparations; see below) or shock frozen after complete removal of the MMR, and stored at −70°C for biochemistry.

Antibodies and antibody purification

The monoclonal antibodies (mAb) 49H2 and X155 which recognize B-type lamins Lh as well as Lh of Xenopus laevis (Lourim and Krohne, 1993), mAb X94 specific for Xenopus lamin A (Höger et al., 1991), and the human antisera designated MAN (Paulin-Levasseur et al., 1996) have been previously described. LAP2/TP antibodies contained in the MAN-serum were affinity purified from immunoblots as described (Harlowe and Lane, 1988; Cordes et al., 1991). As antigens we used the bacterially expressed full-length rat LAP2β, a protein comprising the amino-terminal 187 amino acids of the rat LAP2 (Alsheimer et al., 1998), and XLAP2 of Xenopus A6 cells.

Microscopy procedures

Xenopus A6 cells grown on coverslips were fixed for 10 minutes in methanol at −20°C followed by a fixation for 2 minutes with acetone at −20°C, and then air dried. Staged embryos were squashed between glass slides, and coverslips and rapidly frozen by application to a metal block cooled to −70°C. Coverslips were removed from the slides while frozen, the specimens were air dried for 30-60 minutes, fixed with acetone at −20°C for 5-10 minutes, and then air dried. All specimens were then incubated with the MAN-serum at a dilution of 1:5000 in phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 1.5 mM KH2PO4, 7 mM NaHPO4, pH 7.4) containing 0.3% bovine serum albumine (PBS/BSA) or with affinity purified LAP2/TP antibodies. Bound primary antibodies were visualized with secondary antibodies conjugated with Texas Red (Dianova, Hamburg, Germany) diluted 1:200 in PBS/BSA. For visualization of chromatin, preparations were stained with Hoechst 33258 (2.5 μg/ml) simultaneously with the secondary antibodies. Photographs were taken with a microscope (Axioskop; Zeiss, Jena, Germany) equipped with epifluorescence optics and appropriate filter sets.

Cell fractionations

All buffers and procedures were at 4°C unless otherwise indicated.

Subfractionation of Xenopus oocytes and preparation of egg membranes

Oocyte nuclei were manually isolated in nuclear isolation buffer (NI: 83 mM KCl, 17 mM NaCl, 10 mM Tris-HCl, pH 7.2, 0.1 μg/ml trypsin inhibitor, 0.2 mM phenylmethylsulfonyl fluoride) as described (Krohne and Franke, 1983). Nuclei were manually subfractionated in nuclear envelopes and nuclear contents in NI containing 10 mM MgCl2 (Krohne and Franke, 1983).

Cytoplasmic pellets of oocytes were prepared as follows. Single oocytes were first manually defolliculated in NI with forceps and then enucleated. The cytoplasm of 5-10 oocytes was transferred into a separate 1.5 ml Eppendorf tube (Eppendorfgerätebau, Hamburg, Germany), homogenized by pipetting, and then the homogenate was centrifuged (13,000 g, 10 minutes). The supernatant was removed, the upper layer of the pellet, containing most of the cellular membranes and organelles (Cordes et al., 1995), was resuspended in NI, transferred into a new tube and centrifuged again (see above). The supernatant was removed and the pellet was directly solubilized in lysis buffer for SDS-PAGE. Samples containing total oocyte proteins were prepared by the direct homogenization and boiling of one defolliculated stage V oocyte (Dumont, 1972) in 30 μl lysis buffer for SDS-PAGE.

Xenopus egg membranes (P200) were prepared essentially as previously described (Lourim and Krohne, 1993), except that for the generation of the 200,000 g pellet (P200) and the corresponding supernatant (S200) a low speed egg extract (containing 10 μM GTPγS) was used as starting material instead of the 100,000 g supernatant.

Extractions with Triton, NaCl, and urea

P200

For extraction with high salt solutions, P200 was mixed with an equal volume of lysis dilution buffer (80 mM β‐glycerophosphate, 20 mM EGTA, 15 mM MgCl2, 10 μM GTPγS) containing 2.0 M KCl. For sequential extraction with Triton and high salt buffer, P200 was mixed with an equal volume of lysis dilution buffer (80 mM β‐
glycerophosphate, 20 mM EGTA, 15 mM MgCl₂, 10 μM GTPγS) containing 2% Triton X-100. Samples were incubated for 15 minutes, and fractionated by centrifugation (200,000 g, 60 minutes) in a Beckman TL-100 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA, USA) into pellet and supernatant. The pellet was resuspended in high salt buffer (250 mM NaCl, 10 mM Tris-HCl, pH 7.4), incubated and pelleted again. Proteins of the two supernatants were combined. For urea extractions, P₃₀₀ was brought to final concentrations of 2.0, 4.0, 6.0 or 8.0 M urea using a stock solution of 10 M urea in lysis dilution buffer incubated and fractionated as described above. The pellets were washed once with lysis dilution buffer. Proteins in the supernatants were concentrated by precipitation with chloroform/methanol (Schmidt et al., 1994).

**Xenopus A6 cells**
Approximately 2×10⁷ cells were harvested, washed once with PBS, then resuspended in 3 ml of 8 M urea (20 mM sodium phosphate, pH 7.4, 1 mM DTT) and incubated for 10 minutes. Samples were then fractionated by centrifugation (100,000 g, 60 minutes) into pellet and supernatant. The pellet was washed once with PBS and the proteins of the supernatant were precipitated with chloroform/methanol (Schmidt et al., 1994).

**For the sequential extraction with detergent and high salt solutions, 2×10⁷** cells washed with PBS were resuspended in Triton-buffer (1% Triton X-100, 4 mM EDTA, 1 mM PMSF, 0.1 mg/ml trypsin inhibitor, 10 mM Tris-HCl, pH 7.4), incubated for 10 minutes, and then centrifuged (3000 g, 10 minutes). The supernatant was stored at −20°C. The pellet was resuspended in 1 ml of DNase buffer (4 mM MgCl₂, 4 mM EDTA, 1 mM PMSF, 0.1 mg/ml trypsin inhibitor, 10 mM Tris-HCl, pH 7.4, 500 U of DNase I; Boehringer, Mannheim, Germany), and incubated for 30 minutes at 22-24°C. The sample was then cooled to 4°C, and 1 ml of high salt buffer (500 mM NaCl, 10 mM Tris HCl, pH 7.4) was added. After an incubation for 30 minutes the extract was fractionated by centrifugation (3000 g, 10 minutes). The pellet was washed once with PBS, the supernatants were combined, and proteins of the supernatant precipitated with methanol/chloroform (Schmidt et al., 1994).

**SDS-PAGE and immunoblotting**
Proteins were separated by SDS-PAGE (11% or 12% polyacrylamide; Laemmli, 1970) and transferred to nitrocellulose filters (Schleicher and Schüll, Dassel, Germany) using the semidry method of Kyhse-Andersen (1984). Ascites fluid containing monoclonal antibodies S49, X155, or X94 was diluted 1:1000 with TBST buffer (140 mM NaCl, 0.3% Tween, 10 mM Tris-HCl, pH 8.0) containing 5% nonfat dry milk. Blocking of the filters, dilution of antibodies against *Xenopus* lamins, incubation with antibodies and washing of the filters were performed as described (Schmidt et al., 1994). The MAN-serum was diluted 1:10,000 in TBST buffer, and affinity purified LAP2/TP antibodies were diluted 1:5 to 1:50 in TBST buffer. Bound secondary antibodies coupled to peroxidase (Dianova) were detected using an enhanced chemiluminescence system (Amersham Buchler, Braunschweig/Germany) by exposure to X-ray films (X-OMAT AR; Eastman Kodak Co., Rochester, NY/USA).

Antibodies of the MAN-serum bound to nitrocellulose filters were removed by washing the filters for 10 minutes with TBST, and then two times for 30 minutes with glycine/NaCl (200 mM glycine, 200 mM NaCl, pH 2.5). Filters were subsequently washed for 10 minutes with TBST, reblocked with TBST containing 5% nonfat dry milk, and then incubated either with antibody X155 or with antibody X94.

To detect metabolically labelled proteins after separation by SDS-PAGE, Coomassie blue stained gels were incubated in Amplify (Amersham, Braunschweig, Germany) as recommended by the supplier and exposed to X-ray films. Alternatively proteins were transferred to nitrocellulose, and the dried filters were directly exposed to X-ray films.

**cDNA isolation and characterization**
A *Xenopus* oocyte/ovary Uni-ZAP cDNA library (Stratagene, Heidelberg, Germany) was screened by standard methods (Sambrook et al., 1989) using uncharged nylon filters (Qiagen, Hilden, Germany). A double-stranded cDNA fragment comprising nucleotides 1-956 of the rat LAP2β (Alsheimer et al., 1998) were 32P-labeled using the random priming DNA labelling kit ver. 1.1 (MBI Fermentas, St Leon-Roth, Germany) according to the manufacturer’s instructions. Hybridization was performed at 40°C in 5× SSC (20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 5× Denhardt’s solution (50× Denhardt’s: 1 g Ficoll, 1 g polyvinylpyrrolidone, 1 g bovine serum albumin/100 ml H₂O₂), 1% SDS, 35% formamide, and 10 mg/ml herring sperm DNA. Filters were washed at 40°C in 4× SSC and 0.1% SDS and exposed to X-ray film (Kodak X-Omat AR) with an intensifying screen at −70°C. Positive plaques were isolated through three successive rounds of screening, and the cDNA inserts were subcloned by in vivo excision according to the instructions of Stratagene. Both strands of cDNA inserts were sequenced using the Taq DyeDeoxy Terminator cycle sequencing kit (DNA labelling kit, MBI Fermentas) and the Applied Biosystems automated sequencer (model ABI PRISM™310; Perkin Elmer, Foster City, CA, USA).

**Xenopus A6 cells**

In vitro translation and immunoprecipitations
The cDNAs coding for XLAP2 mRNAs were used for hybridization. The hybridization was performed for 16 hours at 65°C in 5× SSC containing 50% (v/v) deionized formamide (ApliChem, Darmstadt, Germany), 1% SDS, 5× Denhardt’s reagent, and 100 mg/ml yeast tRNA. Washed nylon membranes were exposed to X-ray film (X-Omat AR; Eastman Kodak Co., Rochester, NY) with an intensifying screen at −70°C.

**Expression of rat LAP2β in the pQE expression system**
The cDNA clone rpl.8 and a subclone coding for the amino-terminal 187 amino acids of rpl.8 had been sub cloned in the pQE31 expression vector (Quiagen, Hilden, Germany) and expressed in bacteria as described (Alsheimer et al., 1998).

**In vitro translation and immunoprecipitations**
The cDNAs coding for XLAP2 (this manuscript) and the *Xenopus* nuclear membrane protein p58 (A. Gajewski and G. Krohne, unpublished data) contained in the pBlueScript SK vector were in vitro synthesized by coupled transcription and translation with the TNT system (Promega, Madison, WI, USA) as recommended by the supplier. For the radioactive labelling of the proteins during synthesis 1 μg of plasmid DNA and 40 μCi [35S]methionine (Amersham) were used per experiment. The in vitro translated proteins were immunoprecipitated with antibodies coupled to Protein A-Sepharose (Sigma, Munich, Germany) as follows. Antibodies of the MAN-serum were bound to the Protein A-
Sepharose according to standard methods (Harlowe and Lane, 1988). As a last step the Protein A-Sepharose was washed once with 1× immunoprecipitation buffer (0.1% SDS, 0.5% Na-deoxycholate, 1% NP-40, 20 mM methionine, 50 mM Tris- HCl, 150 mM NaCl, 2 mM EDTA, pH 7.4). One volume of in vitro translation assay (25 μl) were mixed with an equal volume of 2× immunoprecipitation buffer (0.2% SDS, 1% Na-deoxycholate, 2% NP-40, 40 mM methionine, 100 mM Tris-HCl, 300 mM NaCl, 4 mM EDTA, pH 7.4), incubated for 1.5 hours at 4°C with the MAN-antibodies coupled to Protein A-Sepharose, then the Sepharose was pelleted (500 g, 5 minutes). Non-bound proteins remaining in the supernatant were concentrated by precipitation with methanol/chloroform (Schmidt et al., 1994). The Sepharose was then washed three times with 1× immunoprecipitation buffer. The Sepharose pellet was boiled in sample buffer for SDS-PAGE.

RESULTS
Characterization of X LAP2
We screened a Xenopus ovary cDNA expression library with a cDNA fragment of the rat LAP2β and isolated positive clones. The DNA sequencing revealed that two of the clones contained a long open reading frame with an ATG close to the 5’-end of the cDNA. The cDNA is predicted to encode a protein of 518 amino acids with a calculated molecular mass of 58,141 and a pI of 8.95. Comparison of the deduced amino acid sequence of the Xenopus protein with that of rat LAP2β revealed that we had isolated a Xenopus homologue (X LAP2) to the mammalian LAP2β (Fig. 1A). X LAP2 exhibited the highest degree of identity with rat LAP2β in regions (Fig. 1B) that have been shown to mediate chromatin and chromosome binding (Furukawa et al., 1997, 1998), and to interact with the lamina and lamins B1 and B2 (Yang et al., 1997; Furukawa et al., 1998; Furukawa and Kondo, 1998), as well as in the membrane spanning domain. As a major difference, X LAP2 was 66 amino acids longer than the rat protein primarily due to two insertions in the nucleoplasmic domain of the protein (Fig. 1B, marked in black). The apparent discrepancy between the calculated molecular mass of X LAP2 (58,141) and its mobility of Mr 68,000 in SDS-PAGE (Fig. 2B, lane 1) most probably results from the high amount of basic amino acids.

Fig. 1. (A) Amino acid sequence comparison (single letter code) of the Xenopus laevis LAP2 (X) and LAP2β of Rattus norvegicus (R). The membrane spanning domain is underlined in the rat sequence. Bold printed letters represent amino acids identical in both proteins. Proteins contain 518 (X) and 452 (R) amino acids. Gaps (marked by dots) have been introduced in some areas of the sequences to allow their optimal alignment. The X LAP2 sequence has been published under accession no Y17861 in the EMBL Nucleotide Sequence Database. (B) Schematic drawings of rat LAP2β and Xenopus LAP2. Domains of X LAP2 that show a high degree of identity with the chromatin/chromosome binding domain, the lamina/lamin B1/B2 binding domain, and the transmembrane domain (TM) of the rat LAP2β have been marked with the same patterns. The degree of identity between both molecules in these regions is given as a percentage (%) below X LAP2. Two X LAP2 domains not contained in the rat protein are marked in black. The positions of individual amino acids has been marked on top of each molecule.

752  C. Lang and others
To facilitate the biochemical and immunocytochemical investigation of X LAP2, we used a human serum designated MAN that reacts in mammalian cells with three major polypeptides of \( M_r \) 78,000, 58,000, and 40,000 that are localized in the nuclear lamina but are distinct in their behavior from lamins (MAN-antibodies; Paulin-Levasseur et al., 1996). These three mammalian polypeptides were in their electrophoretic mobilities in SDS-PAGE very similar to the LAP2/TP proteins (Alsheimer et al., 1998). In addition these human antibodies recognize proteins localized in the nuclear envelope of other non-mammalian vertebrates. We tested these antibodies on protein blots containing the bacterially expressed full-length rat LAP2β protein (Fig. 2A, lane 1) and a truncated protein comprising the amino-terminal 187 amino acids of this LAP2, a sequence that is present in the mammalian LAP2/TPα, β, and γ (Fig. 2A, lane 2). It is obvious that antibodies of the MAN-serum specifically react with the full-length LAP2β as well as with epitopes in the amino-terminal 187 amino acids of mammalian LAP2/TP proteins.

When the X LAP2 cDNA was in vitro transcribed and translated (Fig. 2B, lane 1), the MAN-antibodies specifically immunoprecipitated the major in vitro translation product of \( M_r \) 68,000 (Fig. 2B, lane 3) but not the smaller polypeptide of \( M_r \) 42,000 that was recovered as a non-bound polypeptide in the supernant (compare lanes 1, 3 and 5 in Fig. 2B). The \( M_r \) 68,000 polypeptide most likely results from the internal start of translation at a triplet on the X LAP2 mRNA coding for methionine 190 (see Fig. 1). The MAN-antibodies did not immunoprecipitate an unrelated Xenopus nuclear membrane protein, p58, that had also been synthesized by in vitro translation (Fig. 2B, lanes 2, 4). These experiments demonstrate the specificity of these human antibodies.

Because we could not exclude the possibility that the MAN-serum contains antibodies against other nuclear proteins beside LAP2/TPα, β, and γ, we affinity purified antibodies of the MAN-serum binding to the bacterially expressed full-length rat LAP2β or the amino-terminal 187 amino acids of the rat LAP2β (see Fig. 2A). When affinity purified LAP2/TP antibodies were used for immunoblotting on total cellular proteins of human tissue culture cells (HeLa cells), we detected with both sets of affinity purified antibodies three major polypeptides of \( M_r \) 78,000, 58,000, and 40,000 that corresponded in their mobility on SDS-PAGE to LAP2/TPα, β, and γ (Fig. 3A, lane 1). In contrast, only one major polypeptide was detected by these affinity purified antibodies on immunoblots of somatic cells from Xenopus (Fig. 3A, lanes 2-5). The detected polypeptide was clearly larger than the mammalian LAP2β (\( M_r \) 68,000 versus \( M_r \) 58,000 in our gel system; see Fig. 3A, lanes 1, 2) but had the same electrophoretic mobility as the in vitro translated X LAP2 (Fig. 3B, lanes 1, 2). As representative somatic tissues of Xenopus we analyzed cultured kidney epithelial cells (A6 cells; Fig. 3A, lane 2), ovary follicle cells (Fig. 3A, lane 3; Fig. 3B, lane 2), spleen (Fig. 3A, lane 4), and liver (Fig. 3A, lane 5). Identical results were obtained when the whole MAN-serum was used for immunoblotting of these fractions (Fig. 3B, lane 2; data not shown for other somatic cells). By immunoblotting we did not detect LAP2/TP proteins comparable in size with the mammalian LAP2/TPα and γ. Our findings strongly indicate that we have cloned with X LAP2 the major LAP2/TP of amphibian somatic cells.

Immunofluorescence microscopy (Fig. 3C) on Xenopus somatic cells with affinity purified LAP2/TP antibodies of the MAN-serum (see above) revealed that they reacted with the nuclear periphery of Xenopus somatic cells resulting in a rimlike staining characteristic for lamins and inner nuclear membrane proteins (Fig. 3C). Identical results were obtained when the whole MAN-serum or MAN-antibodies purified on the immunoreactive \( M_r \) 68,000 polypeptide of Xenopus somatic cells were used for the staining of cells (data not shown).

Extractions performed with Xenopus A6 cells (Fig. 4A) demonstrated that X LAP2 is as expected resistant to the sequential extraction with Triton X-100 and buffers containing 0.5 M NaCl (see Fig. 6B), and 8 M urea (Fig. 4A, lanes 5, 6) whereas B-type lamins could be easily solubilized with urea (Fig. 4A′, lanes 5, 6). The X LAP2 and lamins recovered in the supernatant of cells extracted with 1% Triton and 0.5 M NaCl (Fig. 4A,A′, lanes 4) derived most probably from mitotic cells. These data demonstrate that the somatic lamina-associated protein X LAP2 possess properties characteristic for an integral membrane protein.

**X LAP2 is not detectable in Xenopus oocytes and eggs**

Because we were using in Xenopus oocytes, eggs and egg extracts for the functional analysis of nuclear envelope proteins (for review see Lourim and Krohne, 1994), we were interested in the LAP2/TP proteins expressed in this cell type. A
membranes with 8 M urea we could not detect XLAP2. Even when we analyzed the pellet after extraction of XLAP2 of A6 cells (Fig. 4B, lanes 2, 3) and other somatic cells. By immunoblotting the major polypeptide detected by immunoblotting with the whole MAN-serum (Fig. 4B) we did not detect in membrane enriched fractions of oocytes. By immunoblotting with the DNA dye Hoechst. Bar, 10 μm.

characteristic feature of mammalian LAP2β, γ, and XLAP2 is that these proteins cofractionate with membranes in interphase and mitosis. Therefore we used for our analysis purified membranes derived from unfertilized eggs (P200 fraction) and membrane enriched fractions of oocytes. By immunoblotting with the whole MAN-serum (Fig. 4B) we did not detect in purified egg membranes a polypeptide comparable in size to the XLAP2 of A6 cells (Fig. 4B, lanes 2, 3) and other somatic cells. Even when we analyzed the pellet after extraction of P200-membranes with 8 M urea we could not detect XLAP2. In manually subfractionated oocytes XLAP2 was not detectable in the cytoplasmic pellet containing large membraneous structures like annulate lamellae (Fig. 5A, lane 2), in nuclei (Fig. 5A, lane 3), nor in nuclear envelopes (Fig. 5B, lane 1). These experiments demonstrate that XLAP2 is absent from eggs and oocytes (see above).

A LAP2 protein distinct from XLAP2 is expressed in Xenopus oocytes and eggs

Instead of a protein of Mr 68,000 (XLAP2) LAP2-specific antibodies of the MAN-serum reacted in oocytes (Fig. 6A, lane 2) and on egg membranes (Fig. 6B, lane 3) with a polypeptide of Mr 84,000, and as expected with mammalian LAP2 (Fig. 6B, lane 1) and XLAP2 of somatic cells (Fig. 6A, lane 1; Fig. 6B, lane 2). A Mr 35,000 polypeptide detected in addition to the Mr 84,000 by the MAN-serum (Figs 4B,C, 5) did not react with affinity purified LAP2 antibodies even when concentrated egg fractions were analyzed highly enriched for this protein (Fig. 6B, lane 3).

Extraction experiments with P200-membranes of eggs revealed that the Mr 84,000 possessed properties characteristic for integral membrane proteins (Figs 4B,C). It was resistant to the treatment with 1 M KCl (Fig. 4C, lanes 2, 3), 2-4 M urea (Fig. 4C, lanes 4-7) whereas larger proportions were recovered in the supernatant after extraction with 6 (Fig. 4C, lanes 8, 9) and 8 M urea (Fig. 4B, lanes 6, 7). We observed an identical behavior for the integral inner nuclear membrane protein p58/lamin B receptor of Xenopus when membranes of the P200 fraction were extracted with 6 and 8 M urea (A. Gajewski and G. Krohne, unpublished data) suggesting that during the extraction of meiotic egg vesicles a significant proportion of very small membrane fragments was generated that was no longer pelletable. The Mr 84,000 could be completely solubilized with Triton X-100/high salt buffer (Fig. 4B, lanes 4, 5). In oocytes the Mr 84,000 polypeptides was found in all fractions containing cytoplasmic or nuclear membranes (Fig. 5). The Mr 35,000 polypeptide was in its biochemical properties indistinguishable from the Mr 84,000 LAP protein (Figs 4B,C, 5). By immunofluorescence microscopy on cryostat sections through Xenopus ovary with the whole MAN-serum, we observed a staining of the nuclear envelope of oocytes, follicle cells, and a diffuse staining of the cytoplasm but no reaction with annulate lamellae (data not shown).

Our experiments with affinity purified antibodies indicate that beside XLAP2 at least a second LAP2 protein (Mr 84,000) is expressed in Xenopus. If this interpretation is correct then we should detect by northern blot hybridization with the XLAP2 cDNA a different pattern of mRNA bands in eggs and early embryos than in somatic cells. In A6 cells (Fig. 7) we detected only one mRNA band of ca. 1.8-2.0 kb that corresponded in its size to the cloned XLAP2 cDNA of 1799 base pairs. A mRNA band of similar size was present in embryos of the neurula stage but was barely detectable in eggs and early embryos than in somatic cells. In A6 cells (Fig. 7) we detected only one mRNA band of ca. 1.8-2.0 kb that corresponded in its size to the cloned XLAP2 cDNA of 1799 base pairs. A mRNA band of similar size was present in embryos of the neurula stage but was barely detectable in earlier developmental stages. In unfertilized eggs, gastrulae, and neurulae the XLAP2 probe hybridized with two mRNA bands of 2.8-3.0 kb and 1.1-0.9 kb that were absent from somatic cells. Taking together the northern blot confirmed and extended our results obtained with LAP2-specific antibodies in Xenopus indicating that the XLAP2 probe hybridized to three different mRNAs. The mRNA of 1.8-2.0 kb codes for XLAP2, one of the other two mRNAs is large enough that it can code for the Mr 84,000 (2.8-3.0 kb band)

![Fig. 3.](image-url)

(A) Total cellular proteins of HeLa cells (HeLa, lane 1), Xenopus cultured A6 cells (A6, lane 2), and Xenopus tissues (lane 3, follicle cells [Fol]; lane 4, spleen; lane 5, liver) after separation by SDS-PAGE (12% acrylamide) and immunoblotting with affinity purified antibodies of the MAN-serum specific for LAP2/TP proteins. Antibodies had been affinity purified on the 187 amino-terminal amino acids of the rat LAP2β. (B) Comparison of the electrophoretic mobility of in vitro translated XLAP2 (lane 1) with the major polypeptide detected by immunoblotting with the whole MAN-serum in Xenopus follicle cells (lane 2). The in vitro translation products were synthesized by coupled in vitro transcription and translation of the cDNA coding for XLAP2. Polypeptides of the two samples shown in B were separated in neighbouring lanes in the same SDS-PAGE, and transferred to nitrocellulose. The nitrocellulose strip containing lane 1 was directly processed for fluorography, whereas the second strip (lane 2) was incubated with the whole MAN-serum. Molecular masses of reference proteins (in kDa) are marked in A and B. (C,C') Indirect immunofluorescence microscopy (C) on Xenopus A6 cells with affinity purified LAP2/TP antibodies of the MAN-serum. Antibodies had been affinity purified on the full-length rat LAP2. (C') Staining with the DNA dye Hoechst. Bar, 10 μm.
and the third mRNA could code for a protein of not more than 40 kDa.

**Expression of XLAP2 during development**

We have demonstrated that XLAP2 is expressed in somatic cells but not in oocytes and unfertilized eggs. To determine the onset of the XLAP2 expression during development and the fate of the $M_r$ 84,000 and $M_r$ 35,000 polypeptides we analyzed total proteins of 8 different developmental stages by immunoblotting with the MAN-serum (Fig. 8A). The results shown in Fig. 8A clearly demonstrate that a polypeptide with the electrophoretic mobility of XLAP2 is first detectable with the MAN-antibodies in the initial gastrula (stage 10) and that the intensity of this blotting signal is constantly increasing with the age of the embryos (Fig. 8A, lanes 3-8). The drastic increase of the XLAP2 protein in neurula embryos relative to embryos in the gastrula stage is paralleled by the increase of the XLAP2 mRNA in the neurula stage (see Fig. 7, lanes 2, 3). As an internal control we compared the expression of lamin A in these embryos and could demonstrate that the *Xenopus* A-type lamin is, as expected (Wolin et al., 1987), first synthesized in the swimming tadpole of stage 40 (Fig. 8A').

In contrast to the XLAP2 the amount of the $M_r$ 84,000 polypeptide apparently decreases during development and is no longer detectable in swimming tadpoles of stage 40 (Fig. 8A, lane 7). When the X-ray film was exposed 10 times longer to the nitrocellulose than shown in Fig. 8A very faint amounts of the $M_r$ 84,000 were also detected in embryos of stage 40 and 44 (data not shown). Concomitant with the decrease of the $M_r$ 84,000 polypeptide we have seen an additional immunoreactive polypeptide with an electrophoretic mobility intermediate between the $M_r$ 84,000 and $M_r$ 68,000 (XLAP2) polypeptides (Fig. 8, lanes 3-5). We suggest that this polypeptide might represent a natural degradation product of the $M_r$ 84,000 polypeptide. Our inability to detect the $M_r$ 35,000 polypeptide in whole cell extracts of embryos indicate that its abundance does not increase significantly during early embryonic development relative to the situation in the oocyte.

When squashed embryos of early developmental stages that do not express XLAP2 were processed for immunofluorescence with the MAN-serum we observed that the nuclei of embryos as early as the 2-cell stage are brightly

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**Fig. 4.** Extraction properties of *Xenopus* antigens recognized by the antibodies of the MAN-serum in somatic cells (A,A') and egg membranes (B,C). Proteins were separated by SDS-PAGE and immunoblotted with the whole MAN-serum (A,B,C). Molecular masses of reference proteins (in kDa) are marked (A-C). (A) *Xenopus* A6 cells were sequentially extracted with 1% Triton, DNase, and 250 mM NaCl (lanes 3, 4) or with 8 M urea (lanes 5, 6), and fractionated by centrifugation into supernatants (S) and pellets (P). For comparison total proteins of non-extracted A6 cells (A6, lane 2) and HeLa cells (HeLa, lane 1) were analyzed. (A') The same nitrocellulose shown in A was incubated with lamin antibody X155 after removal of bound MAN-antibodies. Antibody X155 does not react with mammalian lamins. (B) Aliquots of the membrane fraction ($\alpha_{200}$, lane 3; non-extracted membranes) of unfertilized *Xenopus* eggs were sequentially extracted with 1% Triton and 250 mM NaCl (lanes 4, 5) or with 8 M urea (lanes 6, 7), and fractionated by centrifugation into supernatants (S) and pellets (P). For comparison total proteins of A6 cells (A6, lane 2) and HeLa cells (HeLa, lane 1) were analyzed. (C) Aliquots of the membrane fraction ($\alpha_{200}$, lane 1; non-extracted membranes) of unfertilized *Xenopus* eggs ($\alpha_{200}$) were incubated with 1 M KCl (lanes 2, 3), 2 (lanes 4, 5), 4 (lanes 6, 7) or 6 M urea (lanes 8, 9) and fractionated by centrifugation into supernatants (S) and pellets (P). Note that XLAP2 ($M_r$ 68,000) is not detectable in egg membranes (A-C).
Fig. 5. *Xenopus* oocyte polypeptides detected by MAN-antibodies. Proteins were separated by SDS-PAGE (12% acrylamide) and immunoblotted with the whole MAN-serum. Molecular masses of reference proteins (in kDa) are marked. (A) Total proteins of oocyte follicle cells (lane 1), of the oocyte cytoplasmic pellet (lane 2; protein of 5 defolliculated and enucleated oocytes), and manually isolated oocyte nuclei (lane 3; 40 nuclei) after separation by SDS-PAGE. (B) Manually subfractionation of oocyte nuclei into nuclear isolated oocyte nuclei (lane 3; 40 nuclei) after separation by SDS-protein of 5 defolliculated and enucleated oocytes), and manually follicle cells (lane 1), of the oocyte cytoplasmic pellet (lane 2; reference proteins (in kDa) are marked. (A) Total proteins of oocyte immunoblotted with the whole MAN-serum. Molecular masses of proteins were separated by SDS-PAGE (12% acrylamide) and immunoblotting with antibodies of the MAN-serum. Molecular masses of reference proteins (in kDa) are marked. (A) Total cellular proteins of HeLa cells (HeLa, lane 1), and defolliculated oocytes (Oocyte, lane 2) after separation by SDS-PAGE (12% acrylamide) and immunoblotting with antibodies of the MAN-serum. Molecular masses of reference proteins (in kDa) are marked. (A) Total cellular proteins of HeLa cells (HeLa, lane 1), and defolliculated oocytes (Oocyte, lane 2) after separation by SDS-PAGE (12% acrylamide) and immunoblotting with antibodies of the MAN-serum. Molecular masses of reference proteins (in kDa) are marked. (A) Total cellular proteins of HeLa cells (HeLa, lane 1), and defolliculated oocytes (Oocyte, lane 2) after separation by SDS-PAGE (12% acrylamide) and immunoblotting with antibodies of the MAN-serum. Molecular masses of reference proteins (in kDa) are marked. (A) Total cellular proteins of HeLa cells (HeLa, lane 1), and defolliculated oocytes (Oocyte, lane 2) after separation by SDS-PAGE (12% acrylamide) and immunoblotting with antibodies of the MAN-serum. Molecular masses of reference proteins (in kDa) are marked. (A) Total cellular proteins of HeLa cells (HeLa, lane 1), and defolliculated oocytes (Oocyte, lane 2) after separation by SDS-PAGE (12% acrylamide) and immunoblotting with antibodies of the MAN-serum. Molecular masses of reference proteins (in kDa) are marked. (A) Total cellular proteins of HeLa cells (HeLa, lane 1), and defolliculated oocytes (Oocyte, lane 2) after separation by SDS-PAGE (12% acrylamide) and immunoblotting with antibodies of the MAN-serum.

stained indicating that the $M_r$ 84,000/$M_r$ 35,000 polypeptides are located in the nuclear envelope (Fig. 8B).

**DISCUSSION**

We have clearly demonstrated by cDNA cloning in conjunction with LAP2/thymopoietin specific antibodies contained in the human serum designated MAN (Paulin-Levasseur et al., 1996) that the mammalian LAP2β and XLP2 proteins exhibit a high degree of identity and that the expression pattern of members of this protein family is, in the amphibian *Xenopus laevis*, distinct from that of mammals. All tested mammalian somatic cells express as major polypeptides LAP2/TPα, β, and variable amounts of LAP2/TPγ (Harris et al., 1994; Alsheimer et al., 1998). In contrast, somatic cells of *Xenopus* express one major member of the LAP2/TP protein family with an electrophoretic mobility of approximately $M_r$ 68,000 but no LAP2/TP polypeptides comparable in size with mammalian LAP2/TPα and γ.

The sequence comparison of the mammalian LAP2β and the *Xenopus* LAP2 suggests that XLAP2 could possess some of the characteristic binding affinities described for the rat LAP2β. The domain of the rat protein (amino acids 1-85) reported to bind in vitro to metaphase chromosomes (Furukawa et al., 1998) and in vivo to the chromatin of interphase cells (Furukawa et al., 1997) is to 69% identical with the comparable region (amino acids 1-69) of XLAP2. The nucleoplasmic domain of rat LAP2β (amino acids 298-373) essential for the targeting of the LAP2β protein to the nuclear envelope (Furukawa et al., 1995, 1998; Yang et al., 1997), the binding to lamins B1 and B2 (Furukawa et al., 1998; Furukawa and Kondo, 1998), the increase of nuclear volume, and the progression into S-phase (Yang et al., 1997) is also present in the *Xenopus* protein. However, in contrast to the rat protein XLAP2 contains in this domain a long insertion of 68 amino acids. The degree of identity between both proteins in this region is 64% when the 68 amino acid long insertion was not included in the sequence comparison (see Fig. 1B).

The comigration of the in vitro translation product of the XLAP2 cDNA in SDS-PAGE with the $M_r$ 68,000 polypeptide detected by our affinity purified LAP2/TP antibodies in somatic cells of *Xenopus* and northern blot analysis demonstrate that the XLAP2 cloned by us is expressed in somatic cells and embryos from the gastrula/neurula stage on. However, we do not know whether a *Xenopus* LAP2 protein that is 39 amino acids larger (GenBank accession no. af048815) than the XLAP2 described by us is contained in the $M_r$ 68,000 polypeptide band. This larger XLAP2 is at the amino acid level identical with our XLAP2 sequence except for amino acid insertions at three positions comprising one (between amino acids 264 and 265 of our XLAP2), two (between amino acids 339 and 340 of our XLAP2), and 37 (between amino acids 199 and 200 of our XLAP2) amino acids, and the deletion of one amino acid (amino acid 189 of our XLAP2).

Our northern blot analysis in combination with immunoblots performed with affinity purified LAP2 antibodies and the unpublished *Xenopus* LAP2 cDNA (GenBank accession no. af048815) demonstrate that the LAP2 protein family of *Xenopus* contains other members beside the protein sequenced by us. Our data indicate that the three mRNAs hybridizing to the XLAP2 cDNA are coding for three distinct members of the *Xenopus* LAP2 protein family that possess at least one common domain and are developmentally expressed. We have strong evidence that the $M_r$ 84,000 polypeptide expressed in oocytes
and the early embryo is one of these members. First, the detection of a mRNA of 2.8-3.0 kb in eggs and early embryos but not in somatic cells that is large enough to code for a protein of this size favours the idea that this mRNA is coding for the \( M_r 84,000 \) polypeptide. Second, affinity purified LAP2 antibodies reacting with the amino-terminal sequence of 187 amino acids contained in each member of the mammalian protein family (Harris et al., 1994, 1995) also recognize the \( M_r 84,000 \) polypeptide and XLAP2. We can exclude for two reasons that the \( M_r 84,000 \) polypeptide represents a XLAP2 with an unusual mobility in SDS-PAGE due to cell cycle dependent phosphorylations. First, when we analyzed with LAP2 antibodies proteins of a A6 cell culture enriched for mitotic stages we did not detect the \( M_r 84,000 \) polypeptide beside XLAP2 (data not shown). Second, Xenopus oocytes and unfertilized eggs represent interphase and dividing cells respectively but contain only one major immunoreactive polypeptide with identical electrophoretic mobility of \( M_r 84,000 \) and no detectable amounts of XLAP2. These data indicate that the \( M_r 84,000 \) polypeptide is distinct from XLAP2. We can also exclude that the \( M_r 84,000 \) polypeptide is the Xenopus homologue to the mammalian LAP2α because the mammalian protein is not an integral membrane protein (Harris et al., 1994, 1995; Dechat et al., 1998).

The XLAP2 cDNA hybridized also to a small mRNA (0.9-1.1 kb) of oocytes, eggs and early embryos that can code for a protein with a molecular mass of not more than 40 kDa. It is intriguing that the whole MAN-serum reacted with a membrane protein of \( M_r 35,000 \) in oocytes and eggs. However, we have no evidence that the \( M_r 35,000 \) polypeptide contains sequences specific for the Xenopus LAP2 protein family.

We can only speculate on the functions of the \( M_r 84,000 \) and \( M_r 35,000 \) polypeptides during oogenesis and early embryonic development. We suggest that both proteins provide specific functions needed in the oocyte and early embryos that cannot be accounted by XLAP2. In this respect it is interesting to note...
that *Xenopus* oocytes also express a special B-type lamin, lamin L\(\text{III}\), that is absent from the majority of somatic cells (Benavente et al., 1985; Stick, 1988; for review see Krohne and Benavente, 1986). It is possible that these integral membrane proteins are involved during early embryogenesis in the targeting of membranes at the end of mitosis to the chromosome surface as it has been described for lamina-associated proteins (Foisner and Gerace, 1993; Yang et al., 1997; Maion et al., 1997), the lamin B receptor (Pyropospelou et al., 1996; Buendia and Courvalin, 1997), and a cross-reacting nuclear membrane protein of sea urchins (Collas et al., 1996). Presently we cannot exclude the possibility that the \(M_r\) 84,000 LAP2 is expressed in the adult organism in specialized cells of few organs as it has been shown for *Xenopus* lamin L\(\text{II}\) (Benavente et al., 1985).

Our data allow us to conclude that XLAP2 is not expressed in traceable amounts in oocytes, eggs, and early developmental stages up to the gastrula and that it is not synthesized from maternally stored mRNA. In contrast to XLAP2 it has been shown for the B-type lamin L\(\text{I}\) that it is translated from stored maternal mRNA first during the maturation of the egg (Lourim et al., 1996) and later during the midblastula transition (Stick and Hansen, 1985; Benavente et al., 1985). A second example is the *Xenopus* lamin A which is absent from oocytes and first detectable in the swimming tadpole (Wolin et al., 1987; see also Fig. 8A). With our study on XLAP2 we have presented the first example for the developmentally regulated expression of an integral nuclear membrane protein during early embryogenesis.

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