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Functional analysis of LAP2 β, γ and ω during the development of zebrafish (*Danio rerio*)

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RÉSUMÉ

Trois isoformes de la famille 2 des polypeptides associés à la lamina (LAP2) ont été identifiées chez le zebrafish (*Danio rerio*). Les LAP2 de type β, γ et ω du zebrafish possèdent une région aminoterminale identique incluant le domaine LEM. La région carboxyterminale de ces isoformes contient également un domaine de liaison à la lamina ainsi qu'une séquence transmembranaire. La plus grande des trois isoformes, ZLAP2ω, est synthétisée de façon maternelle et est détectée comme un polypeptide ayant une mobilité relative de 84 000. Les isoformes somatiques, ZLAP2β et γ, sont de plus petits polypeptides ayant une mobilité relative de 66 000 et de 45 000. Les analyses par Western et Northern blot ont indiqué que ZLAP2ω est la seule LAP2 présente durant les premiers stades embryonnaires jusqu'au stade de la gastrulation. En progressant au cours du développement embryonnaire, la quantité de ZLAP2ω diminue tandis que la quantité d'isoformes somatiques (ZLAP2β et γ) augmente. En utilisant la technique de microscopie par immunofluorescence sur des cellules somatiques, ZLAP2β et γ sont observés à la périphérie du noyau durant l'interphase. À la fin de la prométaphase, on note une redistribution cytoplasmique de ces isoformes. Elles se retrouvent à la surface des chromosomes ségrégés à la fin de l'anaphase. Cependant, au cours du développement embryonnaire, ZLAP2ω est associée avec les chromosomes mitotiques avant le stade de l'anaphase et la formation de karyomères est également observée. En utilisant la technologie des morpholinos, des expériences de "knock-down" effectuées

pour inhiber la synthèse des ZLAP2 au cours du développement suggèrent une forte corrélation entre la perte d'expression des ZLAP2 et l'apparition de phénotypes anormaux observables 24 heures après la fertilisation. En somme, mes résultats indiquent que les protéines ZLAP2 jouent probablement un rôle clé au cours des premiers stades du développement.

ABSTRACT

Three isoforms of the lamina-associated polypeptide 2 (LAP2) family, have been identified in zebrafish (*Danio rerio)*. Zebrafish LAP2 (ZLAP2) β, γ and ω have identical aminoterminal regions containing the LEM domain. A lamina binding domain and a membrane spanning sequence are also present in the carboxyterminal region. The maternally synthesized ZLAP2ω is the biggest of the three isoforms and is detected as a polypeptide of M_r 84 000. The somatic isoforms, ZLAP2β and γ are represented as smaller polypeptides of M_r 66 000 and 45 000 respectively. Western and northern blot analysis indicated that ZLAP2ω is the only LAP2 present in early embryonic stages up to the gastrula stage. With the progression of embryonic development, the amount of ZLAP2ω decreased whereas the amount of somatic isoforms (ZLAP2β and γ) increased. Using immunofluorescence on somatic cells, ZLAP2β and γ are observed at the nuclear periphery in interphase cells. A cytoplasmic distribution appeared in late prometaphase and the isoforms are located at the surface of segregated chromosomes in late anaphase. However, during early embryonic development, ZLAP2ω is associated with mitotic chromosomes prior to anaphase and the formation of karyomere is also observed. Using the morpholino technology, knock-down experiments on ZLAP2 during the zebrafish development suggest a strong correlation between the loss of ZLAP2 expression and abnormal phenotypes observed 24 hours post-fertilization. Altogether, my data indicate that ZLAP2 proteins may play a key role in early developmental events.

ABREVIATIONS

BAF	Barrier to autointegration factor
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BSA	Bovine serum albumin
CAAX	Cysteine-aliphatic-aliphatic-other
CCD	Charge-coupled device
cDNA	Complementary DNA
CLSM	Confocal laser-scanning microscopy
CMT	Charcot-Marie-Tooth
CY3	Indocarbocyanine
DCM	Dilated cardiomyopathy
DMEM	Dulbecco's-modified Eagle medium
DNA	Desoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate mix
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FPLD	Familial partial lipodystrophy
GFP	Green fluorescent protein
HEPES	N- [2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HM	Hybridization mix
HP1	Human chromodomain protein
hpf	Hours post fertilization
IF	Intermediate filament
kb	Kilobases
kDa	Kilodaltons
LAP 1	Lamina-associated polypeptide 1
LAP 2	Lamina-associated polypeptide 2
LAP2-N	LEM-like motif
LBR	Lamin B receptor
LEM	LAP2, Emerin, MAN1

LGMD	Limb girdle muscular dystrophy
MEOH	Methanol
mGCL	Mouse Germ-cell-less
MO	Morpholino antisense oligonucleotide
M_r	Relative mobility
mRNA	Messenger RNA
NBT	Nitro blue tetrazolium
NCBI	National center for biotechnology information
NE	Nuclear envelope
NLS	Nuclear localization sequence
NPC	Nuclear pore complex
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline Tween
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PMSF	Phenyl methyl sulfonyl fluoride
Rb	Retinoblastoma
RFC	Replication factor complex
RH	Radiation hybrid
RNA	Ribonucleic acid
RT	Room temperature
S1	ZLAP2-serum1
S2	ZLAP2-serum2
SB	Sample buffer
SDS	Sodium dodecyl sulfate
SR	Serine-arginine
SSC	Sodium chloride; Sodium citrate
STD	Sodium tetrathionate dihydrate
STI	Soybean trypsin inhibitor
TCA	Tri-chloro-acetic acid
UV	Ultra-violet
XLAP2	*Xenopus* LAP2
ZFIN	Zebrafish information network
ZLAP2	Zebrafish LAP2

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INTRODUCTION

1. The nuclear envelope

The nuclear envelope (NE) is a complex structure, which separates nucleoplasmic materials from the cytoplasm. The NE of eukaryotic cells is composed of three major domains: the inner and the outer nuclear membranes, the nuclear pore complexes and the nuclear lamina (reviewed by Gerace and Burke, 1988; Gant and Wilson, 1997). The outer nuclear membrane, which is decorated with ribosomes on its outer surface, faces the cytoplasm, is continuous with the endoplasmic reticulum (ER) and is similar or identical to it in composition (Amar-Costesec *et al.*, 1974; Pathak *et al.*, 1986). The inner nuclear membrane is actually a specialized domain of the ER, which contains specific integral membrane proteins that link the membrane to the underlying lamina and the peripheral chromatin. The inner and outer nuclear membranes are separated by the perinuclear space which is continuous with the ER lumen, but join periodically at the pore membrane (Gerace and Foisner, 1994; for review see Georgatos *et al.*, 1994 and Gant and Wilson, 1997). The nuclear pore is a highly specialized membrane domain that is occupied by ~120 000 kDa nuclear pore complex (NPC) of proteins that regulate the signal-mediated import and export across the NE (reviewed by Gant and Wilson, 1997).

A "diffusion-retention" model for the targeting of integral membrane proteins to the inner nuclear membrane was originally proposed by Soullam and Worman (1995). In this model, inner nuclear membrane proteins which are co-translationally inserted into

the ER membrane would diffuse freely to the continuous outer nuclear membrane and would reach the inner nuclear membrane via the nuclear pore membrane and the NPCs. The nucleoplasmic domain of these proteins must pass through the lateral channels of the NPC. The diameter of these channels is approximately 10 nm (Hinshaw *et al.*, 1992), large enough to allow the diffusion of proteins with nucleoplasmic domains less than ~60 kDa. All integral proteins of the inner nuclear membrane have nucleoplasmic domains of less than 60 kDa. Retention in the inner nuclear membrane likely occurs by the binding to nuclear ligands such as lamins and chromatin or via interactions with the transmembrane segment of other resident proteins (reviewed in Worman and Courvalin, 2000). Once immobilized in the inner nuclear membrane, integral proteins cannot freely diffuse back to the ER (Ellenberg *et al.*, 1997; Östlund *et al.*, 1999).

2. The nuclear lamina

The nuclear lamina is an insoluble proteinaceous meshwork located at the interface of the inner nuclear membrane and the chromatin during interphase. Possible functions of the nuclear lamina include providing a structural framework to the nuclear envelope and anchoring sites at the nuclear periphery for the interphase chromatin (McKeon, 1991; Nigg, 1992; Gerace and Burke, 1988; Moir *et al.*, 1991). Nuclear intermediate filament proteins, the lamins, are the major structural elements of the lamina. Nuclear lamins are closely related to the cytoplasmic intermediate filament (IF) protein family (Fisher *et al.*, 1986) and have been classified as type V IFs. In mammals,

lamin proteins are classified into A-type (A/C) and B-type lamins on the basis of their amino acid sequences and biochemical properties. Four lamins, A, B_1, B_2 and C, are found in somatic cells. 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; Gant and Wilson, 1997; Moir *et al.*, 1995). Lamins have been identified in a wide variety of organisms (reviewed in Hutchison, 2002). Characterization of lamins has been done most extensively in mammals, birds and amphibians. However, lamins were also found in different other metazoans such as fish, *Drosophila melanogaster* and *Caenorhabditis elegans* (reviewed in Hutchison, 2002). Invertebrates seem to have fewer lamins than vertebrates and so far no lamins have been found in plant and fungi (reviewed in Hutchison, 2002).

Lamins possess a tripartite structure consisting of a central α-helical rod domain flanked by two globular N-terminal and C-terminal domains (reviewed in Stuurmann *et al.*, 1998). The rod domain of nuclear lamins is 42 amino acids longer than the rod domain of cytoplasmic vertebrate IF proteins. It has been suggested that the lamins occurred early in evolution and that vertebrate cytoplasmic IF proteins evolved from lamins (reviewed in Stuurmann, 1998; Steinert and Roop, 1988). The carboxy-terminal domain of lamins contains a nuclear localization sequence (NLS) for nuclear import (Loewinger and McKeon, 1988). Furthermore, the C-terminal ends of all B-type lamins

and lamin A have a CaaX (cysteine-aliphatic-aliphatic-other) motif, which undergoes a series of post-translational modifications. In a first step, the CaaX motif is isoprenylated by the addition of a farnesyl lipid via a stable thioether linkage to the cysteine residue (Zhang and Casey, 1996). In a second step, a specific protease cleaves the aaX residues from the prenylated proteins and finally, the prenylcysteine is recognized by a specific carboxyl methyltransferase that methylesterifies the carboxyl group. Isoprenylation appears to be required for targeting the lamins to the inner surface of the nuclear envelope (Holtz *et al.*, 1989; Krohne *et al.*, 1989; Weber *et al.*, 1989; Holfemeister *et al.*, 2000). B-type lamins remain isoprenylated throughout their lifetime whereas lamin A is processed further. The C-terminal 18 residues of lamin A are removed by proteolytic cleavage to give the mature lamin A (Sasseville and Raymond, 1995; Weber *et al.*, 1989; Beck *et al.*, 1990). Lamins also possess phosphoacceptor sites flanking both ends of the α-helical rod domain. Specific residues are phosphorylated during mitosis by the mitosis-specific cdc2 kinase. These phosphorylation sites have been shown to play an important role in lamin assembly as well as disassembly (Heald and McKeon, 1990; Moir *et al.*, 1995). The central rod domain of lamins drives the lateral interactions between two lamin protein chains to form parallel homodimers through coiled-coil association. At the next level of structural organization, lamin dimers associate in long chains to form head-to-tail polymers. This kind of association has not been observed during cytoplasmic IF assembly (reviewed in Goldman *et al.*, 2002). In a further step, lamin head-to-tail

polymers associated laterally in an anti-parallel fashion resulting in formation of large paracrystals (Moir *et al.*, 1991). In contrast, most cytoplasmic IF proteins assemble into 10-13 nm filaments and they do not form paracrystals (Quinlan *et al.*, 1995).

In addition to the peripheral lamina, there is evidence that lamins also form nucleoplasmic structures. Internal lamin structures have been reported in G1 cells (Bridger *et al.*, 1993) and in association with DNA replication centres in S phase cells (Moir *et al.*, 1994). It has also been shown that lamin A is part of a diffuse internal skeleton that ramifies throughout the nucleus from the nucleolus to the periphery and forms an extensively branched intra- and transnuclear network (Hozak *et al.*, 1995; Broers *et al.*, 1999). Moreover, a recent study has suggested that lamins are associated with RNA splicing factors in nuclear speckles (Jagatheesan *et al.*, 1999).

Several studies suggest that nuclear lamins are involved in post-mitotic reorganization of the nucleus. All the information available to date indicates that lamins are required for nuclear envelope assembly, but the assembly of a typical lamina is not essential for the formation of nuclear membranes and pores (reviewed in Goldman *et al.*, 2002). During interphase, lamins are polymerized within the nucleus. At this stage of the cell cycle, the nucleus grows very rapidly and it has been suggested that lamina dynamics are required for growth of the NE and progression into S phase (Yang *et al.*, 1997). Moreover, it was shown that the maintenance of a normal nuclear lamin organization is required for the elongation phase of DNA synthesis but not for the

initiation phase. It seems that, in nuclei where the organization of lamins is disrupted, there is a reorganization of the replication factor complex (RFC) and the proliferating cell nuclear antigen (PCNA) and this reorganization is likely to be involved in the inhibition of DNA synthesis (Moir *et al.*, 2000). RCF and PCNA are required cofactors of DNA polymerase δ, the enzyme responsible for DNA synthesis during the elongation phase of replication (Stillman, 1994). Other researchers have suggested another structural role for lamins in supporting nuclear compartments containing proteins involved in RNA splicing (Jagatheesan *et al.*, 1999). However, a recent study has shown that lamin A is redistributed when the nuclear speckles are completely disassembled (Sacco-Bubulya and Spector, 2002). Therefore, the group of Spector has concluded that nuclear speckles are more likely to be maintained by protein-protein interactions of the arginine-serine-rich (SR) protein components of the pre-mRNA-splicing machinery than by attachment to a specific framework. They did not exclude, however, that lamin A may be required as monomers or very short multimers in order to assemble a large number of protein and RNA components into particles (Sacco-Bubulya and Spector, 2002). The nuclear lamina might also help to organize chromatin and therefore could play a role in regulation of gene expression. Lamina proteins have been shown to bind directly to transcriptional regulators. *In vitro* studies demonstrate that lamins A and C bind to retinoblastoma (Rb) protein, which represses the activity of E2F transcription factors by recruiting histone deacetylase (reviewed in Vlcek *et al.*, 2001).

3. Inner nuclear membrane proteins

Recently, several integral membrane proteins have been characterized as permanent residents of the inner nuclear membrane. These proteins include the lamin B receptor (LBR), the lamina-associated polypeptide-1 (LAP1), emerin, MAN1 and the lamina-associated polypeptide-2 (LAP2). The inner nuclear membrane also contains less known proteins such as otefin (p53), nurim and nesprin. Whereas some of these proteins bind to lamins and chromatin (LBR, LAP1, LAP2 and emerin) contributing to the nuclear envelope architecture, the functions and the binding partners of others remain to be explored.

3.1 Inner nuclear membrane proteins in mammalian cells

3.1.1 LBR

The first inner nuclear membrane protein to be identified was lamin B receptor or LBR (Worman *et al.*, 1988; Worman *et al.*, 1990). LBR has a nucleoplasmic amino-terminal domain of approximately 200 amino acids and a hydrophobic C-terminal part that includes eight predicted transmembrane segments (Worman *et al.*, 1988, 1990). The human LBR gene is located on chromosome 1q42.1 and contains 13 exons. The carboxy-terminal domain of LBR is similar in sequence to yeast and plant sterol reductases. Analysis of the exon-intron pattern suggests that the LBR gene may have arise by the recombination between a gene encoding a basic nuclear protein and another gene encoding a sterol reductase (Schuler *et al.*, 1994). Other proteins similar to LBR,

localized in the ER, have been characterized. It was proposed that LBR is part of a multigene family encoding proteins that function in nuclear organization and/or sterol metabolism (Holmer *at al.*, 1998). The N-terminal domain of LBR binds to B-type lamins and chromatin proteins. Those interactions might be responsible for its inner nuclear membrane retention (Soullam and Worman, 1993; Ye and Worman, 1994; Worman *et al.*, 1990; Ye *et al.*, 1997). The B-type lamin binding region of LBR is also the minimal epitope recognized by anti-LBR antibodies from rare patients with primary biliary cirrhosis (Lin *et al.*, 1996). Recent studies have demonstrated that LBR specifically interacts with human chromodomain proteins homologous to HP1, a heterochromatin protein originally identified in *Drosophila melanogaster* (Ye and Worman, 1996; Ye *et al.*, 1997). Thus, it is likely that inner nuclear membrane proteins play a role in the attachment of chromatin to the nuclear envelope, and are part of the nuclear envelope architecture along with lamin proteins.

3.1.2 LAP1

LAP1 is a type II membrane protein that was first identified in rodents and is expressed as three different isoforms: LAP1A, B and C. LAP1A and LAP1B bind to lamins A, C and B_1 (Foisner and Gerace, 1993) while it is suggested that LAP1C needs the presence of lamins A and C in order to accumulate in the nucleus (Powell and Burke, 1990). LAP1C occurs in all cell types, whereas LAP1A and LAP1B are expressed only in well-differentiated cells (Foisner and Gerace, 1993; Martin *et al.*, 1995). LAP1C has a

nucleoplasmic amino-terminal domain followed by one transmembrane segment and a short carboxy-terminal tail in the perinuclear lumen. The other LAP1 isoforms are probably of similar overall structure, arising from the same gene by alternative RNA splicing (Martin *et al.*, 1995). LAP1 gene localizes to the human chromosome 1p36 (reviewed in Holmer and Worman, 2001). Up till recently, only the complete sequence of the rat LAP1C and partially characterized complementary DNAs of other rat and human isoforms were available (Martin *et al.*,1995). Lately, the human coding sequence for LAP1B was cloned (Kondo *et al.*, 2002). The LAP1B sequence corresponds to that of the putative human LAP1C with two additional insertions in-frame. The N-terminal and the transmembrane domain of LAP1B were shown to be necessary and sufficient for its proper localization into the inner nuclear membrane.

3.1.3 Emerin

In 1994, positional cloning of the gene responsible for X-linked Emery-Dreifuss muscular dystrophy led to the identification of emerin that has limited sequence similarity to LAP2 (Bione *et al.*, 1994). Emerin was shown to be localized to the inner nuclear membrane and lacking from this location in most patients with the disease (Manilal *et al.*, 1996; Nagano *et al.*, 1996). In knockout mice for the A-type lamin gene, emerin localized to both the nuclear envelope and ER, suggesting that A-type lamins are implicated into the nuclear localization of emerin (Sullivan *et al.*, 1999). Emerin contains a nucleoplasmic amino-terminal domain followed by a single transmembrane

segment and a short tail in the perinuclear space/endoplasmic reticulum lumen. Several lines of evidence suggest that emerin interacts with A-type and possibly B-type nuclear lamins (Fairley *et al.*, 1999; Sullivan *et al.*, 1999; Clements *et al.*, 2000; Lee *et al.*, 2001). Emerin contains also a conserved sequence of ~40 amino acid residues termed the LEM domain and it was shown that emerin directly binds to BAF (Barrier to Autointegration Factor) via its LEM domain (Lin *et al.*, 2000; Lee *et al.*, 2001). This conserved domain was called LEM because it is shared by a family of proteins including LAP2, emerin and MAN1 (Lin *et al.*, 2000). BAF, a small protein of 10 kDa, was first discovered as a cytoplasmic factor that is acquired by retroviral pre-integration complexes and prevents viral DNA from undergoing suicidal self-integration (Lee and Craigie, 1998). BAF co-localizes with chromosomal DNA during both interphase and mitosis (Furukawa, 1999).

3.1.4 MAN1

The "MAN antigens" are three polypeptides, recognized by the autoantibodies from a patient with a collagen vascular disease and localized to the inner membrane of the nuclear envelope (Paulin-Levasseur *et al.*, 1996). Two of the so-called MAN antigens have been identified as LAP2β (Lang *et al.*, 1999) and MAN1 (Lin *et al.*, 2000). The human MAN1 is an integral membrane protein of 82 kDa that has an amino-terminal domain of approximately 470 amino acids in the nucleoplasm, two transmembrane segments and a C-terminal tail that also faces the nucleoplasm. Protein sequence analysis reveals that MAN1, like emerin, contains the conserved sequence of ~ 40 amino

acids termed the LEM domain (Lin *et al.*, 2000). MAN1 is targeted to the inner nuclear membrane following the "diffusion-retention" model and retention is mediated by its N-terminal domain (Wu *et al.*, 2002). The MAN1 gene is expressed in all tissues tested and is localized to the chromosome 12q14 (Lin *et al.*, 2000). Indirect evidence suggests that MAN1 is associated with the nuclear lamina (Paulin-Levasseur *et al.*, 1996).

3.1.5 LAP2

LAP2 polypeptides, first identified by Foisner and Gerace (1993), are amongst the most studied inner nuclear membrane proteins. LAP2β is the rat homologue to the human protein known as thymopoietin-β (Harris *et al.*, 1994, 1995). Subsequent cloning and sequencing of rat LAP2β cDNAs predicted a protein containing a large nucleoplasmic domain and a single membrane-spanning segment (Furukawa *et al.*, 1995). A single human gene on chromosome 12q22 was shown to encode LAP2/thymopoietins α, β and γ (Harris *et al.*, 1995) and other isoforms by alternative splicing. Additional messenger RNAs highly related to human LAP2 were isolated from mice and it appears that there are six different mammalian LAP2 isoforms (α, β, γ, δ, ε, ζ) (Harris *et al.*, 1994; Berger *et al.*, 1996; for review see Dechat *et al.*, 2000). LAP2α as well as LAP2ζ do not contain a transmembrane segment and are present diffusely throughout the nucleus, while the β, δ, ε and γ isoforms have transmembrane domains and are localized to the inner nuclear membrane (Harris *et al.*, 1994).

The nucleoplasmic LAP2α isoform appears to interact with chromatin (Vlcek *et al.*, 1999) and lamins A/C (Dechat *et al.*, 2000b) and to associate with mitotic chromosomes in *in vitro* nuclear assembly experiments (Vlcek *et al.*, 2002). The nucleoplasmic domain of LAP2β includes a lamina binding region, located between residues 298-373, that binds to lamin B_1/B_2 and shares with all LAP2 isoforms a N-terminal region of 187 amino acids (residues 1-187; LAP2 constant region) which is involved in chromatin binding (Foisner and Gerace, 1993; Furukawa *et al.*, 1997, 1998). Within this constant region reside two structurally homologous globular domains of ~ 40-50 amino acids: the LEM-motif and LAP2-N (LEM-like motif) (Lin *et al.*, 2000; Cai *et al.*, 2001). LAP2 isoforms share the LEM domain with related proteins, emerin and MAN1. The LEM domain of LAP2β and emerin interacts with the DNA binding protein BAF, suggesting that BAF mediates the binding of these two proteins to chromatin (Furukawa, 1999; Cai *et al.*, 2001; Lee *et al.*, 2001; Shumaker *et al.*, 2001). These data also suggest that MAN1 can interact with chromatin via the LEM domain and BAF. However, a newly identified protein named nesprin, that contains an interrupted LEM-like domain, does not bind to BAF (see below, Mislow *et al.*, 2002b). While the LEM domain of LAP2 interacts with BAF (Furukawa, 1999), the analogous "LEM-like" domain (LAP2-N) binds DNA *in vitro* (Cai *et al.*, 2001). Experiments done in *Xenopus* egg extracts have suggested that the LAP2 constant region and BAF are directly or indirectly required for membrane-chromatin attachment and lamina assembly (Gant *et*

al., 1999; Shumaker *et al.*, 2001; Segura-Totten *et al.*, 2002). The MAN serum used in my study recognizes the LEM domain (Paulin-Levasseur and Foisner, personnal communication; unpublished). This means that it can probably detect all members of the LEM family, which include LAP2s, emerin, MAN1 and maybe other unknown proteins.

In mammals, other nuclear proteins are binding to LAP2β such as HA95, a chromatin- and nuclear matrix-associated protein implicated in regulation of NE-chromatin interactions (Martin *et al.*, 2000) and the mouse germ-cell-less (mGCL; Nili *et al.*, 2001). The mGCL protein is the mouse homologue of the *Drosophila* germ-cell-less proteins. Recent studies done on mice have shown that GCL co-localized with LAP2β at the nuclear envelope. It seems that LAP2β alone and together with GCL is capable of reducing the transcriptional activity of the E2F-DP complex (Nili *et al.*, 2001).

3.1.6 Other inner nuclear proteins

Amongst the less known proteins of the inner nuclear membrane, nurim, named for nuclear rim protein, has been recently identified as a nuclear envelope protein in humans. Sequence information suggests that it has a molecular weight of 29 kDa and contains five predicted transmembrane domains. Nurim has been suggested to be amongst the most tightly bound membrane protein on the nuclear envelope but its function is still unknown (Rolls *at al.*, 1999).

Nesprin-1, also known as myne-1, is another novel inner nuclear membrane protein that is expressed in cardiac, skeletal and smooth muscles in mammals (Zhang *et*

al., 2001; Mislow *et al.*, 2002a). Nesprin-1 is a predicted type II transmembrane protein, with a large nucleoplasmic domain containing seven spectrin repeats, a carboxy-terminal membrane spanning domain and an interrupted LEM-like domain (Mislow *et al.*, 2002a). Nesprin-1 was shown to bind directly to lamin A and emerin, but it seems that its LEM-like domain does not bind BAF. It was suggested that the scaffolding of emerin and lamin A mediated by nesprin-1 in muscle may partially account for the tissue specificity of diseases caused by mutations in emerin and lamin A (Mislow *et al.*, 2002b).

3.2 Inner nuclear membrane proteins in non-mammalian cells

The occurrence of inner nuclear membrane proteins in non-mammalian organisms has also been documented in some studies. Otefin is a *Drosophila* nuclear envelope protein with a calculated mass of 45 kDa that probably arises from a single gene. Based on biochemical and microscopic analysis, it has been suggested that otefin is anchored to the inner nuclear membrane by its carboxy-terminal end. Otefin was found at all tested developmental stages of the fruit fly and is probably expressed during oogenesis (Padan *et al.*, 1990).

LBR was found in birds (Worman *et al.*, 1990; Ye and Worman, 1994) and similar proteins were found in plants and yeast (Schuler *et al.*, 1994; Holmer *et al.*, 1998). Moreover, a *Xenopus* homologue to the chicken and mammalian p58/LBR was

characterized recently. It was shown that the *Xenopus* LBR has a different subcellular distribution between oocytes and somatic cells (Gajewski and Krohne, 1999).

Another recent study has demonstrated the presence of the integral membrane proteins, emerin and MAN1, in *Caenorhabditis elegans* (Lee *et al.*, 2000). These proteins designated emr-1 and lem-2 were shown to play a role in the timing of nuclear membrane breakdown in *C. elegans*. In this model organism, the nuclear envelope seems to disassemble very late compared with vertebrates.

No apparent orthologue to LAP2 was detected in *C. elegans* (Lee *et al.*, 2000). However, LAP2β cDNA sequences from *Xenopus laevis* were characterized (Lang *et al.*, 1999; Gant *et al.*, 1999) and LAP2-related poplypeptides were identified in a large number of amphibians and fish (Collas, 1999; Del-Pino *et al.*, 2002). LAP2β- and γ-related proteins are present in somatic cells of fish, frog and salamanders, at varying levels of expression dependent on tissues and species (Del-Pino *et al.*, 2002). In most cases, the LAP2γ-related proteins are predominant in fish somatic cells (except for the rainbow trout), whereas LAP2β-related proteins are the major isoforms in frogs and salamanders (Del-Pino *et al.*, 2002). LAP2α-related proteins were not found in cells of fish and amphibians studied. However, an oocyte-specific LAP2-related protein was identified in oocytes and early embryos of fish and frogs (Lang *et al.*, 1999; Del-Pino *et al.*, 2002). Up to now, such an embryonic LAP2 isoform has not been detected in mammals. It was observed that the transition of LAP2 expression from the oocyte-

specific form to the somatic pattern occurs at gastrulation in many species of fish and frog, independently of the differences in the timing of developmental events (Del-Pino *et al.*, 2002). Since different protein isoforms and expression patterns were observed in different species from fish to mammals, it was suggested that LAP2 proteins may play important functions within cells and could serve as molecular markers in evolutionary studies (Del-Pino *et al.*, 2002). Plants also possess different IMPs, but their roles in nuclear assembly and functions remain to be investigated (reviewed in Holaska *et al.*, 2002).

4. LAP2s as components of an integrated functional structure at the nuclear periphery and the zebrafish as a model system in the functional study of LAP2 proteins

Inner nuclear membrane proteins such as LBR, LAP1, emerin, MAN1 and LAP2 appear to have essential functions in anchoring the nuclear lamina and chromatin to the NE during interphase. It also has been shown that these proteins might play a role during mitotic disassembly and reassembly of the NE (reviewed in Dechat *et al.*, 2000a). Moreover, LAP2β was suggested to play a role in nuclear growth and DNA replication by its interactions with the nuclear lamina (Yang *et al.*, 1997; Gant *at al.*, 1999). However, those studies were performed on cultured cells or cell-free extracts of *Xenopus* eggs and they might not reflect accurately the physiological roles of these proteins *in vivo*. By their interactions with some DNA binding partners such as HP1 for LBR and mGCL for LAP2β, inner nuclear membrane proteins might also be involved in transcriptional

repression (Ye *et al.*, 1997; Nili *et al.*, 2001). Of particular interest, these proteins including LAP2-related polypeptides have been directly linked to pathological processes involved in certain autoimmune diseases (Paulin-Levasseur *et al.*, 1996; Lin *et al.*, 1996) or dysfunctions affecting humans. Most notably, emerin is responsible for the X-linked Emery-Dreifuss muscular dystrophy (Bione *et al.*, 1994; Manilal *et al.*, 1996; Nagano *et al.*, 1996) and A-type lamins have also been implicated in autosomal Emery-Dreifuss muscular dystrophy (Sullivan *et al.*, 1999). Other pathologies such as the limb girdle muscular dystrophy (LGMD), the dilated cardiomyopathy (DCM), the familial partial lipodystrophy (FPLD) and the Charcot-Marie-Tooth (CMT) disorder were also connected to mutations in the A-type lamin gene (reviewed in Hutchison, 2002). Therefore, it has become increasingly evident that inner nuclear membrane proteins and the nuclear lamina constitute an integrated functional structure at the nuclear periphery.

In this thesis, I decided to study the expression patterns and the possible functions of LAP2 proteins during the vertebrate development using the zebrafish, *Danio rerio*, as a model system. This small freshwater fish, a cyprinoid teleost that comes from rivers of India, has a number of valuable features as a model organism for the study of vertebrate development (reviewed by Briggs, 2002). The zebrafish is a common aquarium fish that is found in almost all pet stores around the world. Twenty years ago, George Streisinger recognized many virtues of this experimental system such as its short generation time (2-3 months), the production of large clutches of embryos (100-200 per mating), the easy

mutagenesis and the external fertilization which provides an easy access to all developmental stages (reviewed by Detrich *et al.*, 1999 and Briggs, 2002). The zebrafish is also an attractive model because of its transparency throughout early development which facilitates embryological experiments and rapid morphological screening (reviewed by Detrich *et al.*, 1999 and Briggs, 2002). Furthermore, the sequencing of the zebrafish genome should be completed soon. Sequencing data and additional information about zebrafish genes and mapping are already available through web-based ressources such as the Zebrafish Information Network (ZFIN) and the National Center for Biotechnology Information (NCBI). All this information has already begun to facilitate the isolation and functional analysis of genes required for normal development (reviewed by Briggs, 2002 and Detrich *et al.*, 1999).

Other model systems could have been used also to understand vertebrate development. For example, the mouse is one of the species of choice to study the vertebrate developmental genetics. However, much of the embryogenesis is difficult to follow because it occurs within the mother`s uterus. One of the interesting aspect of the mouse system is the possiblity to create "knock-outs" by homologous recombination, which is not possible in zebrafish (reviewed by Detrich *et al.*, 1999). However, a relatively new "knockdown" technology has been developed in the zebrafish model. This technique is using antisense morpholinos, which have been shown to be effective as specific translational inhibitors in zebrafish embryos (Nasevicius and Ekker, 2000).

Morpholinos are chemically modified oligonucleotides with similar base stacking abilities as natural genetic material, but have a morpholine moiety instead of a ribose (Summerton and Weller, 1997). Antisense morpholinos are designed to bind at the start codon and block the mRNA translation of a specific gene. With the help of this technique, functional analysis can be done effectively *in vivo*.

Another model system, *Xenopus laevis*, has been extensively used previously in developmental studies. However, in my case, the fact that the *Xenopus* embryos are not optically clear compared to the zebrafish was a disavantage in order to observe some developmental abnormalities. Moreover, several molecular and cellular tools to study LAP2 proteins in the zebrafish were available to me at the time that the project was designed. Previous work done in our lab (Dr. Paulin-Levasseur, University of Ottawa) in collaboration with Dr. Krohne (Division of Electron Microscopy, Biocenter of the University of Würzburg, Germany) had led to the isolation and partial characterization of two zebrafish LAP2 cDNAs (ZLAP2) that were homologous to the mammalian LAP2β and LAP2γ. ZLAP2γ is the smallest isoform with 369 amino acids (Fig. 1 and 2), and ZLAP2β is identical to ZLAP2γ but contains an additional insertion of 146 amino acids located right after the common amino-terminal domain (Fig. 1 and 2; amino acids 1-215). The sequences of those two cDNAs had been resolved and a derived oligopeptide from ZLAP2β (amino-terminal region 1-165) was then used to produce two different antisera. The first one, S1, was shown to react with LAP2 proteins of the zebrafish and also to

cross-react with LAP2s from other vertebrates, whereas the second one, S2, was specific to ZLAP2 and did not cross-react with other LAP2 proteins of *Xenopus* and mammals. During the course of my project, a third LAP2 cDNA (ZLAP2ω) was isolated and characterized (Schoft, Beauvais *et al.*, in press). ZLAP2ω is the biggest of the three isoforms and has been created by the insertion of two sequences of 101 and 42 amino acids into the β isoform (Fig. 1 and 2). ZLAP2ω specific sequences share no homology to all known LAP2 isoforms including the mammalian LAP2α. With the help of these molecular and cellular tools combined with the numerous advantages of the zebrafish as a model system, I was able to proceed with the functional analysis of LAP2β, γ and ω during the development of the zebrafish (*Danio rerio*).

5. Objectives

As my project was initiated, only two studies had reported a differential expression of LAP2 proteins during developmental processes. In 1998, a report by Alsheimer and collaborators presented evidence that LAP2 protein isoforms may be differentially regulated during the specific process of rat spermatogenesis. Almost concomitantly, the group of Lang *et al.* (1999) had shown by immunoblotting that only the LAP2β protein isoform (XLAP2β) is detected in somatic cells of *Xenopus* (Lang *et al.*, 1999). They also demonstrated that, while XLAP2β is not expressed in *Xenopus* eggs and embryos prior to gastrulation, an oocyte-specific LAP2-related protein of higher molecular weight is present (Lang *et al.*, 1999). In this previous study, only the *Xenopus*

LAP2β cDNA was characterized and the researchers were able to show that, at the protein level, its expression was developmentally regulated. LAP2α appears to be absent in *Xenopus* (Lang *et al.*, 1999) and it is still unclear whether *Xenopus* expresses LAP2γ. Since there was so little information about the differential expression of LAP2 proteins during developmental processes, I decided to further investigate the process in vertebrates, especially in the zebrafish. The fact that the expression of LAP2 proteins is developmentally regulated implies that it may fulfill a developmental role, which remains to be elucidated. Therefore, in this project, I propose to study several aspects of LAP2 in the zebrafish such as:

-the expression patterns of ZLAP2 proteins during the embryonic development as well as in somatic cell lines and adult tissues;

-the expression patterns of ZLAP2 transcripts in comparison to those of XLAP2 transcripts during embryonic development;

-the partitioning characteristics of ZLAP2 proteins in cultured cells under different extraction/solubilization conditions;

-the distribution of ZLAP2 proteins throughout the cell cycle of somatic and embryonic cells;

-the distribution of ZLAP2 transcripts during the embryonic development;

-the localization of the ZLAP2 gene in the zebrafish genome;

-the effects of inhibiting ZLAP2 protein synthesis on the development of zebrafish

embryos.

In order to reach these objectives, different cellular and molecular techniques such as Western and Northern blot analysis, urea and Triton X-100 extractions, immunolabelling, *in situ* hybridization, mapping and the microinjection of antisense morpholinos have been used.

MATERIALS AND METHODS

Cells, tissues and embryos

AB9 and ZF4 cells were provided by Dr. Marie-Andrée Akimenko (Ottawa Health Research Institute). AB9 is a cell line isolated from the caudal fin of zebrafish. AB9 cells were cultured in Dulbecco's-Modified Eagle Medium (D-MEM; Gibco BRL, Burlington, Ontario, Canada) supplemented with 15% fetal calf serum (FCS; Gibco BRL) and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin; Gibco BRL). The ZF4 cell line has been isolated from 1-day-old embryos (Driever and Rangini, 1993). ZF4 cells were grown in Dulbecco's-Modified Eagle Medium: nutrient mixture F-12 (DMEM/F-12) containing 15mM N-[2-hydroxyethyl] piperazine-N1-[2-ethanesulfonic acid] (HEPES) buffer, L-glutamine and pyridoxine hydrochloride; Gibco BRL) supplemented with 15% FCS and antibiotics. A6 cells (kidney epithelial cells of *Xenopus laevis*) were cultured in DMEM supplemented with 10% FCS and antibiotics. All cells were maintained at 27.5°C in a humidified atmosphere with 5% CO_2.

SDS-PAGE and immunoblotting

Zebrafish embryos of defined stages were obtained as described in Kimmel *et al.* (1995). Zebrafish embryos were directly homogenized in sodium dodecyl sulfate (SDS) sample buffer (SB) 2X in a ratio of 10 embryos/100 µl and 10 µl was loaded on the gel for

analysis by SDS-polyacrylamide gel electrophoresis (PAGE). Small tissue pieces of zebrafish brain, gills, liver and muscles were directly homogenized (~1.43 mg/10 µl) and boiled in SDS sample buffer 2X for analysis by SDS-PAGE (5% stacking and 12% resolving).

Attached cells were scraped from the culture dishes using a rubber policeman and then transferred to centrifuge tubes. Cells were harvested by centrifugation at 1 500 g for 6 min and washed with Tris-acetate buffer, pH 7.5 (10 mM Tris-acetate, 150mM NaCl and 1mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)). Proteins were then solubilized in SDS sample buffer 2X (Laemmli, 1970) (AB9: 1 confluent 100 mm plate in 50µl SB 2X; ZF4: 1 confluent 100 mm plate in 100µl SB 2X) and 10µl was loaded on the gel for analysis by SDS-PAGE (5% stacking and 12% resolving) using the BIO-RAD minigel apparatus.

Protein profiles of zebrafish embryos, tissues and cells, were visualized by Coomassie Blue staining or separated polypeptides were electrophoretically transferred from gels to nitrocellulose membranes and processed for immunoblotting as recommended in the Western Blotting Kit (Amersham Canada, Oakville, Ontario, Canada). Membranes were incubated in 5% milk in phosphate buffered saline (PBS; 130mM NaCl, 5mM Na_2HPO_4, 1.5 mM KH_2PO_4)-Tween (1:200) followed by washes in PBS-Tween (PBST) and incubation with primary antibodies (1h). For visualization of antigen-antibody complexes, immunoblots were reacted with the appropriate biotinylated

secondary antibody in 5% milk/PBST for 1h and after several washes in PBST, were incubated with streptavidin-conjugated with horseradish peroxidase (1:5 000; Amersham Canada) for another hour. Blots were developed using the enhanced chemiluminescence (ECL) kit (Amersham Canada) and the reactivity was visualized on Hyperfilm ECL (Amersham Canada).

Northern hybridization

Total ribonucleic acids (RNA) of zebrafish embryos, from various developmental stages, was isolated using Trizol reagent (Gibco BRL) according to the protocol recommended by the manufacturer. Each RNA pellet was resuspended in 100% formamide. Northern blot analysis was performed according to standard procedures, as described by Sambrook and Russel (2001), but using a modified Church buffer (1mM ethylene diamine tetraacetic acid (EDTA), 0.5 M phosphate buffer (pH 7.0), 7% (w/v) SDS). For each developmental stage, 15 μg of RNA were loaded to formaldehyde gels, transferred to nylon membranes by the capillary method and immobilized by UV irradiation. ^{32}P-labelled probes made from the complementary deoxyribonucleic acids (cDNA) encoding the entire sequence of ZLAP2γ and from the cDNA sequence encoding for the 100 amino acids specific sequence of ZLAP2ω (EDVEEEDWPVLNVKRKLKRSSHRPDQMVPASDDTEN SELSSAECFAVSEDRRRTGPGAGRRETRPLLSDRTSKLSSKSESLRRRSAPVRSVLNEASSPDKD) were used for hybridization. The hybridization was performed at 64°C overnight in a modified Church buffer (see above). Membranes were washed in 0.04M phosphate buffer (pH 7.0) and

0.1% SDS at ;/'.64°C and exposed to X-ray film (Kodak Scientific Imaging film; X-Omat blue, XB-1) with an intensifying screen at -84 °C.

Total RNA from *Xenopus* embryos of the blastula, the gastrula and the neurula stages and the A6 cell line (kidney epithelial cells of *Xenopus laevis*), provided by Dr.Krohne's lab, were isolated using Trizol reagent (Gibco BRL) according to the protocol recommended by the manufacturer. Northern blot analysis was performed according to standard procedures, as previously mentioned for the zebrafish RNA. However, in this case, ^{32}P-labelled probes made from cDNAs coding for the entire sequence of the *Xenopus* LAP2 β (Lang *et al.*, 1999) and ω (Dr. Krohne, personal communication) were used for hybridization. Both cDNAs were provided by Dr. Krohne (Division of Electron Microscopy, Biocenter of the University of Würzburg, Germany).

Extractions with Triton X-100 and urea

Attached cells were scraped from the culture dishes using a rubber policeman and then transferred to centrifuge tubes. Cells were harvested by centrifugation at 1 500 g for 6 min and washed with Tris-acetate buffer, pH 7.5 (10 mM Tris-acetate, 150mM NaCl and 1mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)). For urea extraction, cell pellet was resuspended in 8 M urea (containing 1mM dithiothreitol (DTT)) and incubated for 10 min on ice. Samples were then fractionated by centrifugation (100 000 g, 60 min, 10°C) into pellet and supernatant. Both fractions were dialysed against PBS (pH 7.4) overnight at 4°C. Proteins in the pellet and supernatant

were concentrated by precipitation with trichloroacetic acid (TCA). Samples were brought to final concentration of 10% TCA, heated (40°C, 15 min) and pelleted by centrifugation (13 000 g, 10 min, 5-6°C). The pellet was then resuspended in 5% TCA, pelleted (3 000 g, 10 min), resuspended in cold pure acetone and pelleted again (3 000 g, 10 min).

For Triton X-100 extraction, cell pellet was resuspended in a buffer (1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, 100 mM NaCl, pH 7) containing 1% Triton X-100 and protease inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF); 1mg/ml soybean trypsin inhibitor (STI); 2 mM sodium tetrathionate dihydrate (STD)). Samples were incubated for 15 min on ice and fractionated by centrifugation (13 000 g, 10 min, 4°C) into pellet and supernatant. The pellet fraction was then resuspended in 1 ml of the same buffer without detergent (1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, 100 mM NaCl pH 7), pelleted again (13 000 g, 10 min). Proteins of the two supernatants were combined. Proteins in the supernatant were concentrated by precipitation with TCA.

Indirect immunofluorescence staining

Zebrafish AB9 and ZF4 cells grown on coverslips were fixed, permeabilized and stained as previously described (Chaly *et al.*, 1984). Cells were fixed in 3% paraformaldehyde/PBS for 5 min, exposed to sodium borohydride/PBS (1mg/ml; 3x4 min) and then permeabilized in 0.2% Triton X-100/PBS for 20 min. All antibody incubations were performed at room temperature for 1 hour. Cells were counterstained

for 3 min in 1 µg/ml Hoechst 33258 (Polysciences Inc., Warrington, Pennsylvania, USA) diluted 1:3000 in PBS and they were mounted in 0.1% *p*-phenylene diamine (Sigma-Aldrich Canada Ltd., Oakville, ON) in 50% glycerol/PBS (v/v), pH 7.0. Appropriate controls were performed using the secondary antibodies alone. Conventional epifluorescence microscopy was carried out on a Zeiss Axiophot and observations were recorded on Ilford XP2-400 film.

Zebrafish embryos, at 2, 4 and 24 hpf, were fixed for 2h in 3% paraformaldehyde/PBS, washed in PBS, dehydrated in 100% methanol (MeOH) (2 x 5 min) and stored at -20 °C for 1 day to several weeks. Embryos at 2 and 4 hpf were manually dechorionated in 100% PBS only after rehydratation. Zebrafish embryos at 24 hpf were manually dechorionated in water before being fixed and dehydrated. Embryos were rehydrated in 75% MeOH/25% PBS, 50%MeOH/50% PBS, 25%MeOH/75% PBS (3 x 5 min) and 100% PBS (3 x 5 min). Following rehydration, zebrafish embryos were incubated for 30 min in 1% Triton-X 100/PBS-Tween (1:200). The following steps were performed at 4 °C. Embryos were washed in PBS-Tween (3 x 20 min), incubated for 1 h in 1% bovine serum albumin (PBS/BSA) and overnight with the primary antibody (ZLAP2-serum2 diluted 1:300 in 0.5% BSA/PBS). Embryos were then washed in PBS-Tween (3 x 1h) followed by a 4h incubation with secondary antibodies (anti guinea-pig CY3 diluted 1:1 000 in 0.5% BSA/PBS). For visualization of chromatin, embryos were stained with Hoechst 33258 (diluted 1:1 000 from a stock of 0.2 mg/ml) during the last

two hours of the incubation with secondary antibodies. Washed embryos (2 x 30 min + overnight) were mounted with 0.1% *p*-phenylene diamine (Sigma-Aldrich Canada Ltd., Oakville, ON) in 50% glycerol/PBS (v/v), pH 7.0. Appropriate controls were performed using the secondary antibodies alone. Digital images were taken using a Hamamatsu charge-coupled device (CCD) camera on a Zeiss Axiophot, recorded using Metamorph V. 2.75 Universal Imaging and processed with Adobe Photoshop. The confocal laser-scanning microscopy (CLSM) was carried out on an upright Leica optical system equipped with an Argon-Krypton laser. Images were digitized on a Matrox Meteor-2 image card (written by Ready to Ware) and processed with Metamorph V. 2.75 Universal Imaging and Adobe Photoshop.

***In situ* hybridization**

Embryos of different developmental stages were fixed for 2h at room temperature (RT) in 4% paraformaldehyde, washed in PBS, dehydrated in 100 % MeOH (2 x 5 min) and stored at -20 °C for 1 day to several weeks. Embryos were rehydrated in 75% MeOH/25% PBS, 50%MeOH/50% PBS, 25%MeOH/75% PBS (3 x 5 min) and 100% PBS-Tween (1/200) (3 x 5 min). Embryos at early developmental stages were manually dechorionated in 100% PBS only after rehydratation. Zebrafish embryos at 24 hpf and up were manually dechorionated in water before being fixed and dehydrated. Embryos were then incubated with proteinase K (1/1 000, stock 20 mg/ml) for 1 min, washed in PBS and fixed again for 20 min in 4% paraformaldehyde. Following fixation, embryos were treated with acetylation mix (1.25% triethanolamine, 0.27% acetic anydride in PBS-Tween) for 10 min. Embryos were prehybridized for 2h at 65 °C in hybridization mix

(HM) (50% deionized formamide, 5X sodium chloride; sodium citrate (SSC), 0.1% Tween-20, 50 µg/ml heparin, citric acid (1/100, stock 1M), pH 6.0) and hybridized with the probe overnight at 65 °C in the same mix but with yeast tRNA (1 µl/ml, stock 10 mg/ml). After hybridization, embryos were washed at 65 °C in hybridization mix and 2X SSC in proportion of 75% HM/25% 2X SSC, 50% HM/50% 2X SSC, 25% HM/75% 2X SSC (3 x 10 min) and 100% 2X SSC 10 min plus 2 x 30 min in 0.2X SSC at 60 °C. Then embryos were washed again at RT in 0.2X SSC and PBS-Tween in proportion of 75% 0.2X SSC/25% PBST, 50% 0.2X SSC/50% PBST, 25% 0.2X SSC/75% PBST (3 x 5 min) and 5 min in PBS-Tween. Embryos were preincubated in 10% calf serum and 40 mg/ml BSA in PBS-Tween for 1 h and incubated with anti-digoxigenin (1/2 000 in PBS-Tween with 0.5% calf serum and 2 mg/ml BSA) for 2-4 h at RT and overnight at 4 °C. Anti-digoxigenin was pre-adsorbed in the same conditions with spare embryos for 2-4 h at RT and then stored at 4 °C overnight prior to the incubation. Embryos were then washed 6 x 15 min in PBS-Tween and incubated 2 x 5min in staining buffer (100 mM tris-HCl, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20, 1mM levamisol). Staining was performed for 1h-2h at RT (in the dark) in staining buffer with 175 µg/ml 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) and 337 µg/ml Nitro Blue Tetrazolium (NBT). After staining, embryos were washed in PBS- Tween (2 x 5 min) at RT, post-fixed for 2h in 4% paraformaldehyde, washed again and stored in PBS-Tween with 5 mM EDTA. The complete sequence of ZLAP2γ and the specific region of 100 amino acids of ZLAP2ω were used as digoxigenin-labelled RNA probes.

Mapping of the ZLAP2 gene

The ZLAP2 gene was mapped in the zebrafish genome by polymerase chain reaction (PCR) analysis with the LN54 radiation hybrid cell panel (Hukriede *et al.*, 1999). The panel contained 93 samples of 100 ng of hybrid-cell DNA, plus 100 ng of the DNA from each parental cell lines (zebrafish-AB9 and mouse-B78) and 100 ng of a 1:10 mixture of zebrafish AB9 DNA / B78 mouse DNA. For the PCR reaction, 5 pmoles of the two gene-specific primers, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of 2'-deoxynucleoside 5'-triphosphate mix (dNTP), 1 U Taq polymerase, were added to the DNA for a total volume of 20 µl. PCR was performed starting with 4 min at 94 °C followed by 32 cycles of: 30 sec at 94 °C, 30 sec at 60 °C (appropriate annealing temperature for the primers) and 30 sec at 72 °C; finishing with 7 min at 72 °C. PCR products were separated on 1.5 % agarose gels and the images were captured with Gel Doc 1 000 (Bio Rad). PCR assays were performed in duplicate for each primer set. The results were analysed with the RHMAPPER program by web submission of the data to: http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi.

Injection of morpholinos

The antisense morpholino oligo (Mo) LAP2 was synthesized by Gene Tools (Philomath, USA) to bind to the initiating methionine and the twenty two 5' nucleotides of the ZLAP2 transcript. The sequence of the MoLAP2 is 5'-cgggtcttccagaaattccgacatg-3'. The morpholino was solubilized to the appropriate concentration with 1x Danieau buffer (58

mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES pH 7.6). 3-8 nanograms of morpholinos were injected at the 1-8 cell stage into the yolk, depending on the concentration prepared (0.5mM, 1 mM or 1.5 mM). The injected volume was approximately 1 nl. Non-injected embryos as well as embryos injected with the same amount (3-8 ng) of the standard control morpholino, were used as controls. The standard control consists of a morpholino that does not match the sequence composition of the experimental oligo. The morpholino solution was heated at 65°C for 5 min and centrifuged prior to the microinjection. Images of embryos were obtained using a black and white Q-imaging Retiga 1300C cooled monochrome CCD.

Antibodies

The primary antibodies used were: the MAN antiserum (Paulin-Levasseur *et al.*, 1996) at a dilution of 1:5 000 for immunofluorescence and 1:5 000 for blotting. Also used, the guinea-pig polyclonals ZLAP2-serum1 (S1) and ZLAP2-serum2 (S2) specifically react with all zebrafish LAP2 isoforms and, in addition, ZLAP2-serum1 binds weakly to LAP2 polypeptides from other vertebrates. Moreover, the ZLAP2-serum-1 was shown to display cross-reactivity with some chromatin-associated proteins by immunofluorescence (Schoft, Beauvais *et al.*, in press). The polyclonal S1 and S2 sera were produced from two different guinea-pig, using the amino-terminal part 1-165 as an immunogen. Therefore, each of these two antibodies is reacting against several epitopes. These two sera were used at a dilution of 1:500 for immunofluorescence on cells and 1:3 000 for

blotting.

The following biotinylated secondary antibodies were used for blotting: sheep anti-human Ig (Amersham Canada) at a dilution of 1:4 000; and donkey anti-guinea pig IgG (Jackson Immunoresearch, West Grove, Pa.) at a dilution of 1:5 000. Secondary antibodies used for immunofluorescence were: goat anti-human IgG conjugated with indocarbocyanine (CY3) (Jackson Immunoresearch) at a dilution of 1:400; and donkey anti-guinea pig IgG conjugated with CY3 (Jackson Immunoresearch) at a dilution of 1:1 000.

RESULTS

Characterization of zebrafish LAP2 cDNA sequences

During the course of this study, more information on three ZLAP2 encoding sequences came from collaborative work performed in the laboratories of Dr. Paulin-Levasseur and Dr. Krohne (Schoft, Beauvais *et al.*, in press). In a first step, a zebrafish kidney cDNA library arrayed on filters (Library no.575 of the Resource Center of the German Human Genome Project; Berlin) was probed with the complete nucleotide sequence of the previously characterized *Xenopus* LAP2β cDNA (Lang *et al.*, 1999; accession number Y17861). A clone with a 5'-end showing a high homology to the *Xenopus* LAP2β was isolated and sequenced (clone B4; accession number AJ320189). From this clone, primers were chosen in order to screen, using a PCR-based method, a cDNA library from zebrafish ovary prepared in Dr. Krohne's lab (Schoft, Beauvais *et al.*, in press). Using the same set of primers, three zebrafish LAP2 sequences which encode for 369 (ZLAP2γ), 515 (ZLAP2β) and 657 (ZLAP2ω) amino acids were amplified (Figs. 1 and 2).

The proteins predicted from these cDNA clones have the same amino-terminal segment of 214 amino acids that contains the LEM domain. They also have in common a lamin-binding domain, a transmembrane segment and the carboxy-terminal end. In the ZLAP2γ isoform, the lamin-binding domain is located after the amino-terminal region of 214 amino acids (Fig. 2, ZLAP2γ). However, in the ZLAP2β isoform, there is an insertion of 146 amino acids between the amino-terminal region and the lamin-binding domain (Fig. 2, ZLAP2β). ZLAP2ω is the longest isoform with two additional insertions

Figure 1. Comparison between the amino acid sequences of ZLAP2γ, β and ω. Bold printed letters represent amino acids identical in the three proteins. Proteins contain 369 (γ), 515 (β) and 657 (ω) amino acids. Gaps have been introduced to allow an optimal alignment of the three sequences. The sequences have been published under accession numbers AJ320192 (γ), AJ320191 (β), AJ320190 (ω) in the EMBL Nucleotide Sequence Database. (Adapted from Schoft and Beauvais *et al.* in press)

```
Zgamma  MLEPLEDPSVLTKDKLKSALLANNVALPNGDQRKDVYVQLYLKNLTVQNKKSSGSPDVFS 60
Zbeta   MLEPLEDPSVLTKDKLKSALLANNVALPNGDQRKDVYVQLYLKNLTVQNKKSSGSPDVFS 60
Zomega  MLEFLEDPSVLTKDKLKSALLANNVALPNGDQRKDVYVQLYLKNLTVQNKKSSGSPDVFS 60

Zgamma  SDEELPPAPVVSNRSRSGRKATRKTDKVRPDDVDVTELSNEGLKDLLLKYGLNAGPIVAS 120
Zbeta   SDEELPPAPVVSNRSRSGRKATRKTDKVRPDDVDVTELSNEGLKDLLLKYGLNAGPIVAS 120
Zomega  SDEELPPAPVVSNRSRSGRKATRKTDKVRPDDVDVTELSNEGLKDLLLKYGLNAGPIVAS 120

Zgamma  TRKVYEKRLQKLLDQGPPVAVALPSETSQTDGNQNGNNDSDQYSDREEEPVAPAPVTVPE 180
Zbeta   TRKVYEKRLQKLLDQGPPVAVALPSETSQTDGNQNGNNDSDQYSDREEEPVAPAPVTVPE 180
Zomega  TRKVYEKRLQKLLDQGPPVAVALPSETSQTDGNQNGNNDSDQYSDREEEPVAPAPVTVPE 180

Zgamma  PEVEAELIPVVERPVRSRGKTPVTSRTRSGQHTR ------------------------- 214
Zbeta   PEVEAELIPVVERPVRSRGKTPVTSRTRSGQHTRD ------------------------ 215
Zomega  PEVEAELIPVVERPVRSRGKTPVTSRTRSGQHTREDVEEEDWPVLNVKRKLKRSSHRPDQ 240

Zgamma  ------------------------------------------------------------
Zbeta   ------------------------------------------------------------
Zomega  MVPASDDTENSELSSAECFAVSEDRRRTGPGAGRRETRPLLSDRTSKLSSKSESLRRRSA 300

Zgamma  ------------------------------------------------------------
Zbeta   --------------FSEPSIVKEVSVSLMRMKVQPLTVPKDPKPSKRYSMSATESTKRP 260
Zomega  PVRSVLNEASSPDKDFSEPSIVKEVSVSLMRMKVQPLTVPKDPKPSKRYSMSATESTKRP 360

Zgamma  ------------------------------------------------------------
Zbeta   VSSLNNKHDENTADIPSPPHRQSSREPLVSLINTACVEVDDQGMQDVLSCRSANGGLLAQ 320
Zomega  VSSLNNKHDENTADIPSPPHRQSSREPLVSLINTACVEVDDQGMQDVLSCRSANGGLLAQ 420

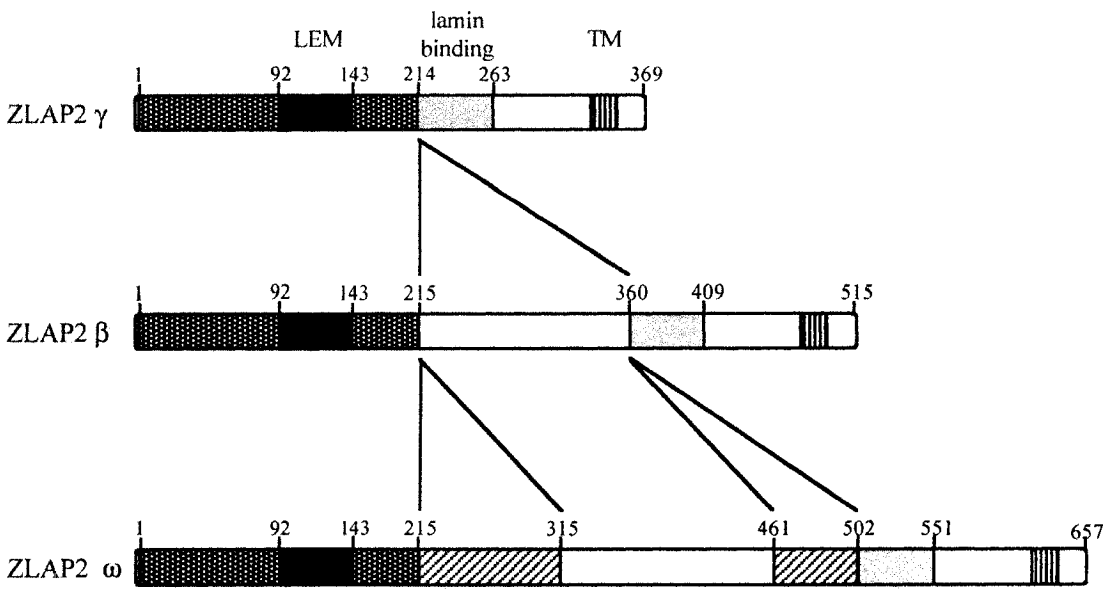
Zgamma  ------------------------------------------------------------
Zbeta   GVRSAAVSKLSKPVLSQSKPSKPLVDMMCCLSPSSDRQKE-------------------- 360
Zomega  GVRSAAVSKLSKPVLSQSKPSKPLVDMMCCLSPSSDRQKEESCGSPQTHPKSRHSKITPF 480

Zgamma  ----------------------VEKVSAIDQTPRAVERDVLKEIFPTENLNTPTGISATC 252
Zbeta   ----------------------VEKVSAIDQTPRAVERDVLKEIFPTENLNTPTGISATC 398
Zomega  LSQITPVRGLDNKLMDMKETMMVEKVSAIDQTPRAVERDVLKEIFPTENLNTPTGISATC 540

Zgamma  RRPIRGAAGRPLIDTWLNESRQRLSDLKQTTSSSSFSSTSSYTESRSVPRVSSIPLSASK 312
Zbeta   RRPIRGAAGRPLIDTWLNESRQRLSDLKQTTSSSSFSSTSSYTESRSVPRVSSIPLSASK 458
Zomega  RRPIRGAAGRPLIDTWLNESRQRLSDLKQTTSSSSFSSTSSYTESRSVPRVSSIPLSASK 600

Zgamma  STAPPTVKSRSRRSLPVWVQLVLLSAVAGFLFFIYQAMETNDVGLFKQSGTDDSTSK 369
Zbeta   STAPPTVKSRSRRSLPVWVQLVLLSAVAGFLFFIYQAMETNDVGLFKQSGTDDSTSK 515
Zomega  STAPPTVKSRSRRSLPVWVQLVLLSAVAGFLFFIYQAMETNDAGLFKQSGTDDSTSK 657
```

Figure 2. Schematic drawings of ZLAP2γ, ZLAP2β and ZLAP2ω. The position of the LEM motif (LEM), lamin binding domain (lamin binding), transmembrane domain (TM), and the position of individual amino acids is marked for each isoform. The insertion sites of the β- and ω-specific sequences are marked by lines. (Adapted from Schoft and Beauvais *et al.* in press)


LEM
lamin binding
TM
1
92
143
214
263
369
ZLAP2 γ
1
92
143
215
360
409
515
ZLAP2 β
1
92
143
215
315
461
502
551
657
ZLAP2 ω

the of 101 and 42 amino acids, respectively, flanking segment of 146 amino acids that is present in ZLAP2β (Fig. 2, ZLAP2ω). Therefore, these two segments are specific to the ZLAP2ω isoform. The amino-terminal (amino acids 1-165 of ZLAP2; 63% identity with mouse LAP2γ) and carboxy-terminal domains (amino acids 229-264 in ZLAP2γ; 72% identity with mouse LAP2γ) of ZLAP2 isoforms show sequence homologies with all other known LAP2 except for the mammalian LAP2α. In mammals, LAP2α only has its amino-terminal domain in common with the other isoforms (reviewed in Dechat *et al.*, 2000). The ZLAP2ω-specific domains do not show any homology to all known LAP2 isoforms. While the size of the ZLAP2ω isoform is similar to that of LAP2α, these proteins are distinct: ZLAP2ω possesses a transmembrane domain but LAP2α does not. Therefore, ZLAP2ω is a novel LAP2 isoform that is present in zebrafish (Schoft, Beauvais *et al.*, in press) and possibly in other organisms (see below the Northern blot analysis of *Xenopus*).

Expression of ZLAP2 proteins during embryonic development

In order to determine the expression pattern of LAP2-related proteins during the development of zebrafish embryos, Western blot analysis using the S1, S2 and the MAN sera was performed on specimens at several developmental stages from 4-8 cells to 54 hours post fertilization (hpf). The analysis reveals that the expression of the three LAP2-related proteins (ZLAP2β, γ and ω) is differentially regulated throughout embryonic development. Using the S1 serum, a polypeptide of M_r 84 000 which corresponds approximately to the predicted molecular weight of ZLAP2ω (72 225 daltons) is detected

in early embryos (4-8 cells) up to the late blastula stage (4 hpf) (Fig. 3A and B). In contrast, polypeptides of M_r ~66 000 and 45 000, which correspond to the predicted molecular weight of ZLAP2β (56 278 daltons) and ZLAP2γ (40 447 daltons) proteins, respectively, are absent from early developmental stages (4-8 cells to 9.5 hpf) and become first detectable at 10 hpf, which corresponds to the end of the gastrulation period. The amount of ZLAP2ω proteins decreases with the progression of embryonic development whereas the amount of ZLAP2β and γ isoforms increases between 10 to 28 hpf until they reach a steady-state after 32 to 54 hpf. At all embryonic stages where it is present, the γ isoform appears as the predominant ZLAP2 protein.

Similar results were obtained using the S2 serum except that, in this case, the ZLAP2ω protein is detected until the gastrula stage (7 hpf) and the reactivity of the ZLAP2β and γ isoforms is weaker than with the S1 serum (Fig. 4A and B). Surprisingly, the MAN serum only recognized the ZLAP2γ protein in embryos and the expression of this isoform is first detectable at 20 hpf (Fig. 5A and B).

Expression of ZLAP2 proteins in somatic cells

In a second step towards deciphering the expression pattern of ZLAP2 proteins, I verified whether the isoforms β, γ and ω were expressed in zebrafish cell lines and adult tissues. Total proteins from two cell lines AB9 and ZF4 and from the liver, brain, muscles and gills of adult fish were analyzed. AB9 and ZF4 cell lines were isolated from cell cultures of zebrafish caudal fin and from 1 day-old embryos, respectively. Protein samples were prepared and separated by SDS-PAGE on a 12% resolving gel. Since the

Figure 3. Expression of ZLAP2 protein isoforms during zebrafish development. Total proteins of 15 developmental stages (from 4-8 cell stage to 54 hpf) were separated by SDS-PAGE (12% acrylamide) and immunoblotted with either the ZLAP2-serum1 (**A**) or the detection system only (**B**). The developmental stage is specified on top of each lane. ZLAP2ω is detected in early embryos up to the late blastula stage (4hpf) and ZLAP2β and γ are detected from 10 to 54 hpf. The positions of ZLAP2ω (M_r 84 000), ZLAP2β (M_r 66 000) and ZLAP2γ (M_r 45 000) are marked by arrows. The black dots correspond to the position of the biotinylated molecular weight markers: phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumine, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa.

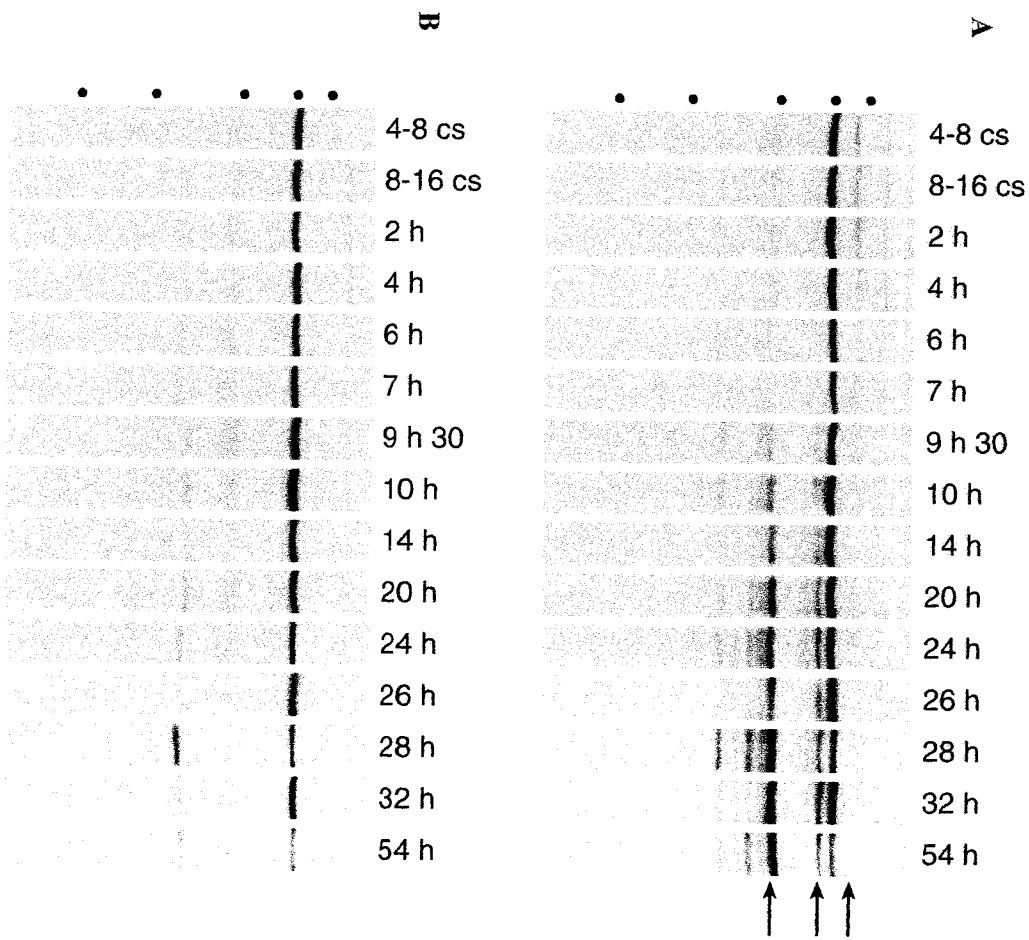

B
A
4-8 cs
8-16 cs
2 h
4 h
6 h
7 h
9 h 30
10 h
14 h
20 h
24 h
26 h
28 h
32 h
54 h

Figure 4. Expression of ZLAP2 protein isoforms during zebrafish development. Total proteins of 15 developmental stages (from 4-8 cell stage to 54 hpf) were separated by SDS-PAGE (12% acrylamide) and immunoblotted with either the ZLAP2-serum2 (**A**) or the detection system only (**B**). The developmental stage is specified on top of each lane. ZLAP2ω is detected in early embryos up to the gastrula stage (7 hpf) and ZLAP2β and γ are detected from 10 to 54 hpf. The positions of ZLAP2ω (M_r 84 000), ZLAP2β (M_r 66 000) and ZLAP2γ (M_r 45 000) are marked by arrows. The black dots correspond to the position of the biotinylated molecular weight markers: phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumine, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa.

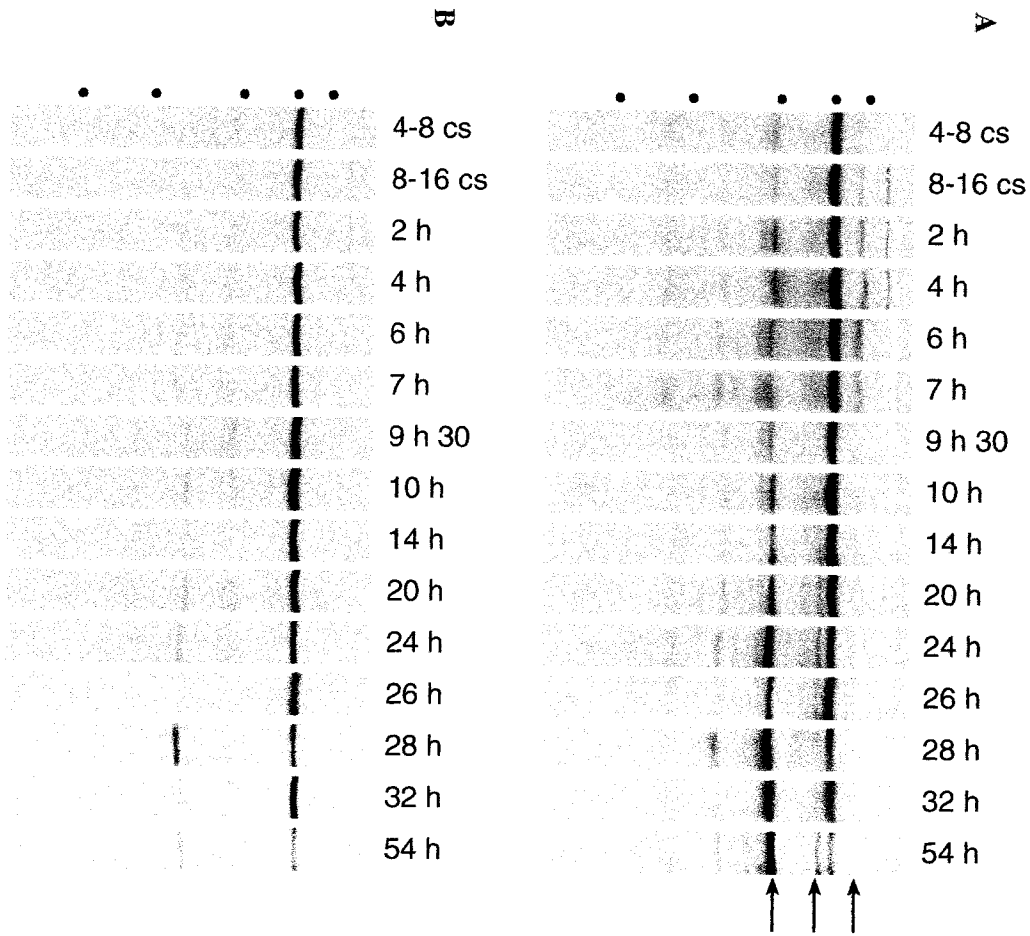
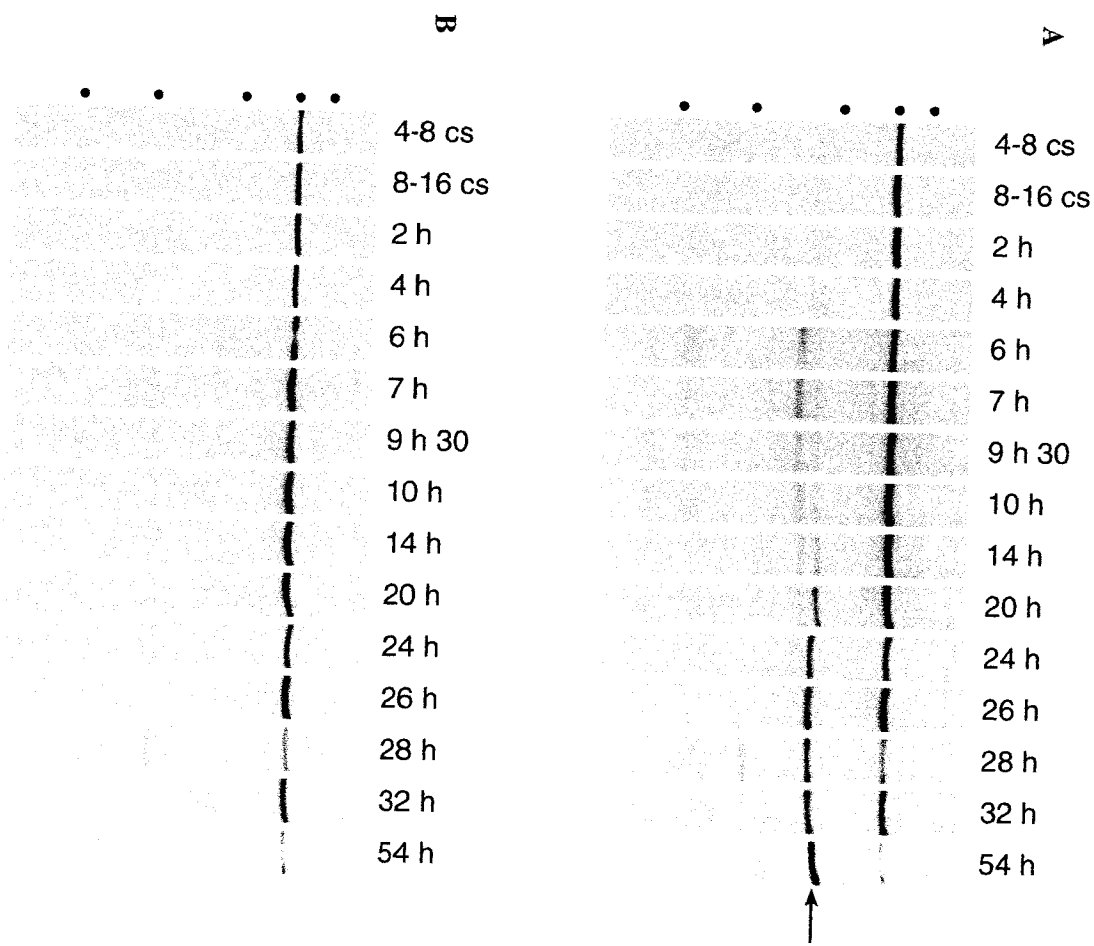

B
A
4-8 cs
8-16 cs
2 h
4 h
6 h
7 h
9 h 30
10 h
14 h
20 h
24 h
26 h
28 h
32 h
54 h

Figure 5. Expression of ZLAP2 protein isoforms during zebrafish development. Total proteins of 15 developmental stages (from 4-8 cell stage to 54 hpf) were separated by SDS-PAGE (12% acrylamide) and immunoblotted with either the MAN serum (**A**) or the detection system only (**B**). The developmental stage is specified on top of each lane. ZLAP2γ is the only isoform detected and it is detected from 20 to 54 hpf. The position of ZLAP2γ (M_r 45 000) is marked by an arrow. The black dots correspond to the position of the biotinylated molecular weight markers: phosphorylase B, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumine, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa.

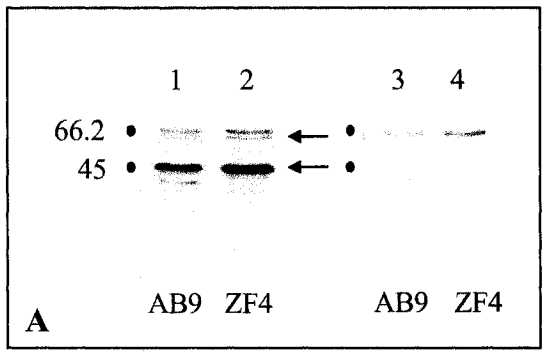

B
4-8 cs
8-16 cs
2 h
4 h
6 h
7 h
9 h 30
10 h
14 h
20 h
24 h
26 h
28 h
32 h
54 h
A
4-8 cs
8-16 cs
2 h
4 h
6 h
7 h
9 h 30
10 h
14 h
20 h
24 h
26 h
28 h
32 h
54 h

S1 and S2 sera showed similar reactivity towards ZLAP2 proteins, the immunoblot analysis was performed here using only the S1 and MAN sera.

Using the S1 serum, only two polypeptides with a relative mobility of M_r 66 000 and M_r 45 000 were detected in the two zebrafish cell lines (Fig. 6A). According to the predicted molecular weight of ZLAP2β and ZLAP2γ, I assumed that these two polypeptides correspond to ZLAP2β and γ (Schoft, Beauvais *et al.*, in press). Using the MAN serum, the same pattern of expression is observed for ZLAP2β and γ (Fig. 7A). However, an extra polypeptide of M_r ~25 000 is also detected in both cell lines and in the gills of adult fish (see below). I did not make any investigation about the nature of this polypeptide.

Using both the S1 and MAN sera, ZLAP2γ is detected in all four tissues tested (liver, brain, muscle and gills), whereas ZLAP2β is only detected in liver and gills (Figs. 6B and 7B). The reactivity of the two sera on tissues differs by the fact that the MAN serum detects again an extra band of M_r 25 000, but only in gills. In muscle tissue, using both sera, two bands with a mobility similar to the ZLAP2γ isoform are seen. The major band exhibits the slower migration and displays some distortion (Figs. 6B and 7B; lane 3). This might be due to the presence of large amounts of actin in muscle, which has a molecular weight of ~40 kDa. The migration of large amounts of actin in the same area of the gel as ZLAP2γ might have interfered with the proper migration of this isoform. The nature of the second faster migrating band detected in the muscle was not investigated. However, it is possible that this band resulted form some degradation in the sample. Using both sera, no larger polypeptide that could correspond to ZLAP2ω was

Figure 6. Immunoblotting analysis of cellular and tissue extracts from the zebrafish. (**A**) Total cellular proteins of AB9 cells (AB9, 1 and 3) and ZF4 (ZF4, 2 and 4) were separated by SDS-PAGE (12% acrylamide) and immunoblotted with either the ZLAP2-serum1 (lane 1 and 2) or the detection system only (lane 3 and 4). (**B**) Total proteins from zebrafish liver (L, lane 1 and 5), brain (B, lane 2 and 6), muscle (M, lane 3 and 7), gills (G, lane 4 and 8) were separated by SDS-PAGE (12% acrylamide) and immunoblotted with either the ZLAP2-serum1 (lane 1-4) or the detection system only (lane 5-8). ZLAP2γ is the major isoform in all tissues and cell lines tested, and only a minor amount of ZLAP2β is detectable in the liver and gills, and both cell lines. The positions of ZLAP2β (M_r 66 000) and ZLAP2γ (M_r 45 000) are marked by arrows in **A** and **B.** Molecular masses of reference proteins (in kDa) are marked in **A** and **B**.


1
2
3
4
66.2
45
AB9
ZF4
AB9
ZF4
A

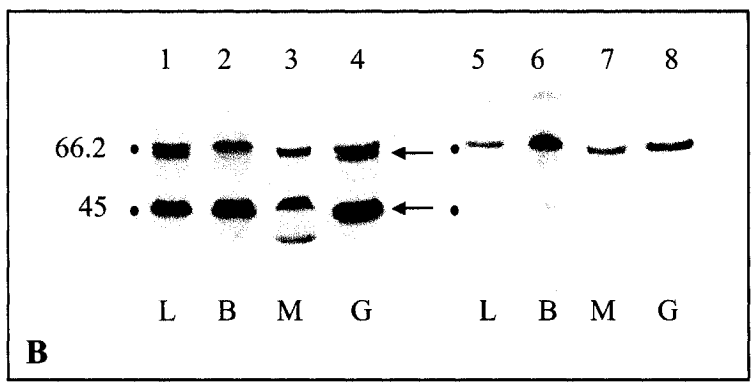
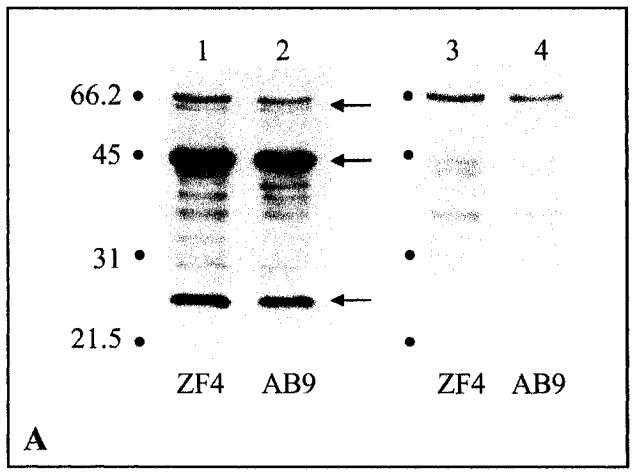
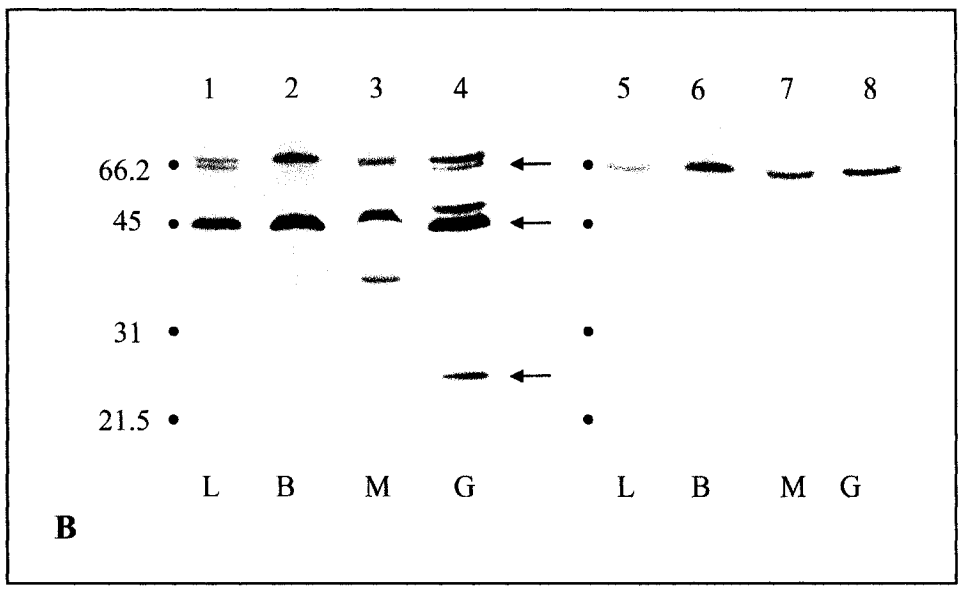

1
2
3
4
5
6
7
8
66.2
45
L
B
M
G
L
B
M
G
B

Figure 7. Immunoblotting analysis of cellular and tissue extracts from the zebrafish. (**A**) Total cellular proteins of ZF4 cells (ZF4, 1 and 3) and AB9 (AB9, 2 and 4) were separated by SDS-PAGE (12% acrylamide) and immunoblotted with either the MAN serum (lane 1 and 2) or the detection system only (lane 3 and 4). (**B**) Total proteins from zebrafish liver (L, lane 1 and 5), brain (B, lane 2 and 6), muscle (M, lane 3 and 7), gills (G, lane 4 and 8) were separated by SDS-PAGE (12% acrylamide) and immunoblotted with either the MAN serum (lane 1-4) or the detection system only (lane 5-8). ZLAP2γ is the major isoform in all tissues and cell lines tested, and only a minor amount of ZLAP2β is detectable in the liver and gills, and both cell lines. The positions of ZLAP2β (M_r 66 000), ZLAP2γ (M_r 45 000) and an unknown polypeptide (M_r 25 000) are marked by arrows in **A** and **B.** Molecular masses of reference proteins (in kDa) are marked in **A** and **B**.


1
2
3
4
66.2
45
31
21.5
ZF4
AB9
ZF4
AB9
A


1
2
3
4
5
6
7
8
66.2
45
31
21.5
L
B
M
G
L
B
M
G
B

detected in the somatic cell lines and adult tissues tested.

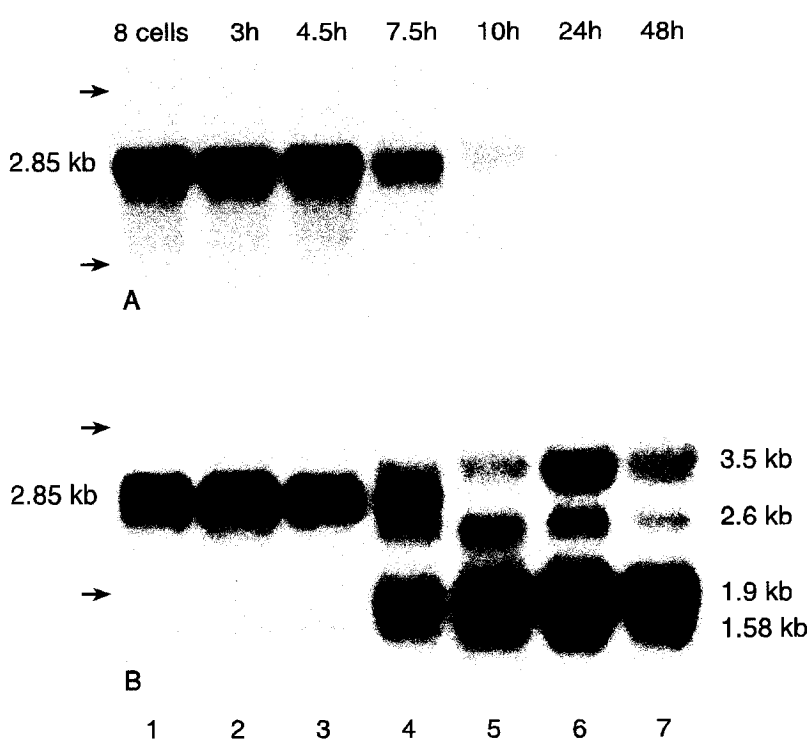
These results indicate that ZLAP2β and γ are the only LAP2-related proteins expressed in the zebrafish cell lines and tissues used in this analysis and that ZLAP2γ is the predominant isoform.

Expression of LAP2 transcripts during embryonic development of the zebrafish and *Xenopus*

Northern blot analysis of RNAs prepared from zebrafish embryos at various developmental stages provided data consistent with immunoblot results. Total RNAs from embryos at the 8 cell-stage, and at 3 hpf, 4.5 hpf, 7.5 hpf, 10 hpf, 24 hpf and 48 hpf were isolated and analyzed by Northern blot using a probe corresponding to the 101 amino acids sequence specific to ZLAP2ω (see Materials and Methods) (Fig. 8A). This probe hybridizes to a band of 2.85 kilobases (kb) in embryos at the 8 cell-stage and blastula stages (3 and 4.5 hpf), identified as ω mRNA. In late gastrula stage embryos (7.5 hpf), lower amounts of the ω mRNA are detected and no corresponding transcript is observed in older developmental stages analyzed (10 to 48 hpf).

When the complete ZLAP2γ cDNA sequence was used as a probe for hybridization, the same pattern was observed for the ω mRNA in embryos at the 8 cell-stage to 48 hpf (Fig. 8B). However, in embryos at the late gastrula stage (7.5 hpf) and in older developmental stages (10 to 48 hpf), four additional bands of 3.5, 2.6, 1.9 and 1.58 kb are present, while the 2.85 kb band, corresponding to the ZLAP2ω transcript, is either reduced in intensity or absent (Fig. 8B, lanes 4-7). Based on their sizes, the 1.9 and 1.58 kb bands are most likely transcripts coding for the ZLAP2β and γ isoforms, respectively.

Figure 8. Northern blot analysis of ZLAP2 transcript expression. Total RNA of zebrafish embryos at the 8-cell stage (lane 1), 3h (early blastula; lane 2), 4.5h (late blastula; lane 3), 7.5h (late gastrula; lane 4), 10h (1-2 somites; lane 5), 24h (30 somites; lane 6) and 48h (lane 7) was hybridized with *in vitro* synthesized ^{32}P-labelled RNA complementary to the ZLAP2 mRNAs. (**A**) Membrane probed with the sequence encoding a region specific to ZLAP2ω (amino acids 215-315 of ZLAP2ω). (**B**) Membrane probed with a sequence complementary to the complete ZLAP2γ mRNA. The positions of the ribosomal RNAs (28S and 18S) are marked by arrows. The sizes of the different hybridizing mRNA bands are marked.


8 cells
3h
4.5h
7.5h
10h
24h
48h
2.85 kb
A
2.85 kb
3.5 kb
2.6 kb
1.9 kb
1.58 kb
B
1
2
3
4
5
6
7

The transcript of 1.9 kb (ZLAP2β) is present in larger amounts than the one of 1.58 kb (ZLAP2γ), which is the opposite of what was observed at the protein level. As for the band of 3.5 kb, since it appears at the same time as the transcripts coding for ZLAP2β and γ (at 7.5 hpf), it is possible that it corresponds to a large unprocessed pre-mRNA. Concerning the 2.6 kb band, it is possible that it corresponds to a degraded form of the 2.85 kb mRNA. Another possibility would be that the 2.6 kb band corresponds to an unknown ZLAP2 isoform.

In addition to the analysis on zebrafish embryos, Northern blot experiments were also performed on *Xenopus laevis* somatic cells and embryos. Total RNAs from *Xenopus* A6 cells (a kidney epithelial cell line) and embryos at the blastula, gastrula and the neurula stages were isolated and probed with two cDNA encoding for the complete sequence of the *Xenopus* LAP2β2 (Lang *et al.*, 1999) and LAP2ω (Dr. Krohne, personal communication; unpublished). Since the two sequences are very similar (Figs. 9 and 10), both probes hybridize to the same bands.

In A6 cells, two bands of 2.7 and 1.8 kb are labelled and they appear to be better detected using the probe for the β isoform (Fig.11 A and B, lane 1). These two bands are also present in embryos at the gastrula (Fig. 11 A and B, lane 3) and the neurula stages (Fig. 11 A and B, lane 4), indicating that these transcripts are first expressed during development at the gastrula stage and are also present in somatic cells from adult fish. Based on previous immunoblot analysis performed on *Xenopus* (Lang *et al.*, 1999), it appears likely that these two bands correspond to transcripts encoding two different XLAP2β isoforms. However, there is also the possibility that one of the two bands corresponds to an uncharacterized XLAP2 isoform. In embryos at the blastula and the

Figure 9. Comparison between the amino acid sequences of three XLAP2 isoforms. Proteins contain 466 (β1), 518 (β2) and 558 (ω) amino acids. Gaps have been introduced to allow an optimal alignment of the three sequences. The sequences have been published under accession numbers Y17861 (β2), AJ514937 (ω) in the EMBL Nucleotide Sequence Database, (β1) unpublished.

```
Xbeta1  MPEFLQDPSVLTKEKLKSELVANNVTLPSGEQRKDVYVQLYLQHLTSQNSGTPDFSSDEE 60
Xbeta2  MPEFLQDPSVLTKEKLKSELVANNVTLPSGEQRKDVYVQLYLQHLTSQNSGTPDFSSDEE 60
Xomega  MPEFLQDPSVLTKEKLKSELVANNVTLPSGEQRKDVYVQLYLQHLTSQNRATPDFSSDEE 60
        ************************************************ .*********

Xbeta1  REATPMRGRGRPPGRKATKKTDKPKAEEKDDPDVTELSNEALKEELLKYGMKPGPILSNT 120
Xbeta2  REATPMRGRGRPPGRKATKKTDKPKAEEKDDPDVTELSNEALKEELLKYGMKPGPILSNT 120
Xomega  REATPMRGRGRPPGRKATKKTDKPRAEEKDDPDVTELSNEALKEELLKYGMKPGPILSNT 120
        ************************:**********************************

Xbeta1  RKLYEQRLFKLKEQGLASSALPADTSKADNKQNGNTDSEQYSDNEEEAKVELTFEKREPL 180
Xbeta2  RKLYEQRLFKLKEQGLASSALPADTSKADNKQNGNTDSEQYSDNEEEAKVELTFEKREPL 180
Xomega  RKLYEQRLFKLKEQGLASSALPADTSKADNKQNGNTDSEQYSDNEEEAKVELTFEKREPL 180
        ************************************************************

Xbeta1  RGNPKPQVTMRNRRTEKTE-------------------------------------TVSE 203
Xbeta2  RGNPKPQVTMRNRRTEKTE-------------------------------------TVSE 203
Xomega  RGNPKPQVTMRNRRTEKTEAEEDTDLIPELNVKRSNRSPPKGFTLDDVEDLSTDHVTVSE 240
        ******************                                      ****

Xbeta1  DVVTEAAWTSGPVKSGPVQTVYKESTKISRRTPRKKVVAPDPVLFDDADISEVPPISELV 263
Xbeta2  DVVTEAAWTSGPVKSGPVQTVYKESTKISRRTPRKKVVAPDPVLFDDADISEVPPISELV 263
Xomega  DVVTEAAWTSGPAKSGPVQTVYKELAKVSRRTPRKKVVAPDPVPFDDADIAEVPPISESV 300
        ************.*********** :*:*************** ******:******* *

Xbeta1  VP-ASNQIFAYAENEHFESSKVVNRVPGSLKHAETLLSVGEISELTRRTPKKQLISEKHL 322
Xbeta2  VP-ASNQIFAYAENEHFESSKVVNRVPGSLKHAETLLSVGEISELTRRTPKKQLISEKHL 322
Xomega  VEPASNQILTYAENEHFESRKVVNQVPESLKHAETLLSVSEFSELTRRTQKKQLISEKHL 360
        *  *****::********* ****:** ***********.*:******* **********

Xbeta1  NVTRETESGSSQIRL---------------------------------------------- 336
Xbeta2  NVTRETESGSSQIRLID--SIGFSNPTDLLNTALIEETKQIIEDSLETPKKTKQLKITKF 380
Xomega  NLTRETDSGSSQIRLIDVHSIGFTNPTDLLNTALIEETKHIIEDSLETPKKTKQLKITKF 420
        *:****:********

Xbeta1  --------VEKNFEARRTERDILKEMFPKEFLTPTGISASCRRPIRGAAGRPLNATDFKT 389
Xbeta2  VTPVKKQIVEKNFEERRTERDILKEMFPKEFLTPTGISASCRRPIRGAAGRPLNATDFKT 440
Xomega  VTPVKKQIVEKTFEERRTERDILKEMFPTEFSTPTGISASCRRPIRGAAGRPLNATDLKI 480
               :***.** *************.** *************************:*

Xbeta1  DETYTSKYVSNVSKYTPAVEAKSAKVKPGRSLPAWIKILLCIILVVFCFLVYQAMETNDG 449
Xbeta2  DETYTSKYVSNVSKYTPAVEAKSAKVKPGRSLPAWIKILLCIILVVFCFLVYQAMETNDG 500
Xomega  NETYTSKYVSNVSKYTPAVEVKSEKVKPGRSLPVWIKILMFLILVVFCLLVYQAMETNEG 540
        :*******************.** *********.*****: :******:*********:*

Xbeta1  IAFSKLFG-ITESNKTEN 466
Xbeta2  IAFSKLFLGITESNKTEN 518
Xomega  MSFSKLLLGITESNKTEN 558
        ::****:  *********
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Figure 10. Schematic drawings of XLAP2β1, XLAP2β2 and XLAP2ω. The positions of the LEM motif (LEM), lamin binding domain (lamin binding), transmembrane domain (TM), and the position of individual amino acids are marked for each isoform. The insertion sites of the β- and ω-specific sequences are marked by lines.

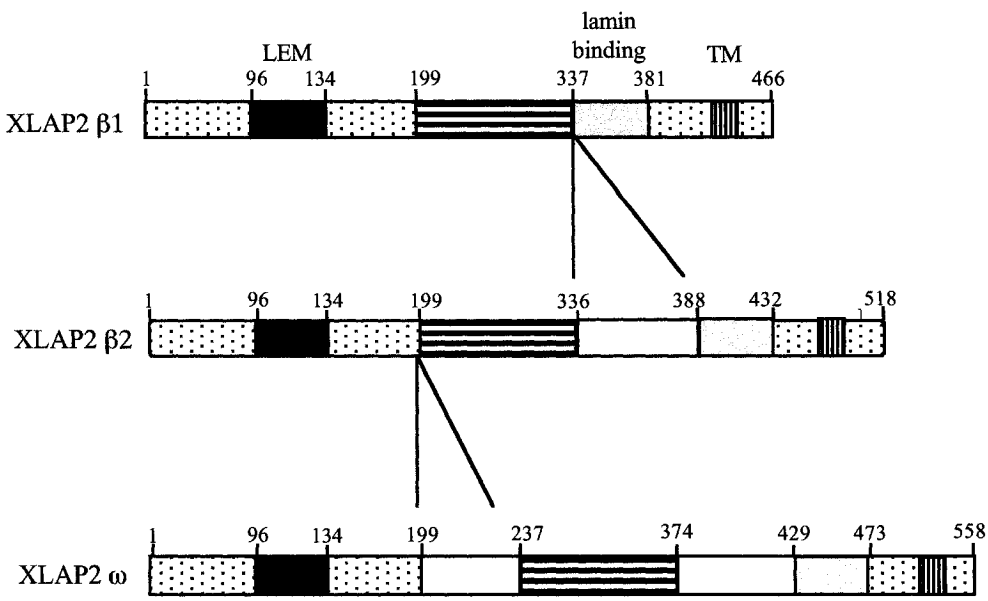
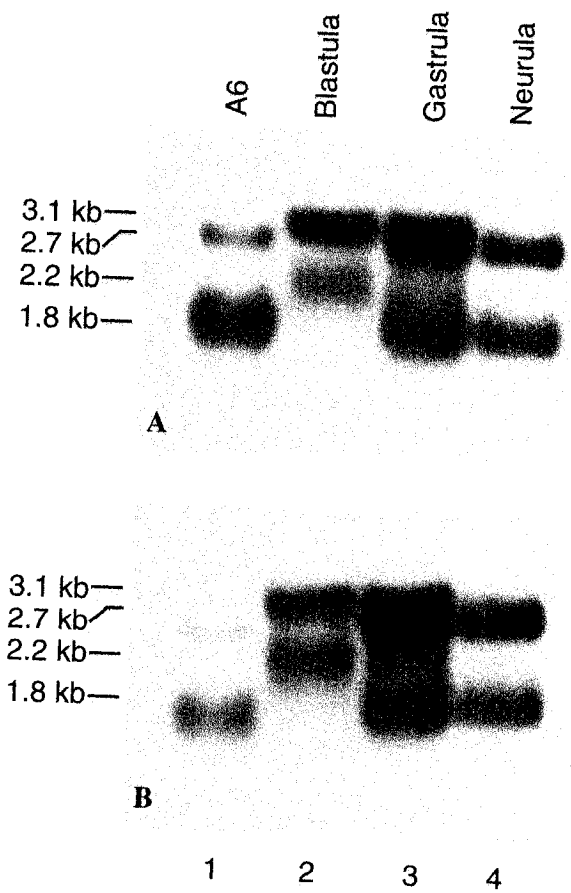

lamin
binding
LEM
TM
1
96
134
199
337
381
466
XLAP2 β1
1
96
134
199
336
388
432
518
XLAP2 β2
1
96
134
199
237
374
429
473
558
XLAP2 ω

Figure 11. Northern blot analysis of *Xenopus* LAP2 transcript expression. Total RNA of *Xenopus* A6 cells (kidney epithelial cells; lane 1) and embryos at the blastula (lane 2), gastrula (lane 3) and neurula (lane 4) was hybridized with *in vitro* synthesized ^{32}P-labelled probes made from DNA complementary to the XLAP2 mRNAs. (**A**) Membrane probed with the cDNA encoding for the entire sequence of the *Xenopus* LAP2β. (**B**) Membrane probed with the cDNA encoding for the entire sequence of the *Xenopus* LAP2ω. The sizes of the different hybridizing mRNA bands are marked.


A6
Blastula
Gastrula
Neurula
3.1 kb
2.7 kb
2.2 kb
1.8 kb
A
3.1 kb
2.7 kb
2.2 kb
1.8 kb
B
1
2
3
4

gastrula stages, another band of 3.1 kb is detected (Fig. 11 A and B, lane 2 and 3). In embryos at the gastrula stage, this band is present together with the one of 2.7 kb (Fig. 11 A and B, lane 3). Based on the size of the transcript and the fact that it is only detected in early embryos until the gastrula stage, this transcript might correspond to a *Xenopus* LAP2ω isoform. This would suggest a similar expression pattern for the LAP2ω isoform in the zebrafish and *Xenopus*. In embryos at the blastula stage, a second band of 2.2 kb is detected (Fig 11 A and B, lane 2). Concerning this band, possible explanations would be that it corresponds to a degraded form of the 3.1 kb transcript or to another uncharacterized XLAP2 isoform.

ZLAP2β and γ possess properties characteristic of integral membrane proteins

To examine the solubility properties of ZLAP2 proteins expressed in cultured cells, AB9 (somatic cell line) and ZF4 (embryonic cell line) cells were subjected to protein extractions with high concentrations of urea, a chaotropic agent, or with the non-ionic detergent Triton X-100 and high salt solutions. Following extraction, the pellet and the soluble protein fractions were separated by SDS-PAGE and immunoblotted using the S1 serum. As mentioned above, two types of extraction/solubilization were performed. The first one is an extraction with urea, which solubilizes all proteins that are not inserted into membranes. Using this type of extraction, integral membrane proteins are recovered in the pellet fraction and the peripheral membrane proteins, such as lamins, are solubilized. In contrast, the second type of extraction, cells are extracted with a non-ionic detergent (Triton X-100) and high salt concentrations, which solubilize the membranes and proteins that are weakly bound to the nuclear lamina. In this case, it is the nuclear matrix (including the nuclear lamina) and the strongly associated proteins

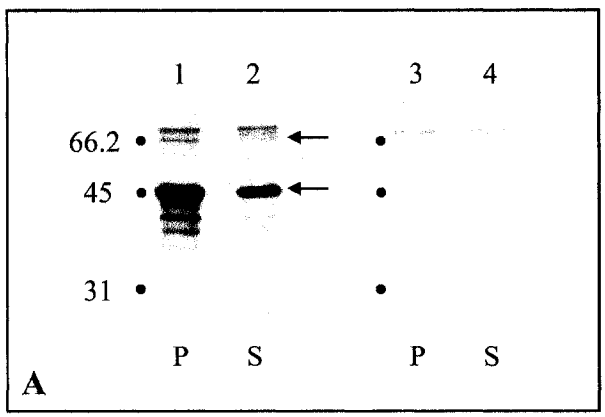
that are found in the pellet fraction. In summary, an inner nuclear membrane protein that is associated with the nuclear lamina is expected to be found in the pellet fraction after both types of extractions.

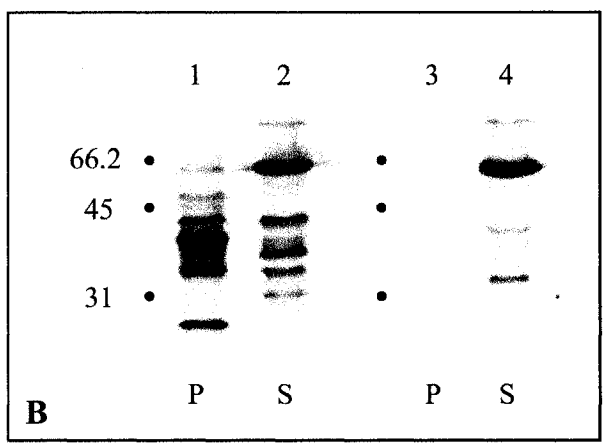
When AB9 cells are extracted with 8M urea, ZLAP2β and γ are mostly recovered in the membrane pellet fraction (Fig. 12A). Some proteins are also recovered in the soluble fraction possibly due to the high stringency of the extraction conditions. Moreover, when ZF4 cells are extracted with a buffered high salt solution containing 1% Triton X-100, ZLAP2β and γ are essentially found in the pellet fraction, but they are degraded (Fig. 12B). Only a small proportion of degraded proteins is recovered in the supernatant. Four major degradation bands with M_r lower than 45 000 are detected. Different protease inhibitors were added to the extraction solution, but they were not effective in blocking degradation. It seems that when integral proteins were extracted from membranes, they were more sensitive to the protease activities. Together, these results indicate that ZLAP2β and γ possess properties characteristic of integral membrane proteins and that they contain sequences mediating strong ionic/hydrophobic interactions with the nuclear matrix/lamina.

Furthermore, urea extraction experiments performed on *Xenopus* A6 cells expressing GFP-ZLAP2ω fusion proteins and on whole cellular membranes of zebrafish ovaries indicated that ZLAP2ω also possesses properties characteristic of integral membrane proteins (Schoft, Beauvais *et al.*, in press).

Distribution of ZLAP2β and γ proteins throughout the cell cycle of somatic cells

Figure 12. Extraction of ZLAP2β and γ proteins from zebrafish AB9 and ZF4 cells with urea and Triton X-100/high salts respectively. Proteins were separated by SDS-PAGE (12% acrylamide) and immunoblotted with either the ZLAP2-serum1 (**A** and **B**; lane 1-2) or the detection system only (**A** and **B**; lane 3-4). (**A**) AB9 cells extracted with 8M urea and fractionated by centrifugation into supernatant (S) and pellet (P). The positions of ZLAP2β (M_r 66 000) and ZLAP2γ (M_r 45 000) are marked by arrows. (**B**) ZF4 cells extracted with 1% Triton X-100/0.1M NaCl and fractionated by centrifugation into supernatant (S) and pellet (P). In both cases ZLAP2β and γ are mostly recovered in the pellet fraction. Molecular masses of reference proteins (in kDa) are marked in **A** and **B**.


1
2
3
4
66.2
45
31
P
S
P
S
A


1
2
3
4
66.2
45
31
P
S
P
S
B

To gain insights into the physiological role of the ZLAP2 proteins, I have investigated the distribution of these proteins throughout the different phases of the cell cycle in cultured somatic cells. Therefore, the S2, MAN and S1 sera were used in immunofluorescence experiments on the AB9 somatic cell line that expresses only the β and γ isoforms (see expression of ZLAP2 proteins in somatic cells).

Using the S2 serum, ZLAP2 proteins are found at the nuclear periphery in interphase (Fig. 13, a-c) and late prophase cells (Fig. 13, d-f). However, in late prophase, the nuclear envelope seems to be more irregular and some staining can also be observed in the nuclear interior. These involutions of the membrane might indicate the initiation of the nuclear envelope breakdown that occurs at prometaphase in mammalian cells. While progressing into mitosis, a cytoplasmic distribution becomes obvious in late prometaphase (Fig. 13, g-i) and is maintained in metaphase (Fig. 13, j-l) until the late anaphase stage. At this stage, ZLAP2 proteins are localized at the external periphery of the chromosomes (Fig. 13, m-o). In late telophase/ early G1, the staining is observed at the nuclear periphery of the reforming daughter cells (Fig. 13, p-r). At this stage, small vesicles surrounding the two daughter cell nuclei are also detected.

The same localization pattern was observed using the MAN serum (Fig. 14, a-r). However, when the S1 serum was used, almost no vesicles are observed in late telophase (Fig. 15, p-r). In addition, some chromosome labeling was detected at metaphase and anaphase stages, suggesting that the S1 serum might react, by immunofluorescence, with some LAP2 non-related antigens associated with the chromatin during mitosis (Fig.15, a-r). This was confirmed when immunolabelling experiments using affinity-purified

Figure 13. Immunofluorescent localization of ZLAP2β and γ proteins during mitosis in zebrafish AB9 cells. Cells were labeled with the ZLAP2-serum2 (**c, f, i, l, o, r**). Preparations were counterstained with Hoechst 33258 (**b, e, h, k, n, q**) to visualize DNA and observed by phase-contrast microscopy (**a, d, g, j, m, p**) to identify mitotic stages. The following mitotic stages are represented: interphase (**a-c**), prophase (**d-f**), late prometaphase (**g-i**), metaphase (**j-l**), late anaphase (**m-o**), telophase (**p-r**). The arrow in panel **o** indicates the staining at the external periphery of chromosomes and the arrow in panel **r** indicates vesicles surrounding the daughter cells nuclei. Bar represents 10μm.

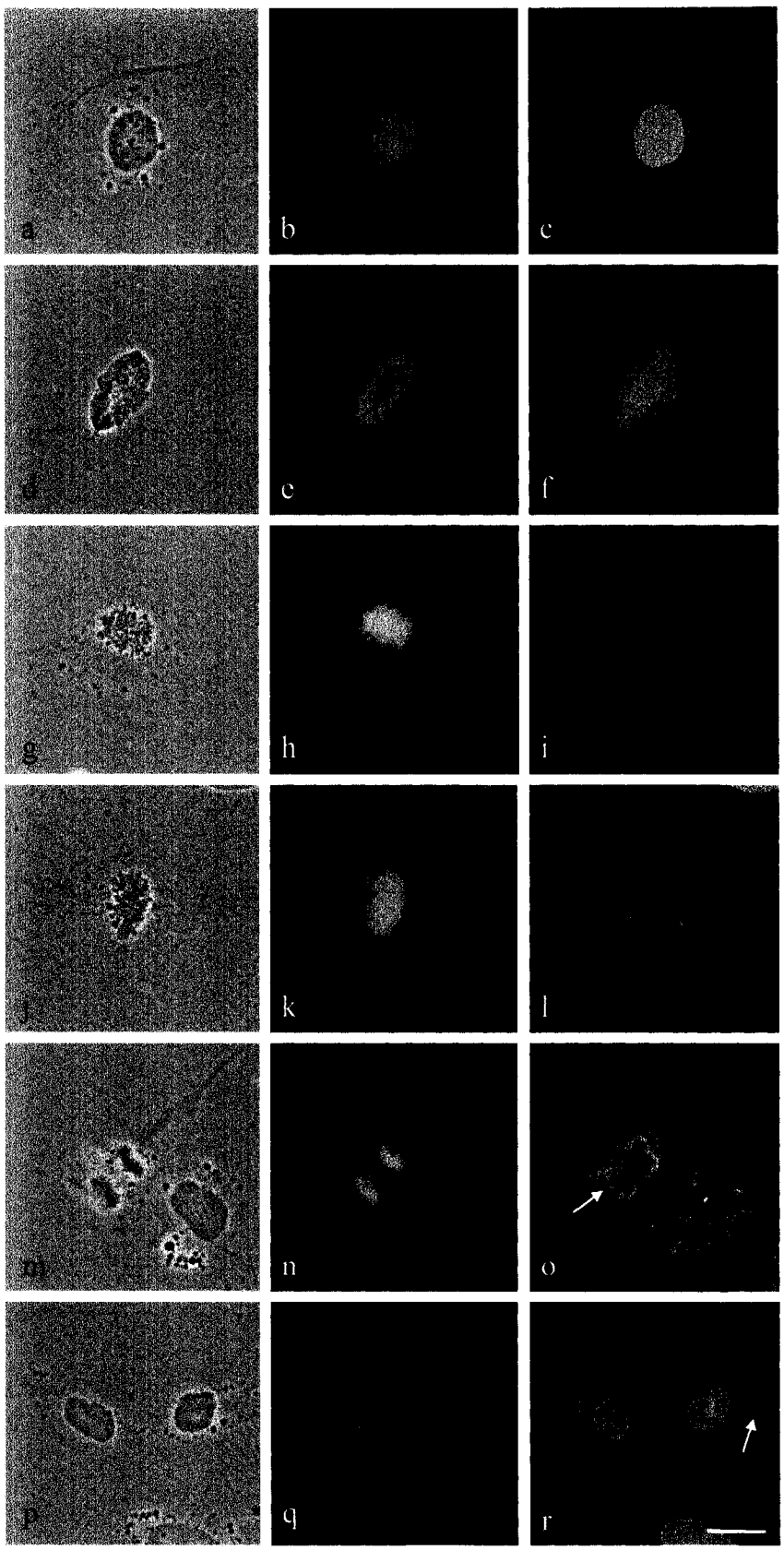

a
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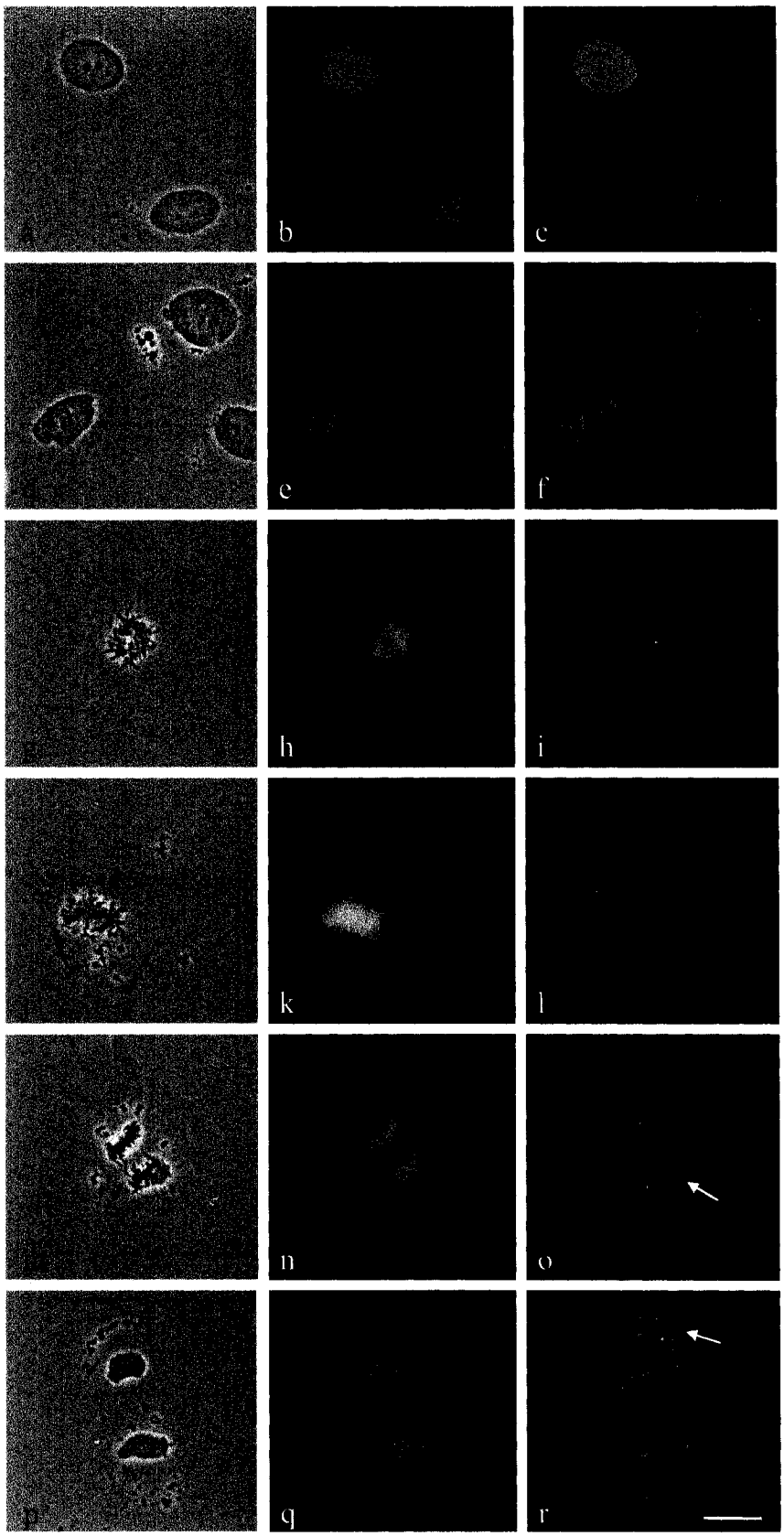
Figure 14. Immunofluorescent localization of ZLAP2β and γ proteins during mitosis in zebrafish AB9 cells. Cells were labeled with the MAN serum (**c, f, i, l, o, r**). Preparations were counterstained with Hoechst 33258 (**b, e, h, k, n, q**) to visualize DNA and observed by phase-contrast microscopy (**a, d, g, j, m, p**) to identify mitotic stages. The following mitotic stages are represented: interphase (**a-c**), prophase (**d-f**), late prometaphase (**g-i**), metaphase (**j-l**), late anaphase (**m-o**), telophase (**p-r**). The arrow in panel **o** indicates the staining at the external periphery of chromosomes and the arrow in panel **r** indicates vesicles surrounding the daughter cells nuclei. Bar represents 10 μm.


b
e
e
f
h
i
k
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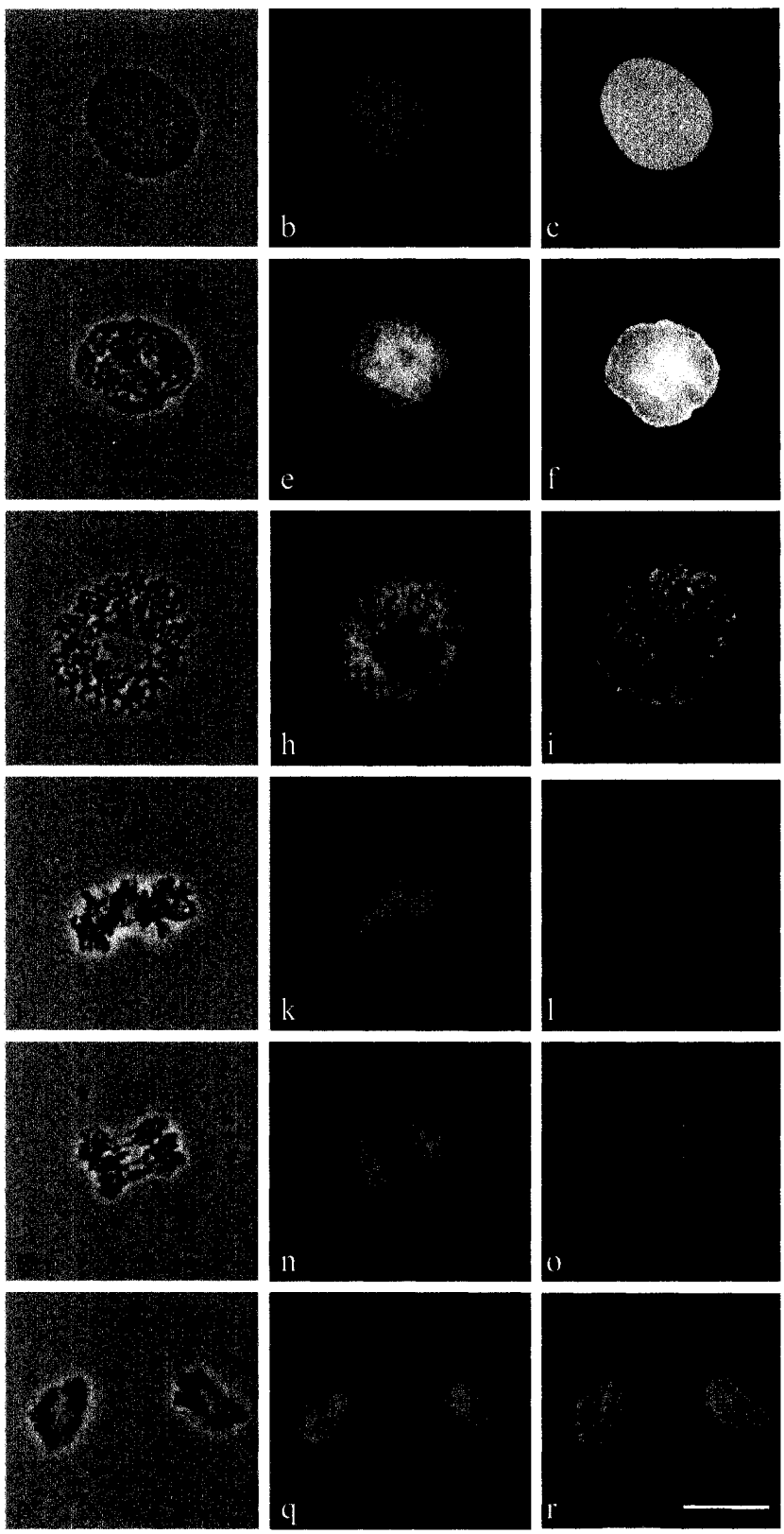
n
o
q
r

Figure 15. Immunofluorescent localization of ZLAP2β and γ proteins during mitosis in zebrafish AB9 cells. Cells were labeled with the ZLAP2-serum1 (**c, f, i, l, o, r**). Preparations were counterstained with Hoechst 33258 (**b, e, h, k, n, q**) to visualize DNA and observed by phase-contrast microscopy (**a, d, g, j, m, p**) to identify mitotic stages. The following mitotic stages are represented: interphase (**a-c**), prophase (**d-f**), late prometaphase (**g-i**), metaphase (**j-l**), late anaphase (**m-o**), telophase (**p-r**). Bar represents 10 μm.


b
c
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i
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l
n
o
q
r

antibodies prepared from the S1 serum were performed: no chromosome labelling was observed during mitosis (Schoft, Beauvais *et al.*, in press).

Distribution of ZLAP2 proteins throughout the cell cycle of embryonic cells

To examine the distribution of ZLAP2 proteins throughout the cell cycle of embryonic cells, immunolabelling of whole embryos at 2, 4 and 24 hpf was performed using the S2 serum. Because the S1 serum cross-reacted by immunofluorescence with other LAP2 non-related proteins associated with the chromatin, it was not used in this series of experiments (see localization throughout the cell cycle of somatic cells).

In 2 and 4 hpf embryos, which only express the ZLAP2ω protein isoform, S2 staining shows that the ω protein is localized at the nuclear periphery during interphase (Fig. 16, a-b). Furthermore, some staining is observed in the nuclear interior of 2 hpf embryos. In mitotic cells of blastula embryos (2 hpf), ZLAP2ω is observed at the surface of the chromosomes in prometaphase, metaphase and anaphase, suggesting that this isoform interacts with the chromatin during early mitotic stages (Fig. 17, a-f). Moreover, in these young embryos, vesicles could be found in association with mitotic chromosomes. It seems that a nuclear envelope assembles around single or few chromosomes at telophase, prior to the formation of a common nuclear envelope enclosing all chromosomes in early G1 (Schoft, Beauvais *et al.*, in press). This phenomenon, termed karyomere formation, is observed at the blastula stage (Fig. 17, g-h). In 24 hpf embryos, where only ZLAP2β and γ proteins are present, staining is detected at the nuclear periphery during interphase (Fig. 16c). Due to the difficulty to observe mitotic figures at this stage, I was not able to determine if there is or not an interaction of these protein isoforms with chromosomes during mitosis. The synchrony

Figure 16. Distribution of ZLAP2 proteins during interphase in zebrafish embryos. Indirect immunofluorescence microscopy of ZLAP2 proteins in whole mount preparations of embryos at the early blastula stage (**a**; 2 hpf), late blastula stage (**b**; 4 hpf) and 24 hpf (**c**). Preparations were stained with the ZLAP2-serum2 and staining is localized at the nuclear periphery. At the blastula stage (**a** and **b**), the cells are only expressing the ZLAP2ω isoform whereas, at 24 hpf (**c**), the cells are expressing the ZLAP2β and γ isoforms. Digital images were taken with a Leica by CLSM in **b** and with a Zeiss Axiophot in **a** and **c**. Bars represents 10 μm.

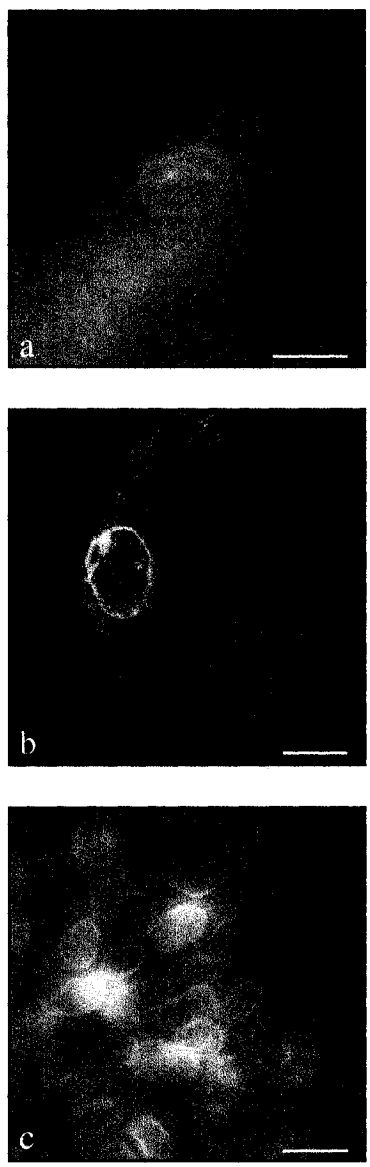
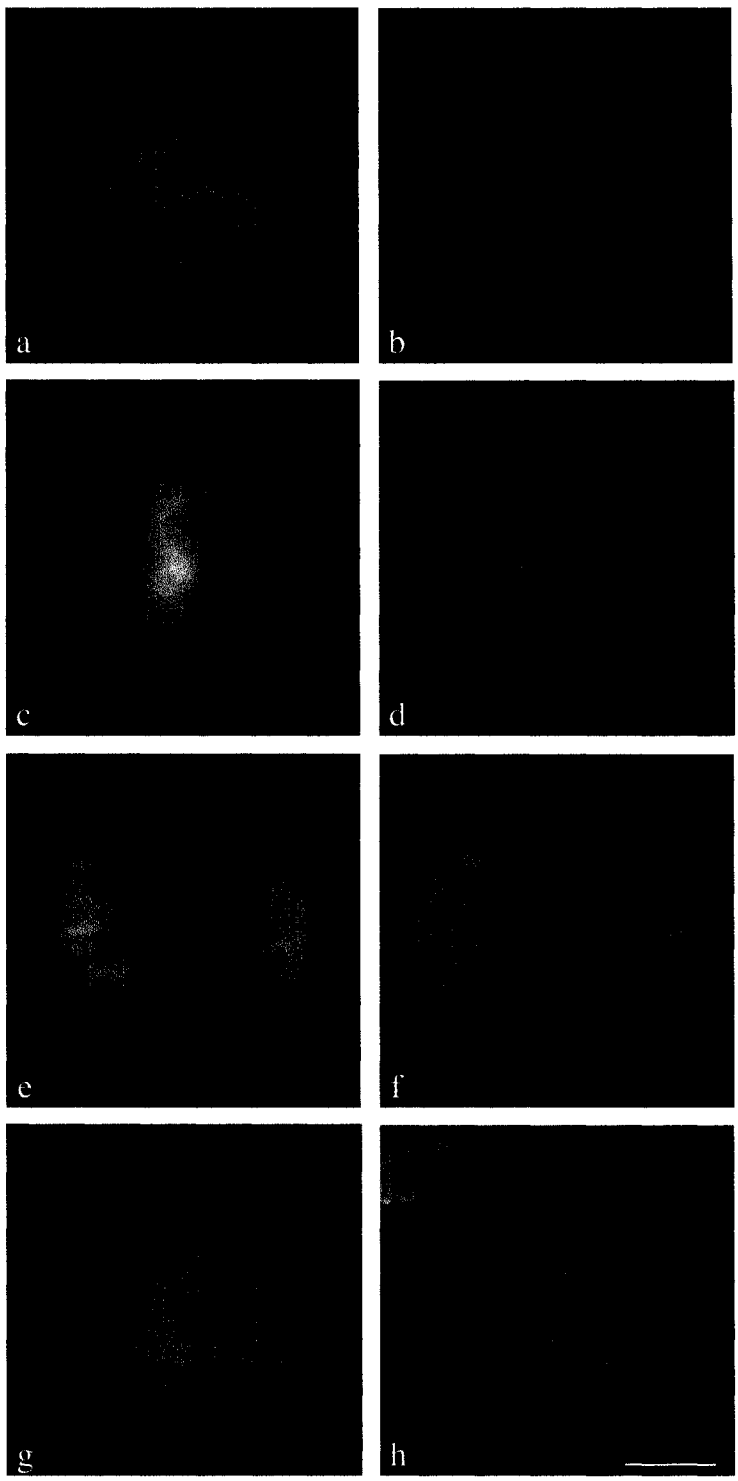

a
b
c

Figure 17. Distribution of ZLAP2 proteins in mitotic cells of zebrafish blastomeres. Indirect immunofluorescence microscopy of ZLAP2 proteins in whole mount preparations of embryos at the early blastula stage (**a-h**; 2 hpf). Preparations were stained with the ZLAP2-serum2 (**b, d, f, h**) and counterstained with Hoechst 33258 (**a, c, e, g**) to visualize DNA. Cells at the prometaphase (**a, b**), metaphase (**c, d**), anaphase (**e, f**) and at the karyomere stage (**g, h**) are shown. At the blastula stage, the cells are only expressing the ZLAP2ω isoform. Staining is observed at the surface of chromosomes during mitosis and surrounding individual chromosomes at the karyomere stage. Digital images were taken with a Zeiss Axiophot (**a-h**). Bar represents 5 μm.


a
b
c
d
e
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g
h

and the length of the cell cycle as well as the cell density in the embryos have made these observations difficult to obtain.

In situ hybridization on whole mount embryos was also attempted on several embryonic stages ranging from 3 to 24 hpf in order to determine the expression pattern of the ZLAP2 transcripts during development. Since the entire sequence of the ZLAP2γ transcript corresponding to the smaller isoform is contained within ZLAP2β and ω transcripts, I used the antisense RNA of ZLAP2γ as probe to detect the expression of all ZLAP2 transcripts. A second antisense RNA probe complementary to the specific region of ZLAP2ω transcript was also used. This probe was supposed to help me to distinguish between the expression pattern of the ω transcript and of the β and γ transcripts. However, after many different trials, I was not able to obtain any specific detection with the different probes. Labelling was observed all over the embryos at all developmental stages between 3 and 24 hpf. Similar results were obtained with sense probes. Therefore, I decided not to pursue this part of the project.

Mapping of the ZLAP2 DNA within the zebrafish genome

Mapping of the ZLAP2 gene was achieved using the LN54 radiation hybrid (RH) panel, which had been constructed by fusing irradiated zebrafish AB9 cells with mouse B78 cells (Hukriede *et al.*, 1999). To screen the panel, a PCR based-method was used. Primers were chosen in introns and specific coding sequences of the ZLPA2ω in order to avoid possible regions of similarity with mouse sequences. The choice of the primers was validated by the absence of PCR amplification products from the control mouse parental cell line DNA. All assays were performed twice.

By submitting the results to the RHMAPPER program, the ZLAP2 gene was

mapped on linkage group 4, 1.01 centiRays from the marker fe47b06 (LOD score: 14.8). In the LN54 panel, 1 centiRay equals 148 kilobases. According to the NCBI search, the marker fe47b06 seems to be similar to the sequence encoding the calcium-transporting ATPase plasma membrane, brain isoform 2 of human. Other close markers around this location are similar to human genes such as B-cell translocation genes 1 and 2 and a DNA sequence for type II cytoskeletal keratin. No relationship was drawn between the ZLAP2 gene and close markers.

Effects of ZLAP2 knock-down on the development of zebrafish embryos

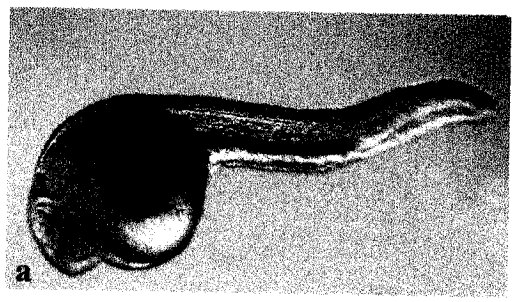
In order to test more directly the role of ZLAP2 proteins during zebrafish development, I used a relatively new technique employing antisense morpholinos (MO). Morpholinos are chemically modified oligonucleotides with similar base stacking abilities as natural genetic material, but have a morpholine moiety instead of a ribose (reviewed by Summerton and Weller, 1997). Morpholino oligos have been shown to be completely resistant to nucleases unlike DNA antisense oligos and S-DNAs which are more easily degraded. In addition, morpholinos have been shown to be effective in long term experiments and do not produce toxic degradation products (reviewed by Summerton and Weller, 1997). Antisense morpholinos are designed to bind at the start codon and block the mRNA translation of a specific gene. Moreover, antisense morpholinos have been shown to be effective and specific translational inhibitors in the zebrafish (Nasevicius and Ekker, 2000). Therefore, by using an antisense morpholino of 25 bp covering the translation initiation site of ZLAP2 transcripts (MOLAP2; Gene Tools), I could observe the effects of the knock-down of ZLAP2 expression on zebrafish development. A standard control (control MO) provided by Gene Tools, that does not

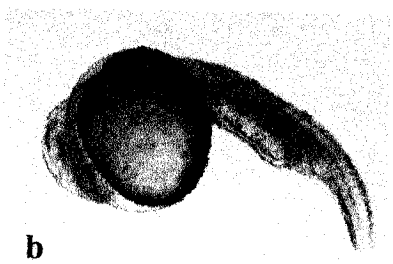
match the composition of the experimental oligo, was also used to test the microinjection system. Approximately 1 nl of morpholinos was microinjected in the yolk of embryos at the 1 to 8 cell stage. After microinjection, embryos were allowed to recover at 27°C and observed periodically for 24 h. Several microinjection series of the MOLAP2 and of the control MO at concentrations of 0.5 mM, 1 mM and 1.5 mM were performed.

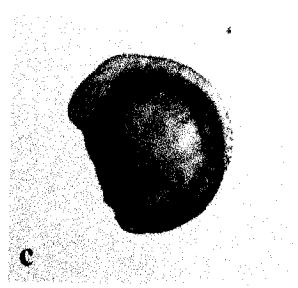
Following microinjection of 0.5 mM MOLAP2, 24 hpf zebrafish embryos displayed a normal phenotype (100%, n≅35) in each experiment (see normal phenotype, Fig. 18a). When these embryos were analyzed by Western blot (total proteins of 10 embryos/100 µl of SB 2x) using the S1 serum, various results were obtained. In some cases, it appears that the expression of the ZLAP2β isoform was almost entirely inhibited whereas the expression level of the ZLAP2γ isoform was almost the same as in the non-injected control embryos (Fig. 19A, lane 1 and 2). However, in other cases, only a really faint detection of both β and γ isoforms was obtained (Fig. 19A, lane 3). These results were obtained from two different microinjection experiments. Therefore, it is possible that the differences in the amounts of morpholino injected was the cause of these various results.

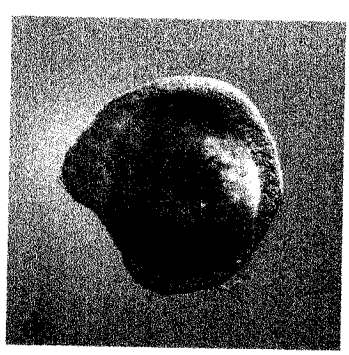
When embryos were microinjected with MOLAP2 at concentrations of 1 mM and 1.5 mM, approximately 40-50 % of the embryos at 24 hpf looked either delayed in development or were severely affected (Fig. 18, b-g). A mosaic of different types of abnormal phenotypes was observed. In some cases, embryos displayed a phenotype similar to non-injected embryos at 14 hpf or a few hours older. However, no investigation was made in order to determine if some structures were specifically affected. Embryos that were the most severely affected displayed a phenotype which,

Figure 18. Effects of ZLAP2 knock-down on the development of zebrafish embryos. Approximately 1 nl of an antisense morpholinos overlaping the translation initiation site of ZLAP2 transcripts was microinjected in the yolk of embryos at 1 to 8 cell stage. Morpholinos were used at concentrations of 0.5 mM, 1 mM and 1.5 mM. After 24h, embryos with a normal phenotype (**a**) and embryos delayed in development or severely affected (**b-g**; frequency: 40-50% with 1 mM and 1.5 mM, n=35) were observed. At a concentration of 0.5 mM all embryos displayed a normal phenotype at 24 hpf. Embryos microinjected with the same concentrations of a standard control morpholinos also displayed a normal phenotype.


a


b


c

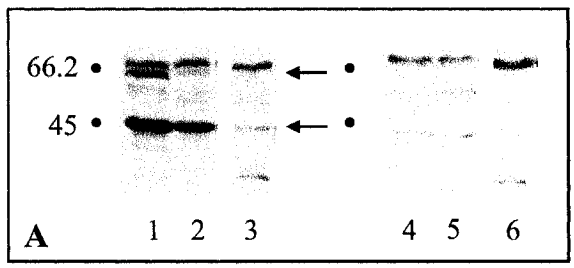



f

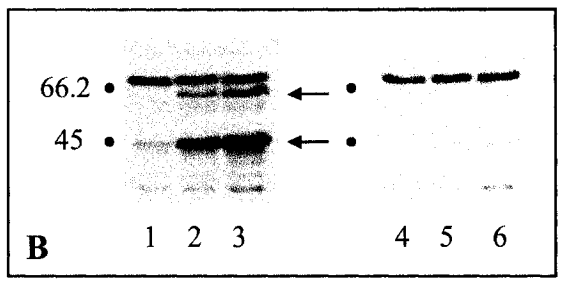

g

Figure 19. Expression of ZLAP2 protein isoforms in morpholino-injected embryos. Total proteins of 10 embryos was homogenized in 100 µl of SB 2X. 10 µl of homogenate was separated by SDS-PAGE (12% acrylamide) and immunoblotted with the ZLAP2-serum1 (**A** and **B**: lane 1-3; **C**: lane 1-4; **D**: lane 1-2) or the detection system only (**A** and **B**: lane 4-6; **C**: lane 5-8; **D** lane 3-4). (**A**) 24 hpf embryos injected with 0.5 mM of MOLAP2 that display a normal phenotype (lane 2-3 and 5-6) and control non-injected embryos (lane 1 and 4) were analyzed. In this case, different expression patterns were observed in normal embryos. (**B**) 24 hpf embryos injected with 1 mM of MOLAP2 that display an affected phenotype (lane 1 and 4) or a normal phenotype (lane 2 and 5) and control non-injected embryos (lane 3 and 6) were analyzed. (**C**) 24hpf embryos injected with 1.5 mM of MOLAP2 that display a normal phenotype (lane 2 and 6) or an affected phenotype (lane 3 and 7) were analyzed. Non-injected embryos (lane 1 and 5) and embryos injected with 1.5 mM of the control MO (lane 4 and 8) were also analyzed. (**D**) 6hpf embryos injected with 1 mM of MOLAP2 (lane 1 and 3) and control non-injected embryos (lane 2 and 4) were analyzed. The positions of ZLAP2β (M_r 66 000) and ZLAP2γ (M_r 45 000) are marked by arrows in **A-C** and the position of ZLAP2ω (M_r 84 000) is marked by an arrow in **D**. Molecular masses of reference proteins (in kDa) are marked in **A-D**.

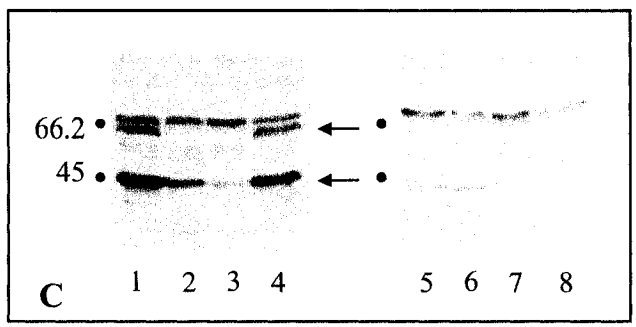
0.5 mM



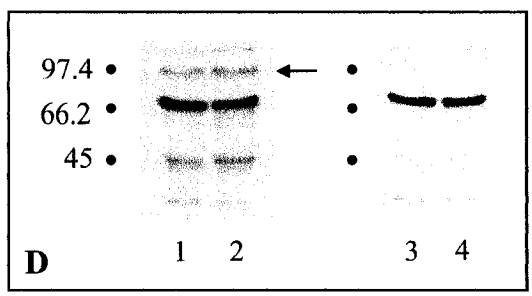
1 mM



1.5 mM



1 mM
6hpf



although it permits to distinguish the head from the tail, looked dramatically underdeveloped. In some rare cases, embryos seemed to develop more normally, but displayed a malformation of the head. All these phenotypes were only investigated by light microscopy observations prior to homogenization for western blot analysis. Therefore, a more extensive evaluation of the different phenotypes will be necessary in order to determine in detail the events leading to these developmental problems.

When performing microinjections with MOLAP2 at a concentration of 1 mM, I obtained some 24 hpf morphants that exhibited abnormal phenotypes and some normal embryos. Embryos injected with the same concentration of the control MO did not display any abnormal phenotype. Using the S1 serum in Western blot analysis, almost no expression of ZLAP2β and γ isoforms was detected amongst total protein extracts prepared from pools of 10 embryos injected with 1 mM MOLAP2 that were developmentally retarded or severely affected at 24 hpf (Fig. 19B, lane 1). In contrast, when protein extracts from embryos displaying a normal phenotype at 24 hpf after microinjection of 1 mM MOLAP2 were analysed, the ZLAP2γ isoform was detected almost at the same intensity as in non-injected embryos whereas the ZLAP2β protein was expressed at a lower level than in the control (Fig. 19B, lanes 2 and 3).

When performing microinjections with MOLAP2 at a concentration of 1.5 mM, again some morphants displayed abnormal phenotypes at 24 hpf and the rest of the embryos was normal. Embryos injected with the control MO at the same concentration did not display any abnormal phenotype. By western blot analysis (total proteins of 10 embryos/100 µl of SB 2x) using the S1 serum, MOLAP2-microinjected embryos with a normal phenotype presented an altered expression of the ZLAP2β and γ protein isoforms

(Fig. 19C, lane 2) comparable to what had been observed following MOLAP2 injection at a concentration of 0.5 mM. In these embryos, no expression of the ZLAP2β proteins was detected and the ZLAP2γ isoform showed a reduced expression compared to embryos injected with the control MO and non-injected embryos (Fig. 19C, lane 1, 2 and 4). However, in embryos that displayed abnormal phenotypes after the microinjection of 1.5 mM MOLAP2, practically no expression of the ZLAP2β and γ proteins was detected (Fig. 19C, lane 3).

In summary, independently of the MOLAP2 concentration used, almost no expression of the ZLAP2β and γ proteins was detected in embryos with an abnormal phenotype at 24 hpf. In microinjected embryos showing a normal phenotype, expression of the ZLAP2β protein was either not detected or reduced and expression of the ZLAP2γ protein was either almost at the same level as the controls or reduced. In all cases, in embryos injected with the control-MO, the expression of ZLAP2β and γ isoforms was detected approximately at the same level as in non-injected embryos (see Fig. 19C, lane 1 and 4). From these experiments, it appears that there is probably a strong correlation between the knock-down of the ZLAP2 expression and and the developmental problems observed.

In order to determine if the microinjection of the MOLAP2 had an effect on the expression of the ZLAP2ω isoform, I have also analysed zebrafish embryos at early developmental stages. No specific effect was observed in the first 8h of development following the injection of the MOLAP2 at a concentration of 1 mM. At 6 hpf, non-injected embryos do not express the ZLAP2β and γ isoforms but only the ZLAP2ω

protein which is detected at a low level (Fig. 19D). Western blot analysis on proteins from pools of 10 MOLAP2-injected embryos (6 hpf) showed that the band corresponding to the ZLAP2ω protein isoform was detected at the same level as in non-injected embryos (Fig. 19D). It appears that the microinjection of the MOLAP2 at a concentration of 1mM had no detectable effect on the expression of the ZLAP2ω isoform, suggesting that this isoform has been maternally deposited in the embryo.

DISCUSSION

Based on the work presented here, I conclude that there are three major LAP2 isoforms (ZLAP2β, γ and ω) in the zebrafish. ZLAP2β and γ are the orthologues of the mammalian LAP2β and γ. However, ZLAP2ω corresponds to a novel LAP2 polypeptide that is expressed in oocytes and during early embryonic development of zebrafish (Schoft, Beauvais *et al.*, in press). ZLAP2ω is the largest of the three isoforms detected in zebrafish and it was shown to possess properties distinct from that of ZLAP2γ, the major zebrafish LAP2 protein (Schoft, Beauvais *et al.*, in press). As this novel isoform was being identified and characterized, evidence did cumulate that LAP2ω-related polypeptides might be present in other organisms. In *Xenopus*, a LAP2-related protein of M_r 84 000 had been previously detected by Western blot in oocytes and during early embryonic development (Lang *et al.*, 1999), and was later shown to possess molecular properties in common with ZLAP2ω (Schoft, Beauvais *et al.*, in press). Moreover, it seems that an oocyte-specific LAP2-related polypeptide might also be present in oocytes and early embryos of a number of fish and frogs (Del-Pino *et al.*, 2002). However, this remains to be further assessed at the molecular level. Up to now, no oocyte-specific isoform has been detected in mammals. Only LAP2α, β and γ isoforms have been found in the mouse ovary (Del-Pino *et al.*, 2002). Homologues to mammalian LAP2α have not been detected in zebrafish. It is interesting to note that homologues to the mammalian LAP2α are also apparently absent from somatic cells of *Xenopus* (Lang *et al.*, 1999) and

other fish and amphibians (Del-Pino *et al.*, 2002). Since we now know that there might be an oocyte-specific isoform in fish and frogs, it would be interesting to see if such a LAP2 isoform is also present in mammalian oocytes.

Expression of ZLAP2 proteins during embryonic development

Immunoblots of proteins isolated from embryonic extracts at early developmental stages (4-8 cell-stage to 7.5 hpf) demonstrate that zebrafish possesses an oocyte-specific LAP2 isoform of M_r 84 000 designated omega (ω) (Schoft, Beauvais *et al.*, in press). The presence of the oocyte-specific isoform ZLAP2ω as early as in 8 cell-stage embryos suggests that this protein might be synthesized from maternally stored mRNA and/or that the maternal protein is deposited in the unfertilized egg. Amphibian oocytes are known to stock large amounts of maternal RNA and proteins during the long phase of oogenesis maturation (Hausen *et al.*, 1985). The oogenesis and the early development of the zebrafish which comprises very rapid cell cycles are similar to those of amphibians (Kane, 1999). As zebrafish embryonic development progresses into gastrulation, a change in ZLAP2 protein expression from the oocyte-specific isoform to the somatic ZLAP2β and γ isoforms occurs. Interestingly, the replacement of LAP2 expression from the oocyte-specific to the somatic isoforms was also observed during gastrulation in *Xenopus* (Lang *et al.*, 1999), in some other types of frogs (*Gastrotheca riobambae* and *Colostethus machalilla*) and the viviparous fish *Priapichtys panamensis* (Del Pino *et al.*, 2002). These results suggest a specific involvement of the ZLAP2 ω isoform in early

development of the zebrafish. This protein might fulfill specific functions needed in the early embryos that cannot be accomplished by ZLAP2β and γ.

Recently, a germ cell-specific lamin LIII (B3) was found to be expressed in zebrafish oocytes (Hofemeister *et al.*, 2002). The lamin LIII, which is also present in *Xenopus* and the goldfish, represents the major lamina constituent in oocytes and early embryos of these organisms (Stick and Krohne, 1982; Yamaguchi *et al.*, 2001). Homologues of lamin LIII have not been identified in birds and mammals and there is no evidence for an additional lamin gene in these species (Hesse *et al.*, 2001). This new lamin was also suggested to represent an ancestral form of vertebrate lamins and to serve a specialized function in fish and amphibians that would be absent in other vertebrate classes (Hofemeister *et al.*, 2002). Since ZLAP2ω also seems to be expressed only in oocytes and early embryos, it would be relevant in future studies to investigate whether there are some direct or indirect interactions between ZLAP2ω and the lamin LIII in zebrafish embryos during early development.

For the analysis of ZLAP2 proteins during the embryonic development, I have used three different sera: S1, S2 and MAN. These sera, which in theory recognize all three protein isoforms, did not show the same levels of detection. It seems that the S1 serum is better at detecting ZLAP2β and γ than the S2 serum. On the other hand, the S2 serum is better at detecting the ZALP2ω protein than the S1 serum. Since both guinea pig sera are polyclonal and were produced in two different animals, a possible

explanation would be that each of the two sera contains antibodies with a different predominance in epitope specificity. Unexpectedly, the MAN serum only detected the ZLAP2γ protein isoform during embryogenesis. As shown previously, the ZLAP2β and γ proteins were detected by the MAN serum in zebrafish somatic cells (Fig.7A). Moreover, since the MAN serum is directed against the LEM domain, it should be able theoretically to recognize all three protein isoforms. Further investigations will be necessary in order to determine what did happen in that case.

Expression of ZLAP2 proteins in somatic cells

In this study, I have demonstrated that the expression pattern of LAP2 isoforms in zebrafish somatic cells differs from those observed in *Xenopus* and mammals. LAP2β and γ proteins are the only isoforms expressed in somatic cells of zebrafish. It appears however that the expression of ZLAP2β is not ubiquitous amongst all adult tissues tested, but was observed only in liver and gills. In other fishes, LAP2β and γ have also been shown to be the major isoforms of somatic cells and were found at varying levels in different tissues and species (Del-Pino *et al.*, 2002). For example, LAP2γ is the only isoform detected in the liver, heart and spleen of the oviparous fish *Moenkhausia oligolepis* whereas LAP2β is the major isoform found in the gills, brain and heart cells of the rainbow trout (Del-Pino *et al.*, 2002). In contrast, somatic cells of mammals express three isoforms: LAP2α, β and γ (Harris *et al.*, 1994; Alsheimer *et al.*, 1998).

Furthermore, in *Xenopus* and other amphibians the predominant isoform of somatic cells is LAP2β (Lang *et al.*, 1999; Del-Pino *et al.*, 2002).

As mentioned above, LAP2γ would be the predominant isoform in somatic cells of zebrafish and most other fish. It would also seem that the presence of three major somatic LAP2 isoforms is characteristic to mammals. It was previously proposed that LAP2γ may represent an ancestral LAP2 isoform, as it is predominant among fish, and that LAP2β and α could have derived from LAP2γ (Del-Pino *et al.*, 2002).

Using the MAN serum, another polypeptide of Mr ~25 000 was detected in the zebrafish AB9 and ZF4 cell lines as well as in the gills of adult zebrafish. Based on the molecular weight of this polypeptide, I first thought that it might be a zebrafish form of emerin. However, if this was the case, one would expect to detect it in the other tissues tested. In mammals, emerin has been found in all tissue types studied (Nagano *et al.*, 1996; Manilal *et al.*, 1996). Emerin has also been detected in the nuclear envelope of various vertebrate cell lines from human to *Xenopus* (Dabauvalle *et al.*, 1999). If the MAN serum detected emerin in the zebrafish, I would expect to find it at least in muscle tissues, since its mislocalization in this tissue is the cause of a neuromuscular disease, the Emery-Dreifuss muscular dystrophy. Therefore, without excluding this possibility, it appears most likely that I was detecting another small polypeptide immunologically related to LEM proteins. Moreover, considering that ZLAP2 isoforms arise from

alternative splicing and the structure of the gene (see Appendix 1), I cannot exclude the possibility that I am detecting other splice variants of the ZLAP2 gene.

Expression of LAP2 transcripts during embryonic development of the zebrafish and *Xenopus*

At the RNA level, my Northern blot analysis indicates that the transcript coding for ZLAP2ω is present in early developmental stages from 8 cell-stage to 7.5 hpf, supporting the possibility that ZLAP2ω proteins come from maternally stored mRNA. Zebrafish RNAs detected before the midblastula transition (~3hpf) are directly coming from the mother. It is at this stage of the development (midblastula transition) that the zygotic genome is transcriptionally activated (Kimmel *et al.*, 1995). Approximately the same amount of ZLAP2ω transcripts is detected in embryos until 7.5 hpf, where a diminution is observed. Therefore, based on the observed expression pattern of ZLAP2ω transcript, it is possible that there is no RNA synthesis from the zygotic genome for ZLAP2ω. Since ZLAP2ω is the only isoform detected in early developmental stages, these results indicate that there is enough maternal ω-mRNA for the embryos to survive during the first hours of development. Pass 7.5 hpf, zebrafish embryos do not seem to require ZLAP2ω RNA anymore. Therefore, I suggest that the RNA of 2.6kb detected with the ZLAP2γ probe might correspond to a degraded form of ZLAP2ω, since this RNA species is first observed at the period when the amount of ZLAP2ω decreases. The

possibility that the 2.6 kb band might also correspond to another LAP2-related polypeptide (splice variant) cannot be excluded (see Appendix 1).

The ZLAP2γ probe also hybridized to 3 other bands in older embryonic stages (7.5 to 48 hpf). The larger transcript (3.5 kb) may correspond to an unprocessed pre-mRNA that contains the ZLAP2 sequences. As this 3.5 kb transcript is synthesized (~7.5 hpf), two mRNA transcripts of 1.9 and 1.6 kb that correspond in size to ZLAP2β and γ are detected. Since there is no such large band detected at earlier developmental stages, the existence of an unprocessed pre-mRNA would also be consistent with the suggestion that there might be no ZLAP2ω mRNA synthesized by the embryos. Interestingly, it appears that there is a larger amount of transcripts detected for ZLAP2β than γ. However, at the protein level, ZLAP2γ appears to be the predominant isoform. A possible explanation would be that, since there is more ZLAP2γ protein, their mRNAs might be translated faster or more efficiently than ZLAP2β mRNAs.

In Northern blot experiments that I carried out with a *Xenopus* somatic cell line (A6) and early embryos (blastula, gastrula and neurula stages), four LAP2 transcripts of 3.1, 2.7, 2.2 and 1.8 kb were detected. In 1999, Lang and collaborators also performed a Northern blot analysis on a *Xenopus* somatic cell line (A6) and embryos (egg, gastrula and neurula stages). Based on their results, they identified a transcript of 1.8-2.0 kb in embryos of the gastrula and neurula stages and in the somatic cell line (A6), that was corresponding to XLAP2β (Lang *et al.*, 1999). Moreover, two other transcripts of 2.8-3.0

kb and 0.9-1.1 kb were detected in eggs and embryos of the gastrula and neurula stages. These transcripts were not identified. However, the 2.8-3.0 kb transcript has been reported to be large enough to code for the polypeptide of 84 000 Da that was detected at the protein level (Lang *et al.*, 1999). As previously mentioned, the polypeptide of M_r 84 000 detected in *Xenopus* oocytes possesses several properties in common with ZLAP2ω (Schoft, Beauvais *et al.*, in press). Therefore, it is likely that the 3.1 kb transcript that I detected in early embryos and the 2.8-3.0 kb transcript reported by Lang and collaborators (1999) corresponds to a *Xenopus* LAP2ω transcript. My Northern blot analysis on *Xenopus* also indicated that two other transcripts of 2.7 and 1.8 kb were present in the somatic cell line (A6) and embryos of the gastrula and the neurula stages. Based on the results obtained by Lang and collaborators (1999), the transcript of 1.8 kb probably corresponds to a *Xenopus* LAP2β transcript. Concerning the third transcript of 2.7 kb, based on its size and since there is no evidence of the presence of a γ isoform in *Xenopus*, I suggest that this transcript encodes for another LAP2β isoform. A previous study suggested the presence of three isoforms homologous to XLAP2β in *Xenopus* oocytes (Gant *et al.*, 1999). However, I cannot exclude the possibility that this transcript might correspond to a γ isoform or to another uncharacterized XLAP2 isoform. Moreover, further investigations will be necessary in order to determine the nature of the 2.2 kb transcript detected in embryos at the blastula stage. The 2.7 kb transcript that I detected in the A6 cell line and embryos in gastrula and neurula was not apparent on the

Northern blot presented by Lang *et al.* (1999). Nevertheless, this group detected a smaller transcript of 0.9-1.1 kb in eggs and early embryos that was not present in our samples. These discrepancies in data open the door for more investigation in order to determine exactly how many LAP2 isoforms are present in *Xenopus* somatic cells and early embryos.

By comparing my Northern blot data performed in zebrafish and *Xenopus*, it appears that the expression pattern of LAP2 transcripts is similar in both organisms. First, the LAP2ω transcript is the only RNA species expressed in early embryos until the gastrula stage. Second, somatic LAP2 transcripts start to be expressed at the gastrula stage.

ZLAP2β and γ possess properties characteristic of integral membrane proteins

According to the results of my extraction experiments, ZLAP2β and γ proteins mostly remain in the pellet fraction after treatment of zebrafish cells with urea or Triton X-100/high salts. In mammals, LAP2β was also shown to be retained in the membrane pellet fraction after urea extraction and to remain associated with the insoluble nuclear lamina after solubilization/extraction with Triton X-100/high salts (Foisner and Gerace, 1993). Based on these data, mammalian LAP2β was classified as an integral membrane protein that displays a close association with the nuclear lamina (Foisner and Gerace, 1993).

In this study, the observation that ZLAP2β and γ remain in the pellet fraction after extracting peripheral membrane proteins (urea extraction) indicates that ZLAP2β and γ must be integral membrane proteins. Moreover, the fact that ZLAP2β and γ are retained in the pellet fraction after solubilizing the membranes and stripping the proteins weakly bound to the nuclear lamina (Triton X-100/high salts) indicates that ZLAP2β and γ strongly interact with the nuclear matrix/lamina. The fact that small amounts of both proteins are recovered in the soluble fraction after urea extractions might be due to the high stringency (8M urea) of the treatment. In the case of the Triton X-100/high salts permeabilization/extraction, the ZLAP2 polypeptides recovered in the soluble fraction might be residual fragments of degraded proteins that were not pelleted.

In mammals, LAP2 has been shown to bind to B-type lamins (Foisner and Gerace, 1993). Since ZLAP2β and γ possess a lamin-binding domain (Schoft, Beauvais *et al.*, in press), I cannot exclude the possibility that these proteins interact with the zebrafish B1 and B2-type lamins, which have been recently identified (*Danio rerio* lamin B1: unpublished, accession number AJ005936; *Danio rerio* lamin B2: Erber *et al.*, 1999). B-type lamins are present in somatic cells of *Xenopus* (Krohne *et al.*, 1981), mammals and birds (Lehner *et al.*, 1987; Stewart and Burke, 1987). Recently, a clone containing the complete coding sequence of the zebrafish lamin A was isolated from a cDNA library from three-day-old embryos, corresponding to the first lamin A characterized at the molecular level in fish (Hofemeister *et al.*, 2002). Further experiments on possible

interactions between ZLAP2 proteins, zebrafish lamins and other putative binding partners may provide insights into the function of LAP2 proteins.

Distribution of ZLAP2β and γ proteins throughout the cell cycle of somatic cells

Immunofluorescence microscopy on zebrafish AB9 cells demonstrates that ZLAP2β and γ are localized at the nuclear periphery during interphase. This observation is consistent with the previous idea that these two isoforms are integral membrane proteins of the nuclear envelope interacting with the nuclear lamina. In late prophase, the staining is also observed at the nuclear periphery. As the cells progress into mitosis, the β and γ LAP2 proteins relocalize to the chromosome surface in late anaphase and telophase after a cytoplasmic distribution in late prometaphase and metaphase. In mammals, the same localization pattern was observed for LAP2β during mitosis (Foisner and Gerace, 1993).

During late telophase/early G1 in AB9 cells, small vesicles surrounding the daughter cell nuclei were observed when the S2 and the MAN sera were used. It is generally assumed that, during mitosis, the nuclear envelope breaks down through the vesicularization of the nuclear membranes and the disassembly of the lamina as the mitotic spindle forms (Gerace and Blobel, 1980; Gant and Wilson, 1997). Therefore, the vesicles detected during late telophase in AB9 cells might indicate that a fraction of ZLAP2β and γ proteins were still in membrane vesicles and that the nuclear envelope was not completely reformed. Since chromosomes first become enveloped with

membranes at the same time (late anaphase) in mammals (Robbins and Gonatas, 1964), it is possible that ZLAP2β and γ isoforms are involved in the targeting of membranes to the chromosome surface at the end of mitosis, as it was suggested for mammalian lamina-associated proteins (Foisner and Gerace, 1993; Yang *et al.*, 1997). Previous studies have also suggested a role for LAP2 isoforms in nuclear envelope assembly and disassembly. In 1993, Foisner and Gerace have suggested that the LAP2β binding to lamins and chromatin is inhibited by mitotic phosphorylation (Foisner and Gerace, 1993). Furthermore, recent studies have shown that, in mammalian cells, lamins become associated with the peripheral regions of chromosomes during late anaphase to mid-telophase (Moir *et al.*, 2000; Lopez-Soler *et al.*, 2001). It was suggested that lamin oligomers formed during the early stages of nuclear assembly could mediate the associations with chromatin- and membrane-associated LAPs (Lopez-Soler *et al.*, 2001). Moreover, *in vitro* assembly assays have shown that various fragments of the LAP2β amino-terminal domain disrupt nuclear envelope assembly and chromatin expansion (Gant *et al.*, 1999). The lamin-binding domain of LAP2 has also been shown to inhibit the increase in nuclear volume during the cell cycle and the progression into S phase (Yang *et al.*, 1997). Together, all these data suggest that the LAP2 isoforms play a key role in nuclear envelope reformation.

Distribution of ZLAP2 proteins throughout the cell cycle of embryonic cells

Immunolabelling of embryos at early developmental stages (2 and 4 hpf) allowed me to determine the mitotic fate of ZLAP2ω during the embryonic cell cycle. As ZLAP2 β and γ in somatic cells, ZLAP2ω is present at the nuclear periphery during interphase. However, during mitosis, ZLAP2ω presents a peculiar localization pattern as it is observed on the surface of metaphase and anaphase chromosomes. It is possible that this binding of ZLAP2ω to chromosomes during early mitotic stages might facilitate the reformation of the nuclear envelope during the very rapid cell divisions that characterize young embryos (Kimmel *et al.*, 1995). Moreover, in 2 hpf embryos, the observation of vesicle-like structures around the chromosomes suggests the formation of karyomeres at the end of mitosis. This phenomenon, described in Schoft, Beauvais *et al.* (in press) for the zebrafish, consists of the assembly of a nuclear envelope around individual chromosomes at the end of telophase. Moreover, the chromatin of these chromosomes was shown to be less condensed than that of chromosomes free of membranes (Schoft, Beauvais *et al.*, in press). It was suggested that ZLAP2ω might play a role in the binding of these nuclear envelope vesicles to chromosomes and therefore might help orchestrate the nuclear envelope reformation in young embryos (Schoft, Beauvais *et al.*, in press). The staining observed in the nuclear interior of interphase cells in 2 hpf embryos might be due to the fusion of vesicular membranes, in order to recreate a unique nuclear membrane.

The formation of karyomeres was also observed in *Xenopus* prior to the midblastula transition, when the cell cycles are very short (Montag *et al.*, 1988; Lemaitre *et al.*, 1998). Moreover, it had been previously shown that *Xenopus* embryos possess a maternally expressed LAP2-related integral membrane protein that has several properties in common with ZLAP2ω (Lang *et al.*, 1999). Lately, this protein was shown to exhibit a sequence identity of 97% with a previously published *Xenopus* LAP2 isoform (Gant *et al.*, 1999; accession number: AF048815). When the *Xenopus* and the zebrafish LAP2ω are compared, it appears that there is low sequence identity in the central part of both molecules. It was suggested that the differences might account for adaptations to different embryonic development and adulthood of fish and amphibians (Schoft, Beauvais *et al.*, in press). It would be of particular interest to develop strategies to determine if the presence of LAP2ω proteins in early embryonic stages of both species is related to karyomere formation.

As expected, ZLAP2β and γ which are expressed later in development localize at the nuclear periphery during interphase of embryonic cells. Since these cells are expressing the same isoforms as the somatic cells, I was expecting to observe the same localization pattern during mitosis. However, due to longer cell cycle in older embryos and to the difficulties to stain cells in dense tissues (whole squashed embryos), I was not able to observe any mitotic stage. I can only speculate that since ZLAP2β and γ do not

bind chromosomes before anaphase in somatic cells, they probably behave the same way in embryonic cells.

Mapping of ZLAP2 DNA within the zebrafish genome

By using the LN54 radiation hybrid panel, I was able to map the ZLAP2 gene on the linkage group 4 of the zebrafish genome. Few markers were identified around the ZLAP2 gene. However, they were not giving any interesting information. Further investigations on genomic sequences surrounding the ZLAP2 gene allowed us to identify another gene localized in 3' next to the ZLAP2 gene. Based on BLAST search, this gene appears to correspond to a phosphate carrier protein precursor. Moreover, after investigations on genomic data on the human and the mouse genome, I discovered that the same gene is also localized next to the LAP2 gene on the chromosome 12 in the human genome and 10 in the mouse genome. It seems that there is a conservation of the synteny between zebrafish, mouse and human for these genes.

Effects of ZLAP2 knock-down on the development of zebrafish embryos

By microinjection of an antisense morpholino (MOLAP2) against ZLAP2 isoforms in 1-8 cell stage embryos, I was able to observe the effects of inhibiting ZLAP2 translation on zebrafish embryogenesis. By injecting 1 nl of morpholinos at concentrations of 0.5, 1 and 1.5 mM, I obtained embryos that appeared to be delayed in development or severely affected whereas other embryos maintained a normal phenotype.

When MOLAP2-microinjected embryos with a normal phenotype at 24 hpf were analyzed for the presence of ZLAP2 protein isoforms by Western blot, various results were obtained. In some cases, the expression of the ZLAP2β isoform was drastically reduced or almost entirely inhibited, while the expression of ZLAP2γ was only slightly reduced or stayed almost at the same level as the controls. Since ZLAP2γ was the only LAP2 protein isoform present in these microinjected embryos, this suggests that the ZLAP2γ isoform can compensate for the loss of the ZLAP2β expression. It also appears that even when the level of expression of ZLAP2γ was reduced, there was still enough protein to maintain an apparently normal phenotype. ZLAP2γ appears to be highly expressed in zebrafish embryos, therefore it is difficult to completely inhibit its expression. The differences in the expression levels of ZLAP2β and γ in microinjected embryos with a normal phenotype might be due to variability in the effectiveness of microinjections. Moreover, since the samples were prepared by homogenizing 10 embryos together, the detection of a reduced expression for ZLAP2β in embryos microinjected with 1 nl of 1 mM of antisense morpholinos could result from pooling only one embryo with a normal expression while other embryos did not express the ZLAP2β isoform. In a few MOLAP2 microinjected embryos with a normal phenotype, almost no expression of the ZLAP2β and γ isoforms was detected. In these cases, the expression levels of both isoforms might have been too low to be detected, but sufficient for the embryos to display an apparent normal phenotype. However, I do not exclude the

possibility that these embryos might have some defects or abnormalities that did not lead to a detectable phenotype using light microscopy. Therefore, microscopic examination of sections from phenotypically normal embryos and embryos with developmental problems by different techniques such as immunostaining, *in situ* hybridization as well as a closer analysis of the embryonic structures would be necessary to further investigate the phenotypes of these embryos. On the other hand, in all cases where an abnormal phenotype was observed, the expression of ZLAP2β and γ was not detected, suggesting that the loss or significant reduction of the ZLAP2β and γ expression led to developmental problems. The expression of ZLAP2β isoform appears to be more affected by the morpholino injection since this protein is most often not detected. It might be due to the lower amount of ZLAP2β proteins present in embryos compared to ZLAP2γ. A drop in the expression of the ZLAP2β protein isoform might be more easily detectable than a reduction of the ZLAP2γ expression.

No modification of ZLAP2ω expression was detected by Western bloting of 6 hpf MOLAP2 injected embryos. Moreover, no special phenotype was observed in the early stages of development (1 cell to 8 hpf). These results support the hypothesis that there is probably a stock of maternal ZLAP2ω protein in the embryos which is large enough for the survival of the embryos during the first hours of development. Maternal mRNAs for ZLAP2ω are also present in embryos at early developmental stages (8 cell-stage to 7.5 hpf). However, if these mRNA are not translated because of the morpholino (MOLAP2),

there should be enough ZLAP2ω proteins to accomplish the normal functions of the embryo during early developmental stages.

Zebrafish embryos microinjected with MOLAP2 were not analyzed between 8 and 24 hpf. Therefore, I can only speculate on the stage at which embryos start to show developmental defects. Since ZLAP2β and γ transcripts are becoming detectable around 7.5 hpf and protein expression is monitored first around 10 hpf, this period may be critical in the action of MOLAP2. The lack of ZLAP2β and γ proteins probably led to the developmental problems observed in this study. Interestingly, some 24 hpf morphants displayed a developmental stage similar to that observed for ~14 hpf embryos, suggesting that a major event might have been perturbed around this stage. In *Xenopus*, a recombinant polypeptide that contains the chromatin-binding region of LAP2β was shown to enhance the efficiency of DNA replication (Gant *et al.*, 1999). Moreover, in mammalian cells, LAP2β was also shown to mediate transcriptional repression (Nili *et al.*, 2001). LAP2 proteins were shown to directly interact with chromatin via their chromatin binding domain (Foisner and Gerace, 1993, Furukawa *et al.*, 1997,1998) and via different binding partners such as: BAF (Furukawa, 1999), HA95 (Martin *et al.*, 2000) and mGCL (Nili *et al.*, 2001). Therefore, since LAP2 proteins appear to have multiple interactions with chromatin, it might be possible that ZLAP2 isoforms directly or indirectly affect the regulation of genes implicated in embryogenesis. In this case, the

lack or reduction of ZLAP2β and γ protein expression would have a major impact on the embryonic development.

It is also possible that the abnormal phenotype observed in 24 hpf embryos was due to mistargeting of the morpholinos. It has been shown that some morpholinos can successfully target the selected gene but can also display a mistargeting phenotype (Nasevicius and Ekker, 2000). It has been hypothesized that the mistargeting is most commonly caused by the simultaneous inactivation of an essential gene with sequence homology to the target gene. Mistargeting usually occurs at a rate of 15-20% (Ekker and Larson, 2001). However, in my experiments, the abnormal phenotype was usually observed at a rate of 40-50%. In addition, there is a strong correlation between the abnormal phenotypes and the loss of ZLAP2 protein expression. In order to be sure that the phenotypes observed are related to the specific inhibition of ZLAP2 mRNA translation, more experiments have to be done. Usually to conclude that a phenotype is related to the down regulation of the gene of interest, a second morpholino that targets a non-overlapping sequence of the same gene is used. In addition, RNA rescue which would consists in microinjecting synthetic sense LAP2 RNA at the same time as the morpholinos is another control that is often used. However, it is difficult to get a 100 % rescue and the overexpression of the protein might cause abnormal phenotypes. Finally, a control morpholino which consists of the same morpholino but with about 5 nucleotide mismatch might be a good control.

Altogether, my results indicate that ZLAP2 isoforms may play a significant role during zebrafish embryonic development. Further experiments with another morpholino targeting the ZLAP2 gene and other controls morpholinos will have to be done in order to better understand the implications of ZLAP2 isoforms during the development. Moreover, experiments implicating antisense morpholinos in cultured cells might provide interesting information about the functions of the ZLAP2 isoforms.

CONCLUSIONS

In summary, I have provided evidence that there are three major LAP2 isoforms found in the zebrafish : ZLAP2β, γ and ω. ZLAP2β and γ are somatic isoforms and ZLAP2ω is a maternally synthesized embryonic isoform. Of the three isoforms detected in zebrafish, ZLAP2γ appears to be the predominant isoform in somatic cells as well as during embryonic development. The analysis of the expression patterns of the three ZLAP2 isoforms revealed that these isoforms are regulated throughout development at the protein and RNA levels, as suggested in amphibia (Lang *et al.*, 1999). Moreover, the transition of ZLAP2 expression from the oocyte-specific to the somatic isoforms occurs at gastrulation, as demonstrated for *Xenopus* (Lang *et al.*, 1999) and in some other types of frogs and fish (Del-Pino *et al.*, 2002). I have also observed several similarities in the expression pattern of LAP2 transcripts of *Xenopus* and zebrafish during embryonic development. Furthermore, localization analysis suggested that ZLAP2 proteins play a key role in nuclear envelope reformation. Finally, the strong correlation between the abnormal phenotypes and the lost of ZLAP2 protein expression observed during the knock-down experiments, suggests that ZLAP2 isoforms may play a key role during zebrafish embryonic development.

REFERENCES

Alsheimer, M., Fecher, E. and Benavente, R. (1998) Nuclear envelope remodelling during rat spermiogenesis: distribution and expression pattern of LAP2/thymopoietins. J.Cell Sci. **111**: 2227-2234.

Amar-Costesec, A., Beaufay, H., Wibo, M., Thines-Sempoux, D., Feytmans, E., Robbi, M., and Berthet, J. (1974) Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. J.Cell Biol. **61**: 201-212.

Beck, L.A., Hosick, T.J., Sinensky, M. (1990) Isoprenylation is required for the processing of the lamin A precursor. J. Cell Biol. **110**: 1489-1499.

Berger, R., Theodor, L., Shoham, J., Gokkel, E., B.-Simoni, F., Arraham, K., Copeland, N., Jenkins, N., Rechavi, G. and Simon, A. (1996) The characterization and localization of the mouse thymopoietin/lamina-associated polypeptide 2 gene and its alternatively spliced products. Genome Res. **6**: 361-370.

Bione, S., Maestrini, E., Rivella, S., Mancini, M., Regis, S., Romeo, G. and Toniolo, D. (1994) Identification of a novel X-linked gene responsible for Emery-Dreifuss muscular dystrophy. Nature Genet. **8**: 323-327.

Bridger, J.M., Kill, I.R., O'Farrell, M., and Hutchison, C.J. (1993) Internal lamin structures within G1 nuclei of human dermal fibroblasts. J. Cell Sci. **104**: 297-306.

Briggs, J.P. (2002) The zebrafish: a new model organism for integrative physiology. Am. J. Physiol. Regulatory Integrative Comp. Physiol. **282**: R3-R9.

Broers, J.L.V., Machiels, B.M., van Eys, G.J.J.M., Kuijpers, H.J.H., Manders, E.M.M., van Driel, R., Ramaekers, F.C.S. (1999) Dynamics of the nuclear lamin as monitored by GFP-tagged A-type lamins. J.Cell Sci. **112**: 3463-3475.

Cai, M., Huang, Y., Ghirlando, R., Wilson, K.L., Craigie, R., and Marius Clore, G. (2001). Solution structure of the constant region of nuclear envelope protein LAP2 reveals two LEM-domain structure : one binds BAF and the other binds DNA. EMBO J. **20**: 4399-4407.

Chaly, N, Little, J.E. and Brown, D.L. (1984) Localization of nuclear antigens during preparation of nuclear matrix antigens during the mitotic cycle. J. Cell Biol. **99**: 661-671.

Clements, L., Manilal, S., Love, D.R., and Morris, G.E. (2000). Direct interaction between emerin and lamin A. Biochem. Biophys. Res. Commun. **267**: 709-714.

Collas, P. (1999) Sequential PKC- and Cdc2-mediated phosphorylation events elicit zebrafish nuclear envelope disassembly. J. Cell Sci. **112**: 977-987.

Dabauvalle, M.C., Müller, E., Ewald, A., Kress, W., Krohne, G., Müller, C.R. (1999) Distribution of emerin during the cell cycle. Eur. J. Cell Biol. **78**: 749-756.

Dechat, T., Vlcek, S. and Foisner, R. (2000a). Review: Lamina-associated polypeptide 2 Isoforms and related proteins in cell cycle-dependent nuclear structure dynamics. J. Struct. Biol. **129**: 335-345.

Dechat, T., Korbei, B., Vaughan, O.A., Vlcek, S., Hutchison, C.J., and Foisner, R. (2000b). Lamina-associated polypeptide 2α binds intranuclear A-type lamins. J. Cell Sci. **113**: 3473-3484.

Del Pino, E.M., Sáenz, F.E., Pérez, O.D., Brown, F.D., Ávila, M.-E., Barragán, V.A., Haddad, N., Paulin-Levasseur, M., and Krohne, G. (2002). The LAP2 (lamina-associated polypeptide 2) expression in fish and amphibians. Int. J. Dev. Biol. **46**: 227-234.

Detrich, III, H.W., Westerfield, M. and Zon, L. (1999) Overview of the zebrafish system. Methods Cell Biol. **59**: 3-10.

Driever, W. and Rangini, Z. (1993) Characterization of a cell line derived from ebrafish embryos. *In vitro* Cell. Dev. Biol. **29A**: 749-754.

Ekker, S.C., and Larson, J.D. (2001) Morphant technology in model developmental systems. Genesis **30**: 89-93.

Ellenberg, J., Siggia, E.D., Moreira, J.E., Smith, C.L., Presley, J.F., Worman, H.J., and Lippincott-Schwartz, J. (1997) Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis. J. Cell Biol. **138**: 1193-1206.

Erber, A., Riemer, D., Hofemeister, H., Bovenschulte, M., Stick, R., Panopoulou, G., Lehrach, H., Weber, K. (1999) Characterization of the *Hydra* lamin and its gene: A molecular phylogeny of metazoan lamins. J. Mol. Evol. **49**: 260-271.

Fairley, E.A., Kendrick-Jones, J., and Ellis, J.A. (1999). The Emery-Dreifuss muscular Dystrophy phenotype arises from aberrant targeting and binding of emerin at the inner nuclear membrane. J. Cell Sci. **112**: 2571-2582.

Fisher, D.Z., Chaudhary, N., and Blobel, G. (1986) cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. Proc. Nat. Acad. Sci. USA **83**: 6450-6454.

Foisner, R., and Gerace, L. (1993) Integral membrane protein of the nuclear envelope interact with lamins and chromosomes, and binding is modulated by mitotic phosphorylation. Cell **73**: 1267-1279.

Furukawa, K., Panté, N., Aebi, U. and Gerace, L. (1995). The cDNA cloning of lamina-associated peptide (LAP) 2 and identification of regions for targeting to the nuclear envelope. EMBO J. **14**: 1626-1636.

Furukawa, K., Glass, C., and Kondo, T. (1997). Characterization of the chromatin binding activity of lamina-associated polypeptide (LAP)2. Biochem. Biophys. Res. Commun. **238**: 240-246.

Furukawa, K., Fritze, C.E., Gerace, L. (1998). The major nuclear envelope targeting domain of LAP2 coincides with its lamin binding region but is distinct from its chromatin interaction domain. J. Biol. Chem. **273**: 4213-4219.

Furukawa, K. (1999). LAP2 binding protein 1 (L2BP1/BAF) is a candidate mediator of LAP2-chromatin interaction. J. Cell Sci. **112**: 2485-2492.

Gant, T. M. and Wilson, K. L. (1997). Nuclear assembly. Annu. Rev. Cell Dev. Biol. **13**: 669-695.

Gant, T.M., Harris, C.A. and Wilson, K.L. (1999) Roles of LAP2 proteins in nuclear assembly and DNA replication: truncated LAP2β proteins alter lamina assembly, envelope formation, nuclear size, and DNA replication efficiency in *Xenopus laevis* extracts. J. Cell Biol. **144** (6): 1083-1096.

Georgatos, S. D., Meier, J. and Simon, G. (1994) Lamins and lamin-associated proteins. Curr. Opin. Cell Biol. **6**: 347-353.

Gerace, L. and Blobel, G. (1980) The nuclear envelope lamina is irreversibly depolymerized during mitosis. Cell **19**: 277-287.

Gerace, L. and Burke, B. (1988) Functional organization of the nuclear envelope. Annu. Rev. Cell. Biol. **4**: 335-374.

Gerace , L. and Foisner, R. (1994) Integral membrane proteins and dynamic organization of the nuclear envelope. Trends Cell Biol. **4**: 127-131.

Goldman, R.D., Gruenbaum, Y., Moir, R.D., Shumaker, D.K., and Spann, T.P. (2002) Nuclear lamins: building blocks of nuclear architecture. Genes and Development **16** (5): 533-559.

Harris, C. A., Andryuk, P. J., Cline, S., Chan, K., H., Natarajan, A., Siekierka, J. J. and Goldstein, G. (1994) Three distinct human thymopoietins are derived from alternatively spliced mRNAs. Proc. Nat. Acad. Sci. USA. **91**: 6283-6287.

Harris, C.A., Andryuk, P.J., Cline, S.C., Mathew, S., Siekierka, J.J. and Goldstein, G. (1995). Structure and mapping of the human thymopoietin (TMPO) gene and relationship of human TMPO to rat lamin-associated polypeptide 2. Genomics **28**: 198-205.

Hausen, P., Wang, Y.H., Dreyer, C., and Stick, R. (1985) Distribution of nuclear proteins during maturation of the *Xenopus* oocyte. J. Embryol. Exp. Morphol. **89** suppl.: 17-34.

Heald, R. and McKeon, F. (1990) Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. Cell **60**: 579-589.

Hesse, M., Magin, T.M., and Weber, K. (2001) Genes for intermediate filament proteins and the draft sequence of the human genome: novel keratin genes and a surprisingly high number of pseudogenes related to keratin genes 8 and 18. J. Cell Sci. **114**: 2569-2575.

Hinshaw, J.E., Carragher, B.O., and Milligan, R.A. (1992) Architecture and design of the nuclear pore complex. Cell **69**: 1133-1141.

Hofemeister, H., Weber, K., and Stick, R. (2000) Association of prenylated proteins with the plasma membrane and the inner nuclear membrane is mediated by the same membrane-targeting motifs. Mol. Biol. Cell **11**: 3233-3246.

Hofemeister, H., Kuhn, C., Franke, W.W., Weber, K., and Stick, R. (2002) Conservation of the gene structure and membrane-targeting signals of germ cell-specific lamin LIII in amphibians and fish. Eur. J. Cell. Biol. **81**: 51-60.

Holaska, J.M., Wilson, K.L., and Mansharamani, M. (2002) The nuclear envelope, lamins and nuclear assembly. Curr. Opin. Cell Biol. **14**: 357-364.

Holmer, L., Pezhman, A., and Worman, H.J. (1998) The human lamin B receptor/sterol reductase multigene family. Genomics **54**: 469-476.

Holmer, L., and Worman, H.J. (2001) Inner nuclear membrane proteins: functions and targeting. Cell. Mol. Life Sci. **58**: 1741-1747.

Holtz, D., Tanaka, R.A., Hartwig, J., and McKeon, F. (1989) The CaaX motif of lamin A functions in conjonction with the nuclear localisation signal to target assembly to the envelope. Cell **59**: 969-977.

Hozák, P., Sasseville, A.M.-J., Raymond, Y., Cook, P.R. (1995) Lamin proteins form an internal nucleoskeleton as well as a peripheral lamina in human cells. J. Cell Sci. **108**: 635-644.

Hutchison, C. J. (2002) Lamins: Building blocks or regulators of gene expression? Nature Reviewes: Molecular Cell Biol. **3** (11): 848-858.

Hukriede, H.A., Joly, L., Tsang, M.,Miles, J., Tellis, P., Epstein, J.A., Barbazuk, W.B., Li, F.N., Paw, B., Postlethwait, J.H., Hudson, T.J., Zon, L.I., McPherson, J.D., Chevrette, M., Dawid, I.B., Johnson, S.L., and Ekker, M. (1999). Radiation hybrid mapping of the zebrafish genome. Proc. Natl. Acad. Sci. USA **96** (17): 9745-9750.

Jagatheesan, G., Thanumalayan, S., Muralikrishna, Bh., Rangaraj, N., Karande, A.A., Parnaik, V.K. (1999) Colocalization of intranuclear lamin foci with RNA splicing factors. J. Cell Sci. **112**: 4651-4661.

Kane, D.A. (1999) Cell cycles and development in the embryonic zebrafish. Methods Cell Biol. **59**: 11-26.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. (1995) Stages of embryonic development of the zebrafish. Developmental Dynamics **203**: 253-310.

Kondo, Y., Kondoh, J., Hayashi, D., Ban, T., Takagi, M., Kamei, Y., Tsuji, L., Kim, J., and Yoneda, Y. (2002) Molecular cloning of one isotype of human lamina-associated polypeptide 1s and a topological analysis using its deletion mutants. Biochem. Biophys. Res. Comm. **294**: 770-778.

Krohne, G., Dabauvalle, M.C., and Franke, W. (1981) Cell type-specific differences in protein composition of nuclear pore complex-lamina structures in oocytes and erythrocytes of *Xenopus laevis*. J. Mol. Biol. **151**: 121-141.

Krohne, G., Waizenegger, I., and Hoger, T.H. (1989) The conserver carboxy-terminal cysteine of nuclear lamins is essential for lamin association with the nuclear envelope. J. Cell Biol. **109**: 2003-2011.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685.

Lang, C., Paulin-Levasseur, M., Gajewski, A., Alsheimer, M., Benavente, R. and Krohne, G. (1999) Molecular characterization and developmentally regulated expression of Xenopus lamina-associated polypeptide 2 (XLAP2). J. Cell Sci. **112**: 749-759.

Lee, K.K., Gruenbaum, Y, Spann, P., Liu, J., and Wilson, K.L. (2000) *C. elegans* nuclear envelope proteins emerin, MAN1, lamin, and nucleoporins reveal unique timing of nuclear envelope breakdown during mitosis. Mol. Biol. Cell **11**: 3089-3099.

Lee, K.K., Haraguchi, T., Lee, R.S., Koujin, T., Hiraoka, Y., and Wilson, K.L. (2001) Distinct functional domains in emerin bind lamin A and DNA-bridging protein BAF. J. Cell Sci. **114**: 4567-4573.

Lee, M.S., and Craigie, R. (1998) A previously unidentified host protein protects retroviral DNA from autointegration. Proc. Natl. Acad. Sci. USA **95**: 1528-1533.

Lehner, C.F., Stick, R., Eppenberg, H.M., Nigg, E.A. (1987) Differential expression of nuclear lamin proteins during chicken development. J. Cell Biol. **105**: 577-587.

Lemaitre, J.M., Geraud, G., and Mechali, M. (1998) Dynamics of the genome during early *Xenopus laevis* development: Karyomeres as independent units of replication. J. Cell Biol. **142**: 1159-1166.

Lin, F., Noyer, C.M., Ye, Q., Courvalin, J.-C., and Worman, H.J. (1996). Autoantibodies from patients with primary biliary cirrhosis recognize a region within the nucleoplasmic domain of inner nuclear membrane protein LBR. Hepatology **23**: 57-61.

Lin, F., Blake, D. L., Callebaut, I., Skerjanc, I., McBurney, M., Paulin-Levasseur, M. and Worman, H. J. (2000) MAN1, an inner nuclear membrane protein that shares the LEM domain with lamina-associated polypeptide 2 and emerin. J. Biol. Chem. **275**(7): 4840-4847.

Loewinger, L. and McKeon, F. (1988) Mutations in the nuclear lamin proteins resulting in their aberrant assembly inthe cytoplasm. EMBO J. **7**: 2301-2309.

Lopez-Soler, R.I., Moir, R.D., Spann, T.P.,Stick, R., and Goldman, R.D. (2001) A role for nuclear lamins in nuclear envelope assembly. J.Cell Biol. **154** (1): 61-70.

Manilal, S., Nguyen, T. M., Sewry, C. A. and Morris, G.E. (1996) The Emery-Dreifuss muscular dystrophy protein, emerin, is a nuclear membrane protein. Hum. Mol.Genet. **5**: 801-808.

Martin, L., Crimaudo, C. and Gerace, L. (1995) cDNA cloning and characterization of lamina-associated polypeptide 1C (LAP1C), an integral protein of the inner nuclear membrane. J. Biol. Chem. **270**: 8822-8828.

Martins, S.B., Eide, T., Steen, R.L., Jahnsen, T., Skålhegg, B.S., Collas, P. (2000). HA95 is a protein of the chromatin and nuclear matrix regulating nuclear envelope dynamics. J. Cell Sci. **113**: 3703-3713.

McKeon, F. (1991). Nuclear lamin proteins: domains required for nuclear targeting, Assembly and cell-cycle regulated dynamics. Curr. Opin. Cell. Biol. **3**: 82-86.

Mislow, J.M.K., Kim, M.S., Davis, D.B., and McNally, E.M. (2002a) Myne-1, a spectrin repeat transmembrane protein of the myocyte inner nuclear membrane, interacts with lamin A/C. J. Cell Sci. **115**: 61-70.

Mislow, J.M.K., Holaska, J.M., Kim, M.S., Lee, K.K., Segura-Totten, M., Wilson, K.L., and McNally, E.M. (2002b) Nesprin-1α self-associates and binds directly to emerin and lamin A in vitro. FEBS Letters **525**: 135-140.

Moir, R.D., Donaldson, A.D., and Stewert, M. (1991) Expression in *Escherichia coli* of human lamins A and C: Influence of head and tail domains of assembly properties and paracrystal formation. J. Cell Sci. **99**: 363-372.

Moir, R.D., Montag-Lowy, M., and Goldman, R.D. (1994) Dynamic properties of nuclear lamins: lamin B is associated with sites of DNA replication. J. Cell Biol. **125**: 1201-1212.

Moir, R. D., Spann, T. P. and Goldman, R.D. (1995) The dynamic properties and possible functions of nuclear lamins. Int. Rev. Cytol. **162B**: 141-182.

Moir, R. D., Yoon, M., Khuon, S., and Goldman, R.D. (2000) Nuclear lamins A and B1: different pathways of assembly during nuclear envelope formation in living cells. J. Cell Biol. **151**: 1155-1168.

Montag, M., Spring, H., and Trendelenburg, M.F. (1988) Structural analysis of the mitotic cycle in pre-gastrula *Xenopus* embryos. Chromosoma **96**: 187-196.

Nagano, A., Koga, R., Ogawa, M., Kurano, Y., Kawada, J., Okada, R., Hayashi, Y. K., Tsukahara, T. and Arahata, K. (1996) Emerin dificiency at thenuclear membrane in patients with Emery-Dreifuss muscular dystrophy. Nature Genet. **12**: 254-259.

Nasevicius, A., and Ekker, S.C. (2000) Effective targeted gene 'knockdown' in zebrafish. Nature Genet. **26**: 216-220.

Nigg, E. (1992). Assembly-disassembly of the nuclear lamina. Curr. Opin. Cell. Biol. **4**: 105-109

Nili, E., Cojocaru, G.S., Kalma, Y., Ginsberg, D., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Berger, R., Shaklai, S., Amariglio, N., Brok-Simoni, F., Simon, A.J., and Rechavi, G. (2001). Nuclear membrane protein LAP2β mediates transcriptional repression alone and together with its binding partner GCL (germ-cell-less). J. Cell Sci. **114**: 3297-3307.

Östlund, C., Ellenberg, J., Hallgerg, E., Lippincott-Schwartz, J., and Worman, H.J. (1999) Intracellular trafficking of emerin, the Emery-Dreifuss muscular dystrophy protein. J. Cell Sci. **112**: 1709-1719.

Padan, R., Nainudel-Epszteyn, S., Goitein, R., Fainsod, A. and Gruenbaum, Y. (1990). Isolation and characterizationof the Drosophila nuclear envelope otefin cDNA. J. Biol. Chem. **265**: 7808-7813.

Pathak, R.K., Luskey, K.L., and Anderson, R.G.W. (1986) Biogenesis of the crystalloid endoplasmic reticulum in UT-1 cells: evidence that newly formed endoplasmic reticulum merges from the nuclear envelope. J. Cell Biol. **102**: 2158-2168.

Paulin-Levasseur, M., Blake, D.L., Julien, M. and Rouleau, L. (1996) The MAN antigens are non-lamin constituents of the nuclear lamina in vertebrate cells. Chromosoma **104**: 367-379.

Powell, L. and Burke, B. (1990) Internuclear exchange of an inner nuclear membrane Protein (p55) in heterokaryons: *in vivo* evidence for the interaction of p55 with the nuclear lamina. J. Cell Biol. **111**: 2225-2234.

Quinlan, R., Hutchison, C.J., and Lane, B. (1995) Intermediate filament proteins. Protein profile, Vol. 2 (ed. P. Sheterline). Academic Press.

Robbins, E., and Gonatas, N. (1964) The ultrastructure of a mammalian cell during the mitotic cycle. J. Cell Biol. **21**: 429-442.

Rolls, M. M., Stein, P. A., Taylor, S.S., Ha, E., McKeon, F., and Rapoport, T. A. (1999). A visual screen of a GFP-fusion library identifies a new type of nuclear envelope membrane protein. J. Cell Biol. **146**: 29-43.

Sacco-Bubulya, P.,and Spector, D.L. (2002) Disassembly of interchromatin granule clusters alters the coordination of transcription and pre-mRNA splicing. J. Cell Biol. **156** (3): 425-436.

Sambrook, J., Fritsch, T. and Maniatis, T. (1989). Molecular cloning: a laboratory Manual. Second edition, Cold Spring Harbor Laboratory Press, USA.

Sasseville, A.M.-J. and Raymond, Y. (1995) Lamin A precursor is localized to intranuclear foci. J. Cell Sci. **106**: 273-285.

Schoft, V.K., Beauvais, A.J., Lang, C., Gajewski, A., Prüfert, K., Winkler, C., Akimenko, M.-A., Paulin-Levasseur, M., and Krohne, G. The lamina-associated polypeptide 2 (LAP2) isoforms β, γ and ω of zebrafish: developmental expression and behavior during the cell cycle. Submitted for publication.

Senior, A. and Gerace, L. (1988) Integral membrane proteins specifis to the inner nuclear membrane and associated with nuclear lamina. J. Cell Biol. **107**: 2029-2036.

Schuler, E., Lin, F., and Worman, H.J. (1994) Characterization of the human gene encoding LBR, an integral protein of the nuclear envelope inner membrane. J. Biol. Chem. **269**: 11312-11317.

Shumaker, D. K., Lee, K. K., Tanhehco, Y. C., Craigie, R. and Wilson, K. L. (2001) LAP2 binds to BAF-DNA complexes: requirement for the LEM domain and Modulation by variable regions. EMBO J. **20**: 1754-1764.

Soullam, B., and Worman, H.J. (1993). The amino-terminal domain of the lamin B Receptor is a nuclear envelope targeting signal. J. Cell Biol. **120**: 1093-1100.

Soullam, B., and Worman, H.J. (1995) Signals and structural features involved in integral membrane protein targeting to the inner nuclear membrane. J. Cell Biol. **130**: 15-27.

Steinert, P.M., and Roop, D.R. (1988) Molecular and cellular biology of intermediate filaments. Annu. Rev. Biochem. **57**: 593-625.

Stewart, C., and Burke, B. (1987) Teratocarcinoma stem cells and early mouse embryos contain only a single major lamin polypeptide closely resembling lamin B. Cell **51**: 383-392.

Stick, R., and Krohne, G. (1982) Immunological localization of the major architectural protein associated with the nuclear envelope of the *Xenopus laevis* oocyte. Expr. Cell Res. **138**: 319-313.

Stillman, B. (1994) Smart machines at the DNA replication fork. Cell **78**: 725-728.

Stuurman, N., Heins, S., and Aebi, U. (1998) Nuclear lamins: their structure, assembly and interactions. J. Struct. Biol. **122**: 42-66.

Sullivan, T., Escalente-Alcalde, D., Bhatt, H., Anver, M., Naryan, B., Nagashima, K., Stewart, C.L., and Burke, B. (1999). Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy. J. Cell Biol. **147**: 913-920.

Summerton, J., and Weller, D. (1997) Morpholino antisense oligomers: design, preparation and properties. Antisense Nucleic acids Drug Dev. **7**: 187-195.

Vlcek, S., Just, H., Dechat, T., and Foisner, R. (1999). Functional diversity of LAP2alpha and LAP2beta in postmitotic chromosome association is caused by an alpha-specific nuclear targeting domain. EMBO J. **18**: 6370-6384.

Vleck, S., Dechat, T., Foisner, R. (2001) Nuclear envelope and nuclear matrix: interactions and dynamics. Cell. Mol. Life Sci. **58**: 1758-1765.

Weber, K., Plessmann, U., and Traub, P. (1989) Maturation of nuclear lamin A involves a specific carboxy-terminal trimming, which removes the polyisoprenylation site from the precursor; implications for the structure of the nuclear lamina. FEBS letters **257**(2): 411-414.

Worman, H.J., Yuan, J., Blobel, G. and Georgatos, S.D. (1988). A lamin B receptor in the nuclear envelope. Proc. Natl. Acad. Sci. USA **85**: 8531-8534.

Worman, H.J., Evans, C.D. and Blobel, G. (1990). The lamin B receptor of the nuclear envelope inner membrane: a polytopic protein with eight potential transmembrane domains. J. Cell Biol. **111**: 1535-1542.

Worman, H.J., and Courvalin, J.-C. (2000) The inner nuclear membrane. J. Membrane Biol. **177**: 1-11.

Wu, W., Lin, F., Worman, H.J. (2002) Intracellular trafficking of MAN1, an integral protein of the nuclear envelope inner membrane. J. Cell Sci. **115**: 1361-1372.

Yamaguchi, A., Yamashita, M., Yoshikuni, M., and Nagahama, Y. (2001) Identification and molecular cloning of germinal vesicle lamin B3 in goldfish (*Carassius auratus*) oocytes. Eur. J. Biochem. **268**: 932-939.

Yang, L., Guan, T. and Gerace, L. (1997) Lamin-binding fragment of LAP2 inhibits increase in nuclear volume during the cell cycle and progression into S phase. J. Cell Biol. **139**: 1077-1087.

Ye, Q. and Worman, H. (1994) Primary structure analysis and lamin B and DNA binding of human LBR, an integral protein of the nuclear envelope inner membrane. J. Biol. Chem. **269**: 11306-11311.

Ye, Q. and Worman, H. (1996) Interaction between an integral protein of the nuclear envelope inner nuclear membrane and human chromodomain proteins homologous to Drosophila HP1. J. Biol. Chem. **271**: 14653-14656.

Ye, Q., Callebaut, I., Pezhman, A., Courvalin, J.C. and Worman, H. (1997) Domain-specific interaction of human HP1-type chromodomain proteins and inner nuclear membrane protein LBR. J. Biol. Chem. **272**: 14983-14989.

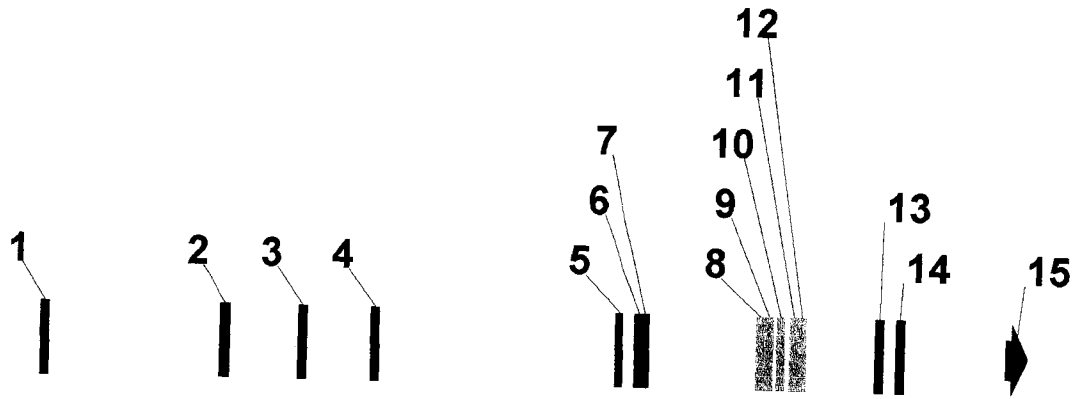
Ye, Q., Barton, R. M. and Worman, H.J. (1998). Nuclear lamin binding proteins. In *Subcellular Biochemistry: Intermediate filaments* (ed. H. Herrmann and J. Robin Harris), pp. 587-610. Plenum Publishing/London, UK.

Zhang, F.L., and Casey, P.J. (1996) Protein prenylation: molecular mechanisms and functional consequences. Annu. Rev. Biochem. **65**: 241-269.

Zhang, Q., Skepper, J.N., Yang, F., Davies, J.D., Hegyi, L., Roberts, R.G., Weissberg, P.L., Ellis, J.A., and Shanahan, C.M. (2001) Nesprins: a novel family of spectrin-repeat-containing proteins that localize to the nuclear membrane in multiple tissues. J. Cell Sci. **114**: 4485-4498.

APPENDIX 1

Schematic representation of the genomic organization of the zebrafish LAP2 gene. BLAST search of the zebrafish genomic sequence (found at www.sanger.ac.uk), using the ZLAP2 cDNA, identified a contig of 22 kb containing the ZLAP2 gene. The gene is organized in 15 exons and 14 introns located on a 22kb DNA sequence. Exons in green are present in the three isoforms (β, γ and ω) so far characterized in zebrafish. Exons in pink are found in the β and γ isoforms and exons in blue are specific to the ω isoform.



ZLAP2

22199 bp

Common to ZLAP2 β, γ and ω

Specific to ZLAP2 ω

Common to ZLAP2 β and ω

APPENDIX 2

Schoft, V.K., Beauvais, A.J., Lang, C., Gajewski, A., Prüfert, K., Winkler, C., Akimenko, M-A., Paulin-Levasseur, M. and Krohne, G. (2003). The lamina-associated polypeptide 2 (LAP2) isoforms β, γ and ω of zebrafish: developmental expression and behavior during the cell cycle. J. Cell Sci. **116**: 2505-2517.

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The lamina-associated polypeptide 2 (LAP2) isoforms β, γ and ω of zebrafish: developmental expression and behavior during the cell cycle

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Summary

Zebrafish lamina-associated polypeptides 2 (ZLAP2) β, γ and ω have in common an N-terminal region with an LEM domain, and in the C-terminal half of the molecule a lamina binding domain and a membrane spanning sequence. The maternally synthesized ω is the largest isoform and the only LAP2 present in the rapidly dividing embryonic cells up to the gastrula stage. ZLAP2ω levels decrease during development, concomitant with the increase of the somatic isoforms ZLAP2β and γ. In somatic zebrafish cells ZLAP2γ is the predominant isoform, whereas only small amounts of ZLAP2β are present.

During early embryonic development, ZLAP2ω becomes associated with mitotic chromosomes before anaphase. The surface of these chromosomes is decorated with vesicles, and each chromosome assembles its own nuclear envelope at the end of mitosis (karyomere formation). Ectopically expressed ZLAP2ω-green fluorescent protein (GFP) fusion protein targets vesicles to mitotic chromosomes in *Xenopus* A6 cells, suggesting that ZLAP2ω is involved in karyomere formation during early zebrafish development.

When ZLAP2β and γ were expressed as GFP fusion proteins in *Xenopus* A6 cells, the β- but not the γ-isoform was found in association with mitotic chromosomes, and ZLAP2β-containing chromosomes were decorated with vesicles. Further analysis of ZLAP2-GFP fusion proteins containing only distinct domains of the ZLAP2 isoforms revealed that the common N-terminal region in conjunction with β- or ω-specific sequences mediate binding to mitotic chromosomes in vivo.

Key words: Zebrafish LAP2, Karyomere, Chromatin binding, Lamina, Mitosis

Introduction

The nuclear envelope is composed of three distinct membrane domains: the outer nuclear membrane, which is in continuity with the endoplasmic reticulum; the membranous wall of the nuclear pore complex, which is located where the outer and inner nuclear membranes fuse to form the pore channel; and the inner nuclear membrane, which is associated with a meshwork of proteins, the nuclear lamina.

Nuclear intermediate filament proteins, the lamins, are the major structural elements of the lamina (for reviews, see Gant and Wilson, 1997; Krohne, 1998; Gruenbaum et al., 2000; Wilson et al., 2001). They are essential architectural proteins implicated in the postmitotic reorganization of the nucleus, including chromatin decondensation and replication (Hutchison et al., 1994; Moir et al., 2000; Lopez-Soler et al., 2001; Holaska et al., 2002; for a review, see Benavente, 1991).

The inner nuclear membrane is structurally and functionally distinct from the other two membrane domains. It contains specific integral membrane proteins (IMPs) that interact with the underlying lamina and the peripheral chromatin during interphase (for reviews, see Collas and Courvalin, 2000; Gruenbaum et al., 2000; Dechat et al., 2000a; Cohen et al., 2001; Goldman et al., 2002).

So far, the best investigated IMPs of the inner nuclear membrane are members of the lamina-associated polypeptide 2 (LAP2) family. In mammals, they have been shown to be generated by alternative splicing from a single gene (Harris et al., 1994; Berger et al., 1996). Six mammalian isoforms (LAP2α, β, γ, δ, ε and ζ) have been identified, and *Xenopus* homologues to the rat LAP2β have been cloned (Gant et al., 1999; Lang et al., 1999). All isoforms except the mammalian LAP2α and ζ are type II IMPs with a short carboxyterminus projecting into the perinuclear cistern and an N-terminal domain localized on the nucleoplasmic side of the inner nuclear membrane (for a review, see Dechat et al., 2000a). All mammalian isoforms possess the same N-terminal segment of 187 amino acids (LAP2 constant region) that is highly conserved in *Xenopus*. Two structural homologous globular domains of approximately 40-50 amino acids in length (LAP2-N and LEM-motif) (Cai et al., 2001) have been located within this sequence. The LEM-motif is also found in otherwise unrelated IMPs of the inner nuclear membrane, like emerin and

MAN1 (Lin et al., 2000). It interacts with the DNA binding protein BAF (barrier to autointegration factor) (Furukawa, 1999; Cai et al., 2001; Lee et al., 2001; Shumaker et al., 2001), suggesting that BAF is mediating the binding of LAP2 and emerin to chromatin. Nuclear reconstitution experiments in *Xenopus* egg extracts indicated that in vitro the LAP2 constant region and BAF are directly or indirectly required for membrane-chromatin attachment and lamina assembly (Gant et al., 1999; Shumaker et al., 2001; Segura-Totten et al., 2002). LAP2-N but not the LEM motif binds to DNA in vitro (Cai et al., 2001).

Other nuclear proteins binding to the mammalian LAP2β are germ-cell-less (Nili et al., 2001), HA95 (Martins et al., 2000) and B-type lamins (Furukawa et al., 1995; Furukawa et al., 1998). The lamina/B-type lamin binding domain has been localized in rat LAP2β between amino acids 298 and 373 (Furukawa et al., 1995; Furukawa et al., 1998). Part of this sequence is highly conserved in *Xenopus* LAP2 isoforms (Gant et al., 1999; Lang et al., 1999).

LAP2α was found to be distributed throughout the nucleus and is not enriched in the lamina (Dechat et al., 1998). It possesses a nuclear targeting/chromatin binding domain (Vlcek et al., 1999), interacts with lamins A/C (Dechat et al., 2000b) and binds to mitotic chromosomes in in vitro nuclear reconstitution experiments (Vleck et al., 2002).

LAP2α, β and γ are expressed in the majority of mammalian cells, whereas in somatic cells of *Xenopus* only LAP2β isoforms have been detected (Lang et al., 1999). The absence of LAP2β from *Xenopus* eggs and embryos before gastrulation, and the expression of a LAP2-related membrane protein of higher molecular weight in oocytes and eggs indicates that this class of IMPs is developmentally regulated in amphibia (Lang et al., 1999).

Here, we report on the molecular characterization of zebrafish LAP2 isoforms, on their expression pattern during embryonic development, and on their behavior during the cell cycle in the embryo and somatic cells.

Materials and Methods

Cells, transfection, animals and tissues

A6 cells (kidney epithelial cells of *Xenopus laevis*) were cultured according to standard procedures (27°C/5% CO_2). Cells were grown on coverslips and transfected with GFP expression vectors (see below) using Rotifect (Roth, Karlsruhe, Germany) according to the manufacturer's instructions. Cells were analyzed 20 to 48 hours after transfection.

Zebrafish ZF4 cells (Driever and Rangini, 1993) were cultured in Dulbecco's modified Eagle's medium/F12 (D-MEM/F12; Gibco BRL, Burlington, ON, Canada) supplemented with 15% fetal calf serum (FCS; Gibco BRL) and 1% antibiotics (50 U/ml penicillin and 50 ug/ml streptomycin; Sigma, St Louis, MO) at 27 °C/5% CO_2. AB9 is a cell line isolated from the caudal fin of zebrafish. AB9 cells were maintained in D-MEM supplemented with 15% FCS, 2 mM L-glutamine and 1% antibiotics under identical conditions.

Zebrafish embryos of defined stages were obtained as described by Kimmel et al. (Kimmel et al., 1995). Zebrafish embryos and small tissue pieces of zebrafish liver, testicle and ovary were directly homogenized and boiled in lysis buffer for analysis by SDS-PAGE.

cDNA isolation and sequence analysis

A zebrafish kidney cDNA library spotted on filters (library no. 575 of the Resource Center of the German Human Genome Project; Berlin) was screened by standard methods. A double-stranded cDNA fragment comprising the complete nucleotide sequence of the *Xenopus* LAP2 (Lang et al., 1999) (Accession No. Y17861) was [^{32}P]-labeled using the random priming DNA labeling kit ver. 1.1 (MBI Fermentas, St Leon-Roth, Germany) according to the manufacturer's instructions. Hybridization was performed as recommended by the supplier of the DNA filters. Both strands of the selected cDNA (zebrafish LAP2-B4, Accession No. AJ320189) were sequenced as described (Lang et al., 1999).

For northern blot analysis, total RNA of zebrafish embryos at the eight-cell stage, and at 3, 4.5, 7.5, 10, 24, and 48 hours postfertilization (hpf) was isolated using the Trizol reagent (Gibco BRL) as described (Gajewski and Krohne, 1999). Northern blot analysis was performed according to standard procedures as described (Sambrook and Russell, 2001), but using a modified Church buffer [1 mM EDTA, 0.5 M phosphate buffer (pH 7.0), 7% (w/v) SDS].

Expression of LAP2 in bacteria and antibody production

A PCR product of the zebrafish LAP2-B4 cDNA coding for amino acids 1-165 was cloned into the pET-21a expression vector (Novagen, Bad Soden, Germany). The targeted sequence was obtained using the following primers:
5′ AGCTTCATATGTTGGAATTTCTGGAAGAC 3′ (5′ end), and
5′ TCTTCCTCGAGGTCGCTGTACTGGTCTGAA 3′ (3′ end).

The sequence was expressed in *Escherichia coli* strain Bl 21 Codon plus (Stratagene, Heidelberg, Germany) and proteins were affinity purified by nickel-chelate affinity chromatography as recommended by the supplier (Qiagen, Hilden, Germany). Two guinea pig antisera against zebrafish LAP2 (ZLAP2) were generated as described previously (Cordes et al., 1991). ZLAP2-serum1 and ZLAP2-serum2 specifically react with all zebrafish LAP2 isoforms and, in addition, ZLAP2-serum1 binds weakly to LAP2 polypeptides from other vertebrates. Human autoimmune antibodies against LAP2 (MAN serum) (Paulin-Levasseur et al., 1996; Lang et al., 1999) and mouse monoclonal lamin antibodies X155 and X223 (Lourim and Krohne, 1993) have been previously described. Mouse monoclonal antibodies against GFP were obtained from Roche (Mannheim, Germany). To control for the specificity of guinea pig antisera, polyclonal ZLAP2 antibodies were affinity purified using bacterially expressed ZLAP2 (amino acids 1-165) coupled to CNBr-activated Sepharose™4B (Amersham Pharmacia, Freiburg/Germany).

RT-PCR, construction of expression vectors and immunoblotting

cDNA from zebrafish ovary and prim-5-stage embryos were prepared as follows. Three ovaries and 50-100 embryos were homogenized in 5 ml of solution D [4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl (*N*-laurosarkosimer), 7.2 μl of β-mercaptoethanol per ml solution D], and then mixed with 50 μl of 2 M sodium acetate (pH 4.0). Five ml of water-saturated phenol and 2.5 ml chloroform/isoamylalcohol (24:1) were added, mixed and incubated on ice for 20 minutes. After centrifugation at room temperature for 10 minutes at 13,000 ***g***, the upper phase was re-extracted with 5 ml chloroform/isoamylalcohol and centrifuged. The RNA was precipitated overnight with two volumes of ethanol, pelleted by centrifugation (30 minutes, 4°C) and washed once with 80% ethanol.

The pellet was redissolved in 300 μl of 'DNAse-Mix' (15 μl of NEB restriction buffer #2 or #3, 3 μl of RQ DNAse I, 1.5 μl of RNAsin, 1.5 μl of 100 mM DTT, 279 μl of water) and incubated at 37°C for 30 minutes. The solution was then mixed with 30 μl of 2 M sodium acetate (pH 4.0), extracted with phenol/chloroform (see above) and then precipitated overnight with two volumes of ethanol. The embryonic RNA was pelleted, and washed (see above). The

Table 1. Primers used for the construction of ZLAP-GFP expression vectors

GFP construct*	Vector	Primer
1-214GFP	pEGFP-N1	5′ TTCTCGAGTGACATG TTGGAATTTCTGGAAGA 3′
(2)		5′ AAGGTACCCTAGTGTGTTGCCCACTGCGT 3′
1-360GFP	pEGFP-N1	5′ TTCTCGAGTGACATG TTGGAATTTCTGGAAGA 3′
(2)		5′ AAGGTACCTTTTGTCGATCACTGGAAGGC 3′
1-502GFP	pEGFP-N1	5′ TTCTCGAGCTTGACATGTTGGAATTCCTGGAA 3′
(3)		5′ CATTGGTACCATCATTGTCTCCTTCATATCCAT 3′
214-314GFP	pEGFP-N1	5′ GATCCTCGAGATGGAGGATGTGGAGGAGGAGGA 3′
(3)		5′ CTCTGGTACCTTGTCAGAGGATGAGGCCTC 3′
315-460GFP	pEGFP-N1	5′ TCTCCTCGAGATGGATTTCTCTGAGCCCTCAATAGTG 3′
(3)		5′ ACTGGGTACCTCTTTTTGTCGATCACTGGAAG 3′
214-502GFP	pEGFP-N1	5′ GATCCTCGAGATGGAGGATGTGGAGGAGGAGGA 3′
(3)		5′ CATTGGTACCATCATTGTCTCCTTCATATCCAT 3′
GFP503-657	pEGFP-C2	5′ AGACAGAATTCGTTGAAAAGGTGTCAGCCAT 3′
(3)		5′ TATTAGGATCCTTATTTGCTGGTACTGTCATC 3′
Full-length ZLAP2γ, β, ω	pEGFP-N1	5′ TTCTCGAGCTTGACATGTTGGAATTCCTGGAA 3′
(1, 2, 3)		5′ AATGGTACCTTGCTGGTACTGTCATCTGTGCCG 3′

*The amino acids contained in the GFP fusion protein are indicated and the position of the GFP in the fusion protein. Numbers in brackets denote the ZLAP2 cDNA that has been used for amplification: (1) ZLAP2γ; (2) ZLAP2β; (3) ZLAP2ω.

pelleted oocyte RNA was dissolved in 500 µl of water, mixed with 500 µl of 8 M lithium chloride and precipitated for 6 hours at –20°C. After centrifugation and washing, the pellet was redissolved in 50 µl of water and the RNA was tested on an agarose gel.

The cDNA was prepared from 5 µg total RNA using the Supercript II reverse transcriptase from Gibco according to the manufacturer instructions. It was then used as a template for PCR amplification. The following primer sequences containing a consensus Kozak site and an in-frame stop codon were selected from the LAP2-B4-cDNA and used for the amplification:

5′ CTTGACATGTTGGAATTTCTGGAAGAC 3′ (5′ end), and
5′ TTATTTGCTGGTACTGTCATCTGTGCC 3′ (3′ end).

The PCR products were inserted into the pCR 2.1 TOPO vector (Invitrogen, Karlsruhe, Germany). Inserts that had been controlled by sequencing, were then excised with *Kpn*I and *Xho*I and cloned into the pBluescript KS vector. Distinct sequences of the three zebrafish LAP2 cDNAs present in the pBluescript KS vector were amplified by PCR and cloned into the eucaryotic expression vectors pEGFP-C2 and pEGFP-N1 (Clontech, Heidelberg, Germany). The PCR primers used are listed in Table 1. Coupled in vitro transcription/translation of cDNAs, SDS-PAGE and immunoblotting were performed as described (Lang et al., 1999).

Microscopic procedures

Transfected *Xenopus* A6 cells grown on coverslips were fixed for 30 minutes with phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, pH 7.4) containing 1.25% glutaraldehyde or 3% formaldehyde. For visualization of chromatin, fixed cells were directly stained for 20 minutes with HOECHST 33258 (2.5 µg/ml PBS). Cells were not dried before mounting to preserve the three-dimensional structure. Some coverslips were processed for immunofluorescence after methanol/acetone fixation as described previously (Lang et al., 1999).

Zebrafish embryos at the 32-128 cell stage, at the late blastula stage (4 hpf) and the 13-somite stage (22 hpf) were manually dechorionated, fixed for 40-60 minutes in 3% formaldehyde/PBS, followed by an extraction with 1% Triton-X 100/PBS for 1 hour. The following steps were performed at 4°C. Tissue pieces and embryos were incubated with PBS containing 0.5-1% bovine serum albumin (PBS/BSA), followed by an overnight incubation with the primary antibody (ZLAP2-serum1, ZLAP2-serum2 diluted 1:300 in PBS/BSA or affinity purified antibodies from ZLAP2-serum1). Specimens were then washed three times each for 1 hour and incubated with secondary antibodies [anti-guinea pig immunoglobulin (Ig) G coupled to Texas Red diluted 1:200 in PBS/BSA] for 5 hours. For visualization of chromatin, preparations were stained with HOECHST 33258 (2.5 µg/ml) during the last 2 hours of the incubation with the secondary antibodies. Washed embryos (see above) were mounted with PBS containing 50% glycerol. The secondary antibodies used (anti-guinea pig IgG coupled to Texas Red; Dianova/Germany) did not stain any structure in zebrafish embryos when the primary antibody was omitted (data not shown). Digital images of transfected cells, living and fixed embryos were taken with a Confocal Laser Scanning Microscope (CLSM; TCS SP, Leica, Heidelberg, Germany) and with a Zeiss Axiophot (Zeiss, Jena, Germany) equipped with a CCD camera (software: CamWare V1.00).

For electron microscopic inspection, embryos were dechorionated, fixed at 4°C for 18 hours with 6.25% glutaraldeyde buffered with 0.1 M phosphate buffer (pH 7.4), washed for 30 minutes with 0.1 M phosphate buffer (pH 7.4), and then fixed for 2 hours with 2% OsO_4 in 50 mM cacodylate (pH 7.2). Embryos were incubated overnight with 0.5% uranyl acetate (in H_2O), dehydrated, embedded in Epon812 and ultrathin sectioned. To allow the electron microscopical analysis of *Xenopus* A6 cells expressing ZLAP2-GFP fusion proteins, cells were grown on CELLocate coverslips (Eppendorf, Hamburg, Germany), fixed 1-2 days after transfection with 1.25% glutaraldehyde in PBS and screened under the fluorescence microscope. Cells were subsequently fixed for 45 minutes with 2.5% glutaraldehyde in PBS and then processed as described for the embryos. Sections were analyzed with a Zeiss EM10 (Zeiss/LEO Oberkochen, Germany). Adobe Photoshop and PowerPoint were used for the preparation of figures.

Preparation of membranes from ovary, extractions of membranes and transfected cells

All buffers were used at 4°C and the experiments were carried out at 4°C unless indicated otherwise. All buffers contained 0.1 µg/ml of trypsin inhibitor and 0.2 mM phenylmethylsulfonyl fluoride. Sucrose-purified total membranes from zebrafish ovaries were prepared as described (Gajewski et al., 1996). Membrane aliquots were mixed with 1 ml of 4 M or 6 M buffered urea (1.5 mM KH_2PO_4, 7 mM Na_2HPO_4), and incubated for 10 minutes at 20°C. Samples were then fractionated by centrifugation (120,000 *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).

Transfected cells grown in Petri dishes (35 mm diameters) were washed three times with PBS and harvested. Cells were then

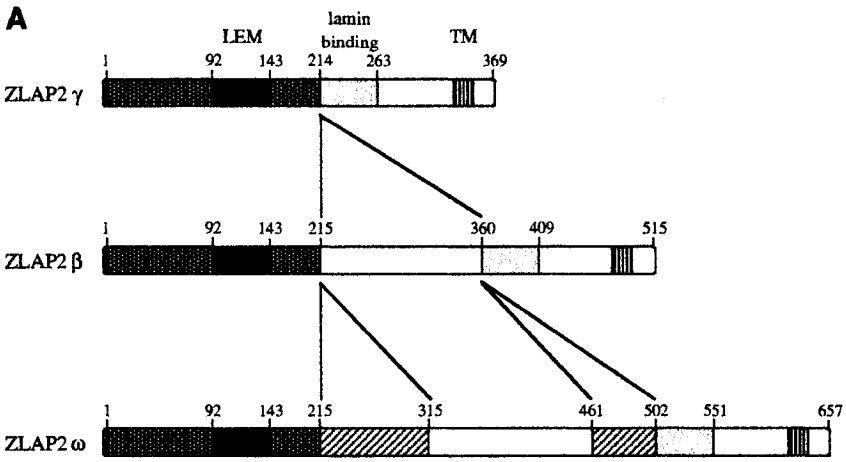

Fig. 1. Isoforms of lamina-associated polypeptide 2 in the zebrafish (ZLAP2). (A) ZLAP2γ, ZLAP2β and ZLAP2ω. The position of the LEM motif (LEM), lamin binding domain (lamin binding), transmembrane domain (TM) and the position of individual amino acids is marked for each isoform. The insertion sites of the β- and ω-specific sequences are marked by lines. (B) Amino acid sequence comparison (single letter code) of ZLAP2γ, β and ω. Bold printed letters represent amino acids identical in the three proteins. Proteins contain 369 (γ), 515 (β) and 657 (ω) amino acids. Gaps have been introduced to allow an optimal alignment of the three sequences. The sequences have been published under Accession Nos AJ320192 (γ), AJ320191 (β) and AJ320190 (ω) in the EMBL Nucleotide Sequence Database.

B

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gamma  MLEFLEDPSVLTKDKLKSALLANNVALPNGDQRKDVYVQLYLKNLTVQNKKSSGSPDVFS 60
beta   MLEFLEDPSVLTKDKLKSALLANNVALPNGDQRKDVYVQLYLKNLTVQNKKSSGSPDVFS 60
omega  MLEFLEDPSVLTKDKLKSALLANNVALPNGDQRKDVYVQLYLKNLTVQNKKSSGSPDVFS 60

gamma  SDEELPPAPVVSNRSRSGRKATRKTDKVRPDDVDVTELSNEGLKDLLLKYGLNAGPIVAS 120
beta   SDEELPPAPVVSNRSRSGRKATRKTDKVRPDDVDVTELSNEGLKDLLLKYGLNAGPIVAS 120
omega  SDEELPPAPVVSNRSRSGRKATRKTDKVRPDDVDVTELSNEGLKDLLLKYGLNAGPIVAS 120

gamma  TRKVYEKRLQKLLDQGPPVAVALPSETSQTDGNQNGNNDSDQYSDREEEPVAPAPVTVPE 180
beta   TRKVYEKRLQKLLDQGPPVAVALPSETSQTDGNQNGNNDSDQYSDREEEPVAPAPVTVPE 180
omega  TRKVYEKRLQKLLDQGPPVAVALPSETSQTDGNQNGNNDSDQYSDREEEPVAPAPVTVPE 180

gamma  PEVEAELIPVVERPVRSRGKTPVTSRTRSGQHTR---------------------------- 214
beta   PEVEAELIPVVERPVRSRGKTPVTSRTRSGQHTRD--------------------------- 215
omega  PEVEAELIPVVERPVRSRGKTPVTSRTRSGQHTREDVEEEDWPVLNVKRKLKRSSHRPDQ 240

gamma  ------------------------------------------------------------
beta   ------------------------------------------------------------
omega  MVPASDDTENSELSSAECFAVSEDRRRTGPGAGRRETRPLLSDRTSKLSSKSESLRRRSA 300

gamma  ------------------------------------------------------------
beta   ---------------FSEPSIVKEVSVSLMRMKVQPLTVPKDPKPSKRYSMSATESTKRP 260
omega  PVRSVLNEASSPDKDFSEPSIVKEVSVSLMRMKVQPLTVPKDPKPSKRYSMSATESTKRP 360

gamma  ------------------------------------------------------------
beta   VSSLNNKHDENTADIPSPPHRQSSREPLVSLINTACVEVDDQGMQDVLSCRSANGGLLAQ 320
omega  VSSLNNKHDENTADIPSPPHRQSSREPLVSLINTACVEVDDQGMQDVLSCRSANGGLLAQ 420

gamma  ------------------------------------------------------------
beta   GVRSAAVSKLSKPVLSQSKPSKPLVDMMCCLSPSSDRQKE-------------------- 360
omega  GVRSAAVSKLSKPVLSQSKPSKPLVDMMCCLSPSSDRQKEESCGSPQTHPKSRHSKITPF 480

gamma  ----------------------VEKVSAIDQTPRAVERDVLKEIFPTENLNTPTGISATC 252
beta   ----------------------VEKVSAIDQTPRAVERDVLKEIFPTENLNTPTGISATC 398
omega  LSQITPVRGLDNKLMDMKETMMVEKVSAIDQTPRAVERDVLKEIFPTENLNTPTGISATC 540

gamma  RRPIRGAAGRPLIDTWLNESRQRLSDLKQTTSSSSFSSTSSYTESRSVPRVSSIPLSASK 312
beta   RRPIRGAAGRPLIDTWLNESRQRLSDLKQTTSSSSFSSTSSYTESRSVPRVSSIPLSASK 458
omega  RRPIRGAAGRPLIDTWLNESRQRLSDLKQTTSSSSFSSTSSYTESRSVPRVSSIPLSASK 600

gamma  STAPPTVKSRSRRSLPVWVQLVLLSAVAGFLFFIYQAMETNDVGLFKQSGTDDSTSK 369
beta   STAPPTVKSRSRRSLPVWVQLVLLSAVAGFLFFIYQAMETNDVGLFKQSGTDDSTSK 515
omega  STAPPTVKSRSRRSLPVWVQLVLLSAVAGFLFFIYQAMETNDAGLFKQSGTDDSTSK 657
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resuspended in 1 ml of 6 M or 8 M buffered urea (1.5 mM KH_2PO_4, 7 mM Na_2HPO_4) and incubated for 10 minutes. All further steps were carried out as described above.

Results

Characterization of zebrafish LAP2 isoforms

We screened a zebrafish kidney cDNA library with a fragment of a previously characterized *Xenopus* LAP2β cDNA (Lang et al., 1999) and isolated one positive clone with a length of 2477 nucleotides (clone B4; GenBank Accession No. AJ320189). DNA sequencing and amino acid prediction revealed that the 5′-end was highly homologous to the *Xenopus* LAP2β. The 3′-end was coding for a putative transmembrane domain and exhibited similarities with the C-terminal end of *Xenopus* LAP2β. The middle part of the cDNA showed little homologies to other LAP2 isoforms and contained a stop codon. To verify whether the 5′ and 3′ ends of the coding region of clone B4 belong to the same gene, we carried out polymerase chain reactions with two different cDNA preparations from *Danio rerio*. Using the same set of primers, we could amplify DNA fragments of 1114, 1554 and 1980 nucleotides, which all contained an open reading frame with a start and stop codon. Comparison of the predicted amino acid sequences revealed that we had amplified three zebrafish LAP2 isoforms (ZLAP2) with lengths of 369 (ZLAP2γ), 515 (ZLAP2β) and 657 (ZLAP2ω) amino acids (Fig. 1A,B). All three proteins possessed the same N-terminal domain (amino acids 1-214) with an LEM module, and the same C-terminal sequence (155 amino acids) with a putative transmembrane domain (TM) and a segment (lamin binding) exhibiting high homologies to the lamina binding domain of *Xenopus laevis* and rat LAP2β (Lang and Krohne, in press; Yang et al., 1997). Segments of the common N-terminal (amino acids 1-165 of ZLAP2; 63% identity with mouse LAP2γ) and C-terminal (amino acids 229-264 in ZLAP2γ; 72% identity with mouse LAP2γ) ZLAP2 domains exhibited high sequence homologies with all other known LAP2 except for the mammalian LAP2α. Comparison of ZLAP2β and ZLAP2γ revealed that the β isoform contained a characteristic insertion of 146 amino acids (β domain) with little homology to the comparable regions of *Xenopus* and mammalian LAP2β. The third protein, ZLAP2ω, was identical to ZLAP2β except for two additional insertions of 101 and 42 amino acids (ω-domains) flanking the β domain.

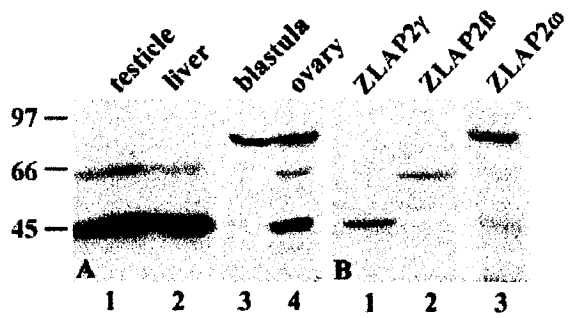


Fig. 2. Characterization of ZLAP2-specific antibodies and proteins encoded by ZLAP2 cDNAs. (A) Total proteins from zebrafish testicle (lane 1), liver (lane 2), blastula (lane 3) and ovary (lane 4) were separated by SDS-PAGE (11% acrylamide) and immunoblotted with the ZLAP2-serum1. A typical radiogram is shown. (B) Coupled in vitro transcription and translation of the cDNAs coding for ZLAP2γ (lane 1), ZLAP2β (lane 2) and ZLAP2ω (lane 3). [^{35}S]-Methionine-labeled polypetides were separated by SDS-PAGE (11% acrylamide) and visualized by fluorography. The additional smaller polypeptides (B, lane 3) with mobilities of ZLAP2γ and ZLAP2β probably result from the internal start of translation at triplets coding for methionine at positions 241 and 447/448 of ZLAP2ω. Molecular masses of reference proteins (in kDa) are marked in (A).

The ω-domains exhibited no homology to all known LAP2 isoforms including the mammalian LAP2α. The exchange of one amino acid in the conserved C-terminal domain of ZLAP2ω (V643A) (Fig. 1B) is a fault of the polymerase during the PCR.

We generated ZLAP2 antibodies (ZLAP2-serum1 and ZLAP2-serum2) specific for epitopes in the common N-terminal domain to verify whether proteins with predicted molecular weights of 40,447 Da (ZLAP2γ), 56,278 Da (ZLAP2β) and 72,225 Da (ZLAP2ω) are expressed in zebrafish. When total proteins of testes, liver, ovary (Fig. 2A) and two zebrafish cell lines (Fig. 7C) were analyzed by SDS-PAGE and immunoblotted using the ZLAP2-specific antibodies, two polypetides with relative mobilities of 45,000 and 63,000 were detected. A third immunoreactive polypeptide of 84,000 was present in embryos of the blastula stage and ovary but was absent from somatic cells (Fig. 2A) and from male germ cells (data not shown). Polypeptides synthesized from the three cloned cDNAs by coupled in vitro transcription/translation showed identical mobilities (Fig. 2B), showing that the proteins detected by immunoblotting represent ZLAP2γ (M_r 45,000), β (M_r 63,000) and ω (M_r 84,000). Antibodies from ZLAP2-serum1 and ZLAP2-serum2 both reacted specifically with the three ZLAP2 isoforms, whereas only antibodies from serum1 weakly recognized, in addition, the *Xenopus* and mammalian LAP2 isoforms (data not shown).

To verify that the three identified zebrafish LAP2 isoforms possess properties characteristic for integral membrane proteins, we extracted ovary membrane fractions and cultured cells with urea. When cultured zebrafish cells (AB9 cells and ZF4 cells) were extracted with 6 M and 8 M urea, ZLAP2β and γ were largely recovered in the membrane pellet (approximately 80-90%, data not shown). Identical results were obtained when *Xenopus* A6 cells expressing the C-terminal domain common to all three ZLAP2 isoforms as a GFP fusion protein (e.g. amino acids 503-657 of ZLAP2ω; GFP503-657) have been extracted with urea (Fig. 3A). GFP503-657 was retained in the membrane pellet, whereas

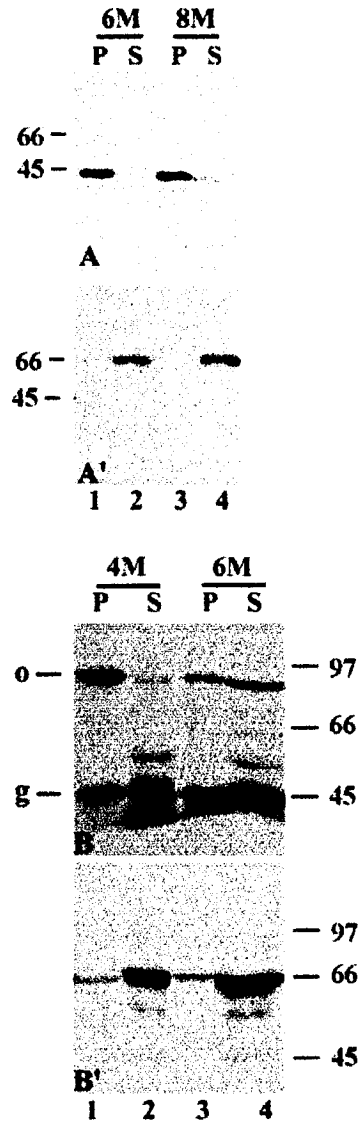


Fig. 3. Biochemical properties of a GFP-ZLAP2ω fusion protein, and of full-length ZLAP2ω. A GFP-ZLAP2ω fusion protein (amino acids 503-657 of ZLAP2ω; GFP503-657) expressed in *Xenopus* A6 cells (A), and ZLAP2ω from zebrafish ovaries (B) was analyzed. The GFP was fused to the aminoterminus of this LAP2 deletion mutant. Proportional amounts of proteins of pellet fractions (P) and supernatants (S) were separated by SDS-PAGE and immunoblotted with GFP antibodies (A) or ZLAP2-specific antibodies (ZLAP2-serum1). The quality of the fractionation was controlled by immunoblotting with antibodies against lamin B2 (A', B'). Lamins should be recovered in the supernatant after urea extraction. (A, A') GFP-ZLAP2ω fusion protein; *Xenopus* A6 cells extracted with 6 M and 8 M urea (A, GFP antibody; B', lamin B2 antibody). (B,B') Membranes of zebrafish ovaries purified by sucrose step gradient centrifugation were extracted with 4 M and 6 M urea. (B, ZLAP2-serum1; B', lamin B2 antibody). The positions of ZLAP2ω (o) and ZLAP2γ (g) are marked in (B) as well as molecular masses of reference proteins (in kDa).

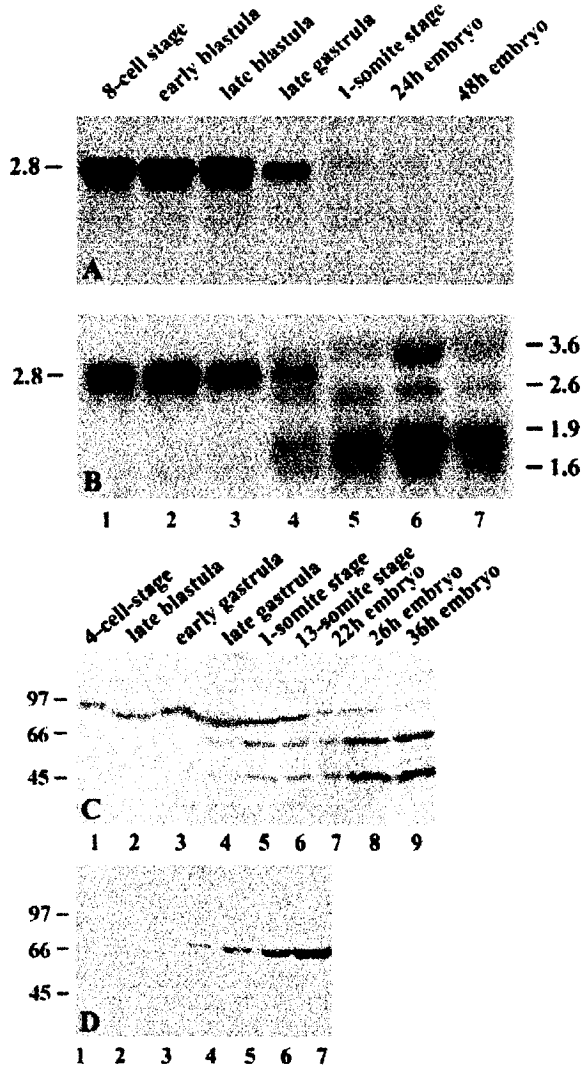


Fig. 4. Expression of ZLAP2 isoforms during zebrafish development. (A,B) Northern blot analysis of the ZLAP2 expression. Total RNA of *D. rerio* embryos at the eight-cell stage (lane 1), early blastula (3 hpf, lane 2), late blastula (4.5 hpf, lane 3), late gastrula (7.5 hpf, lane 4), one-somite stage (10 hpf, lane 5), at the age of 24 hours (24 h embryo, lane 6) and 48 hours (48 h embryo, lane 7) were hybridized with in vitro synthesized [^{32}P]-labeled RNA complementary to ZLAP2 mRNAs. (A) A sequence exclusively present in the ZLAP2ω mRNA (nucleotides 642-942 of ZLAP2ω) or (B) a probe complementary to the complete ZLAP2γ mRNA were used for hybridization. The hybridized mRNAs have estimated sizes of 3600 (3.6), 2800 (2.8), 2600 (2.6), 1900 (1.9) and 1600 (1.6) nucleotides. (C) Total proteins of nine developmental stages were separated by SDS-PAGE (11% acrylamide) and immunoblotted with ZLAP2-serum1. In each lane, total proteins of 2.5 embryos were loaded. The developmental stage is specified on top of each lane. Expression of ZLAP2β and γ was first seen in the late gastrula (lane 4), and ZLAP2ω was only detectable in minor amounts in embryos aged 36 hours (36 h embryo; lane 9). The apparent different mobilities of ZLAP2ω in some lanes is caused by abundant yolk proteins that have slightly lower mobilities than ZLAP2ω. (D) Total proteins of seven developmental stages identical to those in lanes 1-7 of (A) were separated by SDS-PAGE (11% acrylamide) and immunoblotted with lamin antibody X155 reacting in zebrafish with lamin B2. In each lane, total proteins of 2.5 embryos were loaded. Lamin B2 is first detectable in the late gastrula (lane 4). Molecular masses of reference proteins (in kDa) are marked (C,D).

peripheral membrane proteins like lamins were recovered in the supernatant (Fig. 3A'). In transfected *Xenopus* A6 cells, GFP503-657 was localized at the nuclear envelope, and variable amounts were detectable in the endoplasmic reticulum (data not shown). Extractions of whole cellular membranes of zebrafish ovaries with 4 M urea confirmed that ZLAP2ω and γ possesses properties characteristic for integral membrane proteins (Fig. 3B,B'). During the extraction with higher urea concentrations (6 M, Fig. 3B, lanes 3 and 4), apparently a significant amount of very small membrane fragments had been formed that could not be pelleted. A similar behavior of integral membrane proteins of *Xenopus* ovary extracts in the presence of urea has been observed previously (Gajewski and Krohne, 1999; Lang et al., 1999). Also, we noted that the nucleoplasmic domain of ZLAPω is more susceptible to degradation than that of the β and γ isoforms. Therefore, we always detected immunoreactive bands with lower molecular weights on immunblots of the supernatants. Our urea extractions show that the three ZLAP2 isoforms are integral membrane proteins.

The analysis of ZLAP2 expression during development revealed that the isoforms are developmentally regulated both at the mRNA (Fig. 4A,B) and at the protein levels (Fig. 4C), comparable to the situation in amphibia (Lang et al., 1999). In embryos at the eight-cell stage and in embryos during the blastula stage (Fig. 4A, lanes 1-3), we detected only an mRNA band of 2.8 kb that reacted with a ω-specific probe. Lower amounts of the ω-mRNA were also detectable in embryos at the late gastrula stage (7.5 hpf) but not in the older developmental stages tested (Fig. 4A, lanes 5-7). When the complete ZLAP2γ was used for hybridization (Fig. 4B), the ω-mRNA was detected as well as additional mRNAs in embryos at the late gastrula (7.5 hpf) and in older developmental stages. These mRNA bands had sizes of 3.6, 2.6, 1.9 and 1.6 kb. We suggest that these mRNAs in older embryos (Fig. 4B, lane 4) are coding for ZLAP2β and γ. It is not unusual that LAP2 mRNAs possess long non-translated 3′ ends. For the mammalian LAP2β mRNAs, sizes of 3.5 and 2 kb have been reported (Furukawa et al., 1995). We cannot rule out that some of the mRNAs detected (Fig. 4B, lanes 4-7) are coding for additional LAP2 isoforms with mobilities on SDS-PAGE close to that of ZLAP2β, γ or ω.

Immunoblots supported the interpretation of our northern blot data. At the protein level, LAP2ω was the only isoform present in embryos up to the early gastrula stage (Fig. 4C). By contrast, LAP2β and γ were absent from early developmental stages and first detectable at the late gastrula stages (Fig. 4C, lane 4). Interestingly, the expression of LAP2β/γ and somatic B-type lamins started at the same time point during development (Fig. 4D, lane 4) [for the characterization of the B-type lamin specific for the female germ line of the zebrafish see Yamaguchi et al.; see also Hofemeister et al. (Yamaguchi et al., 2001; Hofemeister et al., 2002)]. The amount of LAP2ω per embryo decreased with the progression of development concomitant with the increase of somatic LAP2 isoforms. In embryos aged 36 hours, LAP2ω was barely detectable. In all

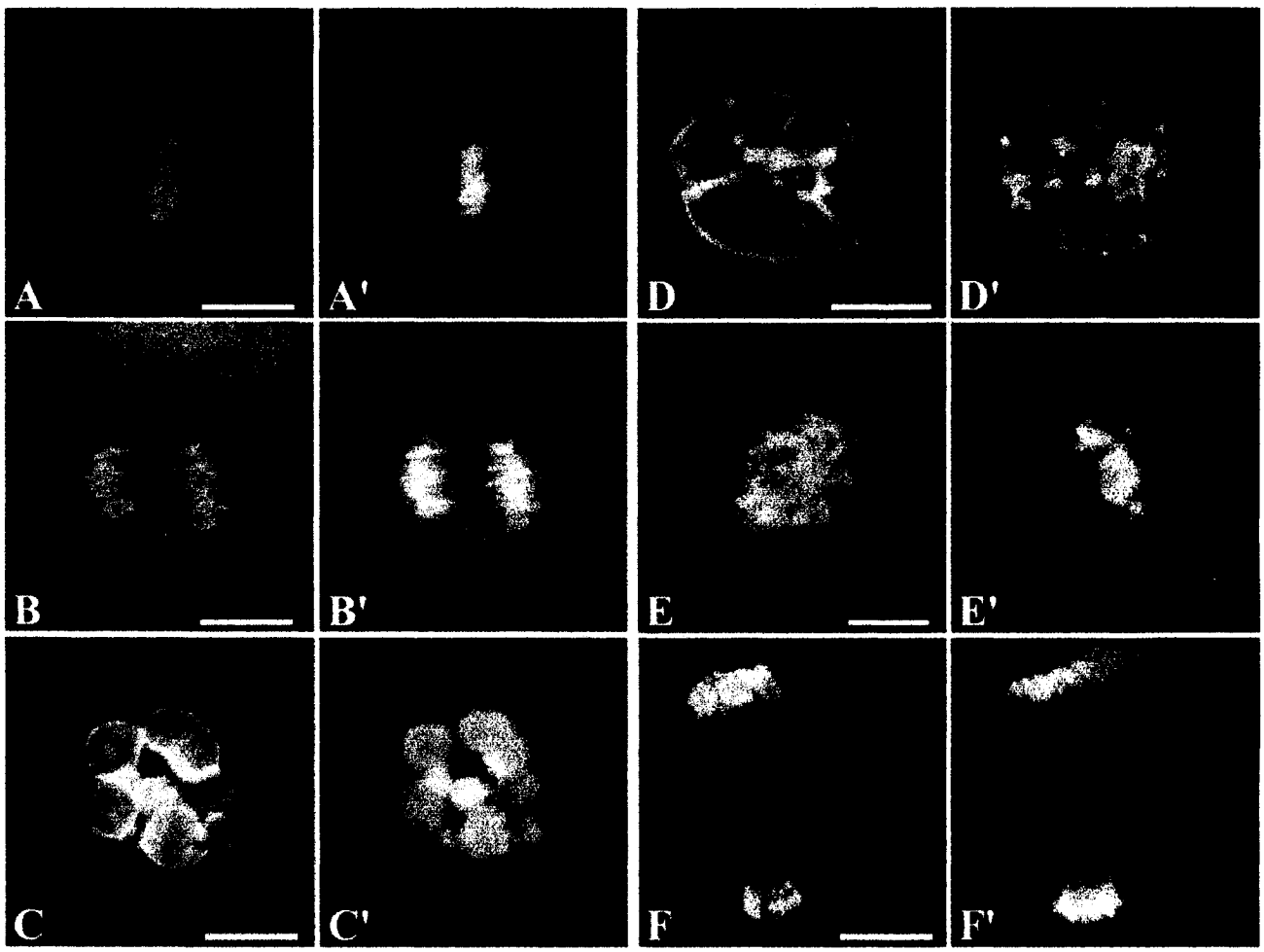


Fig. 5. Distribution of LAP2 during the cell cycle in zebrafish blastomeres. Localization of ZLAP2 in whole-mount preparations of embryos by indirect immunofluorescence microscopy with polyclonal antibodies specific for ZLAP2 (A-F; A'-F', DNA staining by Hoechst). Embryos at the cleavage period (A, B; 1.5 hpf), early blastula stage (C,D; 2.5 hpf) and late blastula stage (E, F; 4 hpf) have been analyzed. Cells at metaphase (A-A', E-E'), anaphase (B-B',F-F'), the karyomere stage (C-C') and interphase (D-D') are shown. (D) The nuclear envelope of the interphase nucleus (D) is highly invaginated, resulting in an apparent intranuclear staining with LAP2 antibodies. This morphology is characteristic for nuclei that have been formed by the fusion of karyomeres. Digital images were taken with a CLSM (C-C',D-D',E-E',F) and a Zeiss Axiophot (A-A',B-B',F'). Bars, 10 μm.

embryonic stages tested the β and γ isoforms were expressed at the same level, whereas LAP2γ was the predominant polypeptide in somatic tissues of adult organism (see Fig. 2A), as well as in two zebrafish cell lines tested (Fig. 7C). Owing to the lack of ZLAP2ω-specific antibodies, we cannot exclude that this isoform is expressed in the adult organism in specialized cells of some organs as it has been shown for a *Xenopus* B type lamin (lamin L_{III}/B3) (Benavente et al., 1985).

The behavior of ZLAP2 isoforms during the cell cycle in the embryo and somatic cells

To learn more about the properties of the ω-isoform in comparison with LAP2β and γ, we analyzed cleavage stages of embryos and cultured cells (Table 2). The immunolabeling of embryos at developmental stages expressing exclusively LAP2ω with our LAP2-specific antibodies indicated that the ω isoform is localized at the nuclear envelope during interphase, as depicted in Fig. 5D. The inspection of mitotic cells from embryos aged 1-2 hours and 4 hours revealed the staining of anaphase chromosomes (Fig. 5B,F). The staining of metaphase chromosomes was clearly visible in the early cleavage stages (Fig. 5A; 1-2 hpf) but not in older embryos (Fig. 5E; 4 hpf). The electron microscopic inspection of blastomeres from the same developmental stages showed that variable numbers of vesicles, which often appeared flattened, were associated with mitotic chromosomes. On metaphase chromosomes, fewer vesicles were seen (data not shown) than on chromosomes at anaphase (Fig. 6A). In addition, numerous vesicles were regularly seen in the cytoplasm bordering the chromosomes (Fig. 6A). The morphology of reforming nuclei at telophase during the blastula stage (Fig. 5C) indicated that a nuclear envelope had been assembled around single or few chromosomes (Fig. 5C; karyomere formation) before the

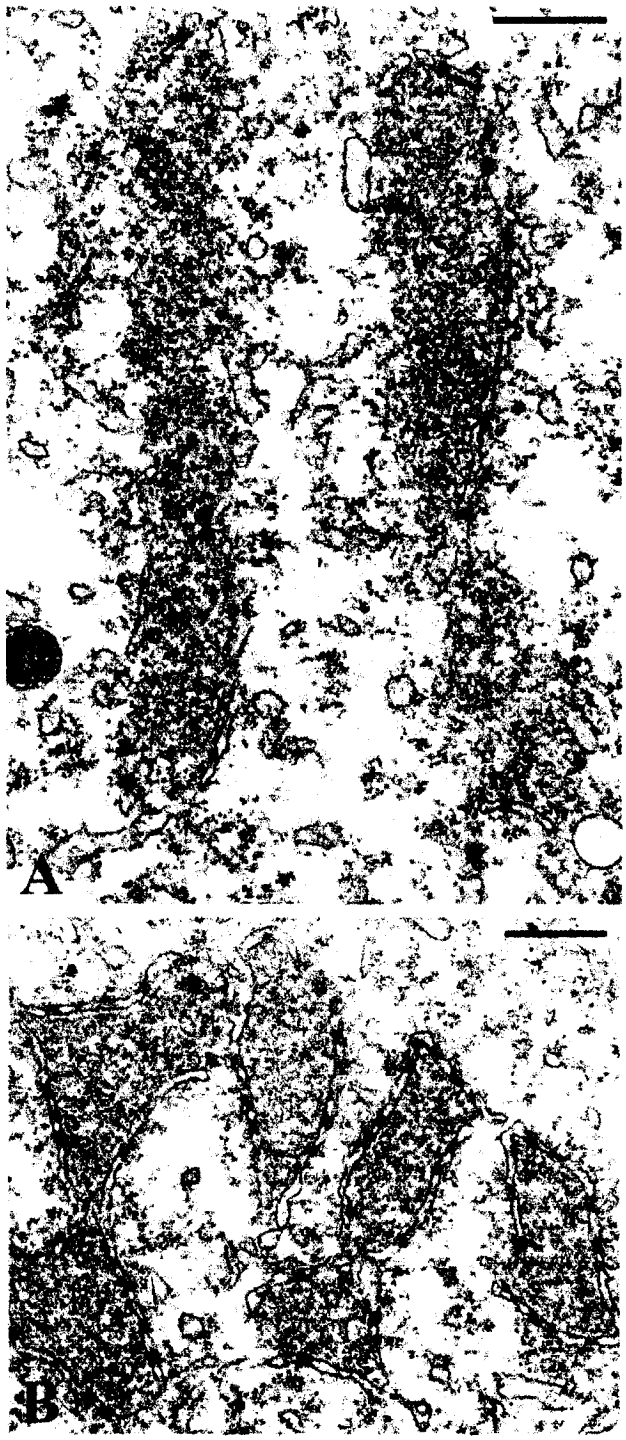


Fig. 6. Morphology of chromosomes during cell division of zebrafish blastomeres at the early blastula stage (2.5 hpf). Electron micrographs showing longitudinal sectioned mitotic chromosomes (A, compare with Fig. 4B) and chromosomes at an early karyomere stage (B, earlier than that shown in Fig. 4C). Numerous flattened vesicles are attached to the surface of mitotic chromosomes (A), and nuclear envelope fragments containing pore complexes are attached to the surface of each chromosome at the early karyomere stage (B). Bars, 0.5 μm.

Table 2. Behavior of zebrafish LAP2-GFP fusion proteins in transfected *Xenopus* A6 cells during mitosis

ZLAP2-GFP fusion protein	Association with mitotic chromosomes
1-214GFP (common domain)	+/–
1-360GFP (β)	+++
1-502GFP (ω)	+++
214-314GFP (ω)	–
315-460GFP (ω)*	–
214-502GFP (ω)	–

*Identical to amino acids 215-360 of zebrafish LAP2β.
Numbers denote amino acids of the zebrafish LAP2 isoforms listed in brackets (see also Fig. 1A). In the fusion proteins used GFP was always localized at the C-terminus of ZLAP2.
+/–, weak staining of chromosomes.
+++, intense staining of chromosomes.
–, no chromosome staining.

formation of a common nuclear envelope enclosing all chromosomes in later interphase (Fig. 5D). Further electron microscopic analysis revealed that during telophase each chromosome assembles its own nuclear envelope (Fig. 6B). In older embryos (11-13 hpf = 5-8 somite stage; see Fig. 4A) that contained beside LAP2ω considerable amounts of the somatic isoforms, only minute amounts of membranes were found in contact with anaphase chromosomes, and no karyomere formation could be seen in telophase. In the embryos aged 12 hours, only very faint, if any, staining of anaphase chromosome was detectable with ZLAP2-specific antibodies.

When we analyzed cultured zebrafish ZF4 and AB9 cells that expressed exclusively the ZLAP2β and γ (Fig. 7C), we noted that γ was the predominant isoform, whereas only minor amounts of the β-isoform were detectable. In these cultured cells, we observed no labeling of metaphase chromosomes during mitosis (Fig. 7A,A') and, in anaphase cells, only areas on the chromosome surface were stained where the reassembly of the nuclear envelope has begun (Fig. 7B,B'). Our data indicate that, during early embryogenesis, a significant amount of ZLAP2ω associates with chromosomes much earlier during the progession of mitosis than LAP2 isoforms in somatic zebrafish cells.

Distinct behavior of GFP-ZLAP2 fusion proteins during mitosis in transfected A6 cells

Our immunofluorescence data suggest that ZLAP2ω possesses properties distinct from those of the β and γ isoforms. To verify our observations by a methodology independent of antibodies, we generated eukaryotic expression vectors allowing the in vivo analysis of the three isoforms as GFP fusion proteins in transfected *Xenopus* A6 cells. We selected *Xenopus* A6 cells for transfection because they are cultured at a temperature that is optimal for the growth of zebrafish embryos and adults. A further reason is that the folding and assembly of at least some zebrafish and *Xenopus* proteins is not optimal at temperatures above their body temperature (Cerda et al., 1998).

The analysis of total proteins from transfected cells by SDS-

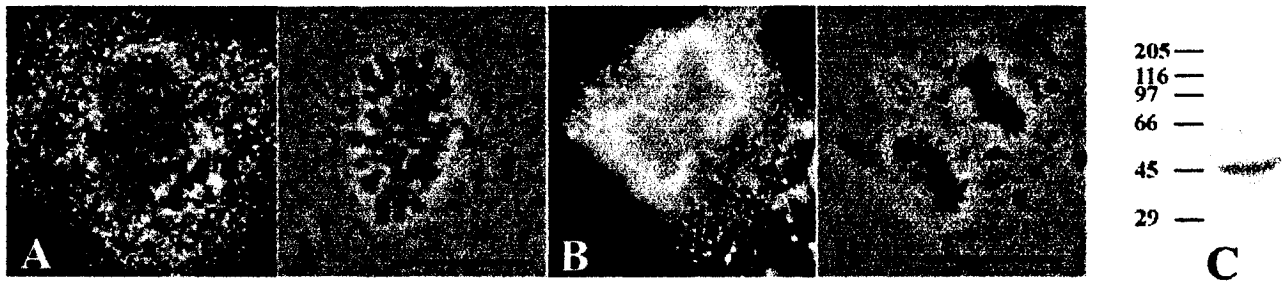
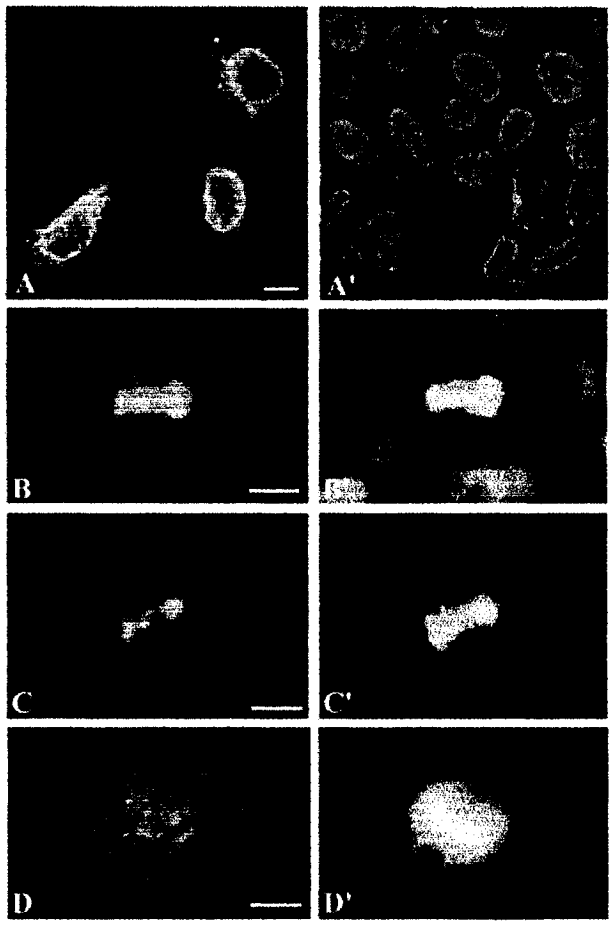


Fig. 7. The behaviour of LAP2 during mitosis in zebrafish AB9 cells. Immunofluorescence microscopy of an AB9 cell at metaphase (A; A', phase contrast) and anaphase (B; B', phase contrast), after staining with ZLAP2-specific antibodies. Note that metaphase chromosomes are not stained by LAP2 antibodies, and that anaphase chromosomes are stained only in regions where the nuclear envelope has begun to reassemble. Bars, 10 μm. (C) LAP2 isoforms of zebrafish AB9 cells. Total proteins of AB9 cells were separated by SDS-PAGE (11% acrylamide) and immunoblotted with ZLAP2-serum1. ZLAP2γ is the major isoform in AB9 cells, and only minor amounts of ZLAP2β were detectable. Identical results were obtained with zebrafish ZF4 cells (data not shown). Molecular masses of reference proteins (in kDa) are marked.

PAGE and immunoblotting with GFP antibodies revealed that each of the three GFP-LAP2 fusion proteins possessed the expected apparent molecular weight (data not shown). During interphase in A6 cells, all three GFP-ZLAP2 fusion proteins were localized in the nuclear envelope (Fig. 8A) and variable amounts in the endoplasmic reticulum. In mitotic cells, ZLAP2ω-GFP (Fig. 8B) and ZLAP2β-GFP (Fig. 8C) were found in association with chromosomes. By contrast, ZLAP2γ-GFP exhibited a diffuse and, in addition, dot-like staining in mitotic cells but did not colocalize with chromosomes (Fig. 8D). The comparative analysis of the three ZLAP2-GFP fusion proteins suggest that the domains common to all three isoforms do not mediate binding to mitotic chromosomes. To verify our hypothesis we generated a number of deletion mutants (see Table 1). A GFP-ZLAP2 fusion protein containing exclusively the N-terminal domain (amino acids 1-214 of ZLAP2; 1-214GFP) common to all three isoforms was homogeneously distributed in the cytoplasm of A6 cells during mitosis and also detectable in regions containing the mitotic chromosomes (Fig. 9A). Two fusion proteins containing the common aminoterminus in conjuction with β-specific sequences (Fig. 9B; amino acids 1-360 of ZLAP2β; 1-360GFP) or β-sequences together with the ω-sequences (amino acids 1-502 of ZLAP2ω; 1-502GFP; data not shown) were predominantly localized on chromosomes in mitotic cells. By contrast, GFP fusion proteins containing exclusively β- and/or ω-specific sequences were excluded from cellular regions containing chromosomes during mitosis in transfected *Xenopus* A6 cells (Fig. 9C; shown for mutant 214-502GFP) (see Table 1). Our results indicate that not a single domain but the common N-terminal domain in conjunction with β- and/or ω-specific sequences mediates binding of the β- and ω-isoforms to mitotic chromosomes.



Ectopically expressed ZLAP2β and ZLAP2ω target vesicles to mitotic chromosomes

We have shown that the nucleoplasmic domain of ectopically

Fig. 8. Intracellular distribution of ZLAP2-GFP fusion proteins in transfected *Xenopus* A6 cells during interphase and cell division. In all constructs GFP was fused to the C-terminus of full-length LAP2 isoforms. Transfected cells expressing ZLAP2ω-GFP (A,B), ZLAP2β-GFP (C) and ZLAP2γ-GFP (D) are shown during interphase (A) and mitosis (B-D). The GFP fluorescence (A-D) and the same cells after staining with lamin B2 antibodies (A') or the DNA dye Hoechst (B'-D') are shown. ZLAP2γ-GFP is distributed in mitotic cells throughout the cytoplasm and forms local aggregates (D), whereas ZLAP2ω-GFP (B) and ZLAP2β-GFP (C) predominantly colocalized with chromatin. Digital images were taken with a Leica CLSM (A, A') and a Zeiss Axiophot (B-D'). Bars, 10 μm.

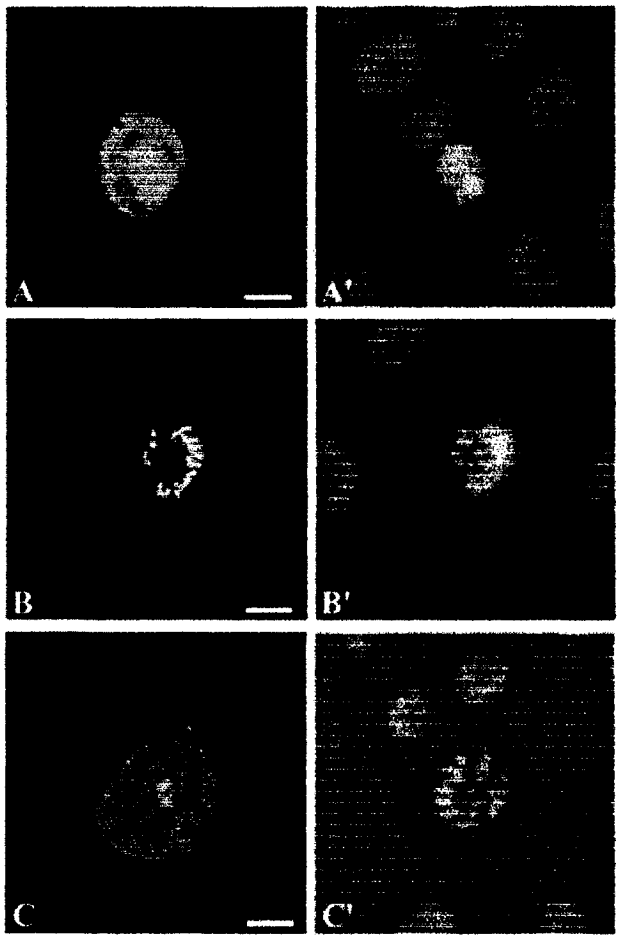


Fig. 9. Intracellular distribution of deletion mutants of ZLAP2 expressed as GFP fusion proteins in transfected *Xenopus* A6 cells during cell division. In all constructs, GFP was fused to the carboxyterminus of LAP2 deletion mutants. Transfected cells expressing mutant 1-214GFP (A; amino acids 1-214 common to all ZLAP2 isoforms), mutant 1-360GFP (B, amino acids 1-360 of ZLAP2β) and mutant 214-502GFP (C; amino acids 214-502 of ZLAP2ω) are shown during mitosis. The GFP fluorescence (A-C) and the same cells after staining with the DNA dye Hoechst (A',B',C') are shown. Note the association of mutant 1-360GFP with mitotic chromosomes and the exclusion of 214-502GFP from regions containing chromosomes. Digital images were taken with a Leica CLSM (A-C) and a Zeiss Axiophot (A'-C'). Bars, 10 μm.

expressed ZLAP2β and ω bind to chromatin and that ZLAP2ω isolated from zebrafish ovaries possesses properties characteristic for integral membrane proteins (see Fig. 3B). To verify whether these two isoforms – in contrast to ZLAP2γ – could be involved in the early targeting of vesicles to mitotic chromosomes, we expressed the three full-length polypeptides as GFP-fusion proteins in *Xenopus* A6 cells. When cells comparable to those shown in Fig. 8B-D were processed for electron microscopy, we noted that mitotic chromosomes of cells expressing ZLAP2ω (Fig. 10A,B,D) or ZLAP2β (data not shown) were associated with numerous vesicles, giving the chromosomes a 'sponge-like' morphology. Vesicles of comparable morphology could not be detected on mitotic chromosomes of cells expressing ZLAP2γ (Fig. 10C,E). The membrane stacks (Fig. 10C,E) visible in the vicinity of the mitotic chromosomes corresponded to the dot-like structures seen by fluorescence microscopy in mitotic (Fig. 8D) and interphase cells (Fig. 8A).

Discussion

We have described with the zebrafish LAP2ω a novel polypeptide that is expressed in oocytes and during early embryonic development. ZLAP2ω possesses properties distinct from that of ZLAP2γ, the major isoform in zebrafish somatic cells.

What are the putative functions of ZLAP2ω during early embryonic development?

ZLAP2ω is a type II membrane protein and the only LAP2 isoform expressed during a developmental stage that is characterized by very rapid cell divisions (cell-cycle length 15-20 minutes) (Kimmel et al., 1995). Further important features of this stage include the association of vesicles with mitotic chromosomes, and the nuclear envelope assembly around individual chromosomes (karyomere formation) at the end of mitosis. By the expression of GFP-LAP2ω fusion proteins in cultured cells, we have provided clear evidence that the ω isoform binds to chromatin and associates with mitotic chromosomes. The mitotic chromosomes of transfected cells ectopically expressing full-length LAP2ω-GFP have similarities with the mitotic chromosomes of rapidly dividing zebrafish early blastomeres. First, many vesicles are associated with the chromosomes, and second, the chromatin of these chromosomes is less condensed than that of chromosomes free of membranes. Our data therefore indicate that ZLAP2ω is involved in the very early binding of nuclear envelope forming vesicles to the surface of individual chromosomes during early embryonic development, thus facilitating the reformation of the nuclear envelope around each chromosome at the end of the very short embryonic cell cycles (see Fig. 5A-C). A protein with comparable properties has so far not been described in mammals.

Karyomere formation is also characteristic of the very short cell cycles before the midblastula transition in *Xenopus* (Montag et al., 1988; Lemaitre et al., 1998) (see also therein for karyomere formation in other organisms). *Xenopus* embryos contain a maternally expressed LAP2-related integral membrane protein that has several properties in common with ZLAP2ω (Lang et al., 1999). The characterization of a *Xenopus* LAP2 cDNA clone (Accession No. AJ514937) that had been isolated in the course of the characterization of *Xenopus* LAP2β (Lang et al., 1999) revealed that the in vitro translated polypeptide encoded by this cDNA is comigrating with the *Xenopus* LAP2 protein of Mr 84,000 expressed in oocytes and during early embryonic development (Lang et al., unpublished). This protein exhibited a sequence identity of 97% with a previously published *Xenopus* LAP2 isoform (Gant et al., 1999) (Accession No. AF048815). The *Xenopus* LAP2ω is 99 amino acids shorter than the zebrafish isoform but much more acidic (XLAP2ω: pI 6.16; ZLAP2ω: pI 9.17). This is most probably the reason for its unusual slow mobility on SDS-PAGE (Lang et al., unpublished). The low sequence identities of the *Xenopus* and zebrafish LAP2ω in the central part of both

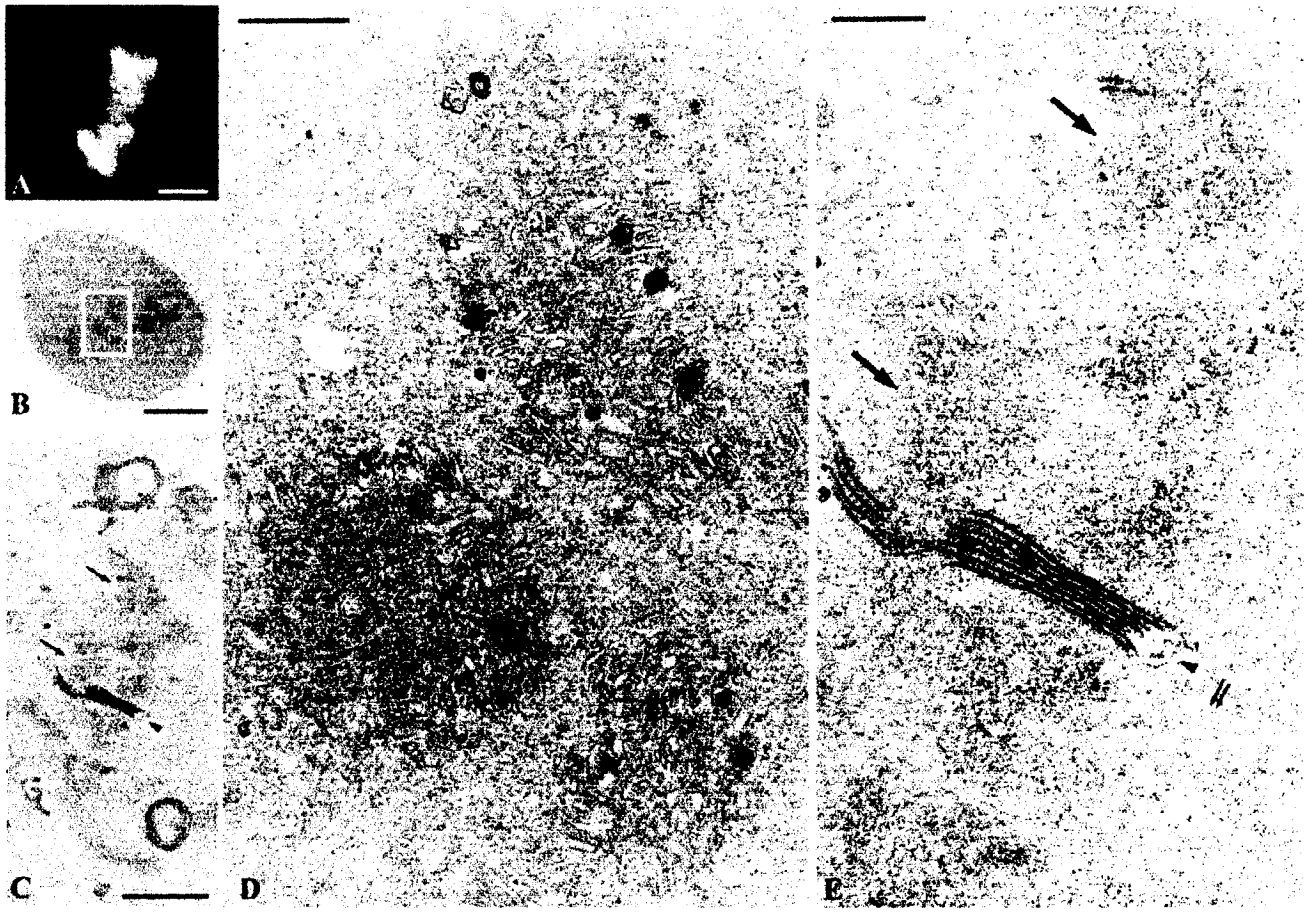


Fig. 10. Mitotic chromosomes of transfected *Xenopus* A6 cells expressing full-length ZLAP2ω-GFP (A,B,D) or ZLAP2γ-GFP (C,E) analyzed by light (A) and electron microscopy (B-E). (A,B,D) The same cell at metaphase is shown by fluorescence microscopy (A) and on electron micrographs of ultrathin sections through this cell (B, D). The metaphase chromosomes are clearly visible at low magnification (B). (D) Higher magnification of the boxed area in (B) shows that chromosomes are associated with vesicles. (C,E) Electron micrographs of an ultrathin section through a transfected cell at metaphase expressing ZLAP2γ-GFP. The chromosomes are shown in an overview (C) and higher magnification (E, central region of C). A membrane stack (arrowheads in C and E) containing ZLAP2γ-GFP, chromosomes (single arrows) and spindle microtubules (double arrows) are marked. Metaphase chromosomes of cells expressing ZLAP2γ-GFP are not associated with vesicles. Bars, 0.5 μm (D,E), 2 μm (C) and 5 μm (A,B).

molecules suggests that these isoforms are adaptations to the different cell cycle length of *Xenopus* and zebrafish blastomeres before the midblastula transition.

Binding of ZLAP2β to mitotic chromosomes

In the cultured zebrafish cells tested, ZLAP2β was present only in minor amounts and could not be distinguished from the abundant γ-isoform due to the lack of isoform-specific antibodies. Therefore the staining of mitotic cells with LAP2 antibodies (see Fig. 7) probably reflects the distribution ZLAP2γ and is in agreement with the behavior of ZLAP2γ-GFP in transfected cells. It is questionable whether chromosome-bound endogenous ZLAP2β could be detected in cultured zebrafish cells with the presently available tools.

The comparison of the behavior of our ZLAP2 fusion proteins during mitosis indicates that the GFP moiety of the fusion protein does not mediate chromosome binding, and that the common carboxyterminus of the three isoforms is not involved in this process. The intracellular distribution of the ZLAP2 deletion mutants tested (see Fig. 9 and Table 1) suggests that the common N-terminal domain alone does possess a weak affinity for mitotic chromatin in transfected *Xenopus* A6 cells. This affinity is enhanced when β- and/or ω-specific sequences are present in the proteins. An observation of Vlcek and coworkers (Vlcek et al., 1999) could help to explain why the common aminoterminus in conjunction with β- and/or ω-specific sequences enhances the binding of ZLAP2 to mitotic chromosomes. Vlcek found that a GST fusion protein containing the common N-terminal domain (amino acids 1-187) of the mammalian LAP2 binds in vitro to mitotic chromosomes (Vlcek et al., 1999). It is known that GST forms dimers and oligomers. It is worth speculating that β- and ω-specific sequences do modulate oligomerization of ZLAP2 thus facilitating the binding of the common aminoterminus to chromatin.

Ectopically expressed LAP2 fusion proteins are often present

in the transfected cells in a higher concentration than the endogenous LAP2 isoforms. If the oligomerization of ZLAP2β was a prerequisite for its binding to mitotic chromosomes, binding to mitotic chromosomes in cells expressing ZLAP2β-GFP fusion proteins would be expected to occur much earlier during the cell cycle than in non-transfected cells because the ectopically expressed protein is present in higher cellular concentrations than the endogenous LAP2. This notion is supported by the observation that ectopically expressed ZLAP2β and ω are found in association with metaphase chromosomes (see Fig. 8B,C). Other than for ZLAP2β, the behavior ZLAP2ω-GFP in transfected cells is in agreement with the distribution of endogenous ZLAP2ω in early embryonic cells during mitosis. In the cleavage period the ω-isoform is associated with chromosomes throughout all mitotic stages (Fig. 5). We conclude from our data that the observed differences in the mitotic behavior of ZLAP2γ compared with the β- and ω-isoforms reflect intrinsic properties of the polypeptides.

How many LAP2 isoforms are expressed in zebrafish?

We have identified by cDNA cloning three different splice products of the ZLAP2 gene: one β, one γ and one ω isoform. To our surprise, several mRNAs of embryos aged 48 hours hybridized to the LAP2-specific probe. Therefore, we cannot rule out that some of the mRNAs detected are coding for additional minor LAP2 isoforms with mobilities on SDS-PAGE close to that of ZLAP2β, γ or ω.

For the following reasons it is unlikely that *D. rerio* is expressing a homologue to the mammalian LAP2α. Antibodies specific for the common N-terminal domain of all so far identified vertebrate LAP2 isoforms (MAN antibodies: Paulin-Levasseur et al., 1996; Lang et al., 1999) (ZLAP2-serum1 and ZLAP2-serum2: this manuscript) did not detect a polypeptide with the size of mammalian LAP2α in protein samples of somatic tissues and cultured cells but only two significant smaller polypeptides that represent zebrafish LAP2β and γ. The only immunoreactive polypeptide of similar size to the mammalian LAP2α is expressed in zebrafish oocytes and during early embryonic development. We have shown that this protein represents ZLAP2ω. In this respect it is interesting that a homologue to the mammalian LAP2α is apparently also absent from somatic cells of *Xenopus* (Lang et al., 1999), and other amphibia and fishes (del Pino et al., 2002). Our present study will now enable us to analyze zebrafish LAP2 functions at the genetic level in this model vertebrate.

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References

Benavente, R. (1991). Postmitotic nuclear reorganization events analyzed in living cells. *Chromosoma* **100**, 215-220.

Benavente, R., Krohne, G. and Franke, W. W. (1985). Cell type-specific expression of nuclear lamina proteins during development of *Xenopus laevis*. *Cell* **41**, 177-190.

Berger, R., Theodor, L., Shoham, J., Gokkel, E., Brok-Simoni, F., Avraham, K. B., Copeland, N. G., Jenkins, N. A., Rechavi, G. and Simon, A. J. (1996). The characterization and localization of the mouse thymopoietin/lamina-associated polypeptide 2 gene and its alternatively spliced products. *Genome Research* **6**, 361-370.

Cai, M., Huang, Y., Ghirlando, R., Wilson, K. L., Craigie, R. and Clore, G. M. (2001). Solution structure of the constant region of nuclear envelope protein LAP2 reveals two LEM-domain structures: one binds BAF and the other binds DNA. *EMBO J.* **20**, 4399-4407.

Cerda, J., Conrad, M., Markl, J., Brand, M. and Herrmann, H. (1998). Zebrafish vimentin: molecular characterization, assembly properties and developmental expression. *Eur. J. Cell Biol.* **77**, 175-187.

Cohen, M., Lee, K. K., Wilson, K. L. and Gruenbaum, Y. (2001). Transcriptional repression, apoptosis, human disease and the functional evolution of the nuclear lamina. *Trends Biochem. Sci.* **26**, 41-47.

Collas, P. and Courvalin, J.-C. (2000). Sorting nuclear membrane proteins at mitosis. *Trends Cell Biol.* **10**, 5-8.

Cordes, V., Walzenegger, I. and Krohne, G. (1991). Nuclear pore complex glycoprotein p62 of *Xenopus laevis* and mouse. cDNA cloning and identification of its glycosylated regions. *Eur. J. Cell Biol.* **55**, 31-47.

Dechat, T., Vleck, S. and Foisner, R. (2000a). Lamina-associated polypeptide 2 isoforms and related proteins in cell cycle-dependent nuclear structure dynamics. *J. Struct. Biol.* **129**, 335-345.

Dechat, T., Korbei, B., Vaughan, O. A., Vlcek, S., Hutchison, C. J. and Foisner, R. (2000b). Lamina-associated polypetide 2α binds intranuclear A-type lamins. *J. Cell Sci.* **113**, 3473-3484.

del Pino, E. M., Sáenz, F. E., Pérez, O. D., Brown, F. D., Ávila, M.-E., Barragán, V. A., Haddad, N., Paulin-Levasseur, M. and Krohne, G. (2002). The LAP2 (lamina-associated polypeptide 2) expression in fish and amphibians. *Int. J. Dev. Biol.* **46**, 227-234.

Driever, W. and Rangini, Z. (1993). Characterization of a cell line derived from Zebrafish embryos. *In vitro Cell. Dev. Biol.* **29A**, 749-754.

Furukawa, K., Panté, N., Aebi, U. and Gerace, L. (1995). Cloning and cDNA for lamina-associated polypeptide 2 (LAP2) and identification of regions that specify targeting to nuclear envelope. *EMBO J.* **14**, 1626-1636.

Furukawa, K., Fritze, C. E. and Gerace, L. (1998). The major nuclear envelope targeting domain of LAP2 coincides with its lamin binding region but is distinct from its chromatin interaction domain. *J. Biol. Chem.* **273**, 4213-4219.

Gajewski, A. and Krohne, G. (1999). Subcellular distribution of the *Xenopus* p58/lamin B receptor in oocytes and eggs. *J. Cell Sci.* **112**, 2583-2596.

Gajewski, A., Lourim, D. and Krohne, G. (1996). An antibody against a glycosylated integral membrane protein of the Xenopus nuclear pore complex: A tool for the study of pore complex membranes. *Eur. J. Cell Biol.* **71**, 14-21.

Gant, T. M. and Wilson, K. L. (1997). Nuclear assembly. *Annu. Rev. Cell Dev. Biol.* **13**, 669-695.

Gant, T. M., Harris, C. A. and Wilson, K. L. (1999). Roles of LAP2 proteins in nuclear assembly and DNA replication: truncated LAP2β proteins alter lamina assembly, envelope formation, nuclear size, and DNA replication efficiency in *Xenopus laevis* extracts. *J. Cell Biol.* **144**, 1083-1096.

Goldman, R. D., Gruenbaum, Y., Moir, R. D., Shumaker, D. K. and Spann, T. P. (2002). Nuclear lamins: building blocks of nuclear architecture. *Genes Dev.* **16**, 533–547.

Gruenbaum, Y., Wilson, K.L., Harel, A., Goldberg, M. and Cohen, M. (2000). Nuclear lamins – structural proteins with fundamental functions. *J. Struct. Biol.* **129**, 313-323.

Harris, C. A., Andryuk, P. J., Cline, S. C., Natarajan, A., Siekierka, J. J. and Goldstein, G. (1994). Three distinct human thymopoietins are derived from alternatively spliced mRNAs. *Proc. Natl. Acad. Sci. USA* **91**, 6283-6287.

Hofemeister, H., Kuhn, C., Franke, W. W., Weber, K. and Stick, R. (2002). Conservation of the gene structure and membrane targeting signals of germ cell specific lamin LIII in amphibians and fish. *Eur. J. Cell Biol.* **81**, 51-60.

Holaska, J. M., Wilson, K. L. and Mansharamani, M. (2002). The nuclear envelope, lamins and nuclear assembly. *Curr. Opin. Cell Biol.* **14**, 357-364.

Hutchison, C. J., Bridger, J. M., Cox, L. S. and Kill, I. R. (1994). Weaving a pattern from disparate threads: lamin function in nuclear assembly and DNA replication. *J. Cell Sci.* **107**, 3259-3269.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Krohne, G. (1998). Lamin assembly in vivo. In *Subcellular Biochemistry: Intermediate filaments* (ed. H. Herrmann and J. Robin Harris), pp. 563-586. London: Plenum Publishing.

Lang, C. and Krohne, G. Lamina-associated polypeptide 2β (LAP2β) is contained in a protein complex together with A- and B-type lamins. *Eur. J. Cell Biol.* (in press).

Lang, C., Paulin-Levasseur, M., Gajewski, A., Alsheimer, M., Benavente, R. and Krohne, G. (1999). Molecular characterization and developmentally regulated expression of *Xenopus* lamina-associated polypeptide 2 (XLAP2). *J. Cell Sci.* **112**, 749-759.

Lee, K. K., Haraguchi, T., Lee, R. S., Koujin, T., Hiraoka, Y. and Wilson, K. L. (2001). Distinct functional domains in emerin bind lamin A and DNA-bridging protein BAF. *J. Cell Sci.* **114**, 4567-4573.

Lemaitre, J. M., Geraud, G. and Mechali, M. (1998). Dynamics of the genome during early *Xenopus laevis* development: Karyomeres as independent units of replication. *J. Cell Biol.* **142**, 1159-1166.

Lin, F., Blake, D. L., Callebaut, I., Skerjanc, I. S., Holmer, L., McBurney, M. W., Paulin-Levasseur, M. and Worman, H. (2000). MAN1, an inner nuclear membrane protein that shares the LEM domain with lamina-associated polypeptide 2 and emerin. *J. Biol. Chem.* **275**, 4840-4847.

Lopez-Soler, R. I., Moir, R. D., Spann, T. P., Stick, R. and Goldman, R. D. (2001). A role for nuclear lamins in nuclear envelope assembly. *J. Cell Biol.* **154**, 61-70

Lourim, D. and Krohne, G. (1993). Membrane-associated lamins in *Xenopus* egg extracts: identification of two vesicle populations. *J. Cell Biol.* **123**, 501-512.

Martins, S. B., Eide, T., Steen, R. L., Jahnsen, T., Skålhegg, B. S. and Collas, P. (2000). HA95 is a protein of the chromatin and nuclear matrix regulating nuclear envelope dynamics. *J. Cell Sci.* **113**, 3703-3713.

Moir, R. D., Spann, T. P., Lopez-Soler, R. I., Yoon, M., Goldman, A. E., Khuon, S. and Goldman, R. D. (2000). The dynamics of the nuclear lamins during the cell cycle – relationships between structure and function. *J. Struct. Biol.* **129**, 324-334.

Montag, M., Spring., H. and Trendelenburg, M. F. (1988). Structural analysis of the mitotic cycle in pre-gastrula *Xenopus* embryos. *Chromosoma* **96**, 187-196.

Nili, E., Cojocaru1, G. S., Kalma, Y., Ginsberg, D., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Berger, R., Shaklai, S., Amariglio, N. et al. (2001). Nuclear membrane protein LAP2β mediates transcriptional repression alone and together with its binding partner GCL (germ-cell-less). *J.Cell Sci.* **114**, 3297-3307.

Paulin-Levasseur, M., Blake, D. L., Julien, M. and Rouleau, L. (1996). The MAN antigens are non-lamin constituents of the nuclear lamina in vertebrate cells. *Chromosoma* **104**, 367-379.

Sambrook, J. F. and Russell, D. W. (2000). *Molecular Cloning: A Laboratory Manual*, 3rd edn. New York: Cold Spring Harbor Laboratory Press.

Segura-Totten, M., Kowalski, A. K., Craigie, R., Wilson, K. L. (2002). Barrier-to-autointegration factor: major roles in chromatin decondensation and nuclear assembly. *J. Cell Biol.* **158**, 475-485.

Shumaker, D. K., Lee, K. K., Tanhehco, Y. C., Craigie, R. and Wilson, K. L. (2001). LAP2 binds to BAF-DNA complexes:requirement for the LEM domain and modulation by variable regions. *EMBO J.* **20**, 1754-1764.

Vlcek, S., Korbei, B. and Foisner, R. (2002). Distinct functions of the unique C terminus of LAP2α in cell proliferation and nuclear assembly. *J. Biol. Chem.* **277**, 18898-18907.

Vlcek, S., Just, H., Dechat, T. and Foisner, R. (1999). Functional diversity of LAP2α and LAP2β in postmitotic chromosome association is caused by an α-specific nuclear targeting domain. *EMBO J.* **18**, 6370-6384.

Wilson, K. L., Zastrow, M. S. and Lee, K. K. (2001). Lamins and disease: insights into nuclear infrastructure. *Cell* **104**, 647-650.

Yamaguchi, A., Yamashita, M., Yoshikuni, M. and Nagahama, Y. (2001). Identification and molecular cloning of germinal vesicle lamin B3 in goldfish (*Carassius auratus*) oocytes. *Eur. J. Biochem.* **268**, 932-939.

Yang, L., Guan, T. and Gerace, L. (1997). Lamin-binding fragment of LAP2 inhibits increase in nuclear volume during the cell cycle and progression into S phase. *J. Cell Biol.* **139**, 1077-1087.