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

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**Abstract.** Humans express three major splicing isoforms of LAP2, a lamin- and chromatin-binding nuclear protein. LAP2β and γ are integral membrane proteins, whereas α is intranuclear. When truncated recombinant human LAP2β proteins were added to cell-free *Xenopus laevis* nuclear assembly reactions at high concentrations, a domain common to all LAP2 isoforms (residues 1–187) inhibited membrane binding to chromatin, whereas the chromatin- and lamin-binding region (residues 1–408) inhibited chromatin expansion. At lower concentrations of the common domain, membranes attached to chromatin with a unique scalloped morphology, but these nuclei neither accumulated lamins nor replicated. At lower concentrations of the chromatin- and lamin-binding region, nuclear envelopes and lamins assembled, but nuclei failed to enlarge and replicated on average 2.5-fold better than controls. This enhancement was not due to rereplication, as shown by density substitution experiments, suggesting the hypothesis that LAP2β is a downstream effector of lamina assembly in promoting replication competence. Overall, our findings suggest that LAP2 proteins mediate membrane-chromatin attachment and lamina assembly, and may promote replication by influencing chromatin structure.

**Key words:** nuclear envelope • chromatin structure • prereplication complex • emerin • MAN

The nuclear envelope generates a unique structural and functional environment for chromosomes inside the nucleus. Molecules move through the nuclear envelope via nuclear pore complexes (NPCs) which regulate nucleocytoplasmic transport while permitting the passive diffusion of small molecules (<40 kD) and ions (reviewed by Ohno et al., 1998). The inner nuclear membrane and NPCs are anchored to the lamina, which is a polymeric network of nuclear-specific intermediate filament proteins named lamins (reviewed by Hutchison et al., 1994; Gant and Wilson, 1997). There are two major types of lamins, B type and A/C type, which are encoded by different genes. B lamins remain membrane-associated throughout the cell cycle, predominantly through their association with lamin-binding membrane proteins (see below), whereas lamins A/C become soluble and are dispersed throughout the cytosol during mitosis. During interphase, B type lamins have also been detected immunologically inside the nucleus at sites of DNA replication (Moir et al., 1994). Biochemical studies suggest that the tail domains of lamins can bind to DNA and core histones (Burke, 1990; Glass et al., 1993; Taniura et al., 1995; reviewed in Gant and Wilson, 1997).

The lamina is a key structural element of the nucleus. When nuclei are assembled in cell-free extracts immunodepleted of soluble lamins, the lamin-depleted nuclei fail to undergo DNA replication (Newport et al., 1990; Meier et al., 1991; Jenkins et al., 1993), suggesting that lamina assembly is linked to the assembly or function of replication complexes. However, this link is likely to be indirect. When nuclei are exposed to dominant negative mutant lamin A proteins, DNA replication sites (Mills...
et al., 1989; Hozak et al., 1994) become physically and functionally disrupted (Spann et al., 1997). However, dominant lamin B mutants have different effects: nuclei with a preexisting lamina can remain replication-competent even when their lamina is gradually dissolved by the mutant lamin B proteins (Elliis et al., 1997). In both cases, the mutant lamin B are thought to sequester depolymerized wild-type lamins and prevent them from recycling (Schmidt et al., 1994). It was not clear why DNA replication would depend, either initially or in an ongoing capacity, on the integrity of the lamina. Furthermore, lamina assembly may not be essential. New results show that plasmid DNA can be fully replicated in vitro if it is incubated first in Xenopus laevis cytosol and subsequently in concentrated nucleosol, suggesting that high concentrations of factors inside the nucleus, rather than nuclear structure per se, are essential for replication competence (Walter et al., 1998).

The inner nuclear membrane contains several unrelated resident proteins that bind to lamins (reviewed by Gerace and Foisner, 1994; Gant and Wilson, 1997), including the lamin B receptor (LBR, also known as p58; Worman et al., 1988, 1990), three isoforms of the lamina-associated polypeptide-1 (LAP1; Martin et al., 1995), and several isoforms of LAP2 (Foisner and Gerace, 1993; Harris et al., 1994, 1995; Berger et al., 1996). Because LBR, the C isoform of LAP1, and α and β isoforms of LAP2 are phosphorylated during mitosis, these proteins are postulated to play structural roles that must be modified for nuclei to disassemble at mitosis (Simos and Gergottos, 1992; Foisner and Gerace, 1993; Ye and Worman, 1994; Martin et al., 1995; Dechat et al., 1998). LAP1, LAP2β, and LBR do not appear to associate with each other. Instead, LBR and LAP1 form separate complexes, each of which has a distinct protein kinase (Simos and Gergottos, 1992; Nikolakaki et al., 1996; Maison et al., 1997). It is not known if LAP2 proteins associate with a kinase. Two proteins related to LAP2, named emerin (Bione et al., 1994; Martin et al., 1996; Nagano et al., 1996) and MAN (Paulin-Levasseur et al., 1996; H. Worman, personal communication), also reside at the inner nuclear membrane. Loss of emerin causes Emery-Dreifuss muscular dystrophy in humans (see Bione et al., 1994; Berger et al., 1996). Because LBR, the C isoform of two recombinant polypeptides derived from human LAP2, on nuclear assembly in cell-free extracts of Xenopus eggs. X. enopus egg extracts are an efficient system for assembling replication-competent nuclei in vitro (Wilson and Wiese, 1996). X. enopus extract components also interact efficiently with nuclear proteins from other species; for example, X. enopus membranes readily incorporate into rat liver nuclei, X. enopus extract removes transport into all exogenous nuclei tested (e.g., Newmeyer et al., 1986), and X. enopus extracts can assemble nuclei around isolated mitotically condensed mammalian chromosomes (e.g., Lawlis et al., 1996). Our results, presented here, show that human LAP2 fragments are functional in the X. enopus extracts, and inhibit nuclear assembly in distinct ways. Our most unexpected finding was that LAP2β fragment 1–408 can influence the efficiency of DNA replication.

Materials and Methods

Reagents and Solutions

Membrane wash buffer (MWB) consisted of 250 mM sucrose, 50 mM KCl, 2.5 mM MgCl₂, 50 mM Hepes, pH 8.0, 1 mM dithiothreitol, 0.5 mM ATP, 1 µg/ml aprotinin, and 1 µg/ml leupeptin. Sonication buffer consisted of 50 mM NaPO₄, pH 8.0, and 300 mM NaCl. Protease inhibitors benzamidine (5 mM final concentration; Sigma Chemical Co.), PM-SF (0.5 mM final concentration; Sigma Chemical Co.), and pepstatin A (1 µg/ml final concentration; Sigma Chemical Co.) were included in the sonication buffer during sonication, but were not added in subsequent steps. Stop buffer consisted of 80 mM Tris-HCl, pH 8.0, 8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% SDS, and 0.2% Bromophenol blue. TBS consisted of 100 mM Tris-HCl, pH 7.5, plus 0.9% (wt/vol) NaCl. WGA (Sigma Chemical Co.) was kept frozen at 10%.
A phicodrin (Sigma Chemical Co.; catalog number A-0781) was kept as a 2.5 mg/ml stock in D M SO at −20 °C.

In Vitro Nuclear Assembly Reactions and Import Assays

Membrane and cytosol fractions were prepared from unactivated Xenopus eggs as previously described (Newmeyer and Wison, 1991; Boman et al., 1992). De membranated Xenopus sperm chromatin was also prepared as previously described (Lohka and Masui, 1983; Newmeyer and Wilson, 1991). Chromatin, at a final concentration of 1–4000 sperm/ml, was stored at −80 °C. For nuclear assembly reactions, 2 μl membranes (1–30 mg protein/ml), 20 μl cytosol (25–30 mg protein/ml, supplemented with 10 mM phosphatecrome, 1 mM ATP, and 50 μg/ml creatine phosphokinase as an ATP regenerating system), and 1 μl de membranated sperm chromatin were mixed on ice and transferred to 22–24 °C to initiate nuclear assembly. All reactions were done using components that had been frozen and thawed once. For reactions that contained recombinant LAP2 fragments, 1 μl of LAP2 protein (in M MB) was added to mixed cytosol and membranes to yield the indicated final concentration of LAP2 fragment. Chromat in was added, and reactions were mixed again and transferred to 22–24 °C to initiate assembly.

To assay for nuclear import, rhodamine-labeled nucleoplasmin was prepared according to Newmeyer et al. (1986), and added to nuclei after 2 h of assembly in the presence or absence of LAP2 fragment 1–408. Nuclei were imaged by epifluorescence microscopy 30 min later (time = 2.5 h). As a negative control for import, WGA (final concentration, 1 mg/ml) was added 5 min before adding fluorescent nucleoplasmin. WGA inhibits active transport by binding to O-GlicNA-modified nucleoporfins at the NPC (see Finlay and Forbes, 1990, and references therein).

Preparation of Recombinant LAP2 Proteins

Esherichia coli cells, strain BL21(DE3)pLYSs (Novagen, Inc.), were transformed with the pET-23a expression vector (Novagen, Inc.) containing inserts coding for either residues 1–408 or residues 1–187 of human LAP2b, or residues 1–164 of Xenopus LAP2A (see below). We followed the convention of numbering amino acids that excludes the initiating methionine, consistent with previous papers (Fosner and G erace, 1993; H arris et al., 1994). The pET-23a expression vector adds a His tag (Leu-Glu-His6) to the COOH terminus of the expressed protein. Thus, LAP2b fragment 1–408 is a 417-amino-acid, 46,49-kD protein, and LAP2A fragment 1–187 is a 196-amino-acid, 21,71-kD protein. To produce each recombinant protein, an overnight culture of a single colony was diluted 1:60 in fresh isopropyl-β-D-thigalactopyranoside (IPTG) for 3 h, and the bacteria were pelleted by centrifugation (6,000 g for 15 min at 4 °C). The pellet was frozen in liquid N and stored at −80 °C. To purify each recombinant fragment, the pellet was resuspended in sonication buffer, subjected to pulse sonification, and centrifuged (20,000 g for 20 min at 4 °C). The supernatant was applied to a Ni-NTA–agarose column (Qiagen, Inc.), which was washed successively with 10 column volumes each of sonication buffer and sonication buffer plus 10 mM imidazole. Recombinant His-tagged proteins were eluted with sonication buffer containing 100 mM imidazole.

The proteins were concentrated and desalted using Centriprep-30 units (Amicon, Inc.). Small aliquots were frozen in liquid N and stored at −80 °C; the thawed proteins were stable at 4 °C for at least a week. Moc purifications were done in parallel using uninduced bacteria to provide a control for potential nonspecific effects due to either imidazole or bacte rial proteins. However, in no case did the effect of mock-purified proteins differ from that of buter alone.

Light Microscopy and Photography

A lightq of assembly reactions were fixed in M MB containing 3.7% form aldehyde, 20 μg/ml H ocist 33342 (a D N A stain; Calbiochem Corp.). The samples were observed using a Nikon M iropolis fluorescence microscope and photographed with Kodak Tri-X Pan 400 film. In some cases samples were imaged using a Photometrics SenSys cooled CCD camera and images were processed and printed using IPLabSpectrum software.

Transmission Electron Microscopy

Samples for electron microscopy were fixed for 30 min on ice in 1.5% (vol/ vol) glutaraldehyde and 1% (vol/vol) paraformaldehyde in 0.1 M cacody late buffer, pH 7.4. Samples were pelleted for 1 min in an Eppendorf cen trifuge at 4 °C, and the chromatin/nuclear pellet was rinsed in cacodylate buffer. Pellets were postfixed for 30 min at 4 °C in 1% reduced tetroxide, dehydrated, and embedded in Spurr’s medium. Samples were sectioned (90-nm sections) and poststained in uranyl acetate followed by lead citrate. Electron micrographs of thin sections were taken on a TEM 10 microscope (Carl Zeiss, Inc.) at 60 or 80 kV.

In Vitro Replication Assays

DNA replication was assayed by incorporation of [α-32P]dCTP (see Pow ers et al., 1995). In brief, 1 μl of [α-32P]dCTP (R edive, 3,000 C/mmoll; Nyc omeds A mershaw) was added to 24–2 μl nuclear assembly reactions (20 μl cytosol, 2 μl membranes, 1 μl chromatin [stock concentration ~40,000Ci/μl], plus 1 μl buffer or recombinant LAP2b polypeptides). A negative con trol, aphidocin was added at a final concentration of 50 μM; this agent inhibits the activity of D NA polymerase α. A ternatively, independent neg ative control reactions were made 1 mM in GTP–γ–S to inhibit nu clear membrane formation (Boman et al., 1992) and, indirectly, D NA replication. After 3 h, samples were combined with an equal volume of stop buffer, protease K (Boehringer Mannheim G mbH) was added to 1 mg/ml final concentration, and samples were incubated at 37 °C for 2 h. To de tect incorporated [α-32P]dCTP, the protease-digested samples were mixed thoroughly by pipetting to ensure homogeneity, and 5-μl aliquots were electrophoresed through 0.8% agarose gels. Note that all reactions contained equal numbers of nuclei, and samples were processed without pel leting steps that might cause loss of material. Gel loading was monitored by ethidium stain contained in the gel. Dried gels were exposed to x-ray film, and signals quantitated with scanning densitometry using the M icro imaging D evice (Imaging Research Inc.). In one experiment, the signal was quantitated by both phosphorimaging and densitometry, with the same results.

To measure the time course of replication, 10-μl aliquots were removed every 15 min from 200-μl reactions (190 μl crude extract, 10 μl chromatin, 5 μl [α-32P]dCTP, plus 6 μl of buffer or purified LAP2 fragment 1–408), digested with proteinase K, and the D NA was separated on agarose gels and quantitated by PhosphorImager as described above.

Bromodeoxyuridine (BrDU) Density Substitution

D density substitution experiments were done essentially as described by Hu and Newport (1998), using both freshly prepared crude nuclear as sembly extracts (10,000 g cytoplasmic fraction), and high speed fractionated, frozen, reconstituted extracts, with the same results. Nuclei were as sembled for 90–120 min in the presence or absence of 3.3 μM fragment 1–408. Crude reactions containing 60 μl cytoplasm with 2,000 sperm per μl, plus 0.5 mM BrdU (Sigma Chemical Co.), 0.5 mM MgCl2, and 0.2 μCi of [α-32P]dCTP per μl of extract. Fractionated/reconstituted reactions contained 20 μl of cytosol, 12 μl of membrane, and a total of 100,000 sperm chromatin, plus 0.2 μCi/μl of [α-32P]dCTP and 0.5 mM BrdU. Reactions were stopped by adding 1 ml ice cold buffer A (50 mM KCl, 50 mM Hepes KOH, pH 7.4, 5 mM MgCl2, and 1 mM DTT), incubated on ice for 5 min, centrifuged 5 min at 16,000 g in a microfuge, resuspended in 100 μl buffer A, made 0.5% in SD S and 0.4 mgml in proteinase K, and digested for 2 h at 37 °C. D NA was then extracted three times with phenol-chloroform, once with chloroform, and ethanol precipitated using 0.3 M sodium acetate. Each ethanol pellet was resuspended in 100 μl TE, mixed with 12.7 ml of 1.75 g/ml CsCl, loaded into a Beckman 16 × 76 mm Quick-Seal tube, and centrifuged 45 h at 30,000 rpm at 20 °C in a Beckman Ti-70 rotor. 46–50 fractions (250–300 μl each) were collected by needle puncture from the bottom of each tube. To quantitate radioactivity, an aliquot of each fraction was counted by liquid scintillation (Beckman LS-7800). To determine the refractive index of each fraction was measured using a refractometer (Bausch & Lomb Inc.), and converted to density using the equation: d (in units of g/ml) = 0.98923 × d, where d is the specific gravity of solution at 20 °C. Values for d as cesium chloride at each index of refraction (n) are provided in the CRC A handbook (Weast, 1967).

Immunoblotting

To detect lamin accumulation by immunoblotting, nuclei were as sembled for 3 h in the presence or absence of human LAP2b fragments, diluted with 100 μl M MB, and then pelleted at top speed in an Eppifuge for 1 min and washed with 200 μl M MB. The washed nuclear pellet was resuspended in SDS-sample buffer, subjected to SDS-PA GE (12% gel), and

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proteins transferred to Immobilon PVDF membrane (Millipore Corp.). The Immobilon was blocked with 5% dry milk in TBS 0.1% Tween-20 (TBS-T-w) for 30 min, rinsed briefly in TBS-T-w, and incubated overnight at 4°C with either of two mAbs: antibody 46F7 (1:750 dilution in TBS-T-w; a kind gift from Prof. Georg Krohne; Lourim and Krohne, 1993), which is specific for Xenopus lamin B3 (formerly known as lamin L3), the major lamin found in Xenopus eggs (Lourim et al., 1996), and a monoclonal directed against human lamin B1 (Calbiochem; final concentration 100 μg/ml in TBS-T-w). Blots were rinsed six times (5 min each) with TBS-T-w and then incubated for 1 h at 22–24°C with HRP-conjugated anti-mouse secondary antibody in TBS-T-w (Newcom A mersham). The blots were washed again (six times for 5 min) and developed using enhanced chemiluminescence (ECL) reagents (Newcom A mersham). Both antibodies gave identical results, detecting a single band of ~70 kD that was present in Xenopus cytosol and (much less abundantly) in Xenopus membrane fractions.

Indirect Immunofluorescence of In Vitro Assembled Nuclei

To visualize nuclear lamins by immunofluorescence, nuclei were assembled in the presence or absence of LAP2β fragments for 3 h. A 2.5-μl aliquot of each assembly reaction was placed on a slide and covered with the siliconized side of an 18 mm square coverslip. The slide was plunged into liquid N2 for 10 s. Subsequent steps were performed at 22–24°C. The coverslip was quickly peeled off, and the sample was fixed/dehydrated in 100% methanol for 1 h. The slide was then rehydrated by incubation for 5 min each in 70, 50, and 30% methanol, then in PBS. A After washing twice in PBS/0.1% Triton, blocking for 5 min in PBS/0.1% Triton/2% BSA, the sample was incubated with 100 μg/ml mouse anti-human lamin B mAb (Calbiochem-Novabiochem Corp.) in PBS/0.1% Triton/2% BSA for 1 h. After extensive washes with PBS/2% BSA, the sample was incubated for 30 min with Texas red-conjugated goat anti-mouse antibody (Oligon Teknika), washed twice with PBS/2% BSA, and incubated with the DNA dye Hoechst 33342 for 5 min. After three more washes with PBS/2% BSA, the sample was overlayd with 5 μl glycerol, covered with an 18 mm square coverslip, and viewed by phase-contrast and immunofluorescence on a Nikon M ircoiphot fluorrecsce microscope.

Cloning Xenopus LAP2 Isolforms

A Xenopus stage V1 oocyte cDNA library in the UniZap vector (Stratagene Cloning Systems) was screened using full-length human LAP2b DNA as a probe. The probe was radiolabeled with α[32P]dCTP using the Multiscribe DNA labeling system (Newcom A mersham). The Xenopus cDNA library was a kind gift from D. Patterton and A. Wolfe (National Institutes of Health, Bethesda, MD). For the primary screen of 106 plaques, we used moderate stringency hybridization (30% formamide, 5× SSC, 42°C), and obtained ~300 positives. 20 strong positives were rescreened, and 12 remained positive. Single positive plaques from the tertiary screen were picked and converted to phagemids. GenBank accession numbers for the phagemid isolates are: clone 1 (AF048815), clone 2 (AF048816), clone 3 (AF048817), and clone 4 (AF048818).

To construct Xenopus LAP2b fragment 1–164, a 5’ primer was designed to code for an NdeI site followed by the first five amino acids 5’(GGGGCATACTGGCGAATTTCTG3)3, and a 3’ primer designed to encode the six terminal amino acids followed by an XhoI site 3’(CCCTCACTTGTTTTTACG3)3. These primers were used in a PCR reaction to obtain a 510-bp fragment using clone 2 as template. This PCR product was digested with NdeI and XhoI (Life Technologies, Inc.; Gibco BRL) and ligated into corresponding sites in pcET23a (Novagen, Inc.). In this case, the vector is predicted to express Xenopus LAP2b amino acids 1-164 followed by the six-His tag (173 amino acids; predicted mass of 19,749 D). The Xenopus fragment was expressed and purified as described above for the human LA P2b fragments.
clear assembly reactions at concentrations ranging from 0.16 to 54 μM (Fig. 2a). Fragment 1–408 had no detectable effect on vesicle binding, envelope enclosure, or nuclear import (see below), but inhibited envelope growth at concentrations as low as 1–3 μM (Fig. 2a). The nuclei became enclosed by an intact nuclear envelope at the same time as control nuclei (~30 min) but did not increase in size for at least 4.5 h. The final size of the arrested nuclei correlated inversely with the amount of fragment 1–408 in the reaction: at higher concentrations, the nuclei were smaller. At concentrations of 1.5–3 μM, fragment 1–408 reproducibly inhibited nuclear growth in all assembly extracts tested (three independent preparations), and virtually all nuclei were similar to those seen in Fig. 2a. Inhibition was not due to residual imidazole, since control nuclei, which were assembled with proteins purified from uninduced bacteria, assembled and grew normally (Fig. 2a, control).

**Nuclei Growth-arrested by Fragment 1–408 Are Active for Nuclear Import**

Three lines of evidence showed that nuclei growth-arrested by fragment 1–408 were not defective for nuclear import. First, they contained prenucleolar coiled bodies (Fig. 2a), the formation of which is dependent on nuclear import (Bell et al., 1992; Bauer et al., 1994). Second, they were active for DNA replication (see below), which also requires nuclear import. Third, we directly tested for import activity by first assembling nuclei for 2 h in the presence or absence of fragment 1–408, then adding a fluorescent karyophilic protein (rhodamine-conjugated nucleoplasmin), and imaging 30 min later. Nucleoplasmin accumulated at the nuclear rim and interior of the positive controls, and 1–408-arrested nuclei (Fig. 2b, left and middle). Negative control nuclei, pretreated with the transport inhibitor WGA for 5 min before adding the nucleoplasmin, failed to accumulate the transport substrate (Fig. 2b, right). Because fragment 1–408 had no detectable effect on nuclear import in three independent assays, we concluded that the nuclear expansion defect was probably a direct effect of fragment 1–408 on other nuclear structures or pathways.

**Inhibition by LAP2 Fragment 1–187: Scalloped, Nonenclosed Nuclear Envelopes**

Nuclei assembled for 3 h in the presence of purified fragment 1–187 appeared very different from 1–408-arrested nuclei, as seen by comparing Fig. 2a (panel labeled 3.1 μM) with Fig. 3a (upper right). Nuclei inhibited by fragment 1–187 remained small, and did not acquire a typical enclosed nuclear envelope. These effects were titratable over the low micromolar range. Nuclei assembled in the presence of 2–3 μM fragment 1–187 were smaller than positive controls. At 5–10 μM, the nuclear membranes consistently had an unusual scalloped morphology: some regions

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**Figure 2. Inhibition of nuclear growth by human LAP2b fragment 1–408.** (a) Nuclei assembled for 1.5 h in reactions containing the indicated concentration of fragment 1–408. The small phase-dense black dots (center of control nucleus) are coiled bodies, which form only in transport-competent nuclei. Upper panels show phase-contrast images of representative nuclei; lower panels show corresponding DNA, as visualized by Hoechst staining. (b) LAP2-arrested nuclei import a fluorescent karyophilic substrate. Nuclei were assembled for 2 h in the presence or absence of 3 μM LAP2 fragment 1–408, supplemented with rhodamine-conjugated nucleoplasmin, and viewed by epifluorescence 30 min later. A negative control, WGA, which inhibits nuclear import, was added to samples 5 min before adding nucleoplasmin (right panels). Upper panels show phase-contrast images of representative nuclei; lower panels show the corresponding nuclei by rhodamine fluorescence, indicative of transport activity.
both the enclosure and morphology of the nuclear envelope had quite different effects, interfering with lamina attachments (5–10 μM) of fragment 1–187. These results showed that the putative chromatin-binding fragment of LAP2 inhibited membrane attachment to chromatin at a concentration of 30 μM. However, at lower concentrations the NH2-terminal fragment had quite different effects, interfering with both the enclosure and morphology of the nuclear envelope.

**Ultrastructure of Arrested Nuclei**

To study their morphology in greater detail, nuclei were examined by transmission electron microscopy (TEM; Fig. 4). As expected, control nuclei were enclosed by two nuclear membranes and studded with NPCs (Fig. 4 a; inset arrows point to NPCs). Nuclei inhibited by fragment 1–408 had an enclosed nuclear envelope (Fig. 4 b), confirming our phase-contrast observations (Fig. 2 a). Although the particular cross-sections shown in Fig. 4, a and b, are similar in size, the magnifications are different, and 1–408-inhibited nuclei were much smaller than control nuclei (Fig. 2). TEM further revealed that 1–408-inhibited nuclei had numerous NPC-containing invaginations of the inner nuclear membrane (Fig. 4 b; arrows). These invaginations were morphologically distinct from the nuclear tunnels described by Fricker et al. (1997) in which the entire envelope invaginates to form tubules extending into the nuclear interior. Nuclei inhibited by 1.5–3 μM fragment 1–408 also appeared to have a higher density of NPCs than control nuclei, consistent with ongoing NPC assembly into growth-arrested nuclei.

**Lamins Accumulate in 1–408-arrested Nuclei, but Not 1–187-arrested Nuclei**

To ask if LAP2 fragments affected lamina assembly, we assayed the inhibited nuclei for lamin accumulation (Fig. 5 a). Nuclei were assembled for 3 h in reactions supplemented with either buffer, 3 μM fragment 1–408, or 5 μM fragment 1–187. Nuclei were then pelleted, subjected to SDS-PAGE, and immunoblotted with mAb 46F7, which is specific for lamin B3, the major lamin found in Xenopus eggs (Lourim and Khrone, 1993; Lourim et al., 1996) (Fig. 5 a). Identical results were obtained using an mAb raised against human lamin B (data not shown). Nuclei inhibited by fragment 1–408, but not those inhibited by fragment 1–187, accumulated lamins over time as shown by immunoblotting of pelleted nuclei (3-h time points shown; Fig. 5).
a). The lamin signal in 1–408-inhibited nuclei was sometimes slightly stronger than in control nuclei (two of five experiments), perhaps reflecting the presence of additional lamin-binding proteins (i.e., fragment 1–408) in these nuclei. These lamin results were consistent with the structural results, since 1–408-inhibited nuclei were enclosed and active for nuclear protein import, and 1–187-inhibited nuclei were not enclosed and did not accumulate imported proteins such as lamins.

We also used indirect immunofluorescence to detect lamins in inhibited nuclei (Fig. 5 b). Consistent with the lamin blots, lamins were not detected by indirect immunofluorescence in 1–187-inhibited nuclei. Lamins were detected both in control nuclei and nuclei inhibited by fragment 1–408, in close association with the nuclear envelope (Fig. 5 b). However, because the 1–408-inhibited nuclei were small, and the immunofluorescent signal quite bright (see Fig. 5 b), we could not determine unambiguously whether lamins were associated exclusively with the envelope, or if they might have also accumulated at inappropriate sites inside the nucleus.

**Fragment 1–187 Blocks Lamina Assembly**

As a negative control for the DNA replication experiments described below, we used GTPγS to inhibit vesicle fusion (Boman et al., 1992; Newport and Dumphry, 1992). No replication was observed in GTPγS-treated reactions, as expected, since these nuclei consist of small vesicles bound to the chromatin surface. However, the GTPγS-arrested nuclei accumulated a low level of lamins (Fig. 6 b; see also Wiese et al., 1997), suggesting that some interactions involving lamins may have proceeded to a limited extent even though vesicle fusion was inhibited by GTPγS. The modest accumulation of lamins on GTPγS-arrested nuclei contrasted significantly with 1–187-inhibited nuclei, which had only background amounts of lamins (compare to membranes alone; Fig. 5 a). We concluded that fragment 1–187 blocks lamin recruitment or attachment to the membrane–chromatin interface. Because there is strong evidence that the NH₂-terminal region of LAP2 binds to chromatin (Furukawa et al., 1998), we further concluded that fragment 1–187 binds competitively to, and blocks, the chromatin partners for endogenous LAP2 proteins. These findings therefore suggest that endogenous LAP2 isoforms must engage their chromatin partner as a prerequisite for lamina assembly.

**DNA Replication Assays**

DNA replication normally occurs in in vitro assembled nuclei if the lamina is properly assembled (Blow and Watson, 1987; Newport et al., 1990; Leno and Laskey, 1991; Cox, 1992). Therefore, replication is used as a marker for the assembly of a structurally intact nucleus. Since nuclei inhibited by fragment 1–187 did not accumulate lamins, we predicted that these nuclei would be unable to replicate. We did not know what to expect with 1–408-inhibited nuclei, which accumulated lamins. Fragment 1–408 is predicted to bind lamins (Foisner and Gerace, 1993; Furukawa et al., 1998), and might somehow disrupt lamina assembly, and hence, secondarily disrupt replication.

To test DNA replication, nuclei were assembled in the presence of [³²P]dCTP for 3 h, with or without added
LAP2 fragments, and the DNA was analyzed by agarose gel electrophoresis and autoradiography (Fig. 6 a). Aliquots were processed in parallel for Western blotting with an anti-lamin antibody (Fig. 6 b), and a representative nucleus from each sample is shown by phase-contrast (Fig. 6 c). No replication activity was detected in 1–187-arrested nuclei (Fig. 6 a), as predicted from their lack of enclosure. However, nuclei assembled in the presence of 3 mM LAP2 fragment 1–408 consistently replicated at levels as high (three experiments) or higher (eight experiments) than control nuclei (Fig. 6 a). In the experiment shown, 1–408-inhibited nuclei also accumulated lamins at higher levels than controls (Fig. 6 b). The increased replication signal in 1–408-inhibited nuclei was not due to unequal loading (ethidium stain of DNA; Fig. 6 a, lower panels). No replication was detected in negative controls treated with GTPγS to prevent envelope formation (Boman et al., 1992; Newport and Dunphy, 1992), or in the presence of 50 μM aphidicolin (Fig. 7), a specific inhibitor of DNA polymerase α (Ikegami et al., 1978). Note the modest degree of enhancement by fragment 1–408 in Fig. 7, an example of the low end of experimental variation in the extent of enhancement. We concluded that although the LAP2 fragment 1–408 blocked the expansion of nascent nuclei in vitro, it unexpectedly enhanced DNA replication activity. Based on densitometry quantitation of eight experiments, nuclei arrested by fragment 1–408 replicated an average of 2.5-fold better than controls.

Enhanced [32P]dCTP Incorporation Is Not Due to Rereplication

We considered two different mechanisms for the increase in [32P]dCTP incorporation: fragment 1–408 might cause rereplication, which would represent a loss of cell cycle control, or it might enhance semiconservative DNA replication, the efficiency of which can vary from 30 to 100% in Xenopus egg extracts (Leno and Laskey, 1991).

The first possibility, rereplication, was tested by equilibrium density substitution in the presence of α[32P]dCTP plus either buffer, 3 mM LAP2 fragment 1–408, 5 mM LAP2 fragment 1–187, or 1 mM GTPγS. (a) Total DNA was prepared from aliquots of each sample, electrophoresed on a 0.8% agarose gel, and the gel dried and exposed to film (see Materials and Methods). Upper panel shows the autoradiograph of dried agarose gel; the lower panel shows the ethidium stain of the same gel as a control for DNA loading. (b) Protein from each sample was blotted and probed using a monoclonal anti-lamin antibody (see Materials and Methods). (c) Corresponding phase-contrast images of a typical nucleus from each sample in a.
and one heavy strand, which would migrate as a single peak in a CsCl gradient. If the enhanced replication were due to rereplication, we would detect a second peak of heavy–heavy DNA at a higher density, and we would not expect the heavy–light DNA from LAP2-treated nuclei to have significantly more radioactivity than the positive control. The experiment was done using both fractionated/reconstituted extract (data not shown) and fresh 10,000 g crude cytoplasm (Fig. 8 a). We found that LAP2-arrested nuclei yielded a single peak of radiolabeled DNA that comigrated at exactly the same refractive index, and hence, density, as the positive control, indicating a single round of semiconservative DNA replication. Furthermore, the peak of incorporated nucleotide was at least fourfold higher than the positive control, consistent with fragment 1–408 enhancing the efficiency of semiconservative replication. These results effectively ruled out the possibility that LAP2 fragment 1–408 causes rereplication.

To examine the time course of replication in LAP2-arrested nuclei, nuclei were assembled in reactions containing α[32P]dCTP in the presence or absence of fragment 1–408; aliquots were removed from each reaction every 15 min, and the incorporated radiolabel was quantitated (Fig. 8 b; see Materials and Methods). The time course of replication was initially indistinguishable between LAP2-arrested nuclei and positive controls; there was a lag phase of 45 min, followed by DNA synthesis. The only difference was that α[32P]dCTP incorporation into LAP2-arrested nuclei continued at the same rate for ≈15 min longer than positive controls, reaching a plateau that was, in this case, ≈1.8-fold higher than the positive control (Fig. 8 b).

The replication results collectively led us to two conclusions. First, the majority of our extracts, which were made from unactivated eggs, were not 100% efficient for replication. Based on the amount of replication enhancement seen in these experiments, which ranged from 0 to ≈4-fold (average, 2.5-fold), we estimated that the efficiency of replication in our extracts varied from 20% to 100%. Second, in extracts that were <100% efficient for replication, LAP2 fragment 1–408 increased the efficiency of semiconservative DNA replication, when present at low (2–4 μM) concentrations. Possible mechanisms for the enhancement of replication efficiency by LAP2 are considered in the Discussion.

Xenopus Oocytes Express LAP2 cDNAs Closely Related to Mammalian LAP2β

Given the effects of human LAP2 fragments in Xenopus nuclear assembly extracts, it was essential to determine if Xenopus LAP2 had the same structural effect on nuclear assembly. To do this, and compare the Xenopus and human LAP2 proteins, we screened a stage VI Xenopus oocyte cDNA library using radiolabeled full-length human LAP2β as a probe (see Materials and Methods). We rescreened 20 of nearly 300 positives, and identified three cDNAs coding for Xenopus LAP2β homologues, which were designated clones 2, 3, and 4 (see Fig. 9 a, and Materials and Methods).

The three Xenopus LAP2 proteins are compared schematically to human LAP2β in Fig. 9 a. Clone 2 was the longest Xenopus LAP2 cDNA, encoding a protein of predicted mass 62,841 kD. Except for a single-base deletion at nucleotide 1119 in clone 3, which is either a mutation or the result of an alternative splicing event (see below), the three Xenopus cDNAs were identical at the nucleotide level except for two regions: nucleotides 595–705 and 1068–1278 in clone 2. These two regions encoded polypeptide inserts that we named insert A (37 residues, 198–234), insert B (17 residues, 357–373), and insert C (53 residues, 374–426), as diagrammed in Fig. 9 a. Clone 2 had all three inserts, whereas clone 3 lacked insert C, and clone 4 lacked...
insert A, suggesting that inserts A and C were alternatively spliced exons. Although insert B was present in all three Xenopus cDNAs, it was absent from human LAP2 and is therefore either a new exon, or a nonhomologous extension of the neighboring exon. All three putative new exons were located at exon boundaries in the mouse genomic sequence (Berger et al., 1996): insert A between mouse exons 5 and 6, and inserts B and C between mouse exons 8 and 9. We concluded that inserts A and C (and probably B) represent bona fide LAP2 exons in Xenopus.

Three regions were broadly similar between Xenopus and human LAP2 proteins: an NH2-terminal region (gray and white boxes in Fig. 9 a), a middle region (diagonal bars), and a COOH-terminal region (horizontal stripes). The predicted masses of the proteins encoded by the Xenopus LAP2 cDNAs are 62.841 kD (clone 2), 46.363 kD (clone 3), and 58.674 kD (clone 4). DNA sequence data are available from GenBank under accession numbers AF048815 (clone 2), AF048816 (clone 3), and AF048817 (clone 4). (b) Alignment of human LAP2β and Xenopus LAP2-clone 2 proteins using BOXSHADE (see Materials and Methods). Black shading indicates identity, gray shading indicates similarity.
Xenopus LAP2 Fragment 1–164 Inhibits Nuclear Growth

To assess the effects of Xenopus LAP2 on nuclear assembly, we focussed on the NH$_2$-terminal domain. We expressed and purified the Xenopus LAP2 fragment consisting of residues 1–164, which are homologous to human fragment 1–187, and added it to Xenopus nuclear assembly reactions at concentrations ranging from 1 to 10 µM (Fig. 10). The Xenopus LAP2 fragment had structural effects identical to those of human LAP2 fragment 1–187, producing scalloped, nonenclosed envelopes at concentrations of 1, 2.5, and 5 µM (Fig. 10). At 10 µM, fragment 1–164 interfered with membrane targeting to the chromatin surface (Fig. 10), paralleling the effects of human fragment 1–187 at 30 µM (Fig. 3 b). We noted that Xenopus fragment 1–164 was inhibitory at concentrations two to five times lower than the comparable human fragment. We concluded that the human and Xenopus NH$_2$-terminal fragments of LAP2 had the same effects on nuclear assembly in vitro. These results strongly suggested that the human polypeptides interacted with bona fide LAP2 binding partners in Xenopus extracts, with slightly lower efficiency, presumably due to species-specific differences in amino acid sequence. We concluded that the human LAP2 fragments used in our experiments affected nuclear assembly and DNA replication by competing for the binding partners of endogenous LAP2 proteins.

Discussion

Consistent with previous results from microinjected HeLa cells (Yang et al., 1997), our in vitro results show that a LAP2 fragment capable of binding to lamins has the effect of blocking nuclear expansion after enclosure. This finding confirms the importance of LAP2 and lamins in mediating nuclear growth. We further show that nuclei growth-arrested by the chromatin-and-lamin-binding nucleoplasmic domain of LAP2 (residues 1–408) were enhanced in their efficiency of semi-conservative DNA replication; this result has important new implications for LAP2 function, as discussed below. In contrast to Yang et al. (1997), who found that residues 1–85 of the conserved NH$_2$-terminal domain had no effect in vivo, the complete NH$_2$-terminal chromatin-binding domain of both human and Xenopus LAP2 strongly inhibited nuclear assembly, producing a scalloped envelope morphology and blocking lamina assembly. The implications of this phenotype are discussed below.

Is Chromatin Binding by LAP2 Proteins a Prerequisite for Lamin Assembly?

Several studies suggest that the LAP2 isoforms are collectively responsible for dynamically organizing the lamina: the biochemical demonstration that rat LAP2 binds lamin B (Foisner and Gerace, 1993), the sequential colocalization of LAP2 with lamin B and lamin A during nuclear assembly in vivo (Dechat et al., 1998), the in vitro and in vivo nuclear growth arrest by lamin-binding fragments of LAP2 (Yang et al., 1997; this study), and the block to lamin accumulation caused by the NH$_2$-terminal chromatin-binding domain of LAP2 (human residues 1–187; this study). The finding, that nuclei arrested by fragment 1–187 did not accumulate lamins, was unexpected since this shared domain of LAP2 binds to chromatin, not lamins. We suggest that when the recombinant chromatin-binding domain of LAP2 is added to assembly reactions, it occupies chromatin sites and prevents endogenous LAP2 proteins from attaching to chromatin. To explain how fragment 1–187 then prevents lamin assembly, we propose that the endogenous LAP2 proteins need to bind to chromatin as a prerequisite for binding to lamins and promoting lamina assembly.

We characterized three cDNAs from Xenopus, which appear to be new β-related isoforms of LAP2. These cDNAs include three putative novel exons, referred to as inserts A, B, and C. Insert A is positioned immediately after the conserved NH$_2$-terminal domain. Inserts B and C interrupt the minimal lamin-binding region of LAP2 (identified as residues 298–373 in rat LAP2β; see Yang et al., 1997). Interestingly, the minimal lamin-binding region is not encoded by a single exon, but spans exons 8 and 9 (mice; Berger et al., 1996). Xenopus inserts B and C are located precisely between mice exons 8 and 9, inserting 17 and 53 residues, respectively, into the middle of the lamin-binding region. Lamin-binding activity is influenced by the COOH-terminal region of LAP2, since residues 289–452 of rat LAP2β bind to lamins fivefold better than minimal residues 298–373 in a yeast two-hybrid assay (Furukawa et al., 1998). In view of our hypothesis that LAP2 chromatin-binding activity may regulate or promote lamin recruitment, it will be interesting to determine the lamin-binding and other activities of Xenopus LAP2 isoforms that have, or lack, each new exon.

LAP2 Proteins May Influence Chromatin Structure

One explanation for why fragment 1–408 (the chromatin-and-lamin-binding fragment) arrests the expansion of nascent nuclei is that it may contribute to the formation of excess or unregulated connections between lamins, membranes, and chromatin. This possibility is supported by the extensive invagination of the inner membrane seen in nu-
nuclei arrested by fragment 1–408. However an alternative possibility, that this chromatin-and-lamin-binding LAP2 fragment inhibits chromatin decondensation, is supported by our finding that high concentrations (54 μM) of fragment 1–408 caused the sperm chromatin to remain smaller than the size expected of chromatin swelled by exposure to egg cytosol (Fig. 2 a; see Newport and Dumphry, 1992). The association of LAP2 with chromatin is relatively strong, since immunoaffinity-purified LAP2β binds substantially to mitotic chromosomes with an affinity of 40–80 nM (Foision and Gerace, 1993). We hypothesize that at high concentrations, recombinant LAP2β may either block chromatin decondensation, or promote condensation.

Interestingly, a chromatin binding partner for LAP2 has been provisionally identified by two-hybrid analysis in yeast as BA F (barrier to autointegration factor; Furukawa, 1999). BA F localizes to the nucleus during interphase, and to chromosomes during mitosis (Furukawa, 1999). BA F is a small novel cellular protein (89 residues) that was identified because it facilitates the efficient integration of HIV DNA into the cell’s genome (Chen and Engelman, 1998; Lee and Craigie, 1998); in the absence of BA F, the viral DNA molecule tends to integrate intramolecularly into itself. Lee and Craigie (1998) propose that BA F acts by crossbridging and thereby compacting the viral DNA molecule. The normal cellular role of BA F is not known. If further experiments confirm that BA F and LAP2 are indeed binding partners, it will be interesting to test the idea that LAP2 influences chromatin structure by affecting BA F activity.

**Concentration-dependent Effects of LAP2 Fragment 1–408 on DNA Replication**

The minimal lamin-binding region of LAP2β (rat residues 298–373) inhibits the initiation, but not the progression, of DNA replication in vivo (Yang et al., 1997). Consistent with this, the full nucleoplasmic region of human LAP2β (fragment 1–408) also inhibited DNA replication at concentrations above 6 μM (data not shown). However at lower concentrations (1–3 μM), which reproducibly inhibited nuclear expansion, fragment 1–408 enhanced DNA replication activity by an average of 2.5-fold in ~80% of our experiments. Thus, by varying the concentration of recombinant protein in the reaction, we uncovered a positive role for LAP2β in replication.

Based on density-substitution experiments, we eliminated the possibility that increased nucleotide incorporation was due to rereplication. A second possibility, that fragment 1–408 triggers extreme levels of repair synthesis, seems unlikely, but cannot be ruled out by our present data. Our results favor a third possibility, that LAP2β fragment 1–408 increases the efficiency of semiconservative DNA replication. This increased efficiency could be an indirect effect of LAP2 on nuclear size, since the small arrested nuclei may achieve higher concentrations of imported replication factors. For example, chromatin can replicate efficiently in the absence of nuclei in vitro, by sequential exposure to cytosolic extracts and 25-fold concentrated nucleoplasmic extracts (Walter et al., 1998). We can provisionally rule out such a size-based model for one simple reason: 1–408-arrested nuclei were always small, but replication was not enhanced in ~20% of our experiments (see below). We think this extract-to-extract variation is an important clue about the mechanism of enhancement.

Logically, replication enhancement is only possible in extracts that are less than 100% efficient. Xenopus egg extracts can vary in replication efficiency from 30% to 100% (Cox and Leno, 1990; Leno and Laskey, 1991; Walter et al., 1998). The cause of this variation is not yet known, but we hypothesize that it may reflect differences between extracts in the efficiency with which prereplication complexes assemble onto chromatin. The prereplication complex consists of MCM proteins, thecdc6 protein, and six ORC proteins; this complex can only be assembled when there are no cyclin-dependent kinases (CDKs) active in the cell (see Dillin and Rine, 1998, and references therein). In somatic cells, such a situation only exists for a narrow window of time between anaphase (when the mitotic cyclin-dependent kinases are inactivated) and early G1 (when G1-phase kinases are activated). According to the two-step model for replication control, which is widely supported by evidence from yeast and Xenopus, the prereplication complex is the obligatory precursor of the replication complex, and is removed during replication, thereby providing a mechanism to limit replication to a single round per cell cycle (see Stillman, 1996; Hua et al., 1997; J alepalli and Kelly, 1997).

Eggs have high levels of mitotic CDK activity (also known as maturation promoting factor activity), which maintains them in a metaphase-arrested state until fertilization. Fertilization triggers a wave of intracellular Ca2+ release, which destroys the Ca2+-sensitive cytostatic factor stabilizing maturation promoting factor (Lorca et al., 1993; Matsus, 1996). Many investigators mimic fertilization by either exposing eggs to Ca2+ or electric shock, before making extracts. In contrast, our extracts are from unactivated eggs; activation is not essential to obtain extracts competent for interphase nuclear assembly (Wilson and Newport, 1988), probably because unactivated eggs are exposed to contaminating Ca2+ ion during extract preparation. To explain our extract-to-extract variation in both replication enhancement per se (no enhancement in ~20% of experiments) and the degree of enhancement (up to fourfold; averaging 2.5-fold), we hypothesize that in many of our extracts the mitotic CDK activity is not fully inactivated. Trace CDK activity might decrease the number of prereplication complexes that can assemble, and thus reduce the efficiency of replication. Further experiments are needed to test this hypothesis.

**Hypothesis: LAP2 Proteins Serve as Downstream Effectors of Lamina Assembly in Promoting DNA Replication, Perhaps by Influencing Chromatin Structure**

Nuclei assembled in lamin-depleted extracts cannot undergo DNA replication (Newport et al., 1990; reviewed by Gant and Wilson, 1997), initially suggesting that lamina assembly is required for DNA replication initiation or progression. However as noted above, replication can occur in the absence of nuclei if the chromatin is exposed to concentrated nucleosolic extracts (Walter et al., 1998). The replication-promoting components of these nucleosolic
extracts have not yet been identified, and are likely to include multiple factors potentially including LAP2 isoform(s). Based on our evidence that LAP2 may affect chromatin structure, and that the full nucleoplasmonic portion of LAP2β (fragment 1-408) enhances the efficiency of DNA replication, we hypothesize that LAP2 isoform(s) may act as downstream effectors of lamin assembly by promoting chromatin conformations favorable either to the assembly of prereplication complexes, or the progression of replication complexes. Further experiments are needed to determine if the putative lamin-influencing activities of LAP2 proteins depend on lamin assembly, if they regulate lamin assembly, or both.

The idea that LAP2β might promote replication by affecting chromatin structure has a precedent when one considers LBR, the lamin B receptor. LBR interacts with a chromatin partner named H1p1 (Y e and W orman, 1996; Y e et al., 1997), which mediates repressive higher-order chromatin structure in Drosophila (reviewed by E lgin, 1996). In turn, Drosophila H1p1 is known to bind ORC1 (P ak et al., 1997). Thus, LBR (an inner nuclear membrane protein) binds to H1p1, which can bind a core component of the prereplication complex. The meanings and mechanisms of these interactions are not yet clear. However, it appears that LBR, and perhaps LAP2, may influence chromatin structure in ways that could modulate the competence of chromatin for DNA replication, and also conceivably its competence for transcription. Our identification of new exons in the lamin-binding region of X enopus LAP2 isoforms increases the potential for subtle differences in functions of the various LAP2 isoforms. Further analysis of the effects of LAP2 isoforms on nuclear dynamics, chromatin structure, DNA replication, and potentially the transcriptional competence of chromosomes will be of interest.

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