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GST-lamin fusion proteins act as dominant negative mutants in *Xenopus* egg extract and reveal the function of the lamina in DNA replication

Darren J. Ellis, Hazel Jenkins, William G. F. Whitfield and Christopher J. Hutchison*

The Department of Biological Sciences, The University of Dundee, Dundee DD1 4HN, Scotland, UK

*Author for correspondence

SUMMARY

A cDNA encoding Xlamin B_1 was cloned from a whole ovary mRNA by RT-PCR. GST-lamin fusion constructs were generated from this cDNA by first creating convenient restriction sites within the Xlamin B_1 coding sequence, using PCR directed mutagenesis, and then sub-cloning relevant sequences into pGEX-4T-3. Two expression constructs were made, the first, termed Δ2+ lacked sequences encoding the amino-terminal 'head domain' of lamin B_1 but included sequences encoding the nuclear localization signal sequence (NLS). The second expression construct, termed Δ2–, lacked sequences encoding the amino-terminal 'head domain' as well as sequences encoding the NLS. Purified fusion proteins expressed from these constructs, when added to egg extracts prior to sperm pronuclear assembly, formed hetero-oligomers with the endogenous lamin B_3. The Δ2+ fusion protein prevented nuclear lamina assembly but not nuclear membrane assembly. The resulting nuclei were small (~10 μm in diameter), did not assemble replication centers and failed to initiate DNA replication. When the Δ2– fusion protein was added to egg extracts prior to sperm pronuclear assembly, lamina assembly was delayed but not prevented. The resulting nuclei although small (~12 μm), did form replication centers and initiated DNA replication. When added to egg extracts after sperm pronuclear assembly was completed Δ2+, but not Δ2–, entered the pre-formed nuclei causing lamina disassembly. However, the disassembly of the lamina by Δ2+ did not result in the disruption of replication centers and indeed these centres remained functional. These results are consistent with the hypothesis that lamina assembly precedes and is required for the formation of replication centers but does not support those centers directly.

Key words: Nuclear lamina, *Xenopus* egg extract, DNA replication, Nuclear matrix

INTRODUCTION

The nuclear lamina is a filamentous meshwork composed of type V intermediate filament proteins, the lamins, which lines the nucleoplasmic face of the inner nuclear membrane and which interconnects nuclear pores (Gerace and Blobel, 1980; McKeon et al., 1986; Aebi et al., 1986; Goldberg and Allen, 1992; Zhang et al., 1996). Lamins fall into two sub-groups, the A-type and B-type lamins. One or more B-type lamins are thought to be expressed constitutively in all embryonic and somatic tissues (Lehner et al., 1987; Wolin et al., 1987; Vorburger et al., 1989a) although different tissues may contain different B-type lamins (Stick and Hausen, 1985; Benevente et al., 1985; Höger et al., 1988). In contrast, expression of A-type lamins is restricted to differentiated tissues (Lehner et al., 1987; Rober et al., 1989). Both types of lamin contain a sequence motif CaaX (C, cysteine; a, aliphatic amino acid, X, any amino acid) at the carboxyl terminus. This sequence serves as a site for modification by isoprenylation (Beck et al., 1990; Wolda and Glomset, 1988; Vorburger et al., 1989b; Firmbach-Kraft and Stick, 1993) and methylation (Chelsky et al., 1987). However, A-type and B-type lamins differ in that the modified cysteine residue in the CaaX motif can be removed by proteolytic cleavage of the final 18 amino acids in lamin A (Beck et al., 1990; Vorburger et al., 1989b; Weber et al., 1989). In addition, A-type lamins can be synthesised from an alternatively spliced mRNA species that lacks codons for the final 82 amino acids and therefore lacks the CaaX motif (this shortened lamin is termed lamin C; Fisher et al., 1986; Lin and Worman, 1993). Isoprenylation at the CaaX motif is thought to function in positioning the lamins at the nuclear envelope since the farnesyl residues (the isoprene added to the lamins) incorporated at this site can associate with the inner nuclear membrane probably via an isoprenyl receptor (Firmbach-Kraft and Stick, 1993; Meier and Georgatos, 1994). Consequently, removal of farnesylated and methylated cysteine residues from A-type lamins during interphase may result in these species dissociating from the nuclear envelope at mitosis. In contrast, B-type lamins, which retain their modified cysteine residues, segregate with nuclear envelope vesicles on nuclear envelope breakdown (Gerace and Blobel, 1980; Stick et al., 1988; Meier and Georgatos, 1994). Interestingly, both in vivo and in vitro, B-type lamins segregate in different membrane vesicles from those containing protein markers of the inner nuclear envelope (Chaudhary and Courvalin, 1993; Collas et al., 1996).

The other major modification process which regulates lamin behaviour is phosphorylation/dephosphorylation. At mitosis, in vertebrate cells, the nuclear envelope is disassembled at prometaphase and must therefore be reassembled at telophase

(reviewed by Cox and Hutchison, 1994). Disassembly of the lamina is regulated through phosphorylation (Ottaviano and Gerace, 1984; Miake-Lye and Kirschner, 1985) by cdc2/cyclin B (Peter et al., 1991). Cdc2 phosphorylation occurs at two sites situated at the amino- and carboxyl-ends of the rod domains (Peter et al., 1990). If these sites are mutated by serine to arginine substitutions, lamina disassembly at mitosis is blocked (Heald and McKeon, 1990). Lamina reassembly is regulated by both phosphorylation and dephosphorylation processes. Drugs which inhibit the activity of protein phosphatase 2a also prevent lamina assembly in *Xenopus* eggs (Murphy et al., 1995). In addition, phosphorylation of lamin B_2 by protein kinase C (PKC), at a site adjacent to the nuclear localization signal sequence, prevents translocation of this lamin across the nuclear envelope. Lamins which are transported across the nuclear envelope during interphase are incorporated into the lamina (Goldman et al., 1992). Therefore it seems likely that PKC phosphorylation also regulates lamina assembly processes.

The best evidence for the function of lamina filaments comes from cell-free extracts of *Xenopus* eggs which support the assembly of replication competent nuclei in vitro (Blow and Laskey, 1986). DNA replication in these nuclei is entrained to normal embryonic cell cycle events, initiation occurring once per cell cycle (Hutchison et al., 1988) and being dependent upon the breakdown and reformation of the nuclear envelope (Blow and Watson, 1987; Blow and Laskey, 1988). Indirect immunofluorescence analysis indicates a close temporal and spatial correlation between lamina assembly and the initiation of DNA replication (Hutchison et al., 1988). Furthermore, removal of lamin B_3 (the major lamin sub-type in *Xenopus* eggs) from the extracts, using monoclonal or polyclonal antibodies, permits the assembly of nuclei which lack a lamina and which do not support DNA replication (Newport et al., 1990; Meier et al., 1991; Jenkins et al., 1993). These nuclei have complete nuclear envelopes containing nuclear pores (Jenkins et al., 1995; Goldberg et al., 1995) and support active transport of karyophilic proteins such as the proliferating cell nuclear antigen (PCNA) (Jenkins et al., 1993). However, the assembly of replication centers containing PCNA is prevented (Jenkins et al., 1993). Replication centers are distributed throughout the nucleoplasm (Mills et al., 1989) and are only associated with the lamina indirectly (Hutchison, 1995). Thus the absence of replication centers in nuclei assembled in lamin B_3 depleted extracts probably arises because these nuclei lack a properly organized nuclear matrix. The corollary of this is that the assembly of a nuclear matrix is dependent upon the prior assembly of a nuclear lamina (Hutchison et al., 1994).

It has been suggested recently that lamins are structural components of both nuclear matrix filaments (Hozak et al., 1995) and replication centers (Moir et al., 1994). Therefore a second interpretation consistent with the results of lamin depletion of egg extracts is that lamin B_3 is directly involved in the formation of replication centers. However, in a recent ultrastructural investigation, lamin B_3 was not detected in internal nuclear matrix filaments of sperm pronuclei assembled in vitro (Zhang et al., 1996).

We wished to investigate whether or not lamin polypeptides are components of nuclear matrix filaments which support replication centers directly. To do this we constructed GST-lamin fusion proteins which would act as dominant negative mutants in *Xenopus* egg extracts. Using these mutant proteins we show that lamina assembly precedes and is required for the initiation of DNA replication but that the lamina is not necessary for the elongation phase of DNA replication.

MATERIALS AND METHODS

Cloning of Xlamin B_1 and generation of GST-lamin fusion constructs

First strand cDNA was prepared from whole ovary mRNA, from which a cDNA encoding Xlamin B_1 was amplified by PCR using the sense strand oligonucleotide (5′-CGGTACCGATCCTGTTGTAGA-3′) and the anti-sense strand oligonucleotide (5′-GGATCA-CATCAGTAGGACGT-3′). The amplified lamin B_1 cDNA was sequenced by thermal cycle sequencing, and compared to the sequence for Xlamin B_1, recorded in the EMBO data base (accession no. X06344; Krohne et al., 1989). The Δ2− clone was created by inserting a *Sal*I site at codons 34 and 35 using the mutagenic primer 5′-GAGAAAGTCCAGCTGCAGGAGCTC-3′ and a *Not*I site downstream of codon 411 using the primer 3′-GCTCGACACGCGT-GTTGATCGCCGGCGTCTTTC-5′. The Δ2+ clone was created by inserting a *Sal*I site at codons 34 and 35, as described above, and a *Not*I site downstream of codon 420 using the primer 3′-CTTTCGCT-TAACTACATCGCCGGCGACTCCGG-5′. The resulting *Sal*I-*Not*I fragment were sub-cloned into the expression vector pGEX-4T-3 (Pharmacia) using standard protocols (Sambrook et al., 1989).

Expression of GST-lamin fusion proteins

The expression of lamin B_1 cDNA cloned into pGEX-4T-3 essentially followed the method of Smith and Johnson (1988). Single colonies from transformed *Escherichia coli* (strain BL21) were inoculated into 10 ml of terrific broth (TB) containing 100 μg ml^{-1} ampicillin and grown up overnight. The overnight culture was added to 1 litre of TB containing 100 μg ml^{-1} ampicillin and the bacteria grown at 37°C until they reached an OD_{600} of 0.9, at which point expression was induced by addition of IPTG at 1 mM. Bacterial growth was continued for a further 3 hours. Bacterial cells were pelleted at 5,000 rpm for 5 minutes and washed once in ice-cold PBS. The recombinant proteins were recovered as inclusion granules following lysis of bacterial cells with lysozyme. The inclusion granules were solubilised in 8 M urea, 25 mM Tris-HCl, pH 8.0, 1 mM DTT and purified by ion exchange chromatography using Whatman QMA cartridges.

Preparation and use of egg extracts

Xenopus egg extracts were prepared and frozen as described previously (Hutchison, 1994) and stored in liquid nitrogen. Prior to use each extract was supplemented with GST-lamin fusion proteins. The fusion protein had been dialysed into renaturation buffer before use (500 mM NaCl, 25 mM Tris-HCl, pH 8.0, 1 mM DTT) and was added to a final concentration of 80 μg ml^{-1}. As a control an equivalent volume of renaturation buffer was added to extracts. The extracts were then mixed with an energy regenerating system (Hutchison, 1994), demembranated sperm heads (10^3 μl^{-1}) and biotin-16-dUTP (4 μg ml^{-1}). The extracts were incubated as 20 μl aliquots at 21°C.

Lamina disassembly assay

Sperm pronuclei were assembled in egg extracts supplemented with 25 μg ml^{-1} aphidicholin over ninety minutes. Each extract was supplement with Δ2− or Δ2+ (at a final concentration of 80 μg ml^{-1}) and incubated for a further 90 minutes at 21°C. The extracts were then fixed with 50 mM EGS (ethylene glycol-bis-succinimidyl succinate) and nuclei recovered onto poly-lysine treated coverslips as described below. Alternatively, the extracts were diluted in nuclear isolation buffer (NIB: 60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.4, 1 mM 2-mercaptoethanol, 0.5 mM spermidine and 0.15 mM spermine) and fresh extract (supplemented with biotin-16-dUTP and the appropriate fusion protein but lacking aphidicholin) was added under the

suspension. Nuclei were transferred to the fresh extract by centrifugation for 5 minutes at 600 ***g*** and the NIB was removed. The nuclei were incubated in the fresh extract for a further 60 minutes at 21°C before fixation in 50 mM EGS.

Nuclear isolation and washing

Sperm pronuclei assembled in *Xenopus* egg extracts were isolated according to the method described by Jenkins et al. (1993). Briefly, a minimum of 2×10^5 nuclei were incubated in 100 μl of egg extract at 21°C. Following nuclear assembly, the samples were diluted in 1 ml of ice-cold nuclear isolation buffer (NIB: 60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.4, 1 mM 2-mercaptoethanol, 0.5 mM spermidine, 0.15 mM spermine). The diluted suspension was layered over a 1 ml 60% Percoll cushion (v/v in NIB) and separated by centrifugation at 3,000 ***g*** for 10 minutes at 4°C. The pronuclei recovered at the interface were diluted in 1 ml of ice-cold NIB and layered onto a 0.5 ml 25% sucrose cushion (w/v in NIB). These were recovered in a pellet following centrifugation at 4,000 ***g*** for 10 minutes in an Eppendorf microcentrifuge. The final pellet was washed once in CSK buffer (10 mM Pipes-KOH, pH 6.8, 10 mM KCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EDTA, 1.2 mM PMSF) and suspended in 0.5ml of CSK buffer containing 0.5% Triton X-100. Nuclei were incubated for 10 minutes at RT and then recovered by centrifugation (1 minute at 13,000 ***g***). The Triton washed pellet was suspended in SDS sample buffer and either stored at –70°C or used immediately for SDS-PAGE and immunoblotting.

Indirect immunofluorescence microscopy

20 μl aliquots of extract containing sperm pronuclei were fixed in EGS at 37°C as described previously (Hutchison, 1994). The fixed nuclei were recovered onto poly-lysine treated glass coverslips by sedimentation through a 30% glycerol cushion at 1,500 ***g*** for 15 minutes. The coverslips were stained overnight at 4°C with primary antibodies as follows: the anti-lamin B_3 mouse monoclonal antibody (mAb) L6 5D5 was diluted 1:100 in PBS containing 1% new-born calf serum (PBS/NCS) together with rabbit anti-GST antibody (diluted 1:2,000 in PBS/NCS, a generous gift from Dr Carl Smythe). A human anti-PCNA antibody (diluted 1:10 in PBS/NCS; Immunoconcepts Ltd) was used to detect replication centres.

For nuclei co-stained with L6 5D5 and rabbit anti-GST antibodies, secondary antibody incubations were with FITC-conjugated goat anti-mouse Ig and TRITC conjugated swine anti-rabbit Ig (DAKO, both diluted 1:25 in PCB/NCS). For nuclei incubated with anti-PCNA antibodies, secondary antibodies were FITC rabbit anti-human (ImmunoResearch Laboratories, diluted 1:50 in PBS/NCS) supplemented with Texas Red streptavidin. Secondary antibody incubations were for 4 hours at 4°C. Following each antibody incubation, coverslips were washed five times in PBS. Following the final wash, coverslips were mounted face down in Mowiol (Calbiochem) containing 1 μg ml^{-1} DAPI. Nuclei were viewed using a Hamamatsu CCD camera attached to a Zeiss Axioskop fitted with a ×40 NA 1.4 PlanNeofluor oil immersion objective. Representative images were collected using BioVision software. Alternatively, nuclei were viewed using a Bio-Rad MRC 600 laser scanning confocal microscope attached to a Nikon Labophot fitted with a ×60 NA 1.4 PlanNeofluor oil immersion objective. Background elimination but not contrast stretch was performed on all images.

Immunoprecipitation

Lamin B_3 was recovered from extracts using mAb L6 5D5 conjugated to paramagnetic Dynabeads. L6 5D5 was conjugated to Dynabeads as described previously (Jenkins et al., 1993). The equivalent of 20 μl of Dynabead was mixed with 30 μl of extract by rotation of a blood wheel for 1 hour at RT. The Dynabeads were then recovered as a pellet using a magnetic particle separator and washed three times in PBS. The Dynabead pellet was prepared for SDS-PAGE by suspension in SDS sample buffer.

Gel electrophoresis and immunoblotting

All samples were resolved on 8% SDS-PAGE at a constant current of 30 mA. Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell) at a constant current of 250 mA for 1 hour in blot transfer buffer (25 mM Tris, 200 mM glycine, 20% AnalaR methanol) (electrophoresis and transfer were performed using a Bio-Rad Protean II minigel apparatus). Filters were blocked in Blotto (3% dried skimmed milk powder prepared in blot rinse buffer (BRB), 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20) for 1 hour at RT. The filters were rinsed in BRB prior to incubation with primary antibody. Primary antibody incubations were with mAb L6 8A7 (Stick, 1988) diluted 1:100 in BRB containing 1% NCS or rabbit anti-GST, diluted 1:2,000 in BRB containing 1% NCS overnight at 4°C. Following incubation with primary antibodies, the filters were washed 5 times for five minutes in BRB. Secondary antibody incubations were with alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit Ig for 1 hour at RT. Following incubations with secondary antibodies, the filters were washed 3 times for five minutes with BRB and once with TBS (10 mM Tris-HCl, pH 7.5, 500 mM NaCl) and developed with NBT and BCiP.

RESULTS

Production and expression of GST-lamin fusion peptides

First strand cDNA was prepared from whole ovary mRNA as described above. A cDNA encoding Xlamin B_1 was amplified from this first strand 'library' by PCR. Two fragments were generated (by creating convenient restriction endonuclease sites by PCR mutagenesis) which corresponded to amino acids 34-420 (Δ2+) and 34-411 (Δ2–), respectively. Each fragment was cloned into pGEX-4T-3 (Pharmacia) and expressed with GST-fused to its amino terminus. Fusion proteins expressed in *E. coli* BL21 formed insoluble inclusion granules which were solubilised in 8 M urea and purified by ion exchange chromatography. The purity of each fusion protein was assessed by SDS-PAGE and western blotting. Typical preparations are shown in Fig. 1.

GST-lamin fusion peptides form hetero-oligomers with native lamin B_3 in *Xenopus* egg extracts and inhibit lamina assembly

To determine the function of each GST-lamin fusion protein, both Δ2+ and Δ2– were added separately to egg extract at a final concentration of 80 ng μl^{-1}. Alternatively, extracts were diluted with an equivalent volume of buffer. After incubation for 150 minutes endogenous lamin B_3 was recovered using mAb L6 5D5 (this monoclonal antibody reacts with lamin B_3 specifically (Stick, 1988) and fails to detect GST-lamin B_1 fusion proteins; data not shown) linked to paramagnetic Dynabeads. Proteins were resolved on SDS-PAGE, transferred to nitrocellulose and immunoblotted with mAb L6 8A7 (which detects lamins B_1, B_2 and B_3). Both fusion proteins co-precipitated with lamin B_3 (Fig. 2A and B) indicating the formation of hetero-oligomers between lamin B_3 and the GST-lamin fusion proteins. To determine the influence of the fusion proteins on nuclear assembly, each fusion protein was added separately to egg extracts prior to the addition of demembranated sperm heads. Following a 150 minute incubation period, sperm pronuclei were recovered and prepared for indirect immunofluorescence microscopy. Pronuclei were co-stained with mAb L6 5D5 to reveal the distribution of lamin B_3 and rabbit anti-GST antibodies to reveal the distribution of the fusion proteins. Typical

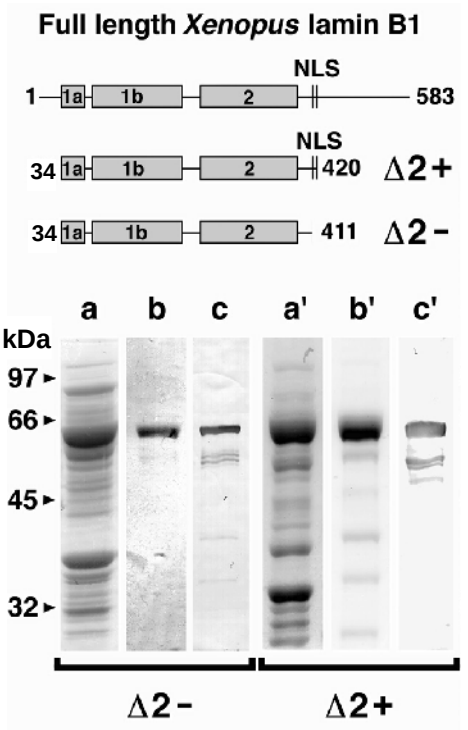

Fig. 1. Expression of Δ2+ and Δ2− GST fusion proteins. The rod domain fragments of *Xenopus* lamin B_1 shown were expressed with GST fused at the lamin's N terminus. Both fusion proteins include the entire coiled-coil forming domain in addition to the C terminus tail sequence which either included the NLS Δ2+ fusion protein) or excluded the NLS Δ2− fusion protein). Fusion proteins expressed in *E. coli* BL21 formed insoluble inclusion granules (tracks a,a′). Washed inclusion granules were solubilised in urea buffer (8 M urea, 25 mM Tris-HCl, pH 8.0, 1 mM DTT) and purified using ion exchange chromatography. The purity of prepared fusion protein was assessed using SDS-PAGE (tracks b,b′) and western blotting analysis using a rabbit polyclonal antibody raised to the GST moiety (tracks c,c′). Key: NLS above vertical bars shows the position of the nuclear localisation signal sequence; 1a, 1b and 2 within grey boxes show the locations of coiled-coil domains 1a, 1b and 2; 1 = residue 1; 34 = residue 34; 411 = residue 411; 420 = residue 420 and 583 = residue 583.

results are shown in Fig. 2C. In control extracts (diluted with buffer) pronuclei were large (having typical diameters varying between 18 and 25 μm) and possessed a well-formed lamina (revealed by perinuclear anti-lamin B_3 immunofluorescence). In contrast, pronuclei assembled in extracts supplemented with Δ2− were smaller (having diameters of ~12 μm). These nuclei did possess a lamina but the intensity of anti-lamin B_3 immunofluorescence was noticeably reduced. While anti-lamin B_3 was perinuclear it appeared to be discontinuous. In addition, the GST-lamin fusion protein was also detected at the nuclear lamina. Pronuclei assembled in extracts supplemented with Δ2+ were also small (having diameters of ~10 μm). However, no peripheral anti-lamin B_3 immunofluorescence was detected in these nuclei indicating the absence of a lamina. Weak nucleoplasmic anti-lamin B_3 and anti-GST immunofluorescence was detected indicating the presence of both lamin B_3 and Δ2+ protein in each pronucleus. These results indicate that both fusion proteins inhibit lamina assembly. Δ2+ prevented lamina assembly entirely whereas at the same concentration, Δ2− had only a partial inhibitory effect.

To confirm these findings we also performed immunoblotting on isolated nuclei. 2×10^5 sperm pronuclei were assembled in extract supplemented with buffer, Δ2− or Δ2+ over 150 minutes. The nuclei were then isolated according to the method of Jenkins et al. (1993), washed in NIB containing 0.5% Triton X-100 to remove soluble lamins and pelleted by centrifugation at 12,000 ***g*** in an Eppendorf microcentrifuge. The nuclear pellets were suspended in SDS sample buffer, resolved on 8% SDS-PAGE and immunoblotted using the mAb L6 8A7 (which detects Xlamins B_1 B_2 and B_3). In nuclei isolated from control extracts (Fig. 3, lane 3) a single band migrating with the expected mobility of lamin B_3 was detected. In contrast, in nuclei isolated from extracts supplemented with Δ2− two bands were detected (Fig. 3, lane 2). The lower band had the same mobility as lamin B_3, while the upper band had the expected mobility of the Δ2− fusion protein. Finally, in nuclei isolated from extracts supplemented with Δ2+ only a single faint band, migrating with the expected mobility of the Δ2+ fusion protein was detected. No insoluble lamin B_3 was detected in this sample. The presence of an insoluble fraction of the Δ2+ fusion protein in this sample probably resulted from the co-sedimentation of cytoplasmic lamin aggregates with the nuclei. In some (but not all) extracts, additions of Δ2+ gave rise to the formation of aggregates of the fusion protein within the cytoplasm (data not shown). These aggregates co-sediment with nuclei through both Percoll and sucrose cushions. Taken together, the results of immunoblotting confirm that Δ2+ but not Δ2− prevents the formation of a lamina in sperm pronuclei assembled in vitro.

Nuclei assembled in the presence of Δ2+ and Δ2− fusion proteins fall into two discrete size classes

Based upon our initial observations, nuclei assembled in the presence of Δ2+ fusion protein lacked a lamina and were ~10 μm in diameter. In contrast, nuclei assembled in the presence of Δ2− fusion protein possessed an incomplete lamina and were ~12 μm in diameter. To determine whether these differences in size were significant the following experiment was performed. First nuclei were assembled in either the presence or absence of fusion proteins. The nuclei were fixed and stained with the DNA intercalating dye DAPI. Phase contrast and fluorescence images were collected with a CCD camera, using BioVision software. Having verified that the DAPI fluorescence filled the entire nucleus, nuclear surface area was determined using the

Table 1. Comparison of nuclear size in control, Δ2− and Δ2+ nuclei

Type of nucleus	Nuclear area ± 95% confidence limits (μm^2)
Control	314.20±29.23
Δ2−	102.08±5.83
Δ2+	78.55±3.65

Sperm pronuclei were assembled for 150 minutes in control extracts, extracts supplemented with Δ2− or extracts supplemented with Δ2+. Samples were prepared as wet mounts and stained with the DNA intercallating dye DAPI. Images were collected with a CCD camera attached to a Zeiss Axioskop fitted with a ×40 Plan Neofluor lens, at a single plane of focus using Biovision software. Nuclear area was determined using the density slice function in NIH Image software. The number of observations for each data set were as follows: Control = 202; Δ2− = 256; Δ2+ = 315. The table shows mean nuclear area ±95% confidence levels, in microns squared. One way analysis of variance shows that there is significant overall variation: $P<0.001$. All pairwise comparisons are also significantly different: $P<0.001$.

density slice function in NIH image software. For populations of >200, the mean area and s.e.m. were determined and compared at a 95% confidence limit using Student's *t*-test. The results (shown in Table 1) revealed that nuclei assembled in control extracts were 307% larger than Δ2– nuclei and 397% larger than Δ2+ nuclei (based on mean area). Interestingly, Δ2– nuclei were significantly larger than Δ2+ nuclei.

The Δ2+ but not the Δ2– fusion protein prevents DNA replication in egg extracts

To determine the influence of each fusion protein on DNA replication, egg extracts were supplemented with Δ2+ or Δ2– prior to the addition of demembranated sperm heads. The extracts were pulse labelled with biotin-16-dUTP at thirty minute intervals and for thirty minute periods. Following each pulse label, sperm pronuclei were recovered for fluorescence microscopy. The nuclei were stained with Texas Red streptavidin to reveal biotin dUTP incorporation. In control extracts (diluted with buffer) DNA replication was first detected after sixty minutes. The fraction of nuclei incorporating biotin-16-dUTP was initially small (~25%), however, this fraction increased to ~75% by 120 minutes before declining to 35% at 150 minutes (Fig. 4B). Replicating nuclei formed in control extracts were large (up to 25 μm in diameter) and surrounded by a nuclear membrane (detected by DIC optics, Fig. 4A). Sperm pronuclei assembled in extracts supplemented with Δ2– also supported DNA replication. Replication was first detected in Δ2– nuclei after 90 minutes. The initial fraction of Δ2– nuclei which incorporated biotin-11-dUTP was 35%. After 150 minutes incubation 80% of Δ2– nuclei were undergoing DNA replication (Fig. 4B). Although Δ2– nuclei incorporated biotin-16-dUTP efficiently, they remained relatively small (~12 μm in diameter). They were, however, surrounded by a continuous nuclear membrane (detected by DIC optics, Fig. 4A). Sperm pronuclei assembled in the presence of Δ2+ did not incorporate biotin-11-dUTP, even after 150 minutes

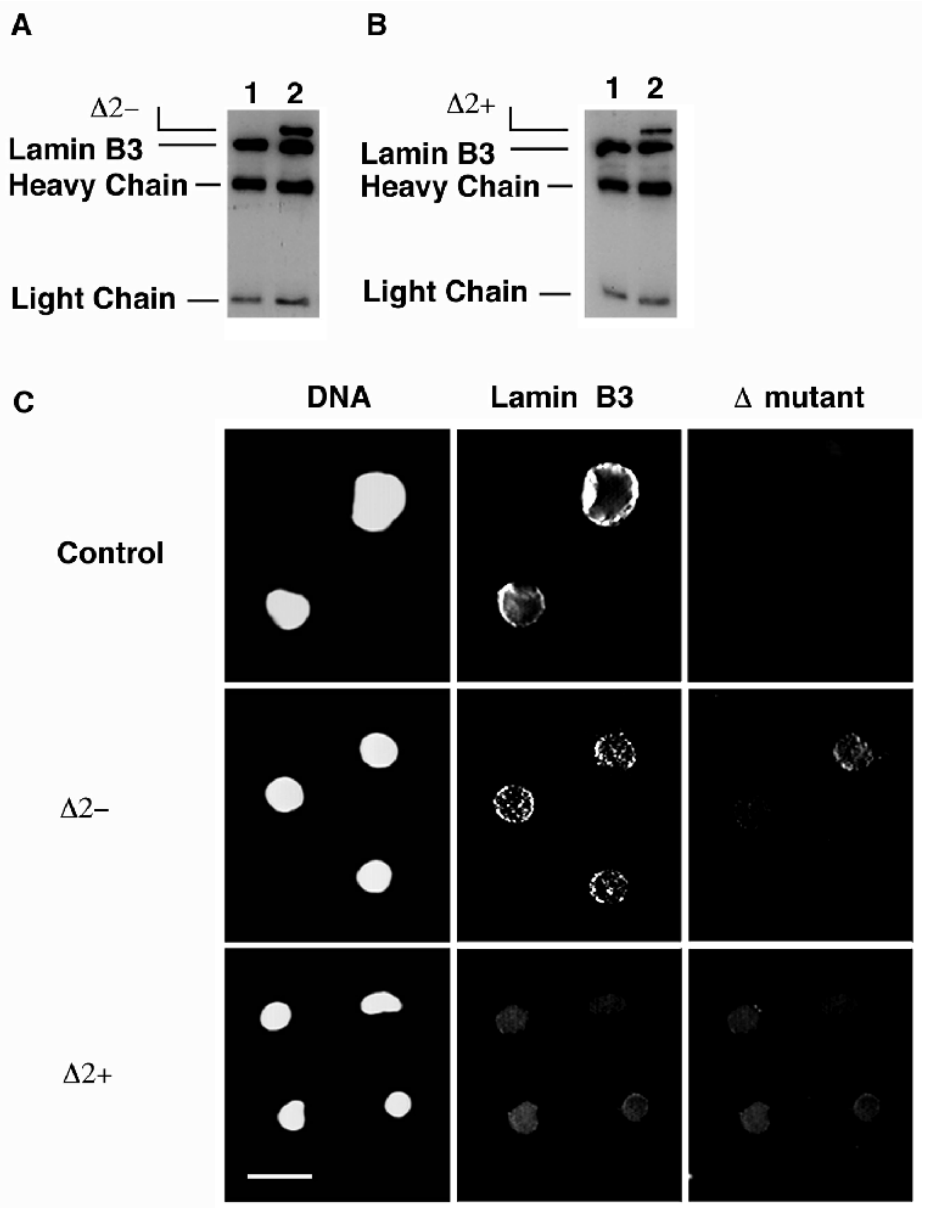

Fig. 2. Pronuclear assembly in Δ2± treated *Xenopus* egg extracts. Lamin B_3 was immunoprecipitated from extracts supplemented with Δ2– (A, lane 2), Δ2+ (B, lane 2) or renaturation buffer (A,B, lanes 1), using mAb L6 5D5 linked to paramagnetic Dynabeads and resolved on 8% SDS-PAGE. The proteins were transferred to nitrocellulose and blotted with mAb L6 8A7. The positions of lamin B_3, GST-fusion proteins and IgG light chains and heavy chains are indicated. (C) The morphology and lamin complement of sperm pronuclei assembled in extracts supplemented with 80 ng μl^{-1} Δ2± fusion protein was investigated by indirect immunofluorescence. Extracts were incubated for 150 minutes, before pronuclei were fixed with EGS and recovered onto glass-coverslips. Coverslips were incubated in the presence of primary antibodies in humid chambers. Mouse monoclonal anti-lamin B3 antibody (L6 5D5) and FITC-conjugated goat anti-mouse IgG were used to reveal endogenous lamina assembly (centre column). Rabbit polyclonal anti-GST antibodies and TRITC-conjugated swine anti-rabbit IgG were used to detect the presence of 2 fusion proteins (right column). The intercalating dye DAPI reveals the presence of DNA (left column). Bar, 20 μm.

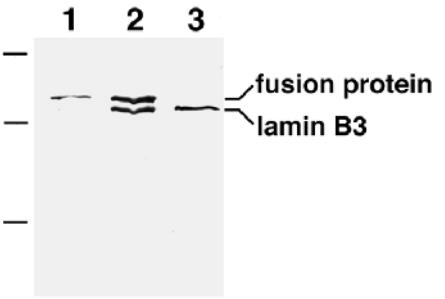

Fig. 3. The influence of Δ2+ and Δ2– on lamina assembly. Nuclei were assembled in extracts supplemented with Δ2+ (lane 1), Δ2– (lane 2) or buffer (lane 3). Nuclei were recovered from each extract, washed in NIB containing 0.5% Triton X-100 and resolved by 8% SDS-PAGE. Protein bands were transferred to nitrocellulose. Filters were probed with mAb L6 8A7 (this monoclonal antibody detects lamins B_1, B_2 and B_3; Stick and Hausen, 1985) followed by alkaline phosphatase-conjugated rabbit anti-mouse Ig. The positions of lamin B_3 and the fusion proteins are indicated. The positions of molecular mass standards are shown at the left hand side of the panel. Pre-stained standards = 139 kDa, 79 kDa and 44.3 kDa.

(Fig. 4B). Throughout the incubation period Δ2+ nuclei remained small (~10 μm in diameter), however, all nuclei were surrounded by a nuclear membrane (detected by DIC optics, Fig. 4A).

Δ2+ fusion protein but not Δ2– fusion protein prevents the assembly of replication foci

One feature of sperm pronuclei assembled in *Xenopus* egg extracts is the formation of replication centres. Each replication centre accommodates approximately 150 replication forks and contains replication proteins such as DNA polymerase alpha and PCNA (Mills et al., 1989; Hutchison and Kill, 1989; Hutchison, 1995). We have previously reported that nuclei assembled in extracts immunodepleted of lamin B_3 fail to assemble replication centres (Meier et al., 1991; Jenkins et al., 1993). Since sperm pronuclei assembled in extracts supplemented with Δ2+ failed to initiate DNA replication, we also wished to investigate the ability of these nuclei to assemble replication centres. Therefore nuclei were first assembled in extracts containing GST-lamin fusion proteins, then fixed, isolated and stained with anti-PCNA antibodies. 30 minutes prior to fixation extracts were pulse labelled with biotin-11-dUTP. Nuclei assembled in control extracts or Δ2– extracts were fixed and isolated during mid S-phase (120 minutes and 150 minutes, respectively). Nuclei assembled in Δ2+ were fixed and isolated after 150 minutes. Replicating nuclei assembled in control extracts or Δ2– extracts displayed a granular distribution of PCNA, which in some cases co-localised precisely with sites of biotin-16-dUTP incorporation (such co-localisation is only observed in nuclei just after initiation of DNA replication; see Hutchison, 1995). indicating the presence of replication centres (Fig. 5). In contrast, nuclei assembled in Δ2+ nuclei displayed three distinct distributions of anti-PCNA immunofluorescence. The majority of nuclei (type I, 78%) displayed only a weakly punctate distribution of anti-PCNA immunofluor-escence. In a smaller fraction of nuclei (type II, 19%), anti-PCNA foci were observed, but the

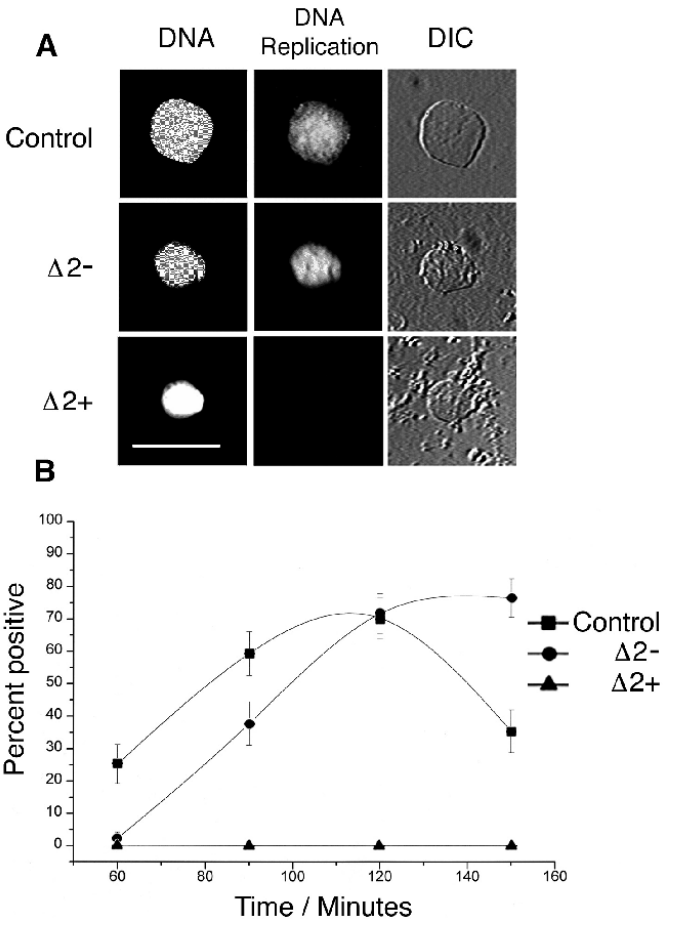

Fig. 4. DNA replication and nuclear membrane assembly in 2 treated *Xenopus* egg extracts. (A) The ability of sperm pronuclei assembled in 2– treated *Xenopus* egg extracts to correctly assemble a nuclear envelope and to maintain replication competence was investigated. *Xenopus* egg extracts were inoculated with 80 ng μl^{-1} of either Δ2– or Δ2+ fusion proteins (control extracts were supplemented to an equivalent dilution with renaturation buffer) and divided into 20 μl aliquots. Extracts were sampled every thirty minutes over 150 minutes. Each extract was pulse-labelled for the final 30 minutes of each time point with 4 μM biotin-16-dUTP. Isolated nuclear samples were fixed and prepared for fluorescence microscopy. Texas Red conjugated to streptavidin was used to detect newly synthesised DNA (central panel). DIC (differential interference contrast) was used to detect nuclear membranes (right hand panel). DAPI reveals the presence of DNA (left hand column). Bar, 20 μm. (B) The graph shows the percentage of nuclei assembled under the conditions described above which were positive for biotin-16-dUTP incorporation at each sample interval (a minimum of 200 nuclei were scored at random for each condition and at each time point).

number of these foci was small and their distribution within nuclei was non-uniform. A very small fraction of nuclei (type III, 3%) had a distribution of anti-PCNA immunofluorescence which was indistinguishable from that observed in control and Δ2– nuclei, except that no biotin-16-dUTP incorporation was observed in these nuclei (Fig. 5). Taken together these results indicated that nuclei assembled in extracts treated with Δ2+ either fail to assemble replication centres or assemble aberrant replication centres.

Δ2+ fusion protein is capable of disassembling a pre-formed lamina without disrupting replication centres

Recent reports have indicated that lamins are components of the internal nucleoskeleton (Hozak et al., 1995) and replication foci (Moir et al., 1994) and may therefore directly support sites of DNA replication. To test this hypothesis we wished to determine whether the Δ2+ fusion protein was capable of disassembling pre-formed lamina filaments and as a result could disrupt replication foci. Sperm pronuclei were assembled in extracts supplemented with aphidicholin. This treatment permits the assembly of replication centres but does not permit their immediate use. As a result nuclei are accumulated at the stage of initiation. Since replication proceeds normally upon the removal of aphidicholin (e.g. Murphy et al., 1995) it is generally assumed that aphidicholin treatment does not alter nuclear sub-structures. Following the assembly of replication competent sperm pronuclei, the extracts were supplemented with either Δ2+ or Δ2− and incubated for a further 90 minutes. Sperm pronuclei were then fixed, isolated and co-stained with L6 5D5 and rabbit anti-GST antibodies or isolated, washed in CSK buffer containing 0.5% Triton X-100 and prepared for immunoblotting. In control nuclei (no fusion protein added) all nuclei were surrounded by a perinuclear anti-lamin B_3 immunofluorescence (Fig. 6A). Nuclei incubated with Δ2− also displayed perinuclear anti-lamin B_3 immunofluorescence. However, in addition these nuclei displayed anti-GST immunofluorescence indicating the presence of the fusion protein in the nucleus. Moreover, the nuclear envelopes had a distinctly roughled appearance and many nuclei were misshapen. No perinuclear anti-lamin B_3 immunofluorescence was detected in nuclei incubated with Δ2+. Instead, anti-lamin B_3 was detected in the nucleoplasm along with anti-GST immunofluorescence. This result indicated that Δ2+ enters preformed nuclei and is able to disrupt the lamina leading to a redistribution of lamin polypeptides from the nuclear envelope to the nucleoplasm. Δ2− also enters nuclei and associates with the

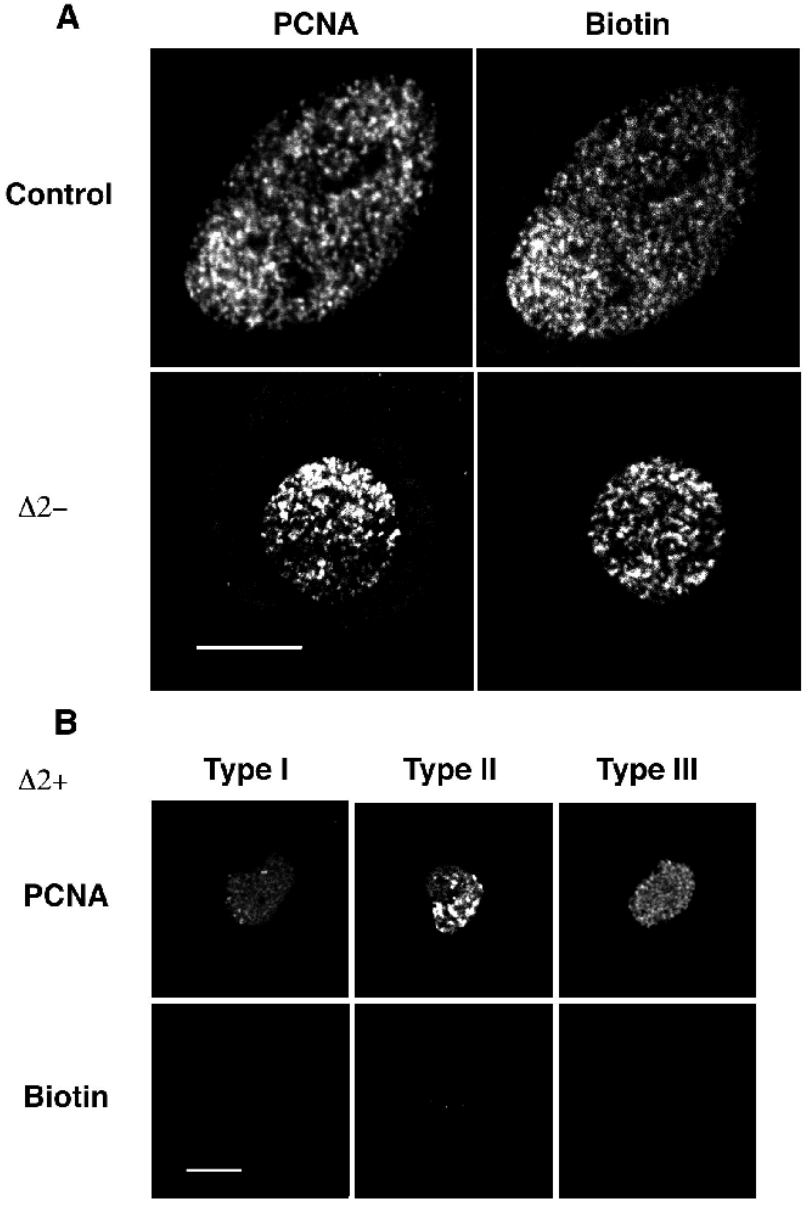

Fig. 5. DNA replication and establishment of PCNA foci in pronuclei assembled in Δ2+/Δ2− treated *Xenopus* egg extracts. The effect of the Δ2 mutant lamins on the ability of pronuclei to assemble PCNA foci was investigated using indirect immunofluorescence and confocal microscopy. *Xenopus* egg extracts were inoculated with 80 ng μl^{-1} of Δ2+ or Δ2− fusion protein (control extracts were supplemented with an equivalent volume of renaturation buffer). Extracts were incubated at 21°C and pulse labelled with 4 M biotin-16-dUTP for the final 30 minutes of each time point. Next, nuclei were isolated and processed for immunofluorescence. Texas Red conjugated to streptavidin was used to detect newly synthesised DNA. Human anti-PCNA antibodies and FITC-conjugated goat anti-human IgG were used to detect PCNA. Representative images were collected, simultaneously in both the FITC and TRITC channels, with a Bio-Rad MRC 600 LSCM using a Kalman averaging programme. (A) The images displayed for control and Δ2− nuclei are typical for initiation nuclei (see Hutchison, 1995) detected at 120 minutes and 150 minutes, respectively. (B) Three images are displayed for Δ2+ nuclei which represent the range of morphologies observed after 150 minutes. These are referred to as type I (representing 78% of nuclei), type II (representing 19% of nuclei) and type III (representing 3% of nuclei). Bars, 10 μm.

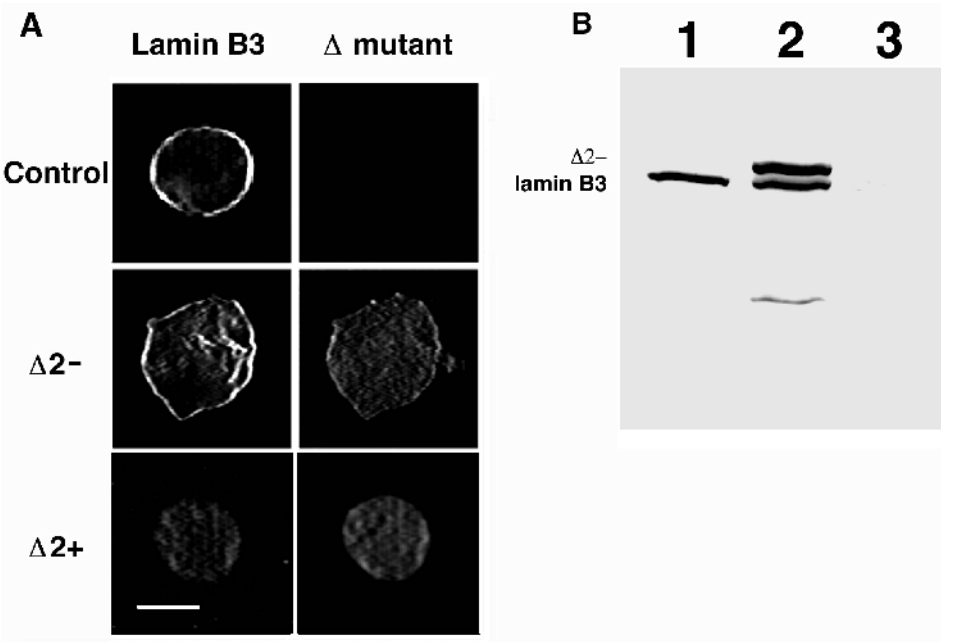

Fig. 6. Lamina disassembly by Δ2+ in aphidicholin arrested *Xenopus* egg extracts. The effect of the addition of the 2 mutant lamins to pre-formed nuclei was investigated using indirect immunofluorescence microscopy and immunoblotting. *Xenopus* sperm pronuclei were assembled for 90 minutes in extracts in which DNA synthesis had been inhibited by the addition of 25 μg ml^{-1} aphidicolin. Extracts were then supplemented with either renaturation buffer (A, control; B, lane 1), Δ2– (A, Δ2–; B, lane 2) or Δ2+ (A, Δ2+; B, lane 3). All exogenous proteins were added at a final concentration of 80 ng μl^{-1}. 90 minutes after inoculation of the fusion proteins (or buffer), nuclei were either fixed with EGS and processed for immunofluorescence or isolated, washed in NIB containing 0.5% Triton X-100 and used for immunoblotting. For immunofluorescence, mAb L6 5D5 and FITC-conjugated goat anti-mouse IgG were used to reveal the lamina (A, left-hand column). Rabbit polyclonal anti-GST antibody (–GST) and TRITC-conjugated swine anti-rabbit IgG were used to detect the presence of fusion proteins (A, right-hand column). For immunoblotting, mAb L6 8A7 followed by AP goat anti-mouse Ig was used to detect both the endogenous lamins and the fusion proteins. In B the positions of lamin B_3 and Δ2– are indicated at the left hand side. Bar, 10 μm.

nuclear lamina. However, while this accumulation of Δ2– alters the morphology of the nucleus it does not disperse the lamina.

These results were confirmed by immunoblotting. On blots, a single band was detected by mAb L6 8A7 in nuclei isolated from control extracts (Fig. 6B, lane 1) This band had the expected mobility of lamin B_3. In contrast, two bands were detected in nuclei isolated from Δ2– extracts (Fig. 6B, lane 2). The lower band had the expected mobility of lamin B_3, while the upper band had the expected mobility of the fusion protein. Finally, no bands were detected in nuclei isolated from Δ2+ extracts. The apparent discrepancy between the detection of nucleoplasmic lamin staining in indirect immunofluorescence and no detection of lamin polypeptides in immunoblotting assays can be explained by the treatment of isolated nuclei with Triton X-100 in CSK buffer. This treatment efficiently removes lamins which are not assembled into polymers. Therefore the results of immunoblotting indicates that there is a complete absence of lamin polymers in nuclei treated with Δ2+ (Fig. 6B, lane 3).

When nuclei incubated in the same extracts were stained with anti-PCNA antibodies, the distribution of PCNA was very similar under all experimental conditions (Fig. 7). In control nuclei and nuclei treated with Δ2–, anti-PCNA immunofluorescence gave the expected granular staining pattern evenly distributed throughout the nucleoplasm. In nuclei treated with Δ2+, anti-PCNA immunofluorescence was still evenly distributed throughout the nucleoplasm, although the texture of the immunofluorescence pattern appeared slightly smoother. The nuclear envelope appeared to be intact (judged by DIC optics). Thus while Δ2+ disrupted the lamina, disruption of this structure did not appear to influence greatly the morphology of replication centres or nuclear envelopes.

To confirm that replication centres remained functional, the following experiment was performed. Nuclei were assembled in extracts supplemented with aphidicholin and biotin-16-dUTP. Following the assembly of nuclei, the extracts were supplemented with buffer or with one of the fusion proteins and incubated for a further ninety minutes. At the end of this incubation period, a sample of extract was removed and the nuclei were fixed and prepared for indirect immunofluorescence (+Aphidicholin). The remaining extract was suspended in NIB. Fresh extract supplemented with the relevant fusion protein and biotin-16-dUTP but lacking aphidicholin was layered under each suspension. Nuclei were transferred into the fresh extract by centrifugation and incubated for a further 60 minutes at 21°C. Finally, the nuclei were fixed and prepared for indirect immunofluorescence (Rescue). In both controls and nuclei treated with Δ2–, strong anti-lamin immunofluorescence was detected (Fig. 8). Biotin-16-dUTP was not incorporated into chromatin before transfer of nuclei to fresh extract. However, in both cases extensive incorporation was detected after transfer and chromatin adopted a prophase-like morphology which is typical of nuclei which have completed DNA replication (Hutchison et al., 1988). As expected, nuclei which had been incubated with Δ2+ for 90 minutes lacked a lamina (Fig. 8). Despite the absence of a lamina, when these nuclei were transferred to fresh extract (also containing Δ2+) extensive biotin-16-dUTP incorporation was detected and in many nuclei a prophase-like distribution of chromatin was adopted. These results indicate that while lamina assembly is required for the assembly of replication centres the lamina does not directly support those centres and is not required for their maintenance.

DISCUSSION

GST-lamin fusion proteins disrupt lamina filaments

In this report we describe the influence of two GST-lamin

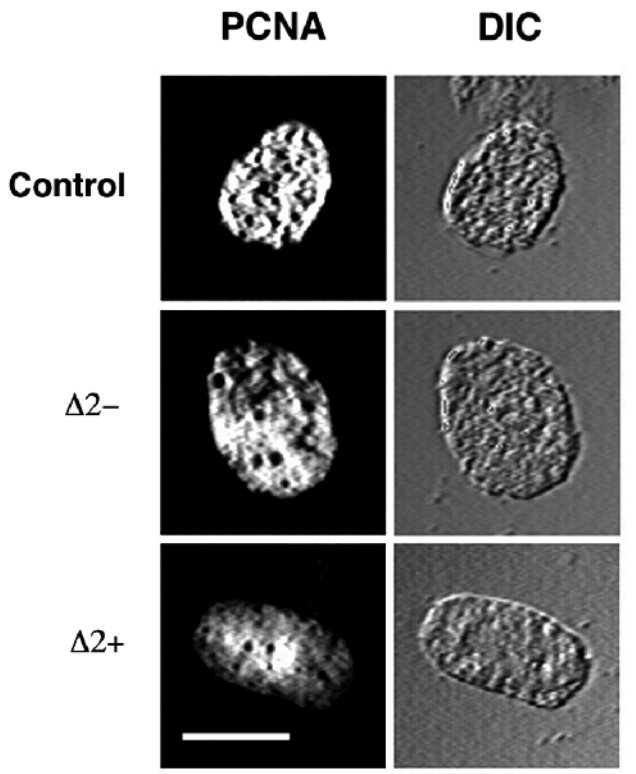

Fig. 7. Lamina disassembly does not alter the distribution of replication centres. *Xenopus* sperm pronuclei were assembled for 90 minutes in extracts in which DNA synthesis had been inhibited by the addition of 25 μg ml^{-1} aphidicolin. Extracts were then inoculated with either renaturation buffer (Control), Δ2– or Δ2+. All exogenous proteins were added at a final concentration of 80 ng μl^{-1}. 90 minutes after inoculation of the fusion proteins (or buffer), nuclei were fixed with EGS and processed for immunofluorescence. Human anti-PCNA antibodies and FITC-conjugated goat anti-human IgG were used to detect PCNA (left-hand column). DIC reveals general nuclear shape (right-hand column). Bar, 10 μm.

fusion proteins on the assembly and stability of lamina filaments. Both fusion proteins form hetero-oligomers with lamin B_3, the major lamin isoform present in *Xenopus* egg extracts. The Δ2– fusion protein, encoding amino acids 34-411 of lamin B_1, lacks a nuclear localisation signal sequence. This protein delays but does not prevent lamina assembly when added to *Xenopus* egg extracts prior to the addition of demembranated sperm heads and indeed becomes incorporated into the lamina. The same protein does not disrupt the lamina of pre-formed nuclei but does alter the morphology of the nuclear envelope. The Δ2+ fusion protein, encoding amino acids 34-420, possesses a nuclear localisation signal sequence. This protein inhibits lamina assembly when added to *Xenopus* egg extracts prior to the addition of demembranated sperm heads. The Δ2+ fusion protein is also able to disrupt the lamina of pre-formed nuclei.

Is the enhanced ability of the Δ2+ fusion protein to disrupt lamina filaments, and to prevent lamina filament assembly, solely due to its possession of a NLS? Both fusion proteins form hetero-oligomers with the endogenous lamin B_3 efficiently and both fusion proteins enter the nucleus (Presumably, Δ2– only enters the nucleus as a hetero-oligomer). However, only the Δ2– fusion protein forms stable associations with lamin B_3 polymers at the nuclear envelope. The dominant negative influence of both fusion proteins must arise from the absence of a head domain which is required for head-to-tail associations (Moir et al., 1991). However, we must conclude that the absence of the head domain alone does not fully explain our results. The differing effects of Δ2+ and Δ2– could be explained as follows. The possession of a NLS permits Δ2+ to accumulate in the nucleus either as a homotypic oligomer or as a heterotypic oligomer in which the molar ratio of Δ2+:lamin B_3 can be greater than 1:1. In contrast, Δ2– can only accumulate in the nucleus as a heterotypic oligomer in which the molar ratio of Δ2–:lamin B_3 is 1:1 or less. Heterotypic oligomers having a low ratio of fusion protein:lamin B_3 can be incorporated into the lamina. In contrast, heterotypic oligomers having a high ratio of fusion protein:lamin B_3 do not assemble into lamina filaments because they cannot form head-to-tail associations.

The ability of Δ2+ to disrupt a pre-formed lamina probably arises in the following way. Previous investigations have revealed that lamin polypeptides exist in a state of dynamic equilibrium between a filamentous state and a soluble nucleoplasmic state (Schmidt et al., 1994). It seems likely that nucleoplasmic Δ2+ 'sequesters' native lamin B_3 into a permanent 'non-filamentous' state over a period of time. Thus as lamin B_3 dimers in lamina filaments turnover, they presumably form stable associations with Δ2+ oligomers in the nucleoplasm and are prevented from re-associating with lamina filaments, eventually resulting in the disassembly of lamina filaments.

Lamina filament assembly is required for the formation but not for the maintenance of replication centers

In previous investigations anti-lamin antibodies have been used to immunodeplete *Xenopus* egg extracts. Sperm pronuclei assembled in lamin-depleted extracts are small, unable to initiate semi-conservative DNA replication (Newport et al., 1990; Meier et al., 1991; Jenkins et al., 1993) and do not form replication centers (Jenkins et al., 1993). Since the capacity to assemble replication competent nuclei could be re-constituted by adding back purified lamin B_3 (Goldberg et al., 1995) it was concluded that lamina assembly is required for the assembly of replication domains. However, an ultra-structural analysis of sperm pronuclei has revealed the presence of two types of filamentous structures. Lamina filaments containing lamin B_3 are located at the nuclear periphery and interconnect nuclear pores. A second network of branched filaments proliferates throughout the nucleoplasm. The branched filaments do not contain lamin B_3 but their organisation is dependent upon the assembly of a lamin B_3 containing lamina (Zhang et al., 1996). Based upon these findings we have proposed that lamina assembly is a pre-requisite for the correct assembly of a nuclear matrix which in turn supports replication centres (Hutchison et al., 1994; Zhang et al., 1996). The results reported here support this hypothesis. Sperm pronuclei assembled in the presence of Δ2+ fail to assemble a lamina and lack replication centres (judged by the absence of PCNA foci). However, when Δ2+ is used to disassemble the lamina of pre-formed nuclei, replication centres are maintained (again judged by the presence of PCNA foci) and these nuclei remain competent for DNA synthesis. Our results can be explained if lamina assembly is

required for the correct assembly of a nuclear matrix but lamina filaments do not themselves support replication centres directly. Thus nuclear assembly in the presence of Δ2+ is arrested prior to the assembly of a nuclear matrix with the result that replication centres do not form. In contrast, preformed nuclei must possess a nuclear matrix which adequately supports replication centres when lamina disassembly is promoted by Δ2+. These results are consistent with results of cell-fusion experiments. When cells in S-phase are fused to cells in mitosis, the nuclear envelopes of the S-phase cells breakdown and chromosomes condense prematurely. However, DNA synthesis in prematurely condensed chromosomes continues (Rao et al., 1978). Similarly, when aphidicholin arrested S-phase nuclei are transferred to mitosis promoting factor (MPF) in vitro, nuclear envelope breakdown, nuclear lamina breakdown and premature chromosome condensation are all induced but DNA replication continues and replication centres are maintained (Hutchison and Kill, 1989).

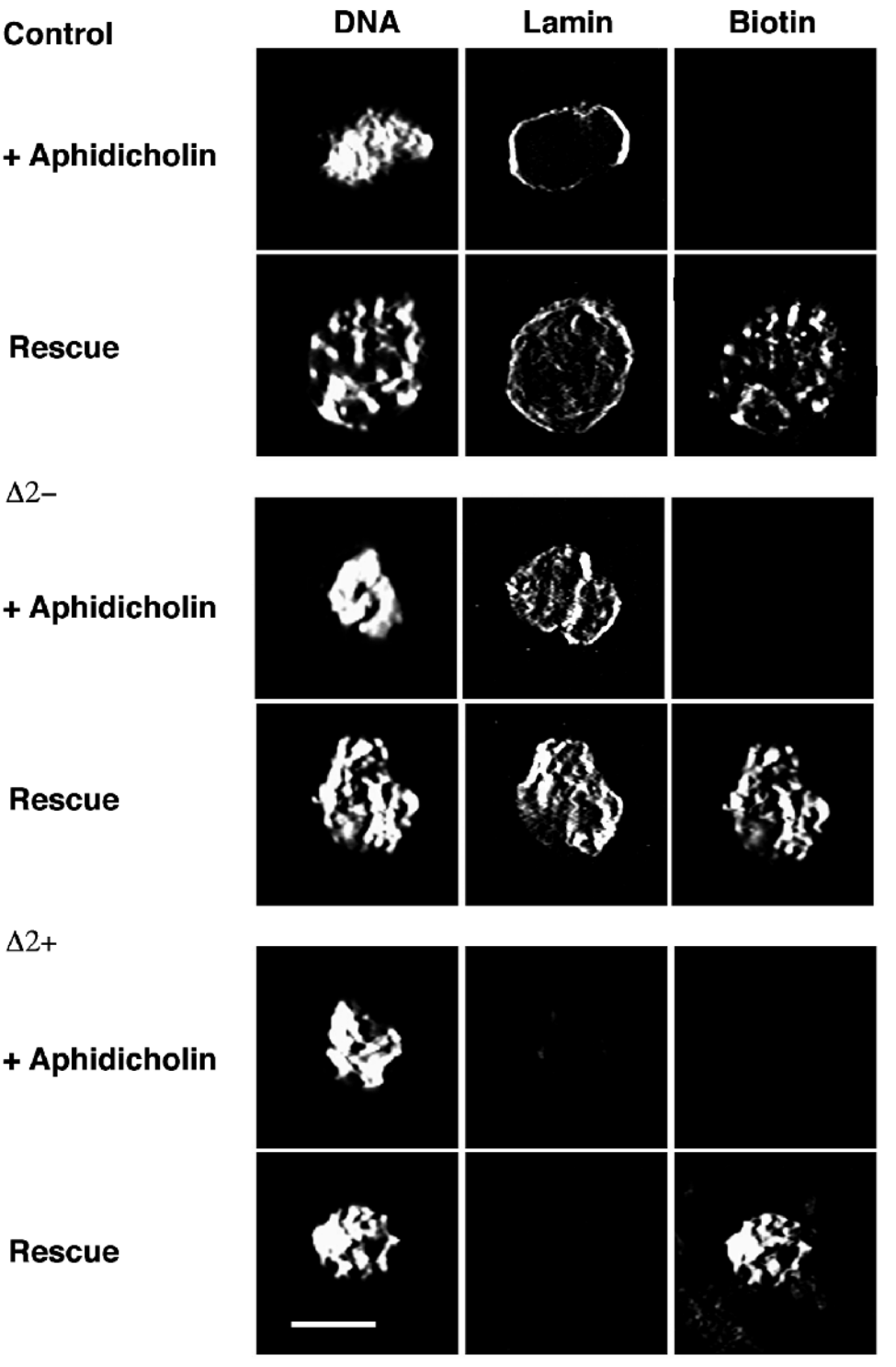

Fig. 8. Lamina disassembly does not alter the function of replication centres. *Xenopus* sperm pronuclei were assembled for 90 minutes in extracts (supplemented with 4 μm biotin-11-dUTP) in which DNA synthesis had been inhibited by the addition of 25 gm μl^{-1} aphidicolin. Extracts were then inoculated with either renaturation buffer (Control), Δ2– or Δ2+. All exogenous proteins were added at a final concentration of 80 ng μl^{-1}. 90 minutes after inoculation of the fusion proteins (or buffer), nuclei were transferred to fresh extract containing the same fusion protein (or buffer) but without aphidicholin. The fresh extract was supplemented with biotin-11-dUTP and incubated for a further 60 minutes. The nuclei were fixed, recovered on glass coverslips and stained with mAb L6 5D5 followed by FITC goat anti-mouse Ig (to reveal the lamina) and Texas Red streptavidin (to reveal biotin incorporation). + Aphidicholin illustrates the morphology of nuclei before transfer to fresh extract. Rescue illustrates the morphology of nuclei after transfer and incubation in fresh extract for 60 minutes. Bar, 20 μm.

Deletion of the N-terminal head domain of human lamina A also gives rise to a dominant negative mutant

In a recent report, Spann et al. (1997) investigated the influence of a dominant negative mutant of human lamin A on nuclear assembly and DNA replication in *Xenopus* egg extracts. This dominant negative mutant (termed ΔNLA) also lacked sequences encoding the N-terminal head domain, but unlike the proteins described here, encoded the entire C-terminal tail domain. When added to *Xenopus* egg extracts ΔNLA, like Δ2+, prevented lamina assembly but not nuclear membrane assembly. However, unlike Δ2+, ΔNLA formed insoluble nucleoplasmic aggregates which also contained the endogenous lamin B_3. By preventing nuclear lamina assembly with ΔNLA, DNA synthesis was also inhibited. Moreover, the distribution of PCNA and RPA (proteins involved in the elongation phase of DNA replication) was disrupted, both proteins co-localising with with the ΔNLA aggregates, rather than sites containing initiation proteins. The data reported by Spann et al. (1997) present both similarities and differences to our own data. Both investigations demonstrate that deletion of the N-terminal head domain of lamins leads to the creation of a dominant negative protein capable of preventing lamina assembly and of disrupting a pre-formed lamina. Both investigations also demonstrate that normal lamina assembly is a requirement for DNA replication. In the case of Δ2+ the dominant negative effect caused endogenous lamins to remain soluble within the nucleoplasm. In contrast, ΔNLA caused the redistribution of all lamins to large aggregations in the nucleoplasm. This difference is probably due to differences in the behaviour of A-type and B-type lamins. A-type lamins bind strongly to chromatin in vitro, via sites of interaction in both the rod domain and in the tail domain (Höger et al., 1991; Glass et al., 1993). In contrast, lamin B_1 displays only weak chromatin binding properties in vitro (Höger et al., 1991). Therefore one explanation for the appearance of ΔNLA (but not Δ2+) in nucleoplasmic aggregates is that in the absence of a lamina, this lamin A mutant accumulates by default on chromatin. Based upon the co-localisation of PCNA and RPA with nucleoplasmic aggregates of ΔNLA, Spann et al. (1997) proposed that the lamina has a direct involvement in the elongation phase of DNA replication. However, despite the fact that ΔNLA is capable of disrupting a pre-formed lamina, Spann et al., (1997) did not test this hypothesis directly. The data presented here, demonstrate that once sites of DNA replication have been established, disruption of the lamina does not inhibit 'run on' synthesis. Therefore, while we confirm that lamina assembly is a pre-requisite for the formation of replication centres, we do not agree with the conclusion that lamins influence the function of replication centres directly.

The initiation of DNA replication is influenced by nuclear size

By manipulating lamina assembly with GST-lamin fusion peptides, we have been able to generate nuclei of predictable sizes. Previous reports have indicated that nuclei start to assemble replication foci after they reach a mean diameter of 10.82±1.76 μm and initiate DNA replication when they have attained a mean diameter of 12.24±2.36 μm (Hutchison, 1995). The data reported here indicate that nuclear size is important for the initiation of DNA replication. In control extracts, nuclei initiate DNA replication after 60 minutes. These nuclei rapidly attain a diameter of 11 μm but continue to grow throughout the incubation period. Nuclei assembled in Δ2− extracts, support partial lamina assembly and become arrested at a size of ~12 μm. These nuclei support DNA replication although the timing of initiation is delayed by 30 minutes when compared to controls. This result suggests that complete lamina assembly is not necessary for the initiation of DNA replication. Instead sufficient lamina assembly to support a critical amount of nuclear growth is important. If this is true then the absence of DNA replication in Δ2+ nuclei may arise through an absence of nuclear growth (these nuclei only attain a diameter of 10 μm). Recent evidence suggests that the function of matrix attachment regions (MARs) is to augment the activities of RNA (and presumably DNA) polymerases by increasing the accessibility of chromatin to these enzymes (Jenuwein et al., 1997). The simplest explanation for the role of lamins in the initiation of DNA replication is that through its interaction with MARs (Luderus et al., 1992) the lamina augments the accessibility of chromatin to replicative polymerases. Furthermore, this function is achieved through a critical increase in nuclear size.

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