Disruption of Nuclear Lamin Organization Blocks the Elongation Phase of DNA Replication

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Abstract. The role of nuclear lamins in DNA replication is unclear. To address this, nuclei were assembled in *Xenopus* extracts containing AraC, a reversible inhibitor that blocks near the onset of the elongation phase of replication. Dominant-negative lamin mutants lacking their NH_2-terminal domains were added to assembled nuclei to disrupt lamin organization. This prevented the resumption of DNA replication after the release of the AraC block. This inhibition of replication was not due to gross disruption of nuclear envelope structure and function. The organization of initiation factors was not altered by lamin disruption, and nuclei resumed replication when transferred to extracts treated with CIP, an inhibitor of the cyclin-dependent kinase (cdk) 2–dependent step of initiation. This suggests that alteration of lamin organization does not affect the initiation phase of DNA replication. Instead, we find that disruption of lamin organization inhibited chain elongation in a dose-dependent fashion. Furthermore, the established organization of two elongation factors, proliferating cell nuclear antigen, and replication factor complex, was disrupted by ΔNLA. These findings demonstrate that lamin organization must be maintained in nuclei for the elongation phase of DNA replication to proceed.

Key words: DNA synthesis • lamina • intermediate filaments • nuclear organization • lamin mutant

Introduction

Nuclear processes such as DNA replication, transcription, and RNA processing show precise spatial and temporal regulation (Nakamura et al., 1986; Nakayasu and Berezney, 1989; O'Keefe et al., 1992; Jimenez-Garcia and Spector, 1993; Spector, 1993; Wansink et al., 1993). In addition, the mapping of interphase chromosomal domains to specific nuclear regions demonstrates that the chromatin templates for these processes are maintained in particular spatial relationships (Schardin et al., 1985; Hadlaczky et al., 1986; Zink et al., 1998). Although these observations suggest that there is an underlying infrastructure that maintains nuclear organization, the composition of this structure and its role in regulating nuclear processes remains controversial. There are several nuclear proteins that are excellent candidates for structural elements of nuclear organization. These include nuclear mitotic apparatus protein (NUMA) (Compton et al., 1992), translocated promoter region (Tpr) (Zimowska et al., 1997), and the nuclear lamins (Moir et al., 1995). In particular, the nuclear lamins have long been thought to provide a structural framework upon which specific nuclear processes are organized.

The nuclear lamins are the major protein components of the nuclear lamina, a thin, electron-dense layer found underlying the inner nuclear membrane (Fawcett, 1966; Gerace et al., 1978; Krohne et al., 1978). Lamins are also found in nucleoplasmic structures that are distinct from the nuclear periphery (Jackson and Cook, 1988; Goldman et al., 1992). The biochemical properties of the lamina along with its close association with the inner face of the nuclear membrane and with pores suggest that the lamina provides structural support for the major components of the nuclear envelope (Aaronson and Blobel, 1975; Aebi et al., 1986; Fisher et al., 1986; Goldman et al., 1986; McKeon et al., 1986; Moir et al., 1995). Furthermore, the observation that a substantial fraction of chromatin lies in close proximity to the lamina suggests that it may also be involved in anchoring, and thereby organizing chromatin (Coggeshall and Fawcett, 1964; Paddy et al., 1990).

Evidence from a number of laboratories suggests that the lamins are required for DNA replication. In these studies, DNA replication was inhibited in nuclei assem-

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bled in *Xenopus* interphase extracts that were immunodepleted of lamin B3 (Newport et al., 1990; Meier et al., 1991). However, these nuclei were smaller and more fragile than those assembled under normal conditions. Further complicating the interpretation of these results, it was shown that other proteins in addition to the lamins were removed in the immunodepletion protocols (Goldberg et al., 1995).

A second approach examining the involvement of lamins in DNA synthesis has made use of a dominant-negative mutant, ΔNLA, which lacks the NH_2-terminal domain of human lamin A. Nuclei assembled in *Xenopus* extracts containing ΔNLA failed to establish normal lamin organization (Spann et al., 1997). Under these conditions, large nucleoplasmic lamin aggregates formed that contained both ΔNLA and the endogenous lamin, *Xenopus* nuclear lamin B3 (LB3).[1] In these lamin-disrupted nuclei, DNA synthesis was inhibited by ~95% and there was a disruption in the organization of the two elongation factors, replication factor complex (RFC) and proliferating cell nuclear antigen (PCNA). However, the distribution of three factors involved in the initiation of DNA synthesis, *Xenopus* minichromosome maintenance factor 3 (XMCM3), *Xenopus* origin replication complex factor 2 (XORC2), and DNA polymerase α, appeared to be unaffected. These observations imply that the lamins play a role in the elongation phase of replication and are consistent with the observation that lamin B colocalizes with PCNA in cultured mammalian cells during mid to late S phase (Moir et al., 1994). In a similar set of experiments, a glutathione *S*-transferase (GST)-lamin B1 fusion construct was used to prevent normal nuclear lamin assembly in *Xenopus* extracts. The *Xenopus* lamin B1 portion of the construct consisted of the rod domain and the COOH-terminal nuclear localization signal (NLS), whereas the NH_2-terminal and most of the COOH-terminal domains were deleted. In this case, DNA replication was also blocked. However, this mutant did not block replication after nuclei were assembled. Based on these findings, the authors concluded that the assembly of a lamina is required to establish replication centers, but once assembled, the maintenance and function of these replication centers is not dependent on the lamins (Ellis et al., 1997).

Other studies suggest that nuclear lamins play a more indirect role in DNA replication through their involvement in the maintenance of nuclear envelope integrity. For example, when sperm chromatin is incubated in interphase extracts depleted of membrane vesicles, nuclear envelope assembly and DNA replication are blocked (Lohka and Masui, 1983; Newport, 1987; Sheehan et al., 1988). This suggests that by acting as a selective barrier, the nuclear envelope establishes the high concentration of replication factors required for DNA synthesis to proceed. If this is the case, depletion of the lamins or the disruption of lamin organization could alter the properties of the envelope. Such alterations could reduce the efficiency of nuclear transport or increase the fragility and leakiness of the membrane, resulting in the loss of replication factors. Consistent with this model, the depletion of a nuclear pore protein or the addition of WGA, an inhibitor of nuclear transport, blocks replication (Powers et al., 1995; Walter et al., 1998). Similarly, the small size of the nuclei assembled in lamin-depleted extracts might be due to an inhibition of nuclear transport, thereby arresting nuclear assembly at an early stage preceding DNA synthesis (Newport et al., 1990; Meier et al., 1991). It has also been shown that a concentrated nucleoplasmic extract prepared from in vitro–assembled nuclei supports efficient DNA replication in the apparent absence of nuclear membranes and lamins. In this case, it is thought that the high concentration of replication factors in the nucleoplasmic extract overcomes the need for a nuclear envelope and lamina (Walter et al., 1998).

The primary purpose of this study is to determine if DNA replication in assembled nuclei requires normal nuclear lamin organization. In addition, we also attempted to determine if nuclear lamins are required for a particular phase of DNA synthesis. To accomplish this, dominant-negative lamin mutants were used to disrupt the organization of lamins after nuclei were assembled in *Xenopus* interphase extracts. Our findings indicate that this disruption blocks DNA synthesis without appearing to alter nuclear transport or nuclear membrane integrity. The effect on replication is reversible and dependent upon the concentration of the mutant lamin. The timing of the arrest of replication was characterized with CIP, which blocks an early cyclin-dependent kinase 2 (cdk2)–dependent initiation step of replication, and AraC, an inhibitor of DNA polymerases that arrests replication at the start of the elongation phase (Harper et al., 1993; Walter and Newport, 1997). We show that the disruption of lamin organization does not block initiation, but instead appears to block near the onset of the elongation phase of DNA synthesis. A comparison of the size of replication products synthesized under these various conditions also confirms that the elongation phase is blocked by ΔNLA-induced disruption of nuclear lamin organization. Finally, we also show that ΔNLA alters the distribution of elongation factors of DNA synthesis.

Materials and Methods

Preparation of Xenopus Nuclear Assembly Extracts

Interphase extracts were prepared from *Xenopus* eggs as described previously, and nuclei were assembled using demembranated sperm chromatin as templates (1,000 nuclei/μl; Spann et al., 1997). To arrest DNA replication during nuclear assembly, the extract contained 0.2 mM AraC (Sigma Chemical Co.) (Walter and Newport, 1997). Approximately 70 min after the addition of chromatin, bacterially expressed protein (0.1 vol; ~2 μM final concentration) was added to the extracts containing assembled nuclei (Spann et al., 1997). The amount of mutant lamin protein added (ΔNLA or ΔNLB3) was the minimal concentration required to disrupt the lamina in 95% of the nuclei after an incubation period of 60 min at 22°C. This concentration varied slightly for different batches of mutant lamin (1–3 μM) and was therefore determined for each preparation. In control samples, an equimolar amount of human lamin A, NLS-vimentin, or an equal volume of buffer (15 mM Tris base, pH 8.8, 300 mM NaCl, 1 mM DTT) was added to extracts containing nuclei assembled in the presence of AraC. Under these conditions, the bacterially expressed proteins remained essentially soluble in the extract as judged by pelleting assays using the conditions described to purify nuclei for immunofluorescence. For

[1]*Abbreviations used in this paper:* cdk, cyclin-dependent kinase; GST, glutathione *S*-transferase; LA, human nuclear lamin A; LB3, *Xenopus* nuclear lamin B3; NLS, nuclear localization signal; NWB, nuclear wash buffer; PCNA, proliferating cell nuclear antigen; RFC, replication factor complex; XMCM, *Xenopus* minichromosome maintenance; factor XORC2, *Xenopus* origin replication complex factor 2.

all experiments, an aliquot of each preparation was processed for immunofluorescence to ensure that the mutant lamin protein had induced lamin disruption (see below).

To transfer nuclei from one extract to another, 60 μl of extract containing nuclei was diluted with 0.5 ml of ice-cold nuclear wash buffer (NWB) (200 mM sucrose, 15 mM Hepes, pH 7.4, 50 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT) and placed in an ultracentrifuge tube (Blow and Laskey, 1988; Spann et al., 1997). The diluted extracts were underlaid with 60 μl of interphase extract. The nuclei were transferred to the second extract by centrifugation at 2,300 *g* for 5 min (TLS55 Beckman rotor at 4°C). After centrifugation, the underlying extract and the nuclei were recovered with a P200 micropipet. The second extract contained 0.2 mM AraC to maintain the replication arrest during transfer and either protein buffer, ΔNLA (2 μM), or GST-CIP (0.4 μM), an inhibitor of cdks that blocks early in the initiation phase of replication (Harper et al., 1993). These reagents were added to the second extract 30 min (21°C) before the transfer protocol. After transfer, DNA replication was assayed by the addition of dCTP and [^{32}P]dATP as described below.

DNA Replication Assay

Replication assays typically consisted of 10–15 μl of extract containing lamin-disrupted or control nuclei (1,000 nuclei/μl). The AraC arrest of replication was relieved by adding dCTP (0.8 mM final concentration) (New England Biolabs, Inc.), and replication products were labeled by the incorporation of α [^{32}P]dATP (75 uCi/ml, 3,000 Ci/mM) (Amersham Pharmacia Biotech). The reactions were stopped by adding 0.5 ml of 4°C NWB, and the nuclei were isolated by centrifugation at 14,000 *g* for 30 s. Pellets of nuclei were resuspended in a buffer containing SDS and proteinase K (1 mg/ml) (Stratagene) (Dasso and Newport, 1990; Walter and Newport, 1997) and incubated at 37°C for 3 h. DNA was resolved on 0.8% agarose TBE gels (Tris-borate, EDTA). Alternatively, the sizes of replication products were determined by alkaline gel electrophoresis (50 mM NaOH, 1 mM EDTA, 1% agarose, 20 V/cm) (Strausfeld et al., 1994). The gels were dried and exposed for autoradiography as described previously (Spann et al., 1997). Quantification of the relative levels of ^{32}P incorporation was carried out using a phosphorscreen and the Molecular Dynamics Storm 860 optical scanner and Image Quant NT4.1 software.

Immunofluorescence

Immunofluorescence observations were carried out as described previously (Spann et al., 1997). In brief, extracts (10–25 μl) containing nuclei were diluted to 0.5 ml with NWB and fixed with either 10 mM ethylene glycolbis(succinimidylsuccinate) (EGS) (Pierce Chemical Co.) or 3% formaldehyde (Tousimis) for 10 min. In experiments for Fig. 10, the NWB buffer contained 0.1% Triton X-100, and the formaldehyde was added 2 min after the extract was diluted. The nuclei were transferred to poly-L-lysine–coated coverslips by centrifugation through a sucrose cushion (30% sucrose, 15 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2.5 mM MgCl, 1 mM DTT) (Mills et al., 1989). The rabbit polyclonal antibodies used were directed against human lamins A and C (Moir et al., 1994) and human vimentin (Prahlad et al., 1998). The mouse mAbs were directed against *Xenopus* lamin B3, L65D5, (Stick, 1988) (a gift of Dr. Reimer Stick, German Cancer Research Center, Heidelberg, Germany), PCNA (PC10; Boehringer), nucleoporins (mAb414; Babco), and the large subunit of RFC (Bunz et al., 1993) (a gift of Dr. Bruce Stillman, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Secondary antibodies used were goat anti–rabbit rhodamine red-X (Jackson ImmunoResearch) and goat anti–mouse FITC (Molecular Probes). Coverslips were mounted in 1 mg/ml p-phenylene diamine, 50 mM Tris-HCl, pH 9.0, and 50% glycerol. To detect DNA, coverslips were treated with 1 μM TOTO-3 (Molecular Probes) for 5 min. Alternatively, Hoechst dye (0.1 μg/ml) (Molecular Probes) was included in the mounting medium.

The confocal images of these preparations were captured using either a Zeiss 410 or a Zeiss 510 confocal microscope equipped with the manufacturer's software. Alternatively, some images were obtained using a Zeiss Axiophot epifluorescence microscope equipped with a Photometrics Sensys digital camera controlled by MetaMorph software (Universal Imaging Corp.).

Assays for the Functional Integrity of the Nuclear Envelope

The functional characteristics of the nuclear envelope of control and ΔNLA-treated nuclei were assayed by several methods. Nuclear membrane integrity was assayed by the ability of nuclei to exclude FITC-labeled 150-kD dextran (Newmeyer and Wilson, 1991). Nuclei were assembled in extract for 75 min and subsequently treated with protein buffer or ΔNLA for an additional 85 min as described above. The dextran (0.8 mg/ml final concentration) (Sigma Chemical Co.) was then added to the extract along with the DNA dye, Hoechst 33258 (10 μg/ml final concentration) (Molecular Probes). After 10 min, a 5-μl drop of the extract was placed on a slide, covered with an 18-mm square #1 coverslip, and observed by conventional epifluorescence. Nuclei were located using a DAPI filter (excitation 359 nm, emission 461 nm), and those that did not fluoresce with an FITC filter (excitation 485 nm, emission 515–565 nm) were scored as excluding dextran.

The ability of nuclei to import molecules after ΔNLA-induced disruption was assayed with TRITC-labeled human serum albumin (HSA) coupled to the NLS of the simian virus 40 large T antigen (Newmeyer and Forbes, 1988; gift of D. Forbes, University of California San Diego, La Jolla, CA). This transport substrate was diluted 1:150 into extracts containing nuclei 85 min after the addition of protein buffer or ΔNLA (see above). The nuclei were fixed 30 min after the addition of the transport substrate by adding an equal volume of NWB containing 4% formaldehyde and 5 μg/ml of Hoechst 33258 (Spann et al., 1997). 5-μl aliquots of the fixed samples were placed on slides and observed as described above. Nuclei that fluoresced when viewed with the rhodamine filter (excitation 546 nm, emission 590 nm) were scored positive for nuclear transport.

Protein Expression and Purification

ΔNLA is derived from human lamin A and lacks the first 33 amino acids of the NH_2 terminus. ΔNLA and lamin A (LA) were expressed in bacteria and purified as described previously (Spann et al., 1997). However, in most cases, a MonoS column was used instead of the MonoQ for ion exchange chromatography (Amersham Pharmacia Biotech). The MonoS column buffer was 15 mM Hepes/NaOH, pH 8.0, 6 M urea, and 1 mM DTT. Elution of ΔNLA was carried out with a 0–1 M NaCl gradient. The ΔNLB3 mutant was made by deleting the first 32 amino acids from the wild-type LB3 using the Quikchange mutagenesis kit (Stratagene). The mutant protein was expressed using the same method as for ΔNLA. The mutant protein was purified on the MonoQ column using a column buffer of 20 mM Tris-HCL, pH 8.0, 6 M urea, 1 mm DTT, and 1 mm EDTA and a 0–1 M NaCl gradient. Peak fractions were dialyzed against protein buffer, 15 mM Tris-HCl, pH 8.8, 300 mM NaCl, 1 mM DTT.

Human vimentin was engineered to contain the lamin B NLS (NLS-vimentin), expressed in bacteria and purified as described previously (Reichenzeller et al., 2000). The protein was dialyzed against 5 mM sodium phosphate, pH 7.4, with 1 mM DTT before addition to the extract.

GST-CIP was expressed in pGEX-2TK (Harper et al., 1993) (ATCC) using the same conditions as ΔNLA. Inclusion bodies were prepared and dissolved in 20 mM Tris, pH 8.1, 6 M urea, and 1 mM DTT, and the protein was further purified using ion exchange chromatography (MonoQ resin with 0–1 M NaCl gradient elution). Peak fractions containing GST-CIP were identified using SDS-PAGE (Laemmli, 1970), dialyzed against 25 mM NaCl, 15 mM Tris-HCl, pH 8.1, and 1 mM DTT for 4 h (500× vol with one buffer change at 2 h). The protein solution was aliquoted and stored at 70°C.

Results

Disruption of Lamin Organization in Nuclei Assembled in the Presence of AraC

In the *Xenopus* egg extract, the onset of DNA replication appears to coincide with the enclosure of chromatin within a nuclear envelope (Lohka and Masui, 1983; Newport, 1987; Sheehan et al., 1988). This makes it technically difficult to determine if normal nuclear lamin organization is required for DNA synthesis only to support proper nuclear envelope assembly and function, or if lamin organization is required for some other aspect of DNA replication. To uncouple DNA replication from nuclear assembly, AraC was added to the extract (Walter and Newport, 1997). This nucleotide analogue reversibly inhibits DNA polymerases but does not affect nuclear assembly or growth. In *Xenopus* interphase extracts, AraC has been shown to arrest replication near the onset of the elongation phase. The addition of dCTP rapidly reverses this inhibition, resulting in the resumption of DNA synthesis (Walter and Newport, 1997). Consequently, the effect on replication of disrupting nuclear lamin organization can be observed by releasing the AraC arrest. In this manner, we can determine if the maintenance of normal lamin organization is required for DNA synthesis after nuclei have assembled.

In our initial experiments, we have used ΔNLA, a mutant derived from human lamin A, to disrupt endogenous lamin organization. The use of the human protein allows us to distinguish the mutant and the endogenous LB3 using our species-specific antibodies. To ensure that ΔNLA disrupts lamin organization of AraC-arrested nuclei, sperm chromatin was added to an interphase extract containing 0.2 mM AraC. After 70 min, 2 μM ΔNLA was added, and 90 min later samples were fixed and processed for immunofluorescence (Materials and Methods). Control nuclei exhibited a typical lamin pattern when observed by immunofluorescence, a bright rim at the periphery of the nucleus with less intense nucleoplasmic lamin staining (Fig. 1 a). This pattern of lamin staining is very similar to that described previously for nuclei assembled in the absence of AraC (see Figure 4 a in Spann et al., 1997).

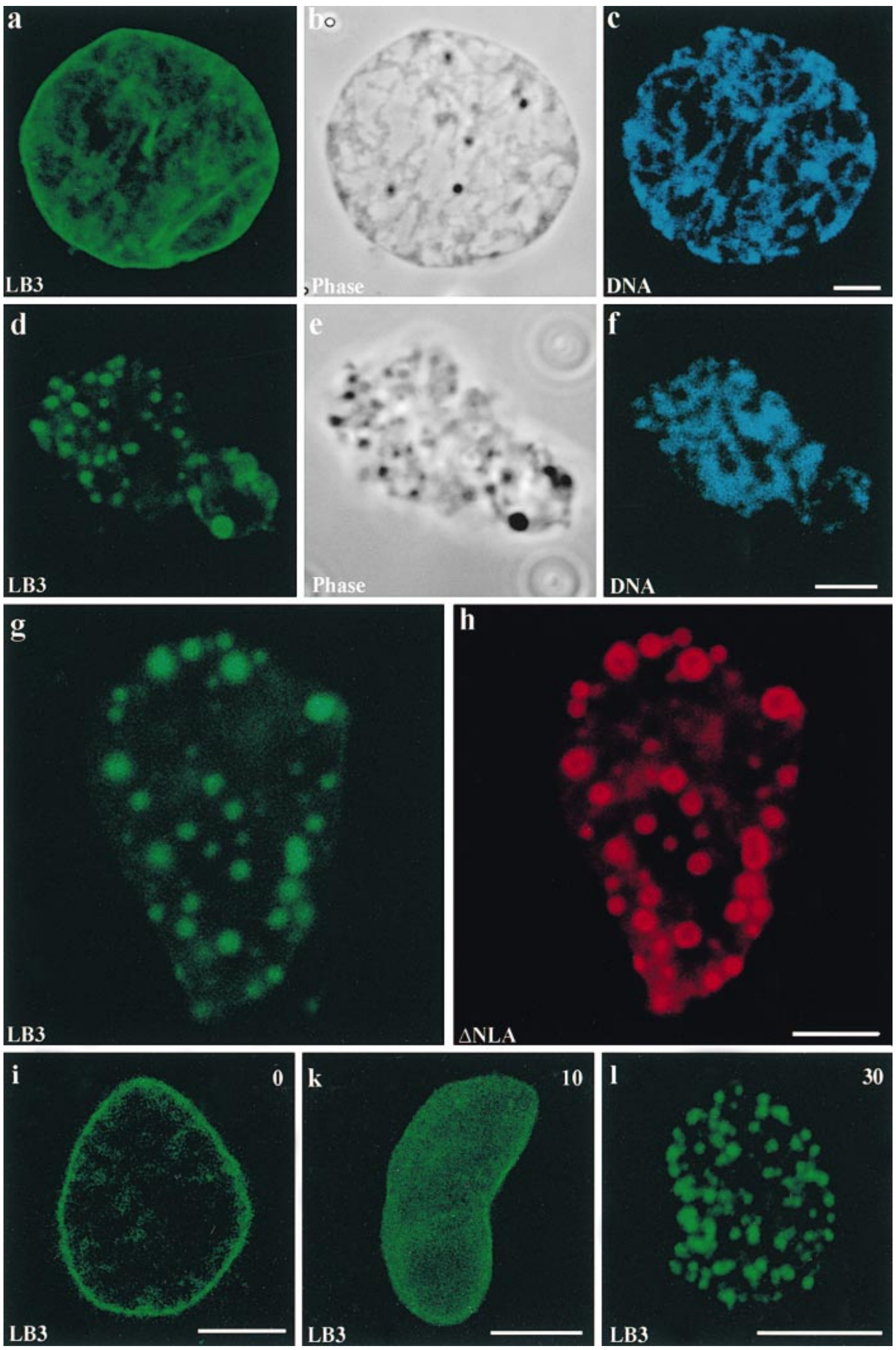

Figure 1. ΔNLA disrupts lamin organization in AraC-arrested nuclei. a–c depict confocal sections from a nucleus exposed to protein buffer, and d–f show a nucleus exposed to ΔNLA. In both cases, nuclei were assembled in interphase extract containing 0.2 mM AraC for 70 min before the addition of an equal volume of ΔNLA or protein buffer, and 90 min later the nuclei were fixed. The control nucleus stained with a mAb directed against lamin B3 (a) reveals a bright rim pattern accompanied by nucleoplasmic staining. In the ΔNLA-disrupted nucleus (d), the LB3 staining is absent from the rim and is instead found in abnormal nucleoplasmic aggregates. These aggregates are clearly visible with phase optics (e). The DNA staining patterns (Hoechst dye) of both the control (c) and disrupted nuclei (f) are also shown. Immunofluorescence using a mAb directed against lamin B3 (g) and a polyclonal antibody directed against human lamin A (h) shows that the ΔNLA protein colocalizes with the endogenous LB3 in nucleoplasmic aggregates. In larger aggregates, the mutant lamin appears to be more concentrated at the periphery, whereas the endogenous lamin is concentrated toward the center. The addition of ΔNLA produced a rapid alteration of lamin organization. 10 min after the addition of the mutant protein, the lamina staining is less apparent and a fine punctate pattern of LB3 staining appears through the nucleus (compare i with k). 30 min after the addition of the mutant protein, LB3 is found in abnormal nucleoplasmic aggregates (l). Each image represents a single optical section through the central region of a nucleus. Bars: 5 μm (a–h); and 10 μm (i–l).

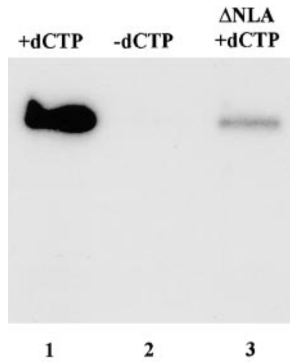

Figure 2. Autoradiograph demonstrating that lamin disruption blocks the resumption of DNA replication in AraC-arrested nuclei. Nuclei were assembled in an interphase extract containing AraC. In lane 1, dCTP was added with [^{32}P]dATP to relieve the AraC block of replication. In lane 2, the dCTP was omitted. In lane 3, dCTP and [^{32}P]dATP were added to AraC-arrested nuclei after disruption by ΔNLA. For all samples, nuclei were allowed to incorporate [^{32}P]dATP for 90 min. The samples were resolved on a 1% TBE agarose gel, and the dried gel was used for autoradiography. Quantitation with a beta imager revealed that the addition of ΔNLA reduced the incorporation of [^{32}P]dATP by >95% (not shown).

In contrast, the addition of the mutant protein, ΔNLA, caused a dramatic reorganization of normal lamin structure in the assembled nuclei such that the endogenous LB3 was found primarily in phase-dense nucleoplasmic aggregates with no obvious nuclear rim staining (Fig. 1, d and e).

The mutant protein, ΔNLA, colocalized with the endogenous LB3 in the nucleoplasmic aggregates (Fig. 1, g and h). When isotype-specific lamin antibodies were used (see Materials and Methods), LB3 was found primarily in the interior of the larger aggregates, whereas the mutant human lamin A was more concentrated at the edge of these aggregates (Fig. 1, g and h). Confocal microscopic observations revealed that the aggregates were distributed throughout the nucleoplasm and often appeared independent of the nuclear envelope (see also Spann et al., 1997). The phase-dense edge at the nuclear periphery was less prominent (compare Fig. 1, b and e) in lamin-disrupted nuclei. Chromatin remained distributed throughout the nucleoplasm of the disrupted nuclei (Fig. 1, c and f).

The degree of disruption was time-dependent as indicated by both the loss of nuclear rim staining and the appearance of nucleoplasmic aggregates. Nuclear rim staining was greatly reduced 10 min after the addition of ΔNLA. Concurrently, nucleoplasmic lamin staining increased, producing a diffuse finely speckled pattern. After 30 min, typical nuclear lamina rim staining disappeared, and the nuclei contained large numbers of nucleoplasmic lamin aggregates (Fig. 1, i–l). Lamin organization was disrupted in ~95% of the nuclei exposed to ΔNLA. In nuclei treated with lower concentrations of ΔNLA (e.g., 0.7 μM), there appeared to be more but smaller aggregates (not shown).

Disruption of Lamin Organization Blocks the Resumption of DNA Replication

To determine whether normal lamin organization must be maintained for DNA synthesis to occur, nuclei were assembled in extracts containing AraC for 70 min and then ΔNLA or protein buffer was added as described above for an additional 70 min. Subsequently, dCTP and [^{32}P]dATP were added to relieve the AraC block and to assay for DNA synthesis, respectively (see Materials and Methods). These reactions were stopped 90 min later and processed for agarose gel electrophoresis and autoradiography (see Materials and Methods). Control nuclei efficiently incorporated [^{32}P]dATP after the addition of dCTP (Fig. 2, lane 1). However, the ΔNLA-treated nuclei failed to resume replication upon release of the AraC block as indicated by an ~95% reduction in the incorporation of [^{32}P]dATP (Fig. 2, compare lanes 1 and 3). The efficacy of the AraC block was demonstrated by the inhibition of [^{32}P]dATP incorporation in the absence of dCTP (Fig. 2, compare lanes 1 and 2). It has been previously shown that the addition of dCTP to extracts containing AraC-arrested nuclei results in the rapid lengthening of accumulated replication products at rates characteristic of the elongation phase of replication (Walter and Newport, 1997). For this reason, AraC is thought to arrest DNA synthesis near the onset of the elongation phase of replication (Strausfeld et al., 1994; Walter and Newport, 1997). The finding that ΔNLA-induced disruption prevents the AraC-arrested nuclei from resuming DNA synthesis suggests that disruption of lamin organization blocks the elongation phase of replication.

As additional controls for the effects of ΔNLA on lamin organization and DNA replication, other purified bacterially expressed proteins were added to the interphase extracts containing nuclei assembled in the presence of AraC (see Materials and Methods). The addition of full-length LA at the same concentrations did not alter the normal bright rim or the less intense nucleoplasmic staining pattern displayed by endogenous LB3 as assayed by immunofluorescence (Fig. 3 a). Furthermore, the lamin A staining was indistinguishable from the LB3 staining pattern (Fig. 3 b). These results are identical to those obtained when lamin A was added to the extract before the onset of nuclear assembly (Spann et al., 1997). In addition, DNA replication was not inhibited in LA-treated nuclei. After the addition of dCTP, the levels of [^{32}P]dATP incorporation by nuclei treated with LA was comparable to that detected in control nuclei (Fig. 3 c*; see Materials and Methods).

As a control for the possible nonspecific effects of abnormal protein aggregates within the nucleoplasm, we added bacterially expressed human vimentin that had been engineered to contain an NLS (Reichenzeller et al., 2000). As has been reported previously in mammalian cells, the NLS-vimentin was imported into nuclei and assembled into nucleoplasmic aggregates. The presence of these structures did not affect the distribution of LB3, and LB3 does not appear to be a constituent of the vimentin aggregates (Fig. 3, d–f). The presence of these aggregates had no detectable effect on DNA replication (Fig. 3 f*). In contrast, the addition of the NH_2-terminal deleted *Xenopus* lamin, ΔNLB3, to extracts containing assembled nuclei disrupted the organization of endogenous lamin B3 (Fig. 3, g–i) and inhibited DNA replication (Fig. 3 i*). The alterations in lamin organization seen in these nuclei were indistinguishable from those seen in nuclei treated with ΔNLA.

Nuclear Envelope Integrity after Lamin Disruption

The nuclear envelopes of lamin-disrupted nuclei appeared

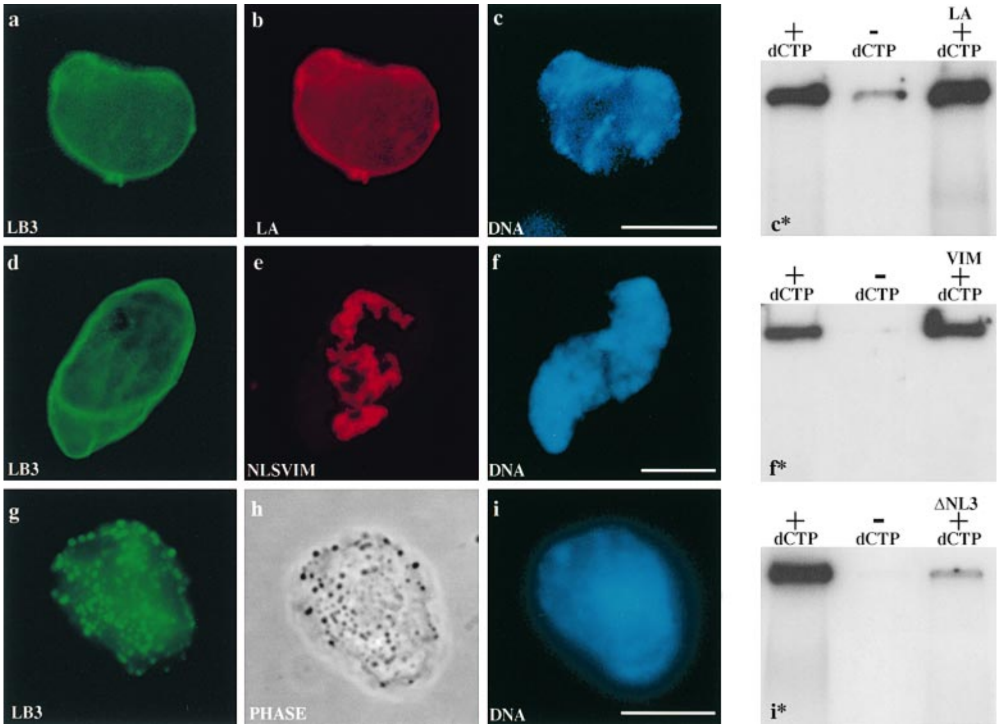

Figure 3. Lamin organization and DNA replication were unaltered by the addition of LA or NLS-vimentin. However, the addition of ΔNLB3 disrupted lamin organization and blocked DNA replication. Each of the bacterially expressed proteins was added to nuclei that had been assembled in extract containing AraC for 70 min. When LA is added, the LB3 staining pattern appears undisrupted (a) and the LA staining pattern was indistinguishable from that of LB3 as shown by immunofluorescence with a lamin A–specific antibody (b). Furthermore, the addition of LA did not prevent the resumption of replication that occurs with the addition of dCTP in DNA replication assays (c*, compare first and third lanes). The addition of NLS-vimentin also does not disrupt the LB3 staining (d), although the NLS-vimentin appears in large aggregates as shown by staining with a vimentin antibody (e). The addition of NLS-vimentin did not prevent the resumption of DNA replication (f*, compare first and third lanes). The addition of the ΔNLB3 disrupts the endogenous lamin network (g), resulting in large nucleoplasmic aggregates seen in immunofluorescence (g) and phase (h). The presence of ΔNLB3 prevents the resumption of DNA replication (i*, compare first and third lanes). All images were obtained using a Zeiss axiovert microscope. Bars, 10 μm.

to be less rigid than control nuclei as evidenced by their wrinkled appearance after centrifugation through a 30% sucrose cushion (compare Fig. 1, b and e). This observation suggested that the postassembly disruption of lamin organization might affect the structure of the nuclear envelope, thereby altering its permeability and transport properties. Since altered transport and permeability could result in an inhibition of DNA replication (see Introduction), we examined the overall distribution of nuclear membranes and pores of nuclei treated with either ΔNLA or LA. Fluorescence microscopy showed that the incorporation of either ΔNLA or LA did not alter the normal distribution of nucleoporins (Fig. 4, a–d). In addition, nuclei treated with either of these proteins were surrounded by membranes as indicated by staining with the dye, $DIOC_6$ (Fig. 4, e–h; see Materials and Methods). It should be noted that these staining patterns are similar to those seen in nuclei assembled in untreated extracts (data not shown). In addition, the nucleoplasmic lamin aggregates in the lamin-disrupted nuclei did not stain with either of these nuclear envelope markers (Fig. 4, c, d, g, and h).

The functional integrity of the envelopes of lamin-disrupted nuclei was assayed by determining whether the nuclei excluded a molecule that does not contain an NLS and is above the size limit for diffusion through nuclear pores (Newmeyer and Wilson, 1991). In these assays, a 150-kD dextran linked to FITC (150 kD) was added to extracts containing either lamin-disrupted or untreated nuclei (see Materials and Methods). 10 min later, Hoechst dye was added to identify nuclei, and these unfixed samples were observed by fluorescence microscopy (see Materials and Methods). We found that 97% of the control nuclei (n = 100) and 98% of the lamin-disrupted nuclei (n = 100) excluded the labeled dextran (Fig. 5, a and d), indicating that the nuclear envelopes of both retained their capacity to exclude large molecules.

The effects of lamin disruption on nuclear import were tested by the addition of rhodamine-tagged serum albumin coupled to an NLS peptide to extracts containing control and lamin-disrupted nuclei (Newmeyer and Wilson, 1991). After a 30-min incubation, the samples were fixed and stained with Hoechst 33258 (see Materials and Methods). Nuclei exhibiting nucleoplasmic rhodamine fluorescence above background were scored as positive for transport. We found that 80–85% of the control (n = 85) and 83% of the lamin-disrupted (n = 85) nuclei were fluorescent (Fig. 6, b and f). No obvious difference was detected between the fluorescence intensities of the control and disrupted nuclei. Controls consisted of untreated nuclei, nuclei treated with protein buffer, and nuclei treated with LA (see Materials and Methods). In all cases the controls yielded indistinguishable results. Based on these criteria, the import of molecules into the nucleus appeared to be unaffected by the disruption of lamin organization.

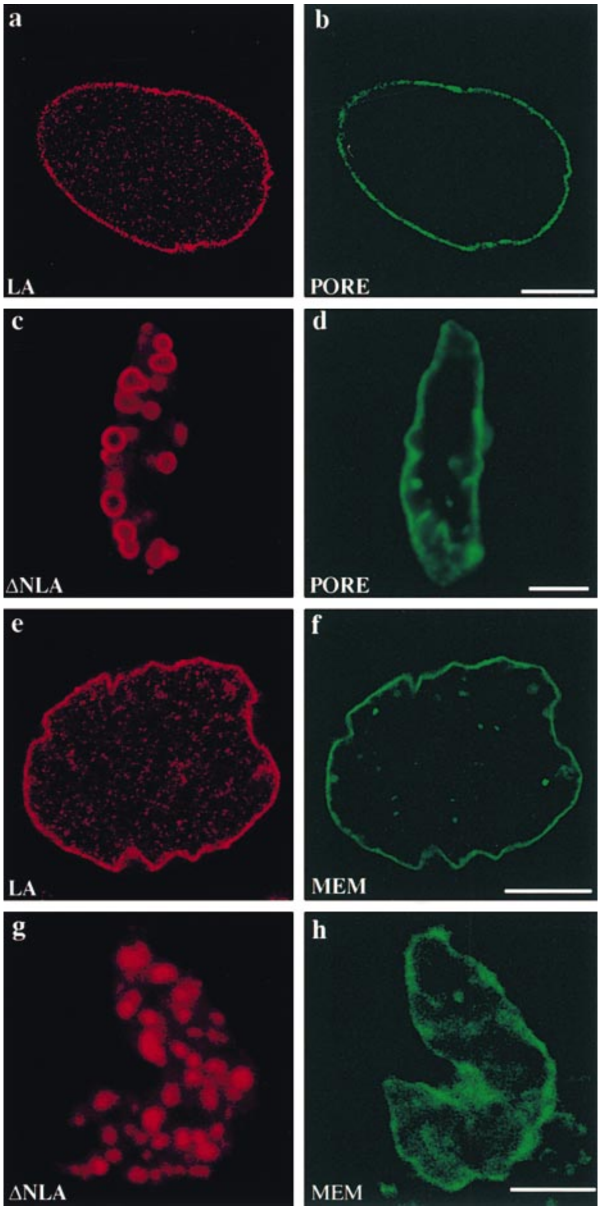

Figure 4. Lamin disruption does not obviously alter the morphology of the nuclear membrane or pores. Control nuclei (treated with LA) and lamin-disrupted nuclei (treated with ΔNLA) were stained with 414 (b and d), a mAb that reacts with the nucleoporins or with the membrane dye $DIOC_6$ (f and h). Both the pore and membrane markers produce a bright rim stain that aligns with the edge of nuclei. The LA (a and e) and ΔNLA (c and g) were stained with an antibody directed against human lamin A. The lamin aggregates did not stain with either the pore (c and d) or membrane marker (g and h). Each pair of confocal images represents an optical section through the central region of a nucleus. Bars, 5 μm.

Disruption of Lamin Organization Prevents the Synthesis of High Molecular Weight DNA

ΔNLA-induced disruption of lamin organization prevents the resumption of replication in AraC-arrested nuclei, suggesting a block of the elongation phase (Fig. 2). In addition, the morphological effects of ΔNLA are concen-

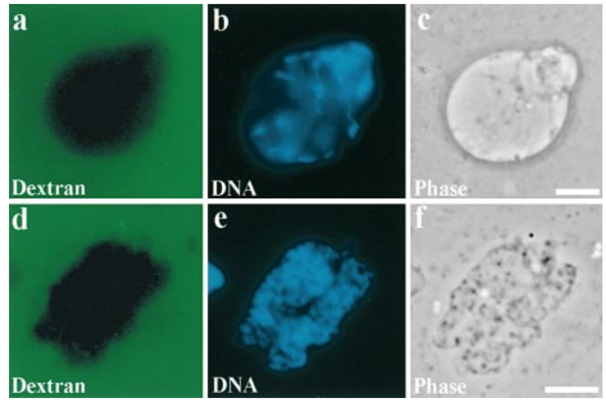

Figure 5. To assess the barrier function of the lamin-disrupted nuclei, FITC-labeled dextran (150 kD) was added to extracts containing normal (a–c) or ΔNLA-disrupted (d–e) nuclei. Aliquots of each reaction were transferred to slides and observed by epifluorescence. Nuclei were located using Hoechst dye (b and e), and observed in the FITC channel (a and d). Both the control and disrupted nuclei appear dark in the FITC channel, demonstrating that both types of nuclei exclude the FITC-labeled dextran. The phase contrast images of these nuclei are seen in c and f. The disrupted nucleus (f) contains phase-dense bodies that represent the lamin aggregates. Over 95% of both control and disrupted nuclei excluded the dextran (n = 100). Bars, 10 μm.

tration-dependent (see above). Based on these findings, there should be a correlation between the concentration of ΔNLA in the extract and the size of the replication products synthesized. To test this possibility, increasing

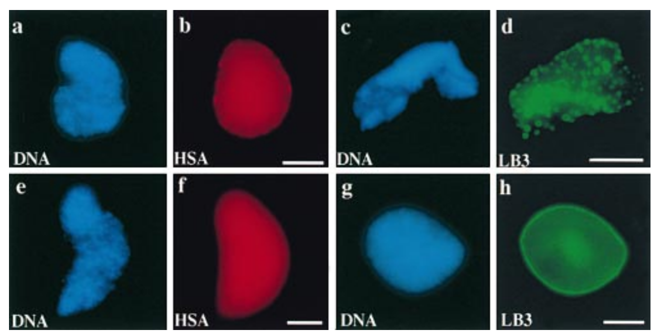

Figure 6. To assess nuclear transport in disrupted nuclei, TRITC-labeled human serum albumin (HSA) coupled to an NLS peptide (see Materials and Methods) was added to extracts containing ΔNLA-disrupted (a–d) and control (untreated, e–h) nuclei. 30 min later, the preparations were fixed and a portion of each was observed by conventional epifluorescence. Nuclei were located using a DNA dye and scored as positive for transport if they also fluoresced in the TRITC channel. A lamin-disrupted nucleus (a and b) and a control nucleus (e and f) are shown. A comparable fraction of nuclei from both reactions imported the labeled HSA over the 30-min time period (a and c, 81% control, 83% ΔNLA; n = 85 for each preparation). There was no detectable difference in the range of fluorescence intensities of the transporting nuclei between these two samples. To ensure the mutant lamin efficiently disrupted lamin organization, a sample of the transport reaction was fixed and stained for the endogenous LB3 distribution. More than 98% of nuclei treated with ΔNLA had disrupted lamin organization compared with control samples (compare d with h). Bars, 10 μm.

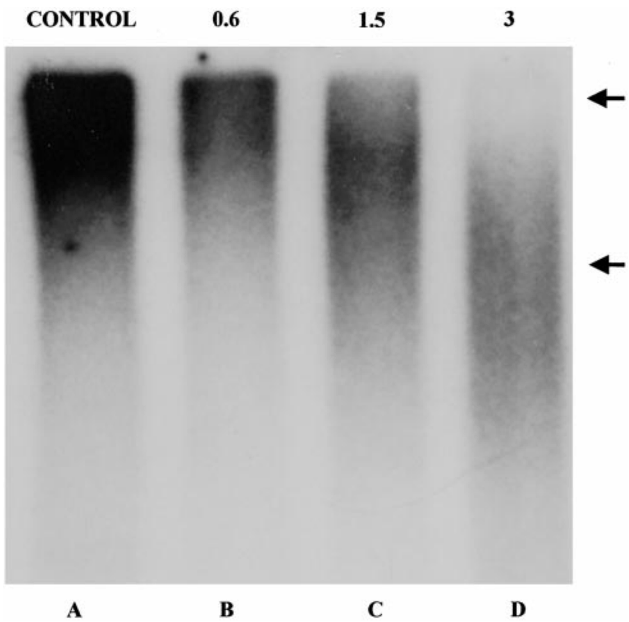

Figure 7. ΔNLA blocks the synthesis of high molecular weight replication products. Increasing concentrations of ΔNLA (0, 0.6, 1.5, and 3 μM) were added to extracts containing AraC-arrested nuclei. After lamin disruption, dCTP was added to each reaction to release the AraC block and replication was allowed to proceed for 10 min. Subsequently [^{32}P]dATP was added to the reactions, and 2 min later the reactions were stopped. The replication products were resolved on a denaturing alkaline agarose gel, and the dried gel was used for autoradiography. The average size of the replication products synthesized in the reaction lacking ΔNLA (lane A) was >10,000 bases. As the concentration of ΔNLA added was increased, the size of the replication products decreased (lanes B–D), such that at 3 μM (lane D), the average size was ~1,500 bases. The arrows on the right are size markers indicating the approximate position of markers 2,027 and 9,416 bases in length (New England Biolabs, Inc.).

amounts of ΔNLA (0–3 μM) were added to extracts containing AraC-arrested nuclei. After lamin disruption, the AraC block was relieved by the addition of dCTP. After 10 min, replicating DNA was labeled with [^{32}P]dATP in a further 2-min incubation (see Materials and Methods). To assess the size of the replication products, the reactions were stopped and processed for denaturing agarose gel electrophoresis (see Materials and Methods). When the AraC block of control nuclei was not released, the size of the replication products was 1,000–2,000 bases (Walter and Newport, 1997; data not shown). However, 10 min after the addition of dCTP, the average size of replication products in the control sample was >10,000 bases (Fig. 7, lane A). As the concentration of ΔNLA was increased, the average size of the products synthesized during this 10-min period was progressively smaller, such that at 3 μM ΔNLA, the average size was ~1,500 bases (Fig. 7, lanes B–D). These results show that increasing concentrations of ΔNLA produce progressively greater effects on chain elongation. Furthermore, 40 min after the release of the AraC block, the average size of replication products continued to be ~1,500 bases in the nuclei disrupted with 3 μM ΔNLA (data not shown). These observations demonstrate that ΔNLA does not simply slow, but rather arrests the process of elongation.

Lamin Disruption Does Not Reverse the Cdk2-dependent Step of the Initiation Phase of Replication

The results described above suggest that the disruption of lamin organization specifically blocks the elongation phase of DNA replication. However, it is also possible that the addition of the ΔN mutant lamins disrupt the function of the initiation complexes involved in DNA replication, thereby inhibiting subsequent replication events, including elongation. CIP, an inhibitor of the cdks, was used to distinguish between these two possibilities (Harper et al., 1993). CIP blocks early initiation events mediated by cdk2 when added during the first few minutes of nuclear assembly (Strausfeld et al., 1994). In addition, CIP does not prevent AraC-arrested nuclei from resuming replication after the addition of dCTP (Fig. 8 a, lane 3; see also Walter and Newport, 1997). This demonstrates that the AraC arrest occurs after the CIP arrest of DNA replication.

AraC-arrested nuclei that were treated with a ΔN lamin mutant to disrupt lamin organization also resumed replication when transferred to extracts containing CIP (Fig. 8 b, lane 6; see Materials and Methods). The presence of CIP caused a slight reduction in the level of [^{32}P]dATP incorporation when compared with that detected in lamin-dis-

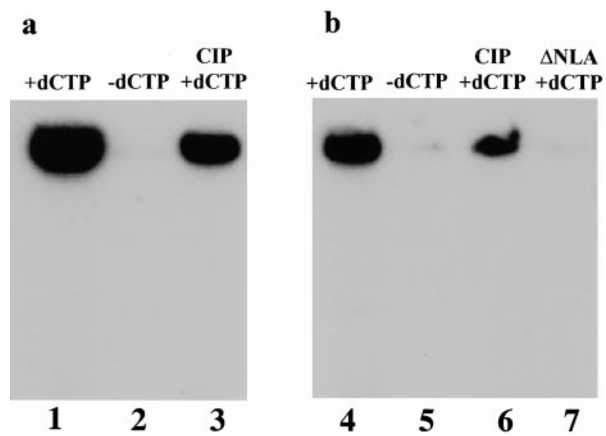

Figure 8. ΔNLA does not disrupt the cdk2-dependent initiation step of replication. Nuclei were assembled in the presence of AraC and subsequently, the protein buffer control (a) or ΔNLA (b) were added. After an additional 90-min incubation, the nuclei were transferred to extract containing AraC and either CIP, ΔNLA, or protein buffer. Replication was monitored with the addition of dCTP and [^{32}P]dATP, and after a 60-min labeling period the reactions were stopped and the replication products resolved on a nondenaturing agarose gel. The image is an autoradiograph of the dry gel. (a) The control nuclei resume replication when transferred to extract containing buffer (lane 1) or CIP (lane 3) as compared with the sample in which the AraC block was not reversed (lane 2). (b) Similarly, ΔNLA-disrupted nuclei resume replication when transferred to extract containing buffer (lane 4) or CIP (lane 6). A sample in which the AraC block was not reversed is shown in lane 5. If the disrupted nuclei were transferred to an extract that also contained ΔNLA, then replication did not resume (lane 7).

rupted nuclei after transfer to extract devoid of CIP (Fig. 8 b, compare lanes 4 and 6). However, CIP caused a similar reduction in the amount of [^{32}P]dATP incorporated by control nuclei (Fig. 8 a; see also Strausfeld et al., 1994; Walter and Newport, 1997). The ability of the lamin-disrupted nuclei to replicate in the CIP-treated extract indicates that resumption of replication after the disruption of lamin organization does not require the early cdk-dependent steps of replication. These results provide additional evidence that the alterations in lamin organization induced by ΔN lamin mutants block DNA synthesis by interfering with the elongation phase and not the initiation phase of replication.

To ensure that the process of transferring the nuclei between extracts did not rescue DNA synthesis, lamin-disrupted nuclei were also transferred to fresh extract containing ΔNLA. These nuclei did not resume replication (Fig. 8 b, lane 7). Similarly, samples to which no dCTP was added did not resume replication (Fig. 8 b, lane 5). These results indicate that both the AraC block and lamin disruption were maintained throughout the transfer procedure.

The Distribution of Elongation Factors Is Altered by Disruption of Lamin Organization

Although disruption of the lamin organization of nuclei appears to block the elongation phase of replication, the molecular interactions responsible for this inhibition are unknown. To begin to determine the mechanism through which this inhibition occurs, we examined the effects of ΔN lamins on the organization of a number of replication factors in AraC-arrested nuclei. The chromatin-associated patterns of three initiation factors, DNA polymerase α, XORC2, and XMCM3, were unaffected by either AraC or the postassembly disruption of lamin organization (data not shown). In contrast, the distributions of both RFC and PCNA were dramatically altered in the lamin-disrupted nuclei. Instead of the normal diffuse staining spread throughout the nucleoplasm of control nuclei (Fig. 9, a and d), both RFC and PCNA colocalized with the lamins in aggregates in postassembly disrupted nuclei (Fig. 9, b, c, e, and f). Furthermore, like the endogenous lamin, LB3, RFC, and PCNA appeared to be concentrated toward the interior of the larger aggregates (Fig 9, b and c; data not shown), whereas the mutant lamin was found at the edge of the aggregates (Fig. 1). In control preparations, LA and NLS-vimentin had no effect on the distribution of any of these factors (data not shown).

In light of the ability of the lamin-disrupted nuclei to resume DNA replication after their transfer to fresh extracts, LB3 and PCNA were also examined to determine whether they recovered their normal distributions during the reversal process. Lamin-disrupted nuclei were transferred to fresh extracts (see Materials and Methods) and fixed at several timepoints. Within 10 min, the nucleoplasmic aggregates became much less apparent and the LB3 staining pattern showed obvious signs of recovery both in the nucleoplasm and in the peripheral region of the nucleus (compare Fig. 10, a and b). After 60 min, a relatively normal LB3 morphology was evident (Fig. 10 d). In the case of PCNA, recovery to a normal distribution was also rapid. In disrupted nuclei, PCNA does not appear to colocalize with chromatin. Instead the PCNA is found in nucleoplasmic aggregates, typically found adjacent to chromatin (Fig. 10, e, i, and m). As early as 15 min after the transfer, the large aggregates of PCNA disappeared, and a typical finely speckled chromatin-associated pattern was seen (Fig. 10, f, j, and n). These morphological features remained evident 60 min after transfer (Fig. 10). In these experiments, the nuclei were extracted with 0.1% Triton X-100 before fixation to ensure that the proteins had re-

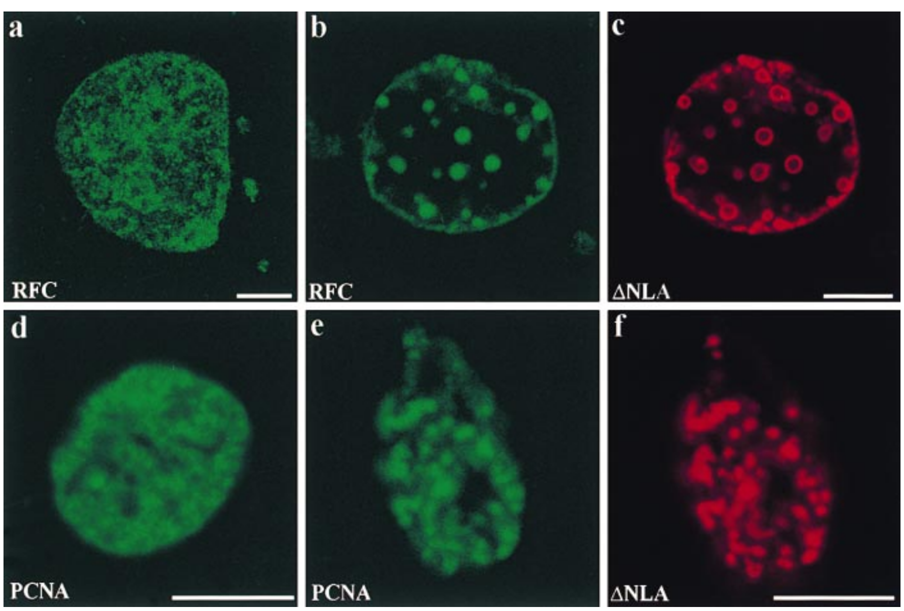

Figure 9. Disruption of lamin organization alters the distributions of RFC and PCNA. Double immunofluorescence of nuclei assembled in the presence of AraC and subsequently treated with buffer or ΔNLA. The nuclei were stained with a mAb directed against the large subunit of RFC (a and b) or PCNA (d and e) and an antibody directed against ΔNLA (c and f). The AraC-arrested control nuclei display normal RFC (a) and PCNA (d) staining patterns, i.e., relatively diffuse staining spread throughout the nuclei. In contrast, both RFC (b) and PCNA (e) are found in abnormal aggregates in the lamin-disrupted nuclei. The mutant lamin is also found associated with these abnormal aggregates (c and f). The size of the aggregates varied slightly between experiments. In cases in which large aggregates are produced (i.e., b) the RFC and PCNA are found in the center of the aggregate with ΔNLA concentrated at the edge of the aggregate. Images represent single confocal optical sections through the central region of a nucleus. Bars, 10 μm.

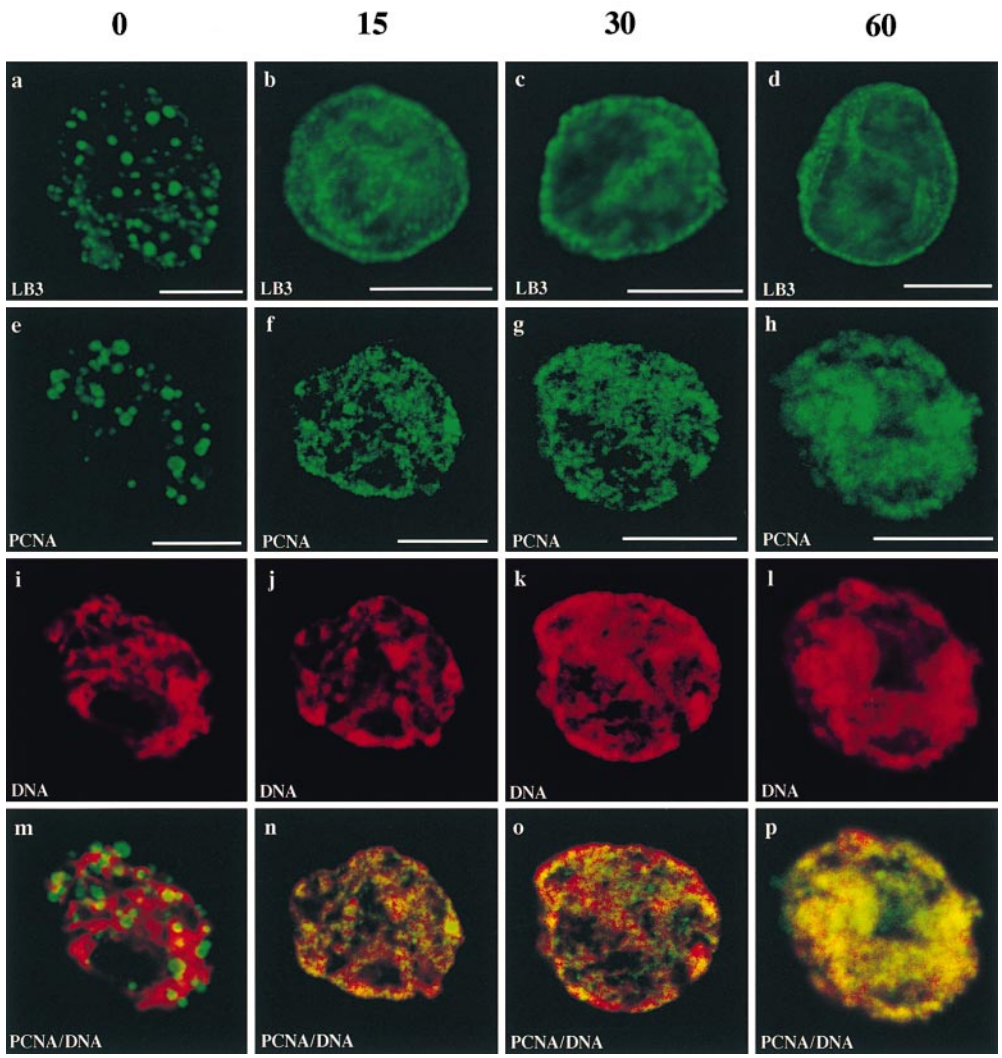

Figure 10. Normal distributions of nuclear lamins and PCNA are rapidly reestablished when lamin-disrupted nuclei are transferred to an untreated *Xenopus* extract. Nuclei were assembled in the presence of AraC and subsequently lamin organization was disrupted by the addition of ΔNLA. The disrupted nuclei were transferred to an untreated extract and fixed at the indicated timepoints (Materials and Methods). Parallel samples were stained for LB3 (a–d) or PCNA and DNA (e–p). The samples in the far left column were fixed before transfer (0 time). At 0 time, LB3 was found in the characteristic nucleoplasmic aggregates (a), but 15 min after transfer to untreated extract, the larger lamin aggregates are no longer visible (b). Instead, a more normal appearance with rim staining and finer speckled nucleoplasmic lamin standing was observed. A nearly normal lamin morphology was apparent 60 min after the transfer (d). Similarly, the PCNA distribution rapidly changed after transfer to untreated extract (e–p). In disrupted nuclei, PCNA appears in large aggregates that do not coalign with chromatin. As early as 15 min after transfer, PCNA staining was no longer found in these aggregates, but instead regained its normal distribution as a finely speckled pattern along the chromatin (f, j, and n). This colocalization with chromatin remained evident 60 min after transfer (h, l, and p). Bars, 10 μm.

constituted stable nuclear structures. Based on these results, it appears that the recovery of normal distribution of both lamins and the elongation factor PCNA are correlated with the resumption of DNA replication.

Discussion

The results of this study demonstrate that the maintenance of normal nuclear lamin organization is required for DNA synthesis. The disruption of lamin organization with mutant lamins results in the arrest of DNA synthesis. Furthermore, the results show that this effect is not due to either an arrest of nuclear assembly or a disruption of the selective barrier function of the nuclear envelope. Instead, the block in DNA synthesis appears to be due to the inhibition of the elongation phase of replication, as shown by the inability of disrupted nuclei to lengthen the short replication products that accumulate in AraC-arrested nuclei (Fig. 7) (Walter and Newport, 1997). This block of DNA synthesis is most likely related to the reorganization of RFC and PCNA that accompanies the disruption of lamin organization (Figs. 9 and 10). RFC and PCNA are required cofactors of DNA polymerase δ, the enzyme responsible for DNA synthesis during the elongation phase of replication (Stillman, 1994). In contrast, the mutant lamins do not alter the distribution of XMCM3, DNA polymerase α, or XORC2, factors thought to be required for the initiation phase of replication (Stillman, 1994; Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995; Carpenter et al., 1996). In addition, lamin-disrupted nuclei resume replication when transferred to an extract containing CIP, which blocks at the initiation phase of DNA replication by inhibiting the early cdk2-mediated events of S phase. These observations show that normal lamin organization is not required for the initiation of DNA synthesis but is required for the elongation phase of DNA synthesis in nuclei.

We showed previously that the addition of ΔNLA to *Xenopus* extracts at the onset of nuclear assembly resulted in a 95% reduction in the amount of DNA synthesized (Spann et al., 1997). Similarly, a *Xenopus* lamin B1 mutant lacking its NH_2-terminal domain has been shown to block lamina assembly and inhibit DNA synthesis in the *Xenopus* system (Ellis et al., 1997). However, nuclei assembled in the presence of these mutant lamins are smaller than control nuclei, raising the possibility that the mutant proteins block replication indirectly by inhibiting some aspect of normal nuclear assembly. A similar explanation has been proposed to explain the inhibition of DNA synthesis in nuclei that are assembled in *Xenopus* extracts after immunodepletion of lamin B3 (Walter et al., 1998). Other evidence also suggests that nuclear lamins play a role in nuclear envelope assembly. The addition of an antibody directed against both LB2 and LB3 to the *Xenopus* interphase extract prevents the formation of the nuclear envelope and results in the formation of a large number of annulate lamellae (Dabauvalle et al., 1991; Lourim and Krohne, 1993). Similarly, an insertional mutation in *Drosophila* leads to a dramatic reduction in the expression level of lamin Dm_0, resulting in a general inhibition of nuclear envelope assembly (Lenz-Böhme et al., 1997). This mutation is lethal in the majority of homozygous offspring. However those that survive display phenotypes that are consistent with an inhibition of nuclear envelope formation, as displayed by the clustering of nuclear pores, the accumulation of annulate lamellae, and in many cases an absence of nuclear membrane.

To avoid inhibiting nuclear envelope formation, we added ΔN lamins to extracts only after AraC-arrested nuclei had assembled to a normal size and possessed normal distributions of nuclear lamins, pores, and membranes. The distribution of RFC, PCNA, and other replication factors also appeared to be normal in the AraC-arrested nuclei before lamin disruption. Therefore, the block of replication that accompanies disruption of lamin organization with the ΔN lamin mutants is not due to a general arrest of nuclear assembly.

The data presented in this study demonstrate that increasing concentrations of ΔN mutants produce increasingly dramatic alterations in lamin organization, which in turn affect both the length of replication products synthesized and the distributions of PCNA and RFC. The reorganization of RFC and PCNA in response to disruption of lamin organization suggests an interaction between these factors and LB3. This is also implied by the colocalization of LB3 with elongation factors inside the larger lamin aggregates. Furthermore, the timecourse of recovery for normal PCNA and lamin organization is very similar when the lamin-disrupted nuclei are transferred to untreated extracts, suggesting an interaction between these molecules. In addition, the presence of elongation factors in the lamin aggregates does not appear to be due to nonspecific trapping, as the formation of abnormal nucleoplasmic aggregates of NLS-vimentin had no effect on the distribution of RFC and PCNA.

A role for the lamins in the organization of PCNA within the nucleus is also supported by results obtained with nuclei assembled in extracts that have been immunodepleted of LB3. In these nuclei, PCNA, which is normally insoluble, is readily extracted with buffers containing a nonionic detergent (Jenkins et al., 1993). These results indicate that the lamins play a specific role in the elongation phase of replication. One explanation of these results would suggest that lamins act as a scaffold upon which replication factors are organized within the nucleus. Such a scaffold could act to organize and increase the local concentration of replication factors such as RFC and PCNA to the critical concentrations required for replication to proceed efficiently within subdomains of the nucleus. The concentration of replication factors at specific sites might explain the precise spatial and temporal regulation of DNA synthesis observed in many cell types (Mills et al., 1989; Nakayasu and Berezney, 1989; Hozak et al., 1993, 1994).

Nuclear lamins were once thought to be found exclusively at the nuclear periphery, whereas DNA synthesis takes place throughout the entire nucleus. These observations appear to be inconsistent with models suggesting that nuclear lamins form a nucleoplasmic scaffold upon which replication factors are organized. However, recent reports from a number of laboratories have provided evidence that lamins are also found within the nucleoplasm, distinct from the lamina at the periphery (Goldman et al., 1992; Hozak et al., 1995; Neri et al., 1999). The models of nucleoplasmic lamin structure proposed in these studies range from distinct nucleoplasmic foci to an internal network throughout the nucleus. In addition, we have found that during mid to late S phase in mammalian cells, lamin B colocalizes with PCNA at sites of DNA synthesis (Moir et al., 1994). We have also observed nucleoplasmic lamin staining in *Xenopus* nuclei undergoing DNA replication (Fig. 1 and unpublished results; Spann et al., 1997). These findings suggest that the elongation phase of replication may be dependent upon interactions between elongation factors and nucleoplasmic lamin structures. This could also help to explain why others have not observed that postassembly disruption of lamin organization prevents the resumption of DNA replication (Ellis et al., 1997). The latter study used a lamin mutant consisting of GST fused to the rod domain but lacking the NH_2 terminus and much of the COOH terminus of *Xenopus* lamin B1. This mutant resulted in a loss of lamina staining when added to assembled nuclei, but it did not prevent replication. However, unlike the ΔN lamin constructs used in this study, the nuclei treated with the GST-lamin did not contain large nucleoplasmic aggregates. A possible explanation consistent with both findings is that the GST fusion protein does not affect normal nucleoplasmic lamin structures. This could be related to the lack of most of the COOH-terminal domain in the GST fusion protein. There is evidence that this domain of lamins binds to chromatin and thereby could mediate interactions between nuclear lamins and proteins that interact with DNA and chromatin such as PCNA and RFC (Hoger et al., 1991). We are also very interested in determining if these differing results are due to the different isotypes of lamins used in the dominant-negative constructs, as has been suggested by Ellis et al. (1997).

In general, only the initiation phase of replication is thought to be regulated in *Xenopus* extracts. This is based largely on the ability of the extract to efficiently replicate M13 phage DNA in the absence of membrane components (Mechali and Harland, 1982). It has been suggested that the elongation machinery is responsible for the replication of this single-stranded template. However, as replication factors have been identified and characterized, it has become clear that most preinitiation and initiation factors normally bind to chromatin before the nuclear membrane is formed (Adachi and Laemmli, 1992; Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995; Yan and Newport, 1995a,b; Carpenter et al., 1996).

In contrast, the elongation factor, PCNA, appears to associate with chromatin only after the nuclear membrane and the lamina have assembled (Hutchison et al., 1989). These results suggest that the elongation phase of DNA replication begins only after the nuclear envelope assembles. The ΔN lamin-induced arrest near the transition from the initiation stage to the elongation phase also suggests that the transition to the elongation phase is a closely regulated process. Other lines of evidence also imply that entry into the elongation phase of replication is more complex than previously envisioned. For example, experiments using kinase inhibitors suggest that the XMCM complex (licensing factor) may help to regulate the switch from the initiation to the elongation phase of replication (Yan and Newport, 1995a). XMCM normally disassociates from chromatin as replication proceeds, but the association is retained when replication is arrested with aphidicolin at the entry to the elongation phase (Chong et al., 1995; Kubota et al., 1995; Madine et al., 1995). Consistent with these findings, XMCM remains associated with chromatin in ΔN lamin-disrupted nuclei (data not shown), even after the release of the AraC block. Similarly, the inhibition of normal lamin assembly in the presence of the ΔNL mutants during nuclear assembly results in the inhibition of replication and the retention of the association between chromatin and XMCM (Spann et al., 1997).

The observation that DNA synthesis requires nuclear envelope assembly has led to another model in which lamins are thought to be indirectly involved in DNA synthesis. In this model, the lamins serve as a support to the nuclear membrane so that it can function as a selective barrier (Walter et al., 1998). Accordingly, the block of DNA synthesis caused by the ΔN mutants used in this study may be due to an alteration of nuclear envelope structure that prevents the concentration of replication factors in the nucleus. However, we have found that disruption of lamin organization in nuclei arrests replication without having an obvious affect on the selective barrier functions of the nuclear envelope (see Figs. 5 and 6). In addition, XORC2, XMCM3, and DNA polymerase α, PCNA, and RFC are all retained in the nucleus after postassembly disruption with ΔN lamin mutants.

Recently, it has been shown that a nucleoplasmic extract prepared from assembled nuclei is capable of DNA replication in the absence of both an intact nuclear membrane and detectable lamin structures (Walter et al., 1998). This nucleoplasmic extract is highly enriched in replication factors relative to the concentrations found in interphase extracts prepared from *Xenopus* eggs. For instance, cyclin E is 25-fold more concentrated in the nucleoplasmic extract. This high concentration of factors may permit DNA synthesis to proceed in the absence of the typical organization and regulation of replication factors that have been observed in nuclei during S phase. In intact nuclei, our data suggest a model in which nuclear lamins are involved in the organization of the molecules required for replication. Consistent with this model is the observation that in mammalian cells, the replication of DNA is highly regulated both spatially and temporally, resulting in individual loci being replicated during defined periods of S phase at known regions in the nucleus (O'Keefe et al., 1992). Analysis of a number of cell types reveals that during S phase, the nucleoplasmic distribution of lamins changes in a predictable manner, consistent with the altering patterns of DNA replication observed as cells progress from the early to later stages of S phase (Moir et al., 1994).

In summary, we find that the disruption of lamin organization blocks nuclear DNA synthesis near the switch from the initiation to the elongation phase of replication. This inhibition of replication is most likely caused by an alteration in the distribution of the elongation factors, PCNA and RFC. Based on these observations and the other data presented in this study, we believe that under normal conditions, nuclear lamins play an important role in the organization and function of elongation factors. The determination of the mechanisms by which the disruption of lamin organization alters the normal organization of RFC and PCNA should provide important new insights into both the temporal and spatial organization of replication centers in the nucleus. This information in turn may lead to the discovery of novel mechanisms through which eukaryotic cells regulate DNA replication.

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