Alteration of nuclear lamin organization inhibits RNA polymerase II–dependent transcription

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Regulation of gene activity is mediated by alterations in chromatin organization. In addition, chromatin organization may be governed in part by interactions with structural components of the nucleus. The nuclear lamins comprise the lamina and a variety of nucleoplasmic assemblies that together are major structural components of the nucleus. Furthermore, lamins and lamin-associated proteins have been reported to bind chromatin. These observations suggest that the nuclear lamins may be involved in the regulation of gene activity. In this report, we test this possibility by disrupting the normal organization of nuclear lamins with a dominant negative lamin mutant lacking the NH_2-terminal domain. We find that this disruption inhibits RNA polymerase II activity in both mammalian cells and transcriptionally active embryonic nuclei from *Xenopus laevis*. The inhibition appears to be specific for polymerase II as disruption of lamin organization does not detectably inhibit RNA polymerases I and III. Furthermore, immunofluorescence observations indicate that this selective inhibition of polymerase II–dependent transcription involves the TATA binding protein, a component of the basal transcription factor TFIID.

Introduction

RNA polymerase II activity may be governed in part by the regulation of the association of genes and transcription factors with structural components of the nucleus, often collectively referred to as the nuclear scaffold or matrix (Davie, 1995). For example, active transcription complexes appear to be bound to a nuclear scaffold, and active genes, along with transcription factors, are reported to be enriched in nuclear matrix preparations (Jackson and Cook, 1985; Stein et al., 1995). These findings, as well as the existence of the matrix itself, are controversial, due in part to the variety of extraction methods used to prepare nuclear matrices/scaffolds (Pederson, 2000). However, the greatest obstacle in determining the role of nuclear structure in transcription has been the failure to identify specific nuclear structural elements involved in transcription.

Nuclear lamins may form such a structural element. These type V intermediate filament (IF)* proteins are found in higher eukaryotic organisms and are subdivided into A types, expressed in differentiated cells, and B types, found in all cells (Moir et al., 1995). The lamins are the major constituents of the lamina, a proteinacious layer located at the inner surface of the nuclear membrane and closely associated with chromatin. The lamins provide structural support for the nuclear envelope and are involved in nuclear assembly (Moir et al., 1995; Gruenbaum et al., 2000). Lamins are also found in the nucleoplasm, distinct from the lamina where they assemble a number of structures, such as distinct foci and a structure, termed the nucleoplasmic veil, that is found throughout the nonnucleolar regions of the nucleoplasm (Bridger et al., 1993; Moir et al., 1994, 2000b; Kennedy et al., 2000; Liu et al., 2000). Live cell observations with GFP–lamins indicate that nucleoplasmic lamin assemblies are integrated into stable structures (Moir et al., 2000b). The nuclear lamins and lamin-associated proteins contain chromatin-binding domains, suggesting that lamins may also be involved in the organization of chromatin (Moir et al., 1995; Wilson et al., 2001).

The involvement of nuclear lamins in transcription has been suggested by recent studies of gypsy, a *Drosophila* insulator element. The effects of this element on enhancers appear to be dependent upon its attachment to a substrate, possibly the nuclear lamins (Gerasimova et al., 2000; Bell et al., 2001). Another *Drosophila* study indicates that loss of function mutants of lamin (Dm_0) result in disruption of directed outgrowth of the cytoplasmic extensions of the tracheal system, and germ line mutant clones yield oocytes in which

mRNA fails to properly localize in the cytoplasm (Guillemin et al., 2001). The authors suggest that the failure of these cytoplasmic processes is due to a novel role for lamins in organizing cytoplasm, or alternatively, lamins may be required for the proper expression of gene products required to carry out these cytoplasmic processes. An involvement of lamins in transcription is also supported by the finding that Rb, a repressor of transcription for a number of cell cycle genes, has been reported to bind lamins (Mancini et al., 1994). In addition, a lamin binding protein, lamin-associated protein 2B (LAP 2B), has been reported to mediate transcriptional repression (Nili et al., 2001). Finally, changes in the expression patterns of lamin isotypes during vertebrate development correlate with the onset of transcription and cell differentiation (Moir et al., 1995). Although these studies are consistent with lamin involvement in transcription, there is little direct evidence to support this possibility.

Like all IF proteins, nuclear lamins contain conserved central α-helical rod domains and nonconserved NH_2- and COOH-terminal domains. The formation of coiled coils by the rod domains drives assembly in vitro (Stuurman et al., 1998). However, the NH_2-terminal domain also appears to play a role in polymerization, as lamin fragments consisting of only the rod and COOH-terminal domains (ΔN) display altered in vitro assembly properties (Moir et al., 1991). In vivo, microinjection of mammalian cells with ΔN human lamin A (ΔNLA) results in the disruption of the organization of both the A- and B-type lamins. Instead of the typical lamina and nucleoplasmic structures (i.e., foci and veil), the mutant and wild-type lamins are sequestered into nucleoplasmic aggregates (Spann et al., 1997). Similarly, transfection studies indicate that expression of a ΔN lamin mutant in mammalian cells disrupts lamin organization (Östlund et al., 2001). Finally, in *Xenopus* interphase extracts, the addition of ΔN lamin mutants disrupts the lamina and the normal distribution of lamins within the nucleoplasm (Moir et al., 2000a).

These ΔN mutants have been used to investigate the role of lamins in DNA replication. For instance, ΔNLA disrupts lamin organization and blocks DNA replication at the transition from the initiation to the elongation phase of DNA replication. This inhibition is accompanied by alterations in the distributions of replication factor complex (RFC) and proliferating cell nuclear antigen (PCNA), two required cofactors of the polymerase (δ) responsible for the rapid processive incorporation of nucleotides that characterizes the elongation phase of replication (Moir et al., 2000a). These results are consistent with other findings suggesting that lamins are involved in DNA replication. For example, it has been reported that lamin A (LA) colocalizes with sites of nucleotide incorporation during early S phase (Kennedy et al., 2000), and nucleoplasmic foci of lamin B (LB) colocalize with PCNA at sites of nucleotide incorporation during mid to late S phase in mammalian cells (Moir et al., 1994). Consistent with these observations, the immunodepletion of lamins from *Xenopus* egg nuclear assembly extracts results in the formation of nuclei that cannot synthesize DNA (Newport et al., 1990). These observations suggest that nuclear lamins interact with replication factor complex (RFC), PCNA, or other associated proteins, perhaps by serving as a scaffold upon which replication complexes assemble. In this report, we investigate whether nuclear lamins play an analogous role in transcription.

Results and discussion

Previously, we determined that the disruption of normal lamin organization blocked the elongation phase of DNA synthesis. To begin to determine if other nuclear functions are also dependent upon lamins, we examined the distribution of RNA splicing factors in BHK 21 cells within 2 h after ΔNLA, a dominant-negative lamin mutant, was microinjected into the cytoplasm. Consistent with previous findings (Spann et al., 1997), immunofluorescence revealed that after the microinjection of ΔNLA, the organization of A- and B-type lamins was altered. This alteration was seen as a loss of the normal distribution of lamins in the lamina and the nucleoplasmic lamin veil. Instead, both types of lamins were found together with the mutant lamin in abnormal nucleoplasmic aggregates (Fig. 1, compare A and B with C and D). Under normal conditions, immunofluorescence using an antibody directed against B′′, a U2-specific binding protein, indicates that splicing factors are distributed in a characteristic pattern of speckles and interconnecting material within

Figure 1. Disruption of lamin organization alters the distribution of splicing factors. The organization of A- and B-type lamins in untreated BHK 21 cells (A and B) and cells microinjected with ΔNLA (C and D) were examined by immunofluorescence with antibodies specific to A- or B-type lamins. Microinjection of ΔNLA disrupted the organization of both B- and A-type lamins (C and D). Immunofluorescence showing the distribution of LA and B′′, a U2-specific binding protein (E–G). In the control cell on the left, splicing factor B′′ is distributed in a characteristic pattern of speckles and interconnecting material within the nucleoplasm. In the ΔNLA-injected cell (upper right), the interconnecting material is absent and the number of B′′ speckles is greatly reduced (E and F). Note that the lamin aggregates and the B′′ speckles do not co-align (G). Bars, 5 μm.
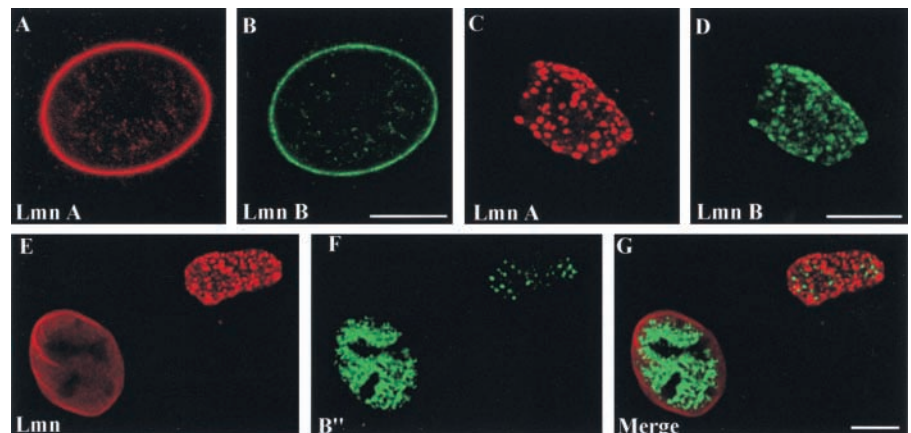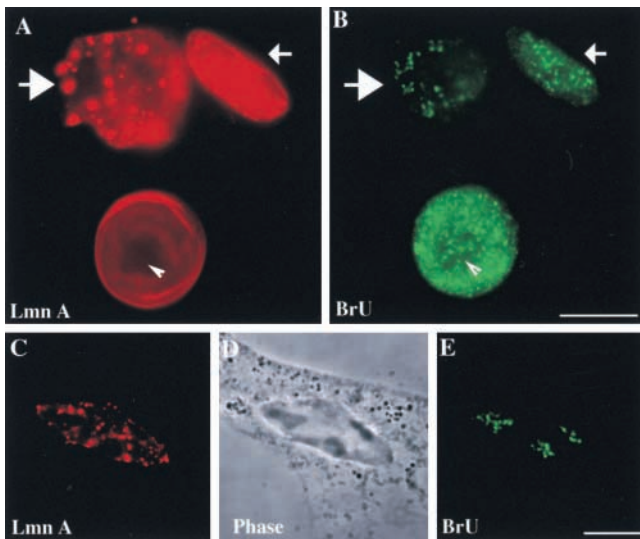
Figure 2. **Disruption of lamin organization results in reduced incorporation of BrUTP.** After microinjection of ΔNLA (arrows in A and B), cells were assayed for transcriptional activity by in situ incorporation of BrUTP. Cells were fixed and stained by immunofluorescence using antibodies directed against LA and BrU. At the bottom is an uninjected cell (A and B). The nucleolus of the uninjected cell is marked with an arrowhead. Lamin (C) and BrU (E) staining of another cell after microinjection of ΔNLA reveals that BrU incorporation is confined to nucleolar regions (D). Bars, 10 μm.

the nucleoplasm. After the disruption of lamin organization with ΔNLA, this pattern is significantly altered with the majority of speckles and the interconnecting material replaced with fewer and distinctly separated speckles (Fig. 1, E and F). A similar reorganization of splicing factors was observed after microinjection of ΔNLA and staining with Y12, an antibody that reacts with snRNPs that contain the SM antigen (unpublished data). In addition, a comparison of the distribution of B″ and the lamin aggregates revealed that these proteins do not colocalize in the ΔNLA-treated cells. The dramatic alteration of the distribution of splicing factors in response to disruption of lamin organization is reminiscent of changes observed after treating cells with α-amanitin, an inhibitor of RNA polymerase II (Spector, 1993). Together, these observations raise the possibility that within the nucleus, transcription depends upon the normal organization of lamins.

To determine whether the disruption of lamin organization affects transcription, ΔNLA was microinjected into the cytoplasm of BHK 21 cells, and 1–4 h later, transcription was assayed by in situ incorporation of BrU into RNA. After a 10-min labeling period, sites of BrU incorporation were detected by immunofluorescence (Huang et al., 1998). Cells injected with ΔNLA displayed greatly reduced BrU staining when compared with the bright, somewhat punctate staining found throughout the nucleus of neighboring uninjected cells (Fig. 2, A and B). The most dramatic reductions in BrU incorporation were seen in cells where lamin organization was most affected, suggesting a dose–response relationship (Fig. 2, A and B, compare large and small arrows). Mouse 3T3 and human HeLa cells injected with ΔNLA displayed virtually identical reductions in BrU incorporation, demon-

strating that the inhibition of transcription was not limited to a particular cell type (unpublished data). Additionally, BrU labeling in cells injected with an equimolar concentration of wild-type LA in the injection buffer was indistinguishable from uninjected cells (unpublished data). Although the overall levels of BrU incorporation were greatly reduced in the nuclei of cells microinjected with ΔNLA, no matter what the degree of disruption of lamin organization, a few clusters of BrU staining remained evident (Fig. 2, B and E). Phase contrast observations revealed that these clusters were associated with nucleoli (Fig. 2, D and E). The intensity of the BrU staining in the nucleolar regions after disruption of lamin organization is comparable to that observed in the nucleolar region of the uninjected cell (Fig. 2, B and E, arrowheads). This retention of BrU staining in nucleolar regions suggests that lamin disruption does not block RNA polymerase I activity. In addition, a normal nucleolar distribution of fibrillarin was detected in nuclei treated with ΔNLA, providing additional evidence that this disruption does not block RNA polymerase I activity (unpublished data; Ochs et al., 1985).

Using the microinjection technique, only a few cells can be studied in each experiment. Therefore, we developed a cell-free system to simultaneously treat large numbers of transcriptionally active nuclei with ΔNLA. *Xenopus* embryos are transcriptionally inactive until the midblastula transition (division 12, ~8 h after fertilization) when RNA polymerases II and III become active (Newport and Kirschner, 1982). We found that early *Xenopus* gastrulae (~10 h after fertilization) could be lysed by centrifugation, yielding intact nuclei in an embryonic extract.
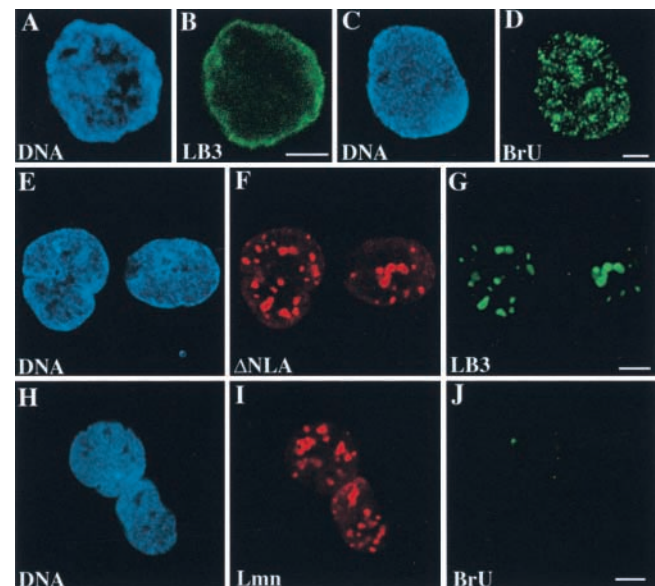

Figure 3. **Disruption of lamin organization in embryonic nuclei inhibits the incorporation of BrU.** Buffer (A–D) or ΔNLA (E–J) was added to embryonic extracts. After 60 min, BrUTP was added, and 15 min later, the cells were fixed. Chromatin was stained with TOTO3 (A, C, E, and H). Immunofluorescence using antibodies against BrU (D and J), LB3 (B and G), and human LA (F and I) was used to monitor the incorporation of BrU and the distribution of lamins. Images are confocal sections through the center of the nuclei. Bars, 5 μm.

The embryonic nuclei possessed a prominent lamina and typical nucleoplasmic lamin structures comprised of the endogenous lamin, LB3 (Fig. 3, A and B). However, 1 h after the addition of ΔNLA, instead of typical lamin structures, abnormal nucleoplasmic aggregates containing both ΔNLA and LB3 were observed in these nuclei (Fig. 3, E–G). The addition of BrUTP revealed that transcriptional activity was dramatically reduced in the ΔNLA-treated nuclei (Fig. 3, H–J), as compared with untreated nuclei (Fig. 3, C–D). The size of the transcription products synthesized by the embryonic nuclei was examined by adding [α^{32}P]UTP to the extract. After 15 min, RNA was prepared and resolved by denaturing agarose gel electrophoresis. In untreated nuclei, ^{32}P was incorporated into tRNA-sized products and higher molecular weight species of RNA (Fig. 4 A). Newly synthesized rRNA-sized products were not detected in these extracts, as RNA polymerase I is not active during early gastrulation (Newport and Kirschner, 1982; Verheggen et al., 2000). The synthesis of the upper molecular weight products was selectively inhibited by the addition of α-amanitin (10 μg/ml), indicating that these are products of RNA polymerase II (Fig. 4 B). ΔNLA treatment of embryonic nuclei also resulted in a dramatic inhibition of the synthesis of upper molecular weight RNA, whereas the synthesis of tRNA-sized products was not detectably altered (Fig. 4, C and D). These results indicate that disruption of lamin organization specifically inhibited RNA polymerase II activity.

To control for possible nonspecific effects on transcription resulting from the introduction of exogenous proteins into the nucleus, an equivalent concentration of full-length human LA was added to embryonic extracts. No alterations in the distribution of endogenous lamins or in the incorporation of BrU were observed (unpublished data). To eliminate the possibility that the inhibition of transcription was due to nonspecific effects of nuclear aggregates of IF proteins, *Xenopus* NLS-vimentin, a cytoplasmic IF protein engineered to
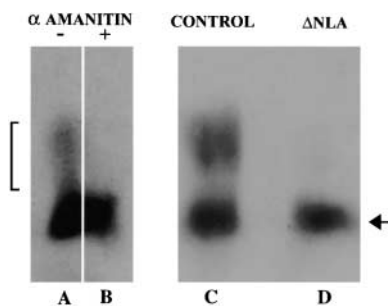

Figure 5. **Disruption of lamin organization does not alter the distribution of Sp1.** BHK 21 cells were microinjected with ΔNLA. Cells were stained with rabbit anti–human LA (A) and a monoclonal anti–human Sp1 (B). The cell on the left in each panel was not injected with ΔNLA and displays normal lamin and Sp1 staining. The cell on the right was injected with ΔNLA, and although lamin organization is disrupted, it also displays a normal distribution of Sp1. Bar, 5 μm.

Figure 4. **Disruption of lamin organization inhibits the synthesis of mRNA-sized products in embryonic nuclei.** The size of transcription products synthesized in the embryonic extract was determined by the addition of [α^{32}P]UTP. After 15 min, total RNA was prepared from each sample and resolved by denaturing gel electrophoresis, and the dried gel was used for autoradiography. The addition of α-amanitin (10 μg/ml) blocked the synthesis of upper molecular weight products (bracket, compare A and B). Alternatively, buffer (control) or ΔNLA (D) was added to embryonic extracts 1 h before the addition of [α^{32}P]UTP. The incorporation of [^{32}P]UTP into upper molecular weight products was inhibited by disruption of LB3 organization, whereas the synthesis of products the size of tRNA (arrow) was not affected.
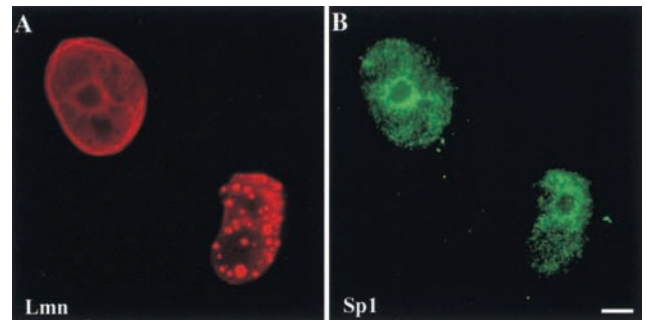
contain a nuclear localization signal, was added to embryonic extracts. This protein was imported into nuclei where it formed nucleoplasmic aggregates (Moir et al., 2000a; Reichenzeller et al., 2000) that did not alter the distribution of LB3 and did not inhibit the incorporation of BrU (unpublished data). Furthermore, chromatin distribution appeared normal in ΔNLA-treated nuclei (Fig. 3, E and H). We have previously demonstrated that ΔNLA disruption of lamin organization does not detectably alter nuclear transport properties (Moir et al., 2000a).

To identify potential mechanisms underlying the inhibition of polymerase II that accompanies the disruption of lamin organization, we examined the distribution of Sp1, a gene-specific transcription factor that binds the GC box found in some polymerase II promoters. In uninjected BHK 21 cells, Sp1 was found throughout the nucleus (Fig. 5 B). Similarly, after injection of ΔNLA and the disruption of lamin organization, Sp1 remained distributed throughout the nucleus in a pattern indistinguishable from that observed in uninjected neighboring cells (Fig. 5 B). Therefore, the disruption of lamin organization does not appear to alter the distribution of the gene-specific transcription factor Sp1.

We also examined the effect of the disruption of lamin organization on the distribution of the TATA binding protein (TBP), a transcription factor for RNA polymerases I, II, and III (Hernandez, 1993). TBP interacts with a number of TBP-associated factors to form the TFIID complex (Albright and Tjian, 2000). This complex is a basal transcription factor thought to be required for forming preinitiation complexes on RNA polymerase II promoters containing a TATA box. We found that in BHK 21 cells, disruption of lamin organization altered the distribution of TBP. Instead of its normal punctate distribution throughout the nucleus (Fig. 6, A and B), most of the TBP colocalized with the nucleoplasmic lamin aggregates (Fig. 6, C and D). However, TBP staining often remained apparent in the nucleolar region of the ΔNLA-treated nuclei, most likely reflecting its involvement in RNA polymerase I–dependent transcription (Fig. 6 D, arrows). In *Xenopus* embryonic nuclei, the disruption of lamin organization also resulted in an alteration of TBP distribution, such that TBP was
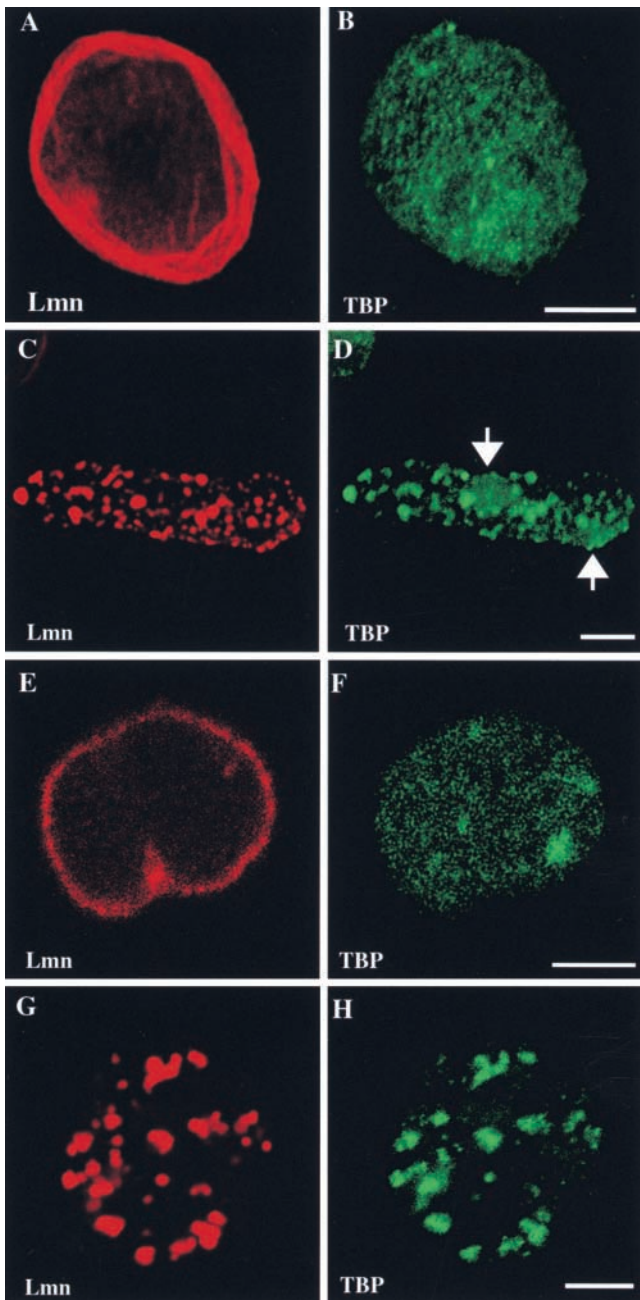
Figure 6. **Disruption of lamin organization alters the distribution of TBP.** BHK 21 cells (A–D) and embryonic nuclei (E–H) stained with a rabbit anti-TBP (B, D, F, and H), rat anti–human LA (A and C), and a monoclonal anti–*Xenopus* LB3 (E and G). An uninjected BHK 21 cell is shown (A and B). An embryonic nucleus in extract treated with buffer is shown (E and F). Both display normal distributions of lamin (A and E) and TBP (B and F). In BHK 21 cells (C and D) and embryonic nuclei (G and H), ΔNLA treatment resulted in TBP (D and H) and lamins (C and G) colocalizing in nucleoplasmic aggregates. Arrows point to the location of nucleolar regions as seen by phase contrast (unpublished data). Images are confocal sections through the center of the nuclei. Bars, 5 μm.

found in nucleoplasmic lamin aggregates (Fig. 6, compare G and H with E and F). In contrast, LA did not alter the distribution of TBP in either BHK 21 cells or embryonic nuclei, and TBP did not colocalize with the NLS-vimentin aggregates in *Xenopus* nuclei (unpublished data).

Therefore, the change in the distribution of TBP accompanying lamin disruption does not appear to simply be the result of adding an exogenous protein that forms nuclear aggregates. The specificity of the interaction between lamins and TBP is also supported by the findings that the transcription factor Sp1 and splicing factors do not colocalize with lamins in the ΔNLA-induced aggregates (Figs. 1 and 6). Splicing factors are very abundant in the nucleus and are not tightly associated with chromatin (Spector, 1993). Therefore, these findings argue against nonspecific trapping as a mechanism to explain the association of TBP with the lamin aggregates. Based on these results, we propose that the distribution of TBP in normal nuclei depends upon the maintenance of normal lamin organization. However, the lack of inhibition of RNA polymerases I and III suggests that lamins may not bind TBP directly. Instead, lamins may interact with TBP through other factors specific for RNA polymerase II, such as the TBP-associated factors of the TFIID complex.

In conclusion, our results demonstrate that disruption of normal lamin organization inhibits RNA polymerase II activity. We believe that these findings provide the first experimental evidence that specific nuclear structural proteins, the lamins, are involved in the synthesis of mRNA. Together with reports that lamins are distributed throughout the nucleoplasm, these findings raise the possibility that lamins may act as a scaffold upon which the basal transcription factors required for RNA polymerase II transcription are organized.

Materials and methods

Protein expression
Myc-tagged human LA (LA–myc), ΔNLA, and *Xenopus* NLS-vimentin were expressed in bacteria and purified as described previously (Spann et al., 1997; Reichenzeller et al., 2000). NLS-vimentin was a gift from H. Herrmann (German Cancer Research Center, Heidelberg, Germany).

Microinjection and in situ transcription assays in mammalian cells
BHK 21, 3T3, and HeLa cells were grown on locator coverslips and microinjected with ΔNLA or LA–myc (15 μM; Goldman et al., 1992). After microinjection (1–4 h), the plasma membrane was permeabilized with 0.1% digitonin and a transcription cocktail containing BrUTP was added (Huang et al., 1998). After 10 min, samples were fixed with 2% paraformaldehyde and processed for immunofluorescence as described previously (Huang et al., 1998).

Preparation of *Xenopus* embryonic extracts
Xenopus laevis eggs were fertilized in vitro, the jelly coats were removed with 2% cysteine, and the embryos were developed in MMR/5 (Newmeyer and Wilson, 1991). In early gastrulation (~10 h after fertilization at 23°C), viable embryos were rinsed four times in extract buffer (250 mM sucrose, 50 mM KCl, 5 mM MgCl$_2$, 1 mM DTT, and 50 μg/ml cycloheximide [ICN Biomedicals]), transferred to a 2-ml straight-sided Eppendorf tube, allowed to settle, and excess buffer was removed. Leupeptin, chymostatin, pepstatin (50 μg/ml; Sigma-Aldrich), and cytochalasin B (5 μg/ml; Sigma-Aldrich) were added. The embryos (1–2 ml) were packed at 200 g for 25 s at 4°C in a refrigerated centrifuge (Allegra GR; Beckman Coulter) and excess buffer was removed. Embryos were lysed by centrifugation at 8,300 g for 9 min at 4°C in a fixed horizontal rotor (H6002; Beckman Coulter). A clear straw-colored phase (the extract) between the lipid layer and the dense pigment granules was collected by puncturing the tube with a 21-gauge needle. Extracts containing ~2,000 nuclei/μl were frozen in N$_{2l}$ and stored at −70°C in 40-μl aliquots.

Transcription assays of *Xenopus* nuclei in extracts
Frozen aliquots were thawed (24°C), buffered with 15 mM Hepes (pH 7.4), and supplemented with an ATP-generating system (Spann et al., 1997), nucleotides (0.5 μM ATP, 0.5 μM CTP, 0.5 μM GTP, and 0.25 μM UTP), 5 mM MgCl$_2$, RNasin (0.25 U/μl; Amersham Pharmacia Biotech), and either

ΔNLA, LA, NLS-vimentin (1 μM), or an equal volume of dialysis buffer (Moir et al., 2000a). Transcription was assayed after 1 h by adding 0.6 μM BrUTP (Sigma-Aldrich) and 0.5 U/μl of RNasin. After 15 min, nuclei were fixed and processed for immunofluorescence as described below. Alternatively, transcription products were sized by substituting 0.3 μM [α^{32}P]UTP (3,000 mCi/mM; Amersham Pharmacia Biotech) for BrUTP in the embryonic extract. 15 min later, total RNA was prepared from 40 μl of extract using a SNAP kit (Invitrogen) and resolved by electrophoresis on a 0.8% agarose denaturing gel. The dried gel was used for autoradiography. Effects of α-amanitin (10 μg/ml; Roche) were tested by adding the drug 20 min before [α^{32}P]UTP.

Immunofluorescence

Cultured cells were fixed for 10 min with 2% paraformaldehyde in PBSa (PBS lacking calcium and magnesium) at 4°C, and subsequently permeabilized with 0.2% Triton X-100 in PBSa at 4°C for 10 min for B″ and Y12 staining. For Sp1 and TBP staining, cells were fixed with 3.7% formaldehyde (EM grade) in PBSa at 4°C and subsequently permeabilized (see above). Embryonic nuclei were fixed with 2% formaldehyde for transcription assays or 3% formaldehyde for other experiments (Moir et al., 2000a) and subsequently permeabilized with 0.1% NP-40. For TBP staining, embryonic nuclei were permeabilized with 0.1% Triton X-100 for 30 s at 24°C before fixation (Moir et al., 2000a). Primary antibodies used were directed against B″, SM antigen (B″ and Y12; 1:50; 1:20; gifts from D. Spector, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), TBP, Sp1 (SC 273 and SC 59; 1:30; Santa Cruz Biotechnology, Inc.), BrU (BU33, 1:150; Sigma-Aldrich), human LA (Moir et al., 1994), human LB (Moir et al., 1994), and *Xenopus* LB3 (Stick, 1988). A rat antibody directed against human LA was used in double labeling experiments with rabbit anti-TBP in BHK 21 cells (Spann et al., 1997). DNA dyes and fluorescent secondary antibodies were used as previously described (Moir et al., 2000a). Images were captured with a ZEISS LSM 510 confocal microscope or a ZEISS axiovert microscope equipped with a Hamamatsu Orca digital camera controlled by Metamorph (Universal Imaging Corp.).

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