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Lamin A/C speckles mediate spatial organization of splicing factor compartments and RNA polymerase II transcription

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The A-type lamins have been observed to colocalize with RNA splicing factors in speckles within the nucleus, in addition to their typical distribution at the nuclear periphery. To understand the functions of lamin speckles, the effects of transcriptional inhibitors known to modify RNA splicing factor compartments (SFCs) were examined. Treatment of HeLa cells with α-amanitin or 5,6-dichlorobenzimidazole riboside (DRB) inhibited RNA polymerase II (pol II) transcription and led to the enlargement of lamin speckles as well as SFCs. Removal of the reversible inhibitor DRB resulted in the reactivation of transcription and a rapid, synchronous redistribution of lamins and splicing factors to normal-sized speckles, indicating a close association between lamin speckles and SFCs. Conversely, the expression of NH_2-terminally modified lamin A or C in HeLa cells brought about a loss of lamin speckles, depletion of SFCs, and down-regulation of pol II transcription without affecting the peripheral lamina. Our results suggest a unique role for lamin speckles in the spatial organization of RNA splicing factors and pol II transcription in the nucleus.

Introduction

The lamins are the major components of a filamentous network underlying the inner nuclear membrane, termed the nuclear lamina. The lamina plays an essential role in maintaining the integrity of the nuclear envelope and provides anchoring sites for chromatin, and is hence considered to be an important determinant of interphase nuclear architecture (for reviews see Gant and Wilson, 1997; Stuurman et al., 1998). Two major kinds of lamins are present in mammalian cells: B-type lamins (B1 and B2), which are found in nearly all somatic cells, and A-type lamins (A and C), which are expressed only in differentiated cells. Lamins A and C and germ cell–specific lamin C2 are alternatively spliced products of the lamin A gene, *LMNA*, whereas lamins B1 and B2 are coded by separate genes. The presence of lamins in the interior of the nucleus has been well documented (Goldman et al., 1992; Bridger et al., 1993; Hozak et al., 1995); in particular their association with DNA replication centers in S phase cells (Moir et al.,

1994) and RNA splicing factor speckles in interphase cells (Jagatheesan et al., 1999). Lamins also interact with inner nuclear membrane proteins such as the lamina-associated polypeptides 1 and 2β (Gerace and Foisner, 1994) and emerin (Clements et al., 2000), as well as intranuclear lamina–associated polypeptide 2α (Dechat et al., 2000).

Lamins belong to the intermediate filament family of proteins and contain a characteristic central α-helical rod domain, flanked by relatively flexible NH_2- and COOH-terminal segments. All three domains are essential for lamin assembly and functions (for review see Stuurman et al., 1998). Deletion of the NH_2-terminal domain of human lamin A (Spann et al., 1997; Moir et al., 2000a) or *Xenopus* lamin B1 (Ellis et al., 1997) leads to defects in lamina assembly, disruption of the lamina, and inhibition of DNA replication. Mutations in human lamin A cause debilitating diseases such as Emery-Dreifuss muscular dystrophy, cardiomyopathy, partial lipodystrophy and axonal neuropathy (Bonne et al., 1999; Fatkin et al., 1999; Cao and Hegele, 2000; Shackleton et al., 2000; De Sandre-Giovannoli et al., 2002). A few of these lamin mutant proteins cause gross defects in the peripheral lamina and also assemble aberrantly, but other mutants do not show an obvious phenotype (Östlund et al., 2001; Raharjo et al., 2001; Vigouroux et al., 2001).

The presence of morphologically distinct nuclear compartments that are enriched for specific proteins is now well

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established (for reviews see Spector, 1993; Lamond and Earnshaw, 1998). RNA splicing factors are present in high concentrations in compartments or speckles called splicing factor compartments (SFCs)* that correspond at the electron microscopic level to interchromatin granule clusters (IGCs) and are also dispersed in the nucleoplasm on perichromatin fibrils (PFs), which contain nascent transcripts (for reviews see Fakan and Puvion, 1980; Spector, 1993; Fakan, 1994). The splicing of pre-mRNAs occurs concomitantly with transcription on PFs (Beyer et al., 1988) and away from, or at the periphery of, SFCs for most transcripts (Jackson et al., 1993; Wansink et al., 1993; Cmarko et al., 1999). Transcription by RNA polymerase II (pol II) has been visualized on hundreds of small foci throughout the nucleoplasm (Jackson et al., 1993; Wansink et al., 1993; Bregman et al., 1995). The SFCs are dynamic compartments involved in the storage/recruitment of splicing factors (Misteli et al., 1997). Their size can change depending on RNA splicing or transcription levels in the cell; for example, they become considerably enlarged due to reduced dissociation of splicing factors in the presence of transcriptional inhibitors (Carmo-Fonseca et al., 1992; Spector, 1993), in pathological conditions (Fakan and Puvion, 1980), or upon inhibition of splicing (O'Keefe et al., 1994).

The gene-specific positioning of transcription sites with respect to SFCs (Smith et al., 1999) and recruitment of splicing factors from SFCs upon gene activation (Misteli et al., 1997) point to significant spatial coordination of transcription and pre-mRNA splicing. A key issue that has not yet been resolved is the importance of nuclear architecture in the spatial organization of transcription and pre-mRNA splicing. It has been proposed that SFCs are generated by interactions with the nucleoskeletal framework (Kruhlak et al., 2000), or, alternatively, that self-organization of splicing factors leads to the assembly of SFCs (Misteli, 2001). The association of transcription sites or active pol II with an insoluble nuclear framework or matrix has been well documented (Jackson et al., 1993; Wansink et al., 1993; Kimura et al., 1999; Wei et al., 1999), several transcription factors have been localized to the nuclear matrix (for review see Stein et al., 2000), and SFCs have also been observed to be attached to a detergent-insoluble nuclear structure (Spector, 1993). Importantly, Hendzel et al. (1999) have demonstrated the presence of an underlying protein architecture in IGCs that physically connects the relatively dispersed granules within the cluster, by using energy transmission electron microscopy in intact cells and thus avoiding the problems associated with typical nuclear matrix isolation protocols (Pederson, 2000; Nickerson, 2001). The main candidate protein constituents of the nuclear framework are the lamins, previously identified at the nuclear periphery but now also observed in the nuclear interior, and actin, which can bind to snRNPs (Nakayasu and Ueda, 1984). A role for lamins in controlling gene expression has been proposed earlier (Wilson, 2000), and in vitro binding of lamin A to the retino-

blastoma protein, an important transcriptional regulator, has been reported (Ozaki et al., 1994). More recently, an NH_2-terminal deletion lamin A mutant, ΔNLA, that disrupts the lamina has been observed to inhibit transcription (Spann et al., 2002). However, the involvement of internal lamins in the spatial organization of transcription or splicing has not been demonstrated.

The aim of this study is to understand the functional role of internal lamin A/C speckles that have been observed to colocalize with SFCs in a variety of cell types using a monoclonal antibody to rat lamin A that has certain unique properties (Jagatheesan et al., 1999). This antibody, mAb LA-2H10, exclusively stains intranuclear speckles in interphase cells without labeling the peripheral lamina, and specifically recognizes only lamins A and C in immunoblots of cellular fractions (the epitope region spanning amino acids 171–246, which has no significant homology with nonlamin proteins, is common to both lamins). mAb LA-2H10 does not cross-react with any other proteins from whole cell lysates in the molecular mass range of 14–250 kD (unpublished data). As the exclusive reactivity of mAb LA-2H10 toward speckles is retained when cells are detergent and nuclease treated to reveal the nucleoskeletal framework, we have attributed this reactivity to the differences in lamin protofilament interactions at the periphery and at internal sites. We have recently demonstrated that muscle differentiation is accompanied by rearrangements in the organization of lamin A speckles, which appear to be controlled by cell cycle–regulated pathways (Muralikrishna et al., 2001). In the present study, we have investigated the possibility of a direct correlation between the organization of lamin A/C speckles, SFCs, and transcriptional events by first examining the effects of inhibitors of pol II transcription. Our results show that lamin A/C speckles reorganize to form enlarged foci in the presence of transcriptional inhibitors, as do the RNA splicing factors SC-35 and U5-116 kD. Removal of a reversible inhibitor results in rapid, synchronous redistribution of lamin A/C speckles and splicing factors with reactivation of transcription. Second, we have observed that expression of NH_2-terminally tagged lamin A (His or FLAG) or lamin C (His), but not FLAG–lamin B1, leads to the disruption of lamin A/C speckles and SFCs, accompanied by a reduction of active pol II and BrUTP incorporation into nascent RNA, without discernible effects on the incorporation of tagged lamin A or C into the nuclear periphery or disruption of the endogenous lamina at the nuclear rim. These results suggest a close association between the internal lamin A/C network and the organization of splicing factors and transcriptional events.

Results

Sensitivity of lamin speckles to transcriptional inhibitors

The effects of pol II inhibitors on the nuclear organization of splicing factors have been well documented (Carmo-Fonseca et al., 1992; Spector, 1993). The incubation of cells with α-amanitin, which specifically inhibits pol II, results in the clustering of SFCs into enlarged foci and the elimination of diffuse nucleoplasmic staining of splicing factors, such as snRNPs and SC-35, in PFs. Cajal bodies are disrupted to elongated cap-like structures, distinct from enlarged SFCs.

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*Abbreviations used in this paper: ASF, alternate splicing factor; DRB, 5,6-dichlorobenzimidazole riboside; IGC, interchromatin granule cluster; NPC, nuclear pore complex; PF, perichromatin fibril; pol II, RNA polymerase II; SFC, splicing factor compartment; TBP, TATA binding protein.
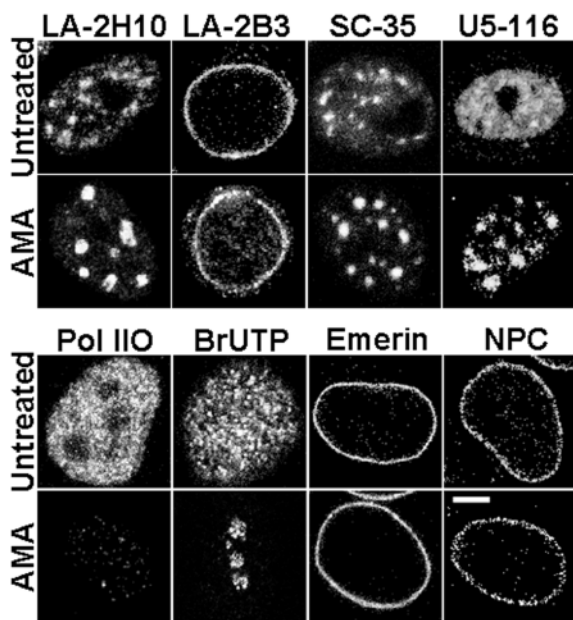
Figure 1. **Lamin speckles form enlarged foci upon α-amanitin treatment.** HeLa cells were treated with α-amanitin (AMA), fixed in formaldehyde, and labeled with antibodies to lamin A (LA-2H10 and LA-2B3), RNA splicing factors (SC-35 and U5-116 kD), RNA pol IIO, BrUTP (after RNA synthesis assay), emerin, and NPC proteins. Bar, 5 μm.

Similar effects are seen with the adenosine analogue 5,6-dichlorobenzimidazole riboside (DRB), but these are reversed upon removal of the drug, unlike α-amanitin. On the other hand, general RNA synthesis inhibitors, such as actinomycin D, cause significant changes in nuclear morphology, including nucleolar breakdown.

We have earlier demonstrated the presence of lamin A/C speckles that are labeled with the antibody mAb LA-2H10 and colocalize with RNA splicing factors such as SC-35 and U5-116 kD in several mammalian cell types (Jagatheesan et al., 1999). SC-35 is an essential non-snRNP splicing factor (Fu and Maniatis, 1990), whereas U5-116 kD is a 116-kD protein that is a component of U5 snRNPs (Fabrizio et al., 1997). Both proteins are concentrated in speckles and also exhibit diffuse nucleoplasmic localization, but to different levels. To explore the possibility that lamin A/C speckles might be modified by transcriptional inhibitors, we have examined the effects of α-amanitin and DRB on the distribution of lamins. When HeLa cells were incubated with α-amanitin and then stained with mAb LA-2H10, a striking redistribution of lamin speckles into enlarged foci was observed (Fig. 1), similar to that seen with SC-35 and U5-116 kD. There were, however, no changes in the typical peripheral pattern of A-type lamins stained with LA-2B3. To confirm the efficacy of α-amanitin treatment, the levels of pol II activity were detected by staining with mAb H5. This antibody recognizes only the active, hyperphosphorylated form of pol II, termed pol IIO, and stains 70–80% of a population of dividing cells in a diffuse pattern of tiny foci that correspond to centers of active transcription, and ~20–30% of cells in a speckled pattern, which is more pronounced in cells prepermeabilized with 1.5% Triton X-100

(Bregman et al., 1995). However, as prepermeabilization weakened the diffuse signal, our samples were fixed under standard conditions. Labeling with mAb H5 was reduced to low levels in cells treated with α-amanitin. Furthermore, the incorporation of BrUTP into newly synthesized RNA transcripts by pol II was abolished in the nucleoplasm of permeabilized cells that had been preincubated with α-amanitin before the addition of ribonucleotides, whereas pol I–generated transcripts continued to be detected in nucleoli, as documented earlier (Wansink et al., 1993). Because lamins A/C bind to the inner nuclear membrane protein emerin (Clements et al., 2000), the effects of α-amanitin on emerin staining were examined. Both treated and untreated cells showed a uniform nuclear rim labeling for emerin. There were also no detectable effects on the distribution of nuclear pore complexes (NPCs), as observed by staining with mAb 414, which labels a major group of O-glycosylated NPC proteins (Davis and Blobel, 1986). Similar effects of α-amanitin on lamin speckles were also observed with BHK-21 cells (unpublished data).

To confirm that the inhibitor-induced enlarged lamin speckles localized with those seen with RNA splicing factors, dual labeling studies were performed with mAb LA-2H10 and antibodies to SC-35 or U5-116 kD, as illustrated in Fig. 2 A. In untreated cells, lamin speckles colocalized with SFCs above a background of diffuse nucleoplasmic staining of splicing factors. After treatment with α-amanitin, this diffuse staining was reduced and the speckles rounded up to form enlarged foci. Importantly, there was complete colocalization of lamin A/C and U5-116 kD or SC-35 staining. On the other hand, enlarged lamin foci were not associated with disrupted Cajal bodies labeled with antibody to p80 coilin, a standard marker for the Cajal body (Bohmann et al., 1995), consistent with the absence of colocalization of lamin speckles with p80 coilin in untreated cells, as observed earlier also (Jagatheesan et al., 1999).

Treatment of HeLa cells with the transcriptional inhibitor DRB also resulted in the enlargement of speckles containing the RNA splicing factors SC-35 and U5-116 kD as well as lamins A/C, as shown in Fig. 2 C. To examine the reversibility of this effect, drug-treated cells were washed with complete medium to remove DRB and kept at 37°C or 4°C for 1 h in complete medium before labeling with antibodies, as described by Bregman et al. (1995). Incubation at 37°C, but not at 4°C, was sufficient to restore transcriptional activity, as revealed by the reappearance of pol IIO staining in the cells (Fig. 2 B). RNA splicing factors were relocated from enlarged foci to a distribution similar to that in untreated cells. Importantly, lamins A/C were also redistributed from enlarged foci to normal-sized speckles. When the time course of this relocalization was analyzed, it was observed that normal speckles containing lamins A/C and SC-35 were formed in a highly coordinate manner. By 10 min after the removal of DRB, 40% of cells began to exhibit a normal pattern of speckles, and both enlarged foci and normal-sized speckles could be seen in the same nucleus (Fig. 2 D). The regular distribution of speckles was reestablished by 30 min. Levels of pol IIO staining were 40–50% of the normal signal by 10 min and restored completely by 30 min (unpublished data). The synchronous

Figure 2. **Localization of lamin speckles and SFCs in enlarged foci upon treatment with transcriptional inhibitors, and their synchronous redistribution after removal of DRB.** (A) HeLa cells treated with α-amanitin (AMA) were fixed in formaldehyde and doubly labeled with mAb LA-2H10 and antibodies to splicing factors or Cajal bodies (SF/CB) as indicated. (B and C) HeLa cells were treated with DRB, washed, incubated for 1 h at 37°C or 4°C in complete DME, formaldehyde fixed, and labeled with antibodies to pol IIO alone (B) or splicing factors and LA-2H10 (double labeling) (C). (D) Time course of redistribution of lamin speckles and SC-35 after DRB treatment, washing, and incubation at 37°C for 10–30 min. Single optical sections of 0.5 μm are displayed. Bar, 5 μm.
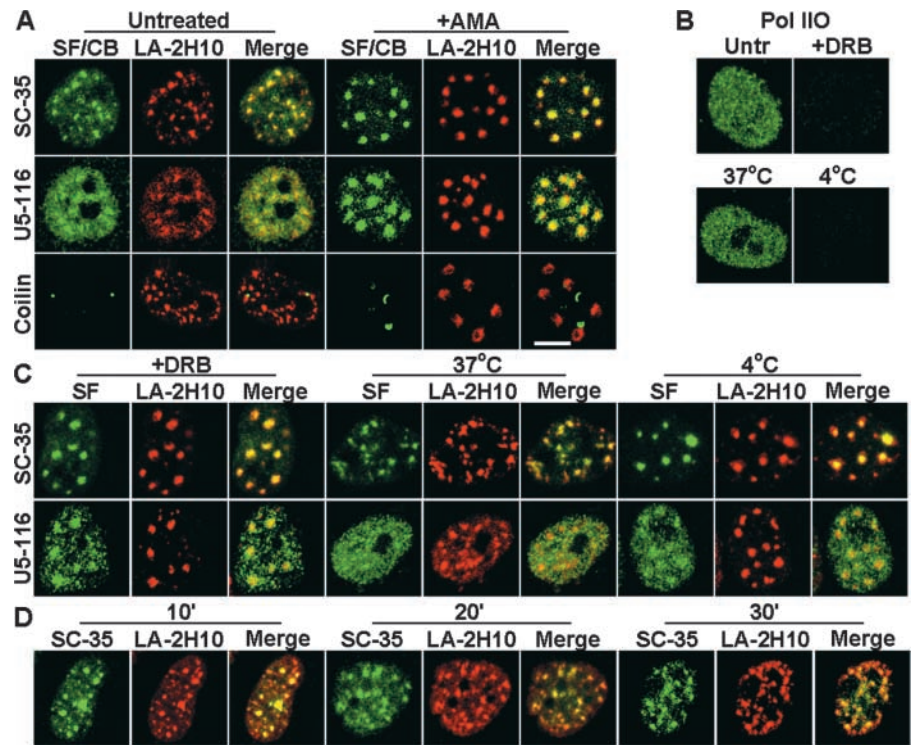
and rapid relocalization of lamins A/C and RNA splicing factors upon transcriptional activation suggests a close association between these events.

Absence of lamin speckles in cells expressing modified lamin A/C

To further understand the functions of lamin A/C speckles, we wished to examine the effects of disrupting the internal lamina organization, without causing gross abnormalities in the peripheral lamina or nuclear morphology, by overexpressing suitably modified lamin A proteins in cultured cells. Both the NH₂-terminal domain and COOH-terminal CaaX motif of lamin A have been shown to be essential for lamin assembly and functions and necessary to direct exogenously expressed lamin A to the peripheral lamina (Spann et al., 1997; Izumi et al., 2000; Moir et al., 2000a). In exploratory experiments with NH₂-terminally tagged mutants deleted in a putative chromatin-binding domain encompassing amino acid residues 396–401 (Taniura et al., 1995), we observed a considerable loss of lamin A/C speckles in transfected cells. Surprisingly, similar observations were made with the wild-type NH₂-terminally tagged constructs. Hence, we have investigated the properties of wild-type tagged lamin A constructs in detail.

The constructs that have been analyzed in this study are as follows. His–lamin A and His–lamin C have an additional six histidine residues at the NH₂ terminus of full-length lamin A or C respectively, whereas FLAG–lamin A has the FLAG epitope in a similar position in lamin A (sequences are given in the Materials and methods). The recombinant proteins have been visualized in transfected cells by using antibodies that detect 6X His or FLAG sequences. The specificity of these antibodies was confirmed in immunoblots of HeLa cell lysates, as shown in Fig. 3 A. The tagged proteins were labeled

by their respective antibodies in transfected cell lysates, and there was no cross-reactivity with untransfected cell lysates. Both pre–lamin A and mature lamin A could be detected in lysates of tagged lamin A–transfected cells. Analysis with
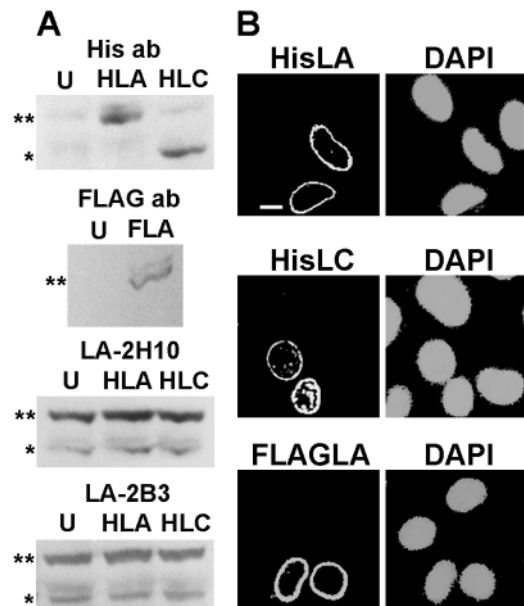
Figure 3. **Expression of exogenous lamins in HeLa cells.** (A) Western blots of equal amounts of untransfected (U) and transfected cell lysates (HLA, His–lamin A; HLC, His–lamin C; FLA, FLAG–lamin A) probed with anti-His, anti-FLAG, and lamin mAbs LA-2H10 and LA-2B3. The positions of lamins A and C are marked with double and single asterisks, respectively. (B) Immunofluorescence of transfected, methanol-fixed HeLa cells stained with anti-His or anti-FLAG antibodies and DAPI (HisLA, His–lamin A; HisLC, His–lamin C; FLAGLA, FLAG–lamin A). Bar, 10 μm.
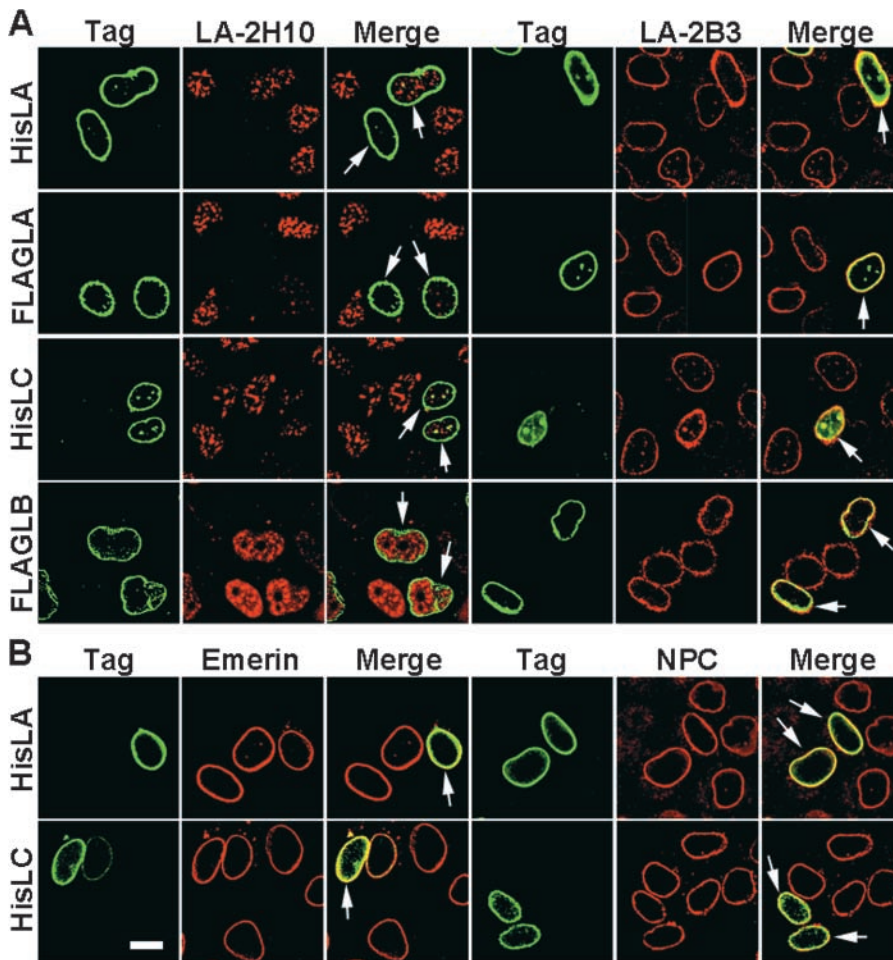
Figure 4. **Disruption of lamin speckles in cells expressing NH$_2$-terminally tagged lamin A or C.** HeLa cells transfected with the indicated lamin constructs were methanol fixed and doubly labeled with antibodies to the His or FLAG epitope tag (Tag) and lamin A (A, LA-2H10 or LA-2B3), emerin, or NPC proteins (B). Arrows indicate transfected cells. Bar, 10 μm.

mAbs LA-2H10 and LA-2B3 did not indicate any obvious changes in the mobilities of lamins in transfected cells as compared with untransfected cells. As the efficiency of transfection was ~20%, a significant increase in the abundance of lamins was not detected in these blots. As shown in Fig. 3 B, exogenous lamins were incorporated into the nuclear periphery to give a smooth rim staining in cells transfected with His–lamin A or FLAG–lamin A. His–lamin C occasionally formed small internal aggregates or round patches toward the periphery in addition to a rim; the internal aggregates were more prominent in samples examined 10–12 after transfection (unpublished data), consistent with earlier reports on the assembly properties of exogenous lamin C (Goldman et al., 1992; Bridger et al., 1993; Pugh et al., 1997).

To examine the effects of expression of exogenous lamins on the endogenous lamina, dual labeling studies were performed with antibodies to the His or FLAG tags and to lamin A/C and are presented in Fig. 4 A. A predominant number of cells expressing His–lamin A (80%), FLAG–lamin A (65%), or His–lamin C (60%) did not display lamin A/C speckles when labeled with mAb LA-2H10. The absence of lamin speckles in transfected cells was not strictly correlated with the levels of expression of tagged lamins, as ~20% of cells expressing equivalent levels of the exogenous protein still displayed normal speckles. We suggest that this variation is due to the timing of DNA entry into each cell and that sufficient exogenous lamin needs to be expressed at a particular stage in order to cause a loss of speckles. His–lamin C-transfected cells occasionally formed aggregates or foci within nuclei. In nuclei displaying both speckles and lamin C foci, 29% of lamin speckles colocalized with these aggregates, suggesting partial overlap of these domains. Labeling with mAb LA-2B3 demonstrated that the peripheral lamina was not significantly altered in transfected cells. As this antibody recognized both tagged and endogenous peripheral lamins A/C, there was markedly higher labeling of transfected cells, but abnormal peripheral lamina structures were not seen. Similar results were obtained with NIH3T3, CHO, and Cos1 cells and with methanol- or formaldehyde-fixed cells (unpublished data). An NH$_2$-terminally tagged FLAG–lamin B1 construct was also checked for its effects on lamin speckles. More than 90% of cells transfected with FLAG–lamin B1 continued to display lamin A/C speckles, though the nuclear envelope was convoluted in these cells. This result is consistent with a recent report on the distinct nature of the A-type and B-type lamin networks (Izumi et al., 2000). Transfection of the tagged lamins did not significantly affect the nuclear rim staining of emerin or NPCs (Fig. 4 B). A mutant lamin A construct in which the nuclear localization sequence had been deleted was unable to enter the nucleus and did not exhibit the properties of His–lamin A (unpublished data).

The absence of lamin speckles in tagged lamin–transfected cells may be attributed to at least two kinds of events: antigen masking or actual lack of speckles. Masked epitopes on
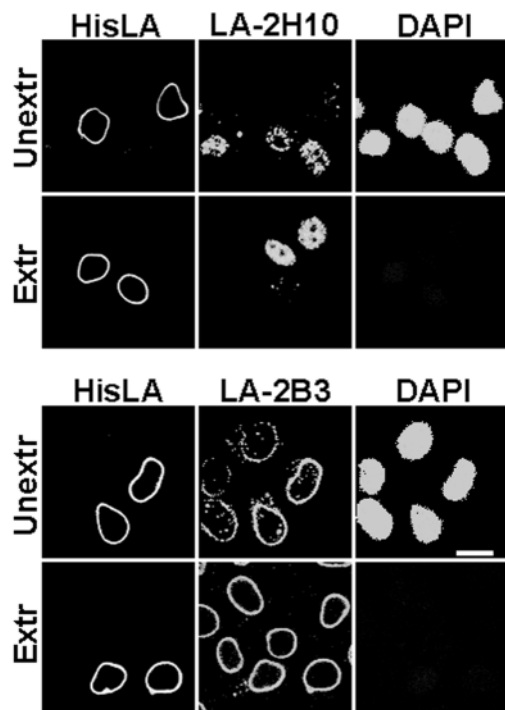
Figure 5. **Extraction of His–lamin A-transfected HeLa cells.** Transfected cells were extracted with detergents, digested with nucleases, treated with 2 M NaCl, fixed with formaldehyde, and doubly stained with anti-His antibody (HisLA) and LA-2H10 or LA-2B3, as well as DAPI. Unextracted cells served as controls. Bar, 10 μm.

lamins can be revealed by the extraction of cell monolayers with detergent, followed by DNase I and salt treatment, which disrupt interactions between lamins and chromatin or other nuclear proteins (Dyer et al., 1997; Muralikrishna et al., 2001). When His–lamin A-transfected HeLa cells were extracted as described in the Materials and methods and then labeled with mAb LA-2H10, antibody reactivity was not regained (Fig. 5), indicating that epitopes were not originally masked due to protein binding. The efficacy of the extraction procedure was confirmed by the lack of staining of DNA with DAPI. Immunolabeling of cells with mAb LA-2B3 or of untransfected cells with mAb LA-2H10 was not altered by the extraction protocol. Similar results were obtained with His–lamin C-transfected cells (unpublished data). Taken together, these findings suggest that the transfection of cells with exogenous lamin A or C that has been minimally modified at the NH$_2$ terminus results in the disappearance of lamin speckles without grossly affecting the peripheral lamina. Although we have not formally ruled out covalent modification of the epitope, we consider it to be unlikely because this sequence does not have a consensus motif for possible modifications such as phosphorylation.

The loss of speckles may occur by one or more mechanisms. If the tagged lamins compete with endogenous lamins for incorporation into speckles (e.g., at the end of mitosis), the tag should be observed within speckles; because this is seen only in 29% of speckles in His–lamin C-transfected cells and not in His–lamin A-transfected cells, the incorporation of NH$_2$-terminally modified lamins might be

inefficient. Another possibility is that subtle changes in the associations between peripheral lamin, emerin, and/or other inner membrane proteins due to overexpression of tagged lamins might prevent speckle formation, if the lamin speckles are part of a network that is anchored to the periphery. However, we do not have firm evidence for this at present. Because the effects on speckles were similar with His– or FLAG–lamin A, there was no dependence on the tag sequence. The absence of significant changes upon expression of FLAG–lamin B1 indicates that the B-type lamina is unlikely to be directly involved in the formation of lamin A/C speckles in these cells.

Disruption of SFCs and pol II transcription upon overexpression of modified lamins A/C

The disappearance of lamin A/C speckles in cells expressing NH$_2$-terminally tagged lamin A or C prompted us to investigate the organization of SFCs in these cells. When HeLa cells were transfected with His–lamin A or C and labeled with antibody to SC-35, 60% of transfected cells showed a reduction of SC-35 staining, as illustrated in Fig. 6 A. Exogenous expression of His–lamin A or C also caused a loss of U5-116 kD in ~60% of transfected cells. The depletion of splicing factors upon the disruption of lamins is unexpected and suggests that lamin speckles are important for the maintenance of SFCs. Splicing factors are also reduced in ΔNLA-injected cells (Spann et al., 2002); on the other hand, other treatments such as overexpression of Clk/STY protein kinase result in the redistribution of splicing factors from speckles to a diffuse pattern (Colwill et al., 1996; Sacco-Bubulya and Spector, 2002). Cells expressing tagged lamins did not exhibit any significant changes in the distribution or morphology of Cajal bodies, visualized by p80 coilin staining, which is consistent with the absence of association of lamin A/C speckles with these compartments and rules out a generalized collapse of intranuclear compartments. The expression of FLAG–lamin A gave results similar to those obtained with His–lamin A; on the other hand, splicing factor distribution was not altered in cells expressing FLAG–lamin B1 (unpublished data).

We reasoned that depletion of splicing factors SC-35 and U5-116 kD due to expression of exogenous lamins A/C might have deleterious effects on pol II transcription in transfected cells. Two different assays were performed to evaluate the transcriptional status of His–lamin A- or C-transfected cells. First, cells were stained with mAb H5, which labels the active form of pol II. Second, the synthesis of RNA transcripts was monitored by the incorporation of BrUTP into nascent RNA. A dramatic reduction in transcription by pol II was observed in transfected cells (Fig. 6 B). A quantitative analysis of the fluorescence of cells stained with mAb H5 (Fig. 6 C) indicated that 90% of cells transfected with His–lamin A or C were stained at very low intensities, on average, approximately fivefold lower than untransfected cells. As shown in Fig. 6 B, BrUTP incorporation was also impaired in transfected cells; pol II transcription was strongly affected, whereas, nucleolar pol I transcription was still evident in 30% of cells expressing exogenous lamins. Reduction of transcription was observed in a higher percentage of cells than effects on SFCs, suggesting that small undetectable changes in these domains were nevertheless inhibitory. No alterations were ob-
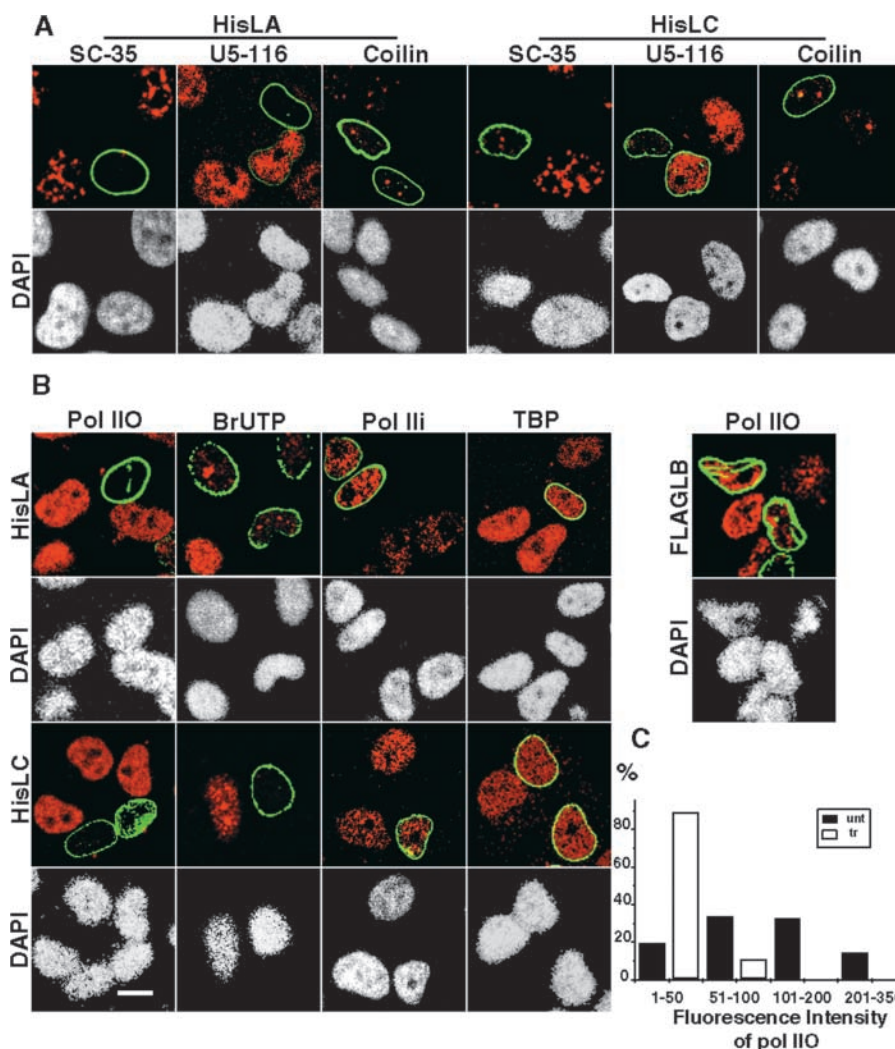
Figure 6.   **Disruption of SFCs and inhibition of transcription in His–lamin A- or C-transfected HeLa cells.** (A) Transfected cells were fixed in formaldehyde and doubly labeled with antibodies to His (green) and to splicing factors or Cajal bodies (red) as indicated. (B) Transfected cells were either formaldehyde fixed and doubly labeled with antibodies to His or FLAG (green) and pol IIO/pol IIi or TBP (red), or were allowed to incorporate BrUTP and then fixed and labeled with antibodies to His and BrUTP (red). The corresponding samples stained with DAPI are also shown. (C) Quantitative analysis of fluorescence intensities of pol IIO-labeled cells (arbitrary units, combined in convenient ranges), displayed as % of His–lamin A/C-transfected (tr, open bar) or untransfected (unt, solid bar) cells. Average intensity for tr cell = 24, unt cell = 118 (n = 100). Bar, 10 μm.

served in the total pool of pol II (pol IIi), detected by a polyclonal antibody to the enzyme, suggesting that the absence of pol IIO staining is a consequence of alterations in pol IIO phosphorylation. His–lamin A- or C-transfected cells were also labeled with an antibody to TATA binding protein (TBP), which is a basal transcription factor component of the preinitiation complex in actively transcribed genes (Burley and Roeder, 1996) and is also bound to inactive chromatin (Chen et al., 2002). There were no discernible differences in the normal staining of this factor in transfected and untransfected cells. Recently, Spann et al. (2002) have reported that the microinjection of cells with ΔNLA, which disrupts lamina organization and forms large intranuclear aggregates, also reduces BrUTP incorporation into nascent RNA and redistributes TBP into ΔNLA-containing aggregates. As we do not observe an effect on TBP, the ΔNLA-induced changes in TBP localization might be short lived and detectable only in injected cells expressing modified lamins, or ΔNLA might have caused more extensive rearrangements within the nucleus. In a control experiment, 60% of cells transfected with FLAG–lamin B1 did not show changes in pol II transcription. To confirm that RNA splicing factors and transcription sites were reduced in transfected cells that lacked lamin A/C speckles, triple labeling studies were performed. As illustrated
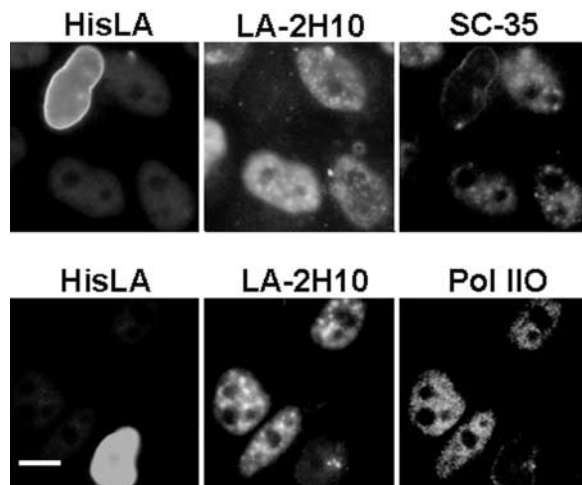
in Fig. 7, His–lamin A-transfected cells depleted in lamin speckles did not stain with anti–SC-35 antibody or mAb H5 to pol IIO in separate experiments.

Discussion

A-type lamins adopt a novel structural organization in the form of 20–50 speckles that colocalize to >90% with RNA splicing factors such as SC-35 and U5-116 kD, which have been visualized using a monoclonal antibody to recombinant rat lamin A, mAb LA-2H10 (Jagatheesan et al., 1999). Our present observations on the synchronous and reversible formation of enlarged foci of lamin A/C and SC-35 or U5-116 kD in the presence of transcriptional inhibitors, and the disruption of lamin speckles as well as SFCs upon overexpression of NH$_2$-terminally tagged lamins A/C, indicate that lamin A/C speckles are closely associated with SFCs.

Dynamic association of lamin A/C and SFCs

SFCs are dynamic compartments involved in the storage and recruitment of splicing factors. Splicing factor assemblies containing SF2/alternate splicing factor (ASF) are recruited from SFCs to transcription sites in vivo upon gene activation (Misteli et al., 1997). However, when transcription is

Figure 7. **Depletion of SC-35 and pol IIO in His–lamin A-transfected cells that lack lamin speckles.** His–lamin A-transfected cells were triply labeled with antibodies to His, lamin A (LA-2H10), and SC-35 or pol IIO. Bar, 10 μm.

inhibited there is a decrease in speckle dynamics, and budding and transport of smaller domains derived from speckles are no longer seen. There is a concomitant reorganization of speckles to larger and more spherical foci. Removal of the reversible transcriptional inhibitor DRB causes restoration of transcription by pol II, and, simultaneously, splicing factors such as SC-35 redistribute within the nucleus to their normal organization (Bregman et al., 1995). We have observed a coordinate redistribution of lamin A/C and SC-35 upon reactivation of transcription beginning within 10 min of removal of DRB. Thus lamins, in association with SFCs, can move relatively rapidly in response to signals that also cause the relocalization of splicing factors to sites of transcription, and might be regulated by similar signaling events. The dynamic reorganization of lamins A/C in response to transcriptional inhibition is consistent with a dynamic remodeling of potential structural components of the nucleoplasm in response to changing nucleoplasmic transcription.

We speculate that the redistribution of lamins may involve the depolymerization and repolymerization of lamin filaments. We have earlier observed that the speckles are resistant to detergent and/or salt extraction and nuclease digestion (Jagatheesan et al., 1999; Muralikrishna et al., 2001); hence speckles are likely to be composed of lamin filaments, though these may be in a different configuration than those found at the nuclear periphery. The head-to-tail arrangement of lamin dimers to form a protofilament has been well established; however, very little is known about the possible structures that can be adopted during lateral assembly of protofilaments to form the mature filament (Stuurman et al., 1998). The comparatively rapid redistribution of lamin A speckles observed in this study suggests that this pool of lamin A is in dynamic equilibrium, unlike the relatively static lamin framework at the nuclear rim, which dissociates only during mitosis. Further examples of lamin redistribution include the reorganization of lamin speckles to a uniform network during muscle differentiation (Muralikrishna et al., 2001); these rearrangements appear to occur specifi-

cally in muscle cells, probably due to the unique morphology of the multinucleated myofiber that is formed. The association of lamins with DNA replication centers in S phase cells is an example of cell cycle–dependent changes in lamina organization (Moir et al., 1994). In live cell studies on lamin dynamics, GFP–lamin A does not form speckles, possibly due to the large size of the GFP tag, and is primarily incorporated into the nuclear periphery (Broers et al., 1999; Moir et al., 2000b). As lamin speckles are highly insoluble structures, they do not copurify with IGCs in stoichiometric amounts, though small quantities of lamins have been detected in purified IGCs (Mintz et al., 1999).

Implications for the spatial organization of RNA splicing and transcription

A close coordination between the processes of RNA splicing and transcription is indicated by several lines of evidence, such as the cotranscriptional splicing of nascent transcripts (Beyer et al., 1988) and the association of the COOH-terminal domain of pol II with a number of multiprotein complexes involved in transcription and pre-mRNA splicing (for review see Hirose and Manley, 2000). Furthermore, studies with inhibitors of splicing or transcription indicate that blocking one process generally affects the other (Carmo-Fonseca et al., 1992; Spector, 1993; O'Keefe et al., 1994). The movement of ASF-enriched domains from SFCs toward specific transcription sites upon BK viral gene activation (Misteli et al., 1997) is suggestive of a directed targeting of the splicing machinery to required locations. The detailed analysis of the mobility of GFP-tagged nuclear proteins within the nucleoplasm of live cells by fluorescence recovery after photobleaching and fluorescence loss in photobleaching techniques indicates substantial mobility of these proteins in the nucleus (Kruhlak et al., 2000; Phair and Misteli, 2000). However, this mobility is considerably less than that of GFP alone, and Kruhlak et al. (2000) suggest that ASF-containing structures behave as though they are physically impeded by frequent and transient associations with nucleoskeletal elements. This is further substantiated by single particle tracking studies with the splicing factor U1 snRNP whose dynamics within SFCs is much slower than in aqueous solution, suggesting transient binding to immobile sites (Kues et al., 2001).

Our observations on the synchronous, reversible changes in lamin speckles and SFCs due to the inhibition of transcription and, conversely, the down-regulation of transcription after disruption of lamin speckles provide compelling evidence for a role for lamin speckles in the organization of transcription and splicing. We propose that lamin A/C speckles are part of a dynamic structure that can be rapidly modulated by specific signaling events to spatially organize mRNA splicing and pol II transcription. Protein kinase activities have been proposed to functionally link the transcription and splicing machineries, primarily by the phosphorylation of arg-ser–rich splicing factors that assist in their recruitment to splicing sites (Misteli et al., 1998). Though lamin A does not have a distinct arg-ser domain, phosphorylation might control lamin localization, albeit indirectly, through interactions with other proteins; for instance, CrkRS has been shown to be tightly bound to the nuclear

matrix and can also phosphorylate the COOH-terminal domain of pol II (Ko et al., 2001). Other structural proteins may perform similar functions to those proposed for lamins A/C; for example, lamins B1/B2 might compensate for the lack of A-type lamins in undifferentiated cells or under pathological conditions, or actin might also play a structural role in the nucleus. The association of factors with a structural framework would undoubtedly allow for greater coordination of regulatory events in the nucleus as well as higher specificity and efficiency.

Materials and methods

Plasmid constructs
All the lamin expression constructs used in this study were expressed from the CMV promoter. A full-length rat lamin A cDNA clone coding for pre–lamin A was prepared by extending an available cDNA clone missing the first 25 amino acids (Hamid et al., 1996); it was tagged at the 5′ end with sequences coding for 6X His or FLAG epitopes by PCR and initially cloned into pBSII-SK. Lamin C was derived from lamin A by PCR and tagged as above. After sequence verification, His- or FLAG-tagged lamin inserts were subcloned into pCI mammalian expression vector (Stratagene). A FLAG–lamin B1 expression vector was obtained by cloning rat lamin B1 cDNA (provided by M.R.S. Rao, Indian Institute of Science, Bangalore, India) into pCruzOctA (Stratagene). The NH$_2$-terminally tagged sequences were MGGS**HHHHHH**ETPS for His–lamin A or C, M**DYKDDDDK**ETPS for FLAG–lamin A, and MGS**DYKDDDDK**EF*MATA* for FLAG–lamin B1 (epitopes in bold and lamin sequences in italics).

Cell culture and DNA transfection
HeLa cells were grown on coverslips to 70% confluency in Petri dishes containing DME supplemented with 10% FCS. DNA transfections were performed with lipofectamine (Invitrogen) according to the manufacturer's instructions. Cells were processed for immunofluorescence microscopy 24 h after transfection. Transfection efficiencies were ∼20%. Cells were treated with the transcriptional inhibitor α-amanitin at 50 μg/ml for 5 h and DRB at 25 μg/ml for 3 h.

Antibodies
Antibodies to recombinant rat lamins used in this study and characterized in detail previously are mAb LA-2H10, which recognizes intranuclear lamin A speckles, and mAb LA-2B3, which stains the nuclear periphery (Jagatheesan et al., 1999). SC-35 mAb (Fu and Maniatis, 1990) was provided by J. Gall (Carnegie Institution of Washington, Baltimore, USA); rabbit polyclonal antibodies to U5-116 kD (Fabrizio et al., 1997) were from R. Lührmann (University of Marburg, Marburg, Germany); and p80 coilin (Bohmann et al., 1995) was from A. Lamond (University of Dundee, Dundee, Scotland). A rabbit polyclonal antibody to recombinant TBP was provided by P. Bhargava (Centre for Cellular and Molecular Biology). His and FLAG mAbs were from CLONTECH Laboratories, Inc. and Sigma-Aldrich, respectively; rabbit polyclonal antibodies to His and pol IIi were from Santa Cruz Biotechnology Inc.; and mAb 414 to NPC proteins and mAb H5 to pol IIO were from Berkeley Antibody Co. An emerin mAb was obtained from Novocastra Laboratories Ltd. and a rat mAb to BrdU was from Sera Laboratories.

Immunofluorescence microscopy
HeLa cells were washed with PBS and then fixed by treatment with 3.5% formaldehyde for 15 min followed by 0.5% (vol/vol) Triton X-100 for 6 min at room temperature, or with methanol at −20°C for 10 min, as indicated. Cells were then incubated with 0.5% gelatin in PBS for 1 h followed by incubation with first antibody for 1 h and then FITC-conjugated second antibody for 1 h at room temperature for single labeling experiments. For double labeling experiments, fixed cells were incubated with the first primary antibody followed by biotinylated second antibody and avidin-Cy3, and then with the next primary antibody and species/subtype-specific FITC-conjugated second antibody. For double labeling experiments with antibodies of the same subtype, one of the primary antibodies was directly biotinylated for detection with Cy3. For triple labeling studies, fixed cells were either stained sequentially with anti-His antibody and anti–mouse AMCA conjugate, followed by mAb LA-2H10 and Texas red–conjugated anti–mouse IgM, and then biotinylated SC-35 antibody and avidin-FITC; or

with anti-His antibody and FITC-conjugated anti–mouse IgG, followed by mAb H5 and Texas red–conjugated anti–mouse IgM, and then biotinylated LA-2H10 and Alexa®-350. Samples were mounted in Vectashield (Vector Laboratories) containing 1 μg/ml DAPI. There was no cross-reactivity of the fluorescent second antibodies in control experiments in which either primary antibody was omitted. Antibody conjugates were from Jackson ImmunoResearch Laboratories, Molecular Probes, or Vector Laboratories. Confocal laser-scanning immunofluorescence microscopy was performed on a Meridian Ultima scan head attached to an Olympus IMT-2 inverted microscope fitted with a 100X, 1.3 NA or 60X, 1.4 NA objective lens, with excitation at 515, 488, and 351–364 nm (Argon ion laser). Image analysis, including crossover subtraction, two-dimensional quantitation of pol IIO fluorescence, and estimation of colocalized speckles, was done using DASY master program V4.19 (Meridian Instruments Inc.), and images were assembled using Adobe Photoshop 5.0®. Quantitative analysis was performed by inspection of $n = 100$ cells per sample. To reduce photobleaching of UV-sensitive dye conjugates, triple-labeled samples were viewed on an Olympus BX60 fluorescence microscope with a cooled camera device, and images were analyzed using ImagePro software.

RNA synthesis
The incorporation of BrUTP into nascent RNA was measured by a standard protocol (Wansink et al., 1993). HeLa cells (untransfected or 24 h after transfection) were rinsed once with PBS and once with a glycerol-containing buffer (20 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$, 25% glycerol, 0.5 mM PMSF, and 0.5 mM EGTA). Cells were then permeabilized in glycerol buffer containing 0.1% Triton X-100 at room temperature for 3 min and washed once with glycerol buffer. Subsequently, cells were incubated in transcription mix (100 mM KCl, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$, 0.5 mM EGTA, 25% glycerol, 1 mM PMSF, 2 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.2 mM BrUTP, and 25 U/ml RNAsin) for 4.5 min at 37°C. At the end of the transcription reaction, cells were rinsed twice with cold PBS and then fixed in 3.5% formaldehyde in PBS. Fixed cells were labeled with anti-BrdU antibody as described above.

Extraction of nuclei
Cells were extracted by the relatively mild protocol described by De Conto et al. (2000), as this was found to be more suitable for cells after transfection. Transfected cells plated on coverslips were rinsed twice with TM buffer (50 mM Tris-HCl, pH 7.5, 3 mM MgCl$_2$) and then incubated for 10 min on ice in TM buffer containing 0.4% Triton X-100, 0.5 mM CuCl$_2$, and 0.2 mM PMSF. Cells were rinsed and incubated with DNase I (20 U/ml) and RNase A (20 μg/ml) for 20 min at 37°C in TM buffer. The samples were then treated with 2 M NaCl for 5 min on ice, washed with TM buffer, fixed with formaldehyde, and stained as described above.

Immunoblot analysis
HeLa cell lysates were analyzed by immunoblotting as described previously (Jagatheesan et al., 1999).

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