Serine/Threonine Phosphatase 1 Modulates the Subnuclear Distribution of Pre-mRNA Splicing Factors

Tom Misteli* and David L. Spector

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Submitted June 17, 1996; Accepted July 25, 1996
Monitoring Editor: Joseph Gall

HeLa cell nuclei were permeabilized and reconstituted with nuclear extract to identify soluble nuclear factors which play a role in the organization of pre-mRNA splicing factors in the mammalian cell nucleus. Permeabilized nuclei reconstituted with nuclear extract were active in transcription and DNA replication and nuclear speckles containing pre-mRNA splicing factors were maintained over several hours independent of soluble nuclear components. The characteristic rounding up of nuclear speckles in response to inhibition of RNA polymerase II seen *in vivo* was reproduced in permeabilized cells and was strictly dependent on a catalytic activity present in the nuclear extract. By inhibitor titration experiments and sensitivity to inhibitor 2, this activity was identified as a member of the serine/threonine protein phosphatase 1 family (PP1). Interference with PP1 activity affected the distribution of pre-mRNA splicing factors in transcriptionally active, permeabilized cells, and excess PP1 activity caused increased dephosphorylation of SR proteins in nuclear speckles. These data show that the dynamic reorganization of the mammalian cell nucleus can be studied in permeabilized cells and that PP1 is involved in the rounding up of speckles as well as the overall organization of pre-mRNA splicing factors in the mammalian cell nucleus.

INTRODUCTION

Intron sequences are removed from pre-messenger RNA in a nuclear splicing reaction (reviewed in Green, 1991; Moore *et al.*, 1993). The excision of intervening sequences is catalyzed within the spliceosome, a complex consisting of five major small nuclear ribonucleoprotein particles (snRNPs) and additional non-snRNP proteins (reviewed in Lührmann *et al.*, 1990; Green, 1991; Lamm and Lamond, 1993; Fu, 1995). *In vitro*, spliceosomes form on nascent pre-mRNA molecules by the stepwise association of first the U1-snRNP with the 5' splice site, followed by the binding of the U2-snRNP near the branchpoint, allowing the entry of the U4/U5/U6 tri-snRNP complex to complete spliceosome assembly. Upon formation of the mature spliceosome, the splicing reaction is thought to occur in a two-step mechanism culminating in the joining of exons and the removal of intervening sequences before resolution of the spliceosome (re-

viewed in Green, 1991; Lamm and Lamond, 1993; Moore *et al.*, 1993; Fu, 1995).

In vivo, splicing occurs cotranscriptionally or very shortly after release of the nascent RNA from the transcription complex and takes place at, or very near, the site of transcription (Beyer and Osheim, 1991; Bauren and Wieslander, 1994). As determined by fluorescence microscopy, snRNPs and non-snRNP splicing factors localize in the mammalian cell nucleus in a characteristic pattern, commonly referred to as the speckled pattern (Northway and Tan, 1972; Spector *et al.*, 1983, 1991; Verheijen *et al.*, 1986; Fu and Maniatis, 1990). The pattern typically contains 20–40 intensely stained speckles which are set against a diffuse nuclear population of splicing factors. Splicing factors localized in speckles are resistant to extraction with non-ionic detergent, salt, or treatment with DNase I, suggesting that they represent distinct nuclear subcompartments, possibly associated with a nuclear scaffold (Fey *et al.*, 1986; Bisotto *et al.*, 1995). By electron microscopy, speckles are observed to consist of two domains which are morphologically and functionally distinct: the larger and more highly concen-

* Corresponding author.

trated regions seen by fluorescence microscopy correspond to interchromatin granule clusters (IGCs), whereas the more diffusely distributed splicing factors and the regions at the periphery of IGCs correspond to perichromatin fibrils (PFs; reviewed in Spector, 1993). Functionally, nucleotide incorporation studies suggest that PFs are the sites of transcription, whereas IGCs are generally not active in transcription (Fakan and Bernhard, 1971; Fakan and Nobis, 1978; Fakan, 1994; Bachellerie *et al.*, 1975). The overall morphological appearance of nuclear speckles reflects the transcriptional activity of the cell. When RNA polymerase II transcription is blocked by the addition of specific inhibitors or by microinjection of antisense oligonucleotides against splicing components, speckles lose their irregular shape, round up, and fuse into larger clusters, demonstrating that they are of a dynamic nature (Sinclair and Brasch, 1978; Spector *et al.*, 1983; O'Keefe *et al.*, 1994).

It is unclear what the mechanism for this reorganization is and what causes splicing factors to assume a particular subnuclear localization. Two protein kinases, SRPK-1 (Gui *et al.*, 1994a,b) and CLK/STY (Colwill *et al.*, 1996), specifically phosphorylate a family of non-snRNP proteins, the SR proteins (Roth *et al.*, 1990; Birney *et al.*, 1993; Fu, 1995). Members of the family share a common motif of repeating arginine-serine dipeptides, which is required for localization to speckles. It has been proposed that phosphorylation of the SR domain controls the subnuclear distribution of SR proteins since overexpression of either kinase results in the displacement of splicing factors from speckles to a diffuse distribution in the nucleus (Gui *et al.*, 1994a; Colwill *et al.*, 1996).

We have used permeabilized cells to identify nuclear factors which affect the distribution of pre-mRNA splicing factors in the mammalian cell nucleus. We show that soluble nuclear factors are required for the redistribution of pre-mRNA splicing factors in response to inhibition of RNA polymerase II transcription. One of the soluble factors required for this redistribution was identified as a member of the protein phosphatase 1 (PP1) family. PP1 also affected the phosphorylation state of splicing factors in speckles and was important for the proper nuclear distribution of these factors.

MATERIALS AND METHODS

All reagents were of analytical grade or higher and purchased from Sigma (St. Louis, MO) unless otherwise stated. All electron microscopy reagents were purchased from Electron Microscopy Sciences (Fort Washington, PA) unless otherwise stated.

Cell Culture

HeLa cells were grown on glass coverslips in DMEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 mg/ml

streptomycin at 37°C in 10% CO₂. All cell culture reagents were purchased from Life Technologies (Gaithersburg, MD).

Protein Phosphatases and Phosphatase Inhibitors

The catalytic subunits of PP1, PP2A, and inhibitor 2 were purified from rabbit skeletal muscle (Yang *et al.*, 1981; De Guzman and Lee, 1988; Beullens *et al.*, 1993) and kindly provided by Drs. Mathieu Bollen (Katholieke Universiteit Leuven, Belgium) and Tim Haystead (University of North Carolina). Activity of phosphatase preparations and inhibitors under all experimental conditions was assayed using *p*-nitrophenyl phosphate (Takai and Mieskes, 1991). The catalytic subunits of phosphatases were added to permeabilized cells at a final concentration of 25 nM, inhibitor 2 at 50 nM. Okadaic acid, tautomycin, and microcystin-LR were obtained from Calbiochem (San Diego, CA) and dissolved in ethanol.

Nuclear Extract Preparation

Nuclear extracts were prepared exactly as described in Krainer *et al.* (1984).

Permeabilization and Incubations of Permeabilized Cells

HeLa cells grown on 22 × 22-mm glass coverslips were permeabilized by incubation for 5 min on ice with 0.1% Triton X-100 in buffer containing 20 mM Tris-KOH, pH 7.4, 40 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol. The cytoplasmic and soluble nuclear components were washed away by three quick rinses in buffer. The coverslips were blotted on Whatman filter paper no. 1, placed in a dry Petri dish with the cells facing up and 100 μl of incubation mixture consisting of 25 μl 4× buffer, 50 μl nuclear extract, 10 μl of a 10× ATP-regenerating system (100 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 10 mM ATP; reagents from Boehringer Mannheim, Indianapolis, IN), and 15 μl of distilled H₂O was added onto the coverslip. Incubation was carried out in a covered Petri dish in a 37°C incubator with 10% CO₂. The transcriptional inhibitor α-amanitin (Calbiochem, San Diego, CA) was used at 50 μg/ml.

Immunofluorescence

Permeabilized cells were fixed for 10 min at room temperature in freshly prepared 2% paraformaldehyde in phosphate-buffered saline (PBS), rinsed three times in PBS, and incubated for 45 min at room temperature with primary antibody in PBS. Intact cells were fixed as described above and permeabilized with 0.2% Triton X-100 for 5 min on ice and washed three times in PBS before incubation with primary antibody. The dilutions of the primary antibodies used were anti-SM (1:2500; gift from Joe Kraft, Yale University), anti-SC35 (1:100; Fu and Maniatis, 1990), anti-U2-B" (1:3; Habets *et al.*, 1986), anti-m₃G (1:7; Krainer, 1988), anti-lamin A/C (1:200; Gerace *et al.*, 1978), anti-centromere (1:200; Moroi *et al.*, 1980). Coverslips were then rinsed three times for 10 min each with PBS and incubated with the appropriate secondary antibody coupled to either Texas Red or fluorescein (Organo-Teknika, Durham, NC) in PBS (1:100) for 45 min at room temperature (RT). Coverslips were washed three times for 10 min in PBS, blotted, and mounted in 90% glycerol in 0.2 M Tris-base (pH 8) with 1 mg/ml *p*-phenylenediamine. Cells were observed using a Nikon-Microphot FXA fluorescence microscope equipped with a 35-mm camera.

In Vitro Transcription and DNA Replication Assay

In vitro transcription assays were performed according to Wansink *et al.* (1993), except that permeabilization buffer was used and permeabilized cells were first incubated for 45 min with nuclear extract as described above, before washing and addition of labeled nucleotides. In vitro DNA replication assays were performed as de-

scribed in the study by Hozak *et al.* (1993), except that permeabilization buffer was used and permeabilized cells were first incubated for 45 min with nuclear extract as described above, before washing and addition of labeled nucleotides. Incorporated Br-UTP was detected using a fluorescein-tagged mouse monoclonal antibody against Br-UTP (Boehringer Mannheim) at a dilution of 1:20 in PBS. Incorporated biotin-dUTP was detected with streptavidin-fluorescein (Organo-Teknika, Durham, NC). For simultaneous detection of splicing factors and transcription sites, the transcription assay, fixation, and detection of the splicing factor were performed as described above. The coverslips were then washed over 60 min in five changes of PBS supplemented with 5% normal goat serum, and the incorporated Br-UTP was detected as described above.

Quantitation of Speckle Shape

All speckles in typically 20–30 randomly chosen cells were evaluated and classified as either irregular shaped or round. Irregular-shaped speckles exhibited at least one extension of more than 0.5× their average radius. Round speckles contained no extensions at all or extensions smaller than 0.5× their average radius. Results represent average values $\pm$ SEMs from at least three experiments.

Electron Microscopy

Cells were grown on thermanox plastic coverslips (Nunc, Naperville, IL). Intact or permeabilized cells were fixed for 20 min at RT in 2% paraformaldehyde and 0.5% glutaraldehyde in PBS (pH 7.4), washed three times in 0.3 M glycine in PBS (pH 7.4), dehydrated in a graded series of ethanol, and embedded in LR-White. Sections through the embedded monolayer were cut on a Reichert-Jung Ultracut E ultramicrotome and picked up on gold grids. For immunolabelling, nonspecific binding was blocked by incubation of sections with 8% normal goat serum in TBS (20 mM Tris-KOH, 150 mM NaCl, 1% Tween 20), followed by overnight incubation of antibody 3C5 (1:200–1:500) at RT. Sections were washed over 15 min in several changes of TBS and incubated for 60 min at RT with goat anti-mouse IgM-5 nm gold (Energy Beam Sciences, Agawam, MA). Grids were washed for 15 min in several changes of TBS and distilled H₂O and poststained using the EDTA regressive method (Bernhard, 1969).

Western Blotting

HeLa cells grown on glass coverslips were permeabilized and the soluble fraction was recovered by aspiration. The remaining cellular fraction on the coverslip was extracted for 30 min at 4°C with 20 mM Tris-HCl (pH 8.0), 2% Triton X-100, 1% deoxycholate, 250 mM NaCl, and 25 mM iodoacetamide and scraped and the fraction further extracted for 60 min at 4°C. The particulate fraction of the extract was removed by centrifugation at $15,000 \times g$ for 10 min at 4°C. The resulting pellet did not contain any significant amounts of splicing factors as determined by extraction of the pellet fraction with 6 M urea followed by Western blotting with splicing factor antibodies. Proteins in the supernatant and particulate fraction were separated on a 12% polyacrylamide gel, transferred to nitrocellulose, and blotted as follows: membranes were blocked for 2 h in 5% fat-free milk in PBS, incubated overnight with the appropriate dilution of primary antibody in 2.5% milk in PBS, washed three times for 20 min in the same buffer, incubated for 2 h at RT with the appropriate secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL), washed three times for 30 min in PBS, and finally for 10 min in water before detected bands were visualized using the ECL system (Amersham) or the SuperSignal system (Pierce, Rockford, IL) according to the manufacturers' specifications using Hyperfilm-ECL (Amersham). The dilutions of the primary antibodies used were 1:2000 for anti-SM, 1:5 for anti-SF2/ASF (gift from A. Krainer, Cold Spring Harbor), 1:3 for anti U2-B", 1:500 for anti-lamin A/C, and 1:2 for monoclonal antibody (mAb) 104.

RESULTS

To assess the feasibility of using permeabilized cells to study the subnuclear organization of pre-mRNA splicing factors, the effect of permeabilization on the distribution of nuclear antigens was determined. HeLa cells grown on coverslips were permeabilized with 0.1% Triton X-100 for 5 min on ice, the soluble nuclear components were removed by washing, and nuclear antigens were detected using indirect immunofluorescence (IF). In permeabilized cells, the non-snRNP splicing factor SC35 (Figure 1B) and snRNPs as monitored by the U2-B" protein (Figure 1D), the SM- (Figure 1F), and m₃G-epitopes (Figure 1H) remained organized in speckles which were not changed in appearance compared with speckles in intact cells (Figure 1A, C, E, and G). The more diffusely distributed pool of splicing factors commonly seen in intact cells was decreased upon permeabilization, except in the case of the SC35 epitope (Figure 1, A and B). This is most likely a reflection of the fact that the SC35 antibody reacts with a phosphoepitope and only recognizes the subset of SC35 which is associated with speckles, but not the more diffusely distributed pool of SC35. The overall chromatin structure in the nuclei was not grossly altered by the permeabilization procedure, since the distribution of centromeres (Figure 1, I and J) was not changed upon permeabilization. Furthermore, immunolabelling of lamins A/C gave a continuous signal along the nuclear rim (Figure 1, K and L). The decreased diffuse fluorescence signal suggested the loss of soluble nuclear material upon permeabilization. This was confirmed by Western blotting with antibodies against SF2/ASF, U2-B", and the SM antigen (Figure 2). After permeabilization ~20% of SF2/ASF, U2-B", and the SM epitope were lost from the nuclear fraction (P) and were found in the supernatant (S). This number correlated well with the decrease in fluorescence intensity. As a control, lamins A and C were retained completely in permeabilized nuclei (Figure 2). These data demonstrated that the majority of pre-mRNA splicing factors organized in nuclear speckles is resistant to detergent extraction, but the more soluble pool of splicing factors is largely removed by permeabilization.

To address the functionality of the permeabilized cells, fluorescence *in situ* assays for transcription and DNA replication were performed (Hozak *et al.*, 1993; Wansink *et al.*, 1993). Permeabilized cells were incubated for 45 min with a nuclear extract routinely used in *in vitro* splicing assays (Krainer *et al.*, 1984), and the cells were washed and supplied for 15 min with either bromo-UTP or biotin-dUTP for incorporation during transcription or DNA replication, respectively. Both transcription (Figure 3A) and replication (Figure 3C) occurred as efficiently in

Figure 1. Permeabilization does not affect the speckles but removes the diffuse nuclear fraction of the fluorescence signal. HeLa cells were either fixed and permeabilized (A, C, E, G, I, and K) or first permeabilized and then fixed (B, D, F, H, J, and L) and processed for fluorescence microscopy. Nuclear antigens were detected using antibodies against SC35 (A and B), U2-B' (C and D), SM (E and F), trimethylguanosine cap (G and H), centromeres (I and J), lamin A/C (K and L). Bar, 10 μ m.

permeabilized cells after 45 min of incubation with nuclear extract as in cells pulsed for 15 min immediately after permeabilization (Figure 3, B and D).

Speckles Do Not Require Soluble Nuclear Factors for Their Maintenance

We tested whether nuclear factors are required to maintain the structure of speckles. In permeabilized cells incubated for 2 h in the presence of nuclear extract, speckles look virtually indistinguishable from speckles in cells immediately fixed after permeabilization (Figure 4A and B). In fact, soluble nuclear factors were not required for the maintenance of speckles since prolonged incubation of permeabilized cells with buffer alone supplemented with 2 mM ATP, 5 mM $MgCl_2$, and an ATP-regenerating system was sufficient to maintain the appearance of speckles (Figure 4C). When Mg^{2+} ions were omitted from the buffer,

speckles appeared to be fewer and more round in shape (Figure 4D).

The speckles also did not dramatically alter their ultrastructure during 2 h of incubation in buffer alone (Figure 5). Electron microscopy of permeabilized cells regressively stained using the EDTA method showed that permeabilized nuclei contained less nucleoplasmic material (Figure 5B), consistent with the loss of nucleoplasmic components detected with fluorescence microscopy and Western blotting. The morphology of IGCs, as defined by immunoreactivity with the 3C5 epitope, which recognizes several SR proteins (Turner and Franchi, 1987), was virtually indistinguishable in permeabilized cells incubated for 2 h with buffer compared with IGCs in intact cells (Figure 5A). We conclude that the maintenance of nuclear speckles in permeabilized cells does not require soluble nuclear factors.

Figure 2. Soluble nuclear factors are lost from permeabilized cells. HeLa cells were permeabilized with a minimal volume of buffer containing 0.1% Triton X-100. The supernatant was removed and the particulate fraction detergent extracted. Proteins in the particulate fraction (P) or the supernatant (S) were separated by SDS-gel electrophoresis and transferred onto nitrocellulose. The blots were probed with antibody against SF2/ASF, U2-B'', the SM epitope, or lamins A/C.

Reorganization of the Speckled Pattern Requires Soluble Nuclear Factors

Nuclear speckles enriched in pre-mRNA splicing factors are not static structures since inhibition of RNA polymerase II transcription causes splicing factors to accumulate in enlarged IGCs, which can be detected as bright, round nuclear structures (Figure 6, A and G; Sinclair and Brasch, 1978; Spector *et al.*, 1983) using fluorescence microscopy. To probe the mechanisms involved in this redistribution, the rounding up was reconstituted in permeabilized cells. As previously reported, when added to intact cells, α -amanitin, a highly specific (K_i of 10^{-8} M) and irreversible inhibitor of RNA polymerase II (Lindell *et al.*, 1970), caused the redistribution of the SC35 and SM antigens (Figure 6, A and G; Sinclair and Brasch, 1978; Spector *et al.*, 1983) as well as the m_3G and U2-B'' antigen into round structures (our unpublished results). The effect of α -amanitin was measured qualitatively using immunofluorescence and quantitatively by determining the percentage of round speckles in randomly selected cells (Table 1). When permeabilized cells were incubated with α -amanitin in buffer alone, extranuclear transcription was efficiently inhibited (Figure 6E), but no significant change in the speckled pattern of non-snRNP (Figure 6B) or snRNP proteins (Figure 6H) compared with untreated cells was observed (see Figure 1 for untreated cells). Quantitatively, the number of round speckles ($5.7 \pm 3.4\%$; Table 1) was not significantly different from untreated, intact cells ($2.7 \pm 1.4\%$) or permeabilized cells incubated in buffer alone ($5.2 \pm 3.8\%$). The effect of the transcriptional inhibitor

could be reconstituted in permeabilized cells when incubation with α -amanitin was performed in the presence of a nuclear extract (Figure 6, C and I). Transcription, with the exception of nucleolar RNA polymerase I transcription, was inhibited completely (Figure 6F) and $\sim 86\%$ of speckles did round up. This value was only slightly lower than that in intact cells, where 98% of the speckles rounded up after α -amanitin treatment. The rounding up was not simply the result of the altered phosphorylation state of the SC35 epitope, since the SM antigen (Figure 6I), U2-B'', and m_3G antigen behaved identically (our unpublished results). The observed rounding up was also not due to the presence of excess SR proteins added with the nuclear extract, as rounding up also occurred when permeabilized cells were incubated with α -amanitin in an S100 extract, which is virtually free of SR proteins, or in a 200-fold diluted nuclear extract (Table 1).

PP1 Activity Is Required for Redistribution

Recently, two kinases have been identified which cause the redistribution of SR proteins from the speckled pattern into a diffuse nuclear distribution (Gui *et al.*, 1994a; Colwill *et al.*, 1996). Since rounding up of speckles upon inhibition of transcription appears to be

Figure 3. Permeabilized cells transcribe and replicate DNA. Transcription was measured by incorporation of bromo-UTP for 15 min at 37°C either in permeabilized HeLa cells after incubation with nuclear extract for 45 min at 37°C (A) or directly after permeabilization (B). DNA replication was detected by incorporation of biotin-16-dUTP for 15 min at 37°C either in permeabilized cells after incubation with nuclear extract for 45 min at 37°C (C) or directly after permeabilization (D). Bar, 10 μm .

Figure 4. Speckles do not require soluble nuclear factors for maintenance. Permeabilized cells were incubated for 0 min (A), 2 h in the presence of nuclear extract (B), 2 h in buffer alone with 5 mM $MgCl_2$ and an ATP-regenerating system (C), or 10 min in buffer in the absence of Mg^{2+} ions or ATP (D). The cells were then fixed, and the SC35 epitope was detected using IF. Bar, 10 μm .

the reverse of this process, we were prompted to evaluate the possibility of an involvement of phosphatases in the rounding up of speckles in response to inhibition of transcription. Permeabilized cells were incubated with nuclear extract and α -amanitin in the presence of phosphatase inhibitors (Figure 7). Rounding up of speckles, as judged by detection of either SC35 or the SM antigen, was completely inhibited by

1 μM okadaic acid (OkA; Figure 7, A and E) or tautomycin (TTM) (Figure 7, B and F). In the presence of the phosphatase inhibitors, the speckles appeared to be generally unaltered, although some speckles seemed slightly enlarged and less well defined at their periphery (Figure 7, A and B). The number of round speckles, $4.6 \pm 2.8\%$ and $5.3 \pm 2.6\%$ for the two inhibitors, respectively, was not significantly different from that

Figure 5. Interchromatin granule clusters do not require soluble nuclear factors for maintenance. HeLa cells were either fixed and permeabilized (A) or permeabilized, incubated for 2 h in the presence of buffer (B), and processed for electron microscopy. The sections were labeled with the 3C5 antibody that recognizes SR proteins. Note the loss of nucleoplasmic material and the unaltered morphology of IGCs in permeabilized cells (B) compared with intact cells (A). Bar, 500 nm.

Figure 6. Rounding up of the speckles can be reconstituted in permeabilized cells. The transcriptional inhibitor α -amanitin was added to intact cells for 4 h (A, D, and G), to permeabilized cells in the absence of nuclear extract for 60 min (B, E, and H), or to permeabilized cells in the presence of nuclear extract for 60 min (C, F, and I). Transcriptional activity was assessed by the addition of bromo-UTP for 15 min at 37°C (D–F). Cells were then fixed and processed for fluorescence microscopy using anti-SC35 antibody (A–C), anti-bromo-UTP antibody (D–F), or anti-SM antibody (G–I). Bar, 10 μ m.

in permeabilized cells incubated with nuclear extract alone ($4.3 \pm 2.6\%$; Table 1), demonstrating the inhibitory effect. Importantly, the phosphatase inhibitors did not restore transcriptional activity (Figure 7, C and D), ruling out the possibility that the prevention of rounding up was an indirect effect due to a reversal of the α -amanitin action.

Both phosphatase inhibitors are specific for members of the families of ser/thr protein phosphatases 1 (PP1) and 2A (PP2A; Bialojan and Takai, 1988; Cohen *et al.*, 1990; MacKintosh and Klumpp, 1990). However, their inhibitory abilities differ between the two families (Takai *et al.*, 1995). OkA inhibits PP2A more efficiently than PP1, whereas TTM inhibits PP1 more efficiently than PP2A. Microcystin-LR, a third inhibitor of PP1 and PP2A, inhibits members of both families with roughly equal specificity (Takai *et al.*, 1995). We took advantage of the differential inhibitory profiles to distinguish which of the two families of phosphatases was responsible for preventing the rounding up of speckles. Dilutions of the three inhibitors in the range between 1 μ M and 1 nM were added to permeabilized cells in the presence of nuclear extract and α -amanitin, the pattern of several components of the

speckled pattern was determined using IF, and the percentage of round speckles was determined. The results from these experiments are summarized in Figure 8 and were identical for the SM antigen, SC35, U2-B", and m₃G antigen. OkA only efficiently prevented rounding up at a concentration of 1 μ M and showed a slight effect at 100 nM. Microcystin-LR was able to prevent rounding up completely at 1 μ M and prevented rounding up more strongly than OkA at 100 nM. In contrast, TTM was still able to prevent rounding up of more than 80% of speckles at concentrations as low as 10 nM. This inhibitory profile suggested that PP1 activity was required for rounding up of speckles in response to inhibition of RNA polymerase II transcription.

To directly test this possibility, the activity of PP1 was specifically inhibited by the addition of inhibitor 2, a cellular inhibitor peptide, which selectively inhibits PP1 activity, including nuclear PP1 (Brautigan *et al.*, 1990; Jagiello *et al.*, 1995). The addition of inhibitor 2 to permeabilized cells in the presence of nuclear extract and 1 μ M α -amanitin completely inhibited the rounding up of speckles (Figure 9, A and C), and only 5% of speckles appeared to be round (Table 1). This clearly

Table 1. Quantitation of speckle shape

	Round speckles (%)	Transcription
Intact cells		
untreated	2.7 ± 1.4	+
+ α -amanitin	98.0 ± 2.3	–
Permeabilized cells		
+ buffer	5.2 ± 3.8	+
+ nuclear extract	4.3 ± 2.6	–
+ buffer/ α -amanitin	5.7 ± 3.4	–
+ nuclear extract/ α -amanitin	86.2 ± 8.2	–
+ S100 (1:1)/ α -amanitin	88.6 ± 5.7	–
+ nuclear extract (1:200)/ α -amanitin	84.3 ± 7.2	–
+ nuclear extract/ α -amanitin 1 μ M	4.6 ± 2.8	–
okadaic acid		
+ nuclear extract/ α -amanitin 1 μ M	5.3 ± 2.6	–
tautomycin		
+ nuclear extract/ α -amanitin, inhibitor-2	5.1 ± 2.9	–

Intact or permeabilized cells were incubated under the conditions indicated, and the percentage of round speckles was determined as described in MATERIALS AND METHODS. Values represent means $\pm$ SEM from at least three experiments. Transcriptional activity was assessed as described in MATERIALS AND METHODS and scored as either positive or negative without quantitation.

demonstrated that PP1 activity is required for the redistribution of splicing factors in response to inhibition of RNA polymerase II transcription. On the other hand, PP1 activity alone was not sufficient to cause rounding up of speckles as in the presence of α -amanitin and the catalytic subunit of PP1 alone; speckles did not round up as detected by either SC35 (Figure 9B) or the SM epitope (Figure 9D). PP1 was active under these conditions because it efficiently dephosphorylated *p*-nitrophenyl phosphate (Takai and Mieskes, 1991; our unpublished results). This suggested that additional soluble nuclear factors, possibly regulatory factors of PP1, were required for the rounding up of speckles (Jagiello *et al.*, 1995).

PP1 Modulates the Distribution of Splicing Factors in the Absence of α -Amanitin

The above experiments demonstrated a role of PP1 in the redistribution of splicing factors in response to inhibition of RNA polymerase II transcription. But does PP1 also affect the distribution of splicing factors in the absence of transcriptional inhibition? To this end, permeabilized cells were incubated for 30 min with nuclear extract supplemented with inhibitor 2 or, alternatively, supplemented with the catalytic subunit of PP1, the cells were fixed, and the distribution of the splicing factor SC35 and the SM antigen was determined using IF (Figure 10). The shorter incubation period was chosen to ensure that

the permeabilized cells were transcriptionally active at all times (see Figure 3). The addition of the catalytic subunit of PP1 to permeabilized cells resulted in the diffuse distribution of SC35 and the SM antigen in the nucleus (Figure 10, B and D). On the other hand, the addition of inhibitor 2 to permeabilized cells resulted in enlarged speckles (Figure 10, A and C). These structures were distinct from the round speckles induced by inhibition of transcription because they were typically more elongated and significantly larger. These data show that interference with PP1 activity affects the distribution of pre-mRNA splicing factors in transcriptionally active mammalian cell nuclei.

PP1 Affects the Phosphorylation State of SR Proteins in Permeabilized Cells

To test whether PP1 can affect the phosphorylation state of SR proteins in speckles, HeLa cells were permeabilized and incubated for 30 min with nuclear extract supplemented with either PP1 or PP2A catalytic subunit. The soluble factors present in the nuclear extract were removed by repeated washing after the incubation and the cellular fraction was exhaustively extracted with detergent as described in MATERIALS AND METHODS. The presence of splicing factors in the permeabilized cells after the washing steps was confirmed using IF, and the phosphorylation state of SR proteins in the speckles was detected on Western blots using antibody mAb104 which recognizes a phosphoepitope present in SR proteins (Roth *et al.*, 1990). In the presence of nuclear extract alone, SR proteins were heavily phosphorylated (Figure 11, left lane). After incubation with PP1, the level of phosphorylation was dramatically reduced and in many experiments no signal could be detected at all (Figure 11, middle lane). The dephosphorylation was specific for PP1 because the catalytic subunit of PP2A added at similar and higher levels of activity was unable to efficiently dephosphorylate SR proteins, possibly with the exception of SRp30 and SRp40 (Figure 11, right lane).

DISCUSSION

To identify soluble nuclear factors which affect the subnuclear distribution of pre-mRNA splicing factors, HeLa cells were permeabilized and then supplemented with nuclear extract to reconstitute the nuclear environment. Similar systems without the readdition of nuclear extracts have previously been used in the study of organization of transcription and DNA replication (Bravo and Macdonald-Bravo, 1987; Jackson *et al.*, 1988; Jackson *et al.*, 1993; Wansink *et al.*, 1993; reviewed in Hughes *et al.*, 1995) and the organization of the nuclear lamina (Högner *et*

Figure 7. Inhibitors of ser/thr protein phosphatases interfere with the rounding up of the speckles in response to inhibition of transcription. Permeabilized cells were incubated with nuclear factors and α -amanitin in the presence of either 1 μ M Oka (A, C, and E) or 1 μ M TTM (B, D, and F). Transcriptional activity in the same cells as SC35 distribution was measured by incorporation of bromo-UTP for 15 min at 37°C (C and D). Either SC35 (A and B) or the SM (E and F) epitope was detected by using indirect IF. Bar, 10 μ m.

al., 1988). Several observations suggest that permeabilized, reconstituted nuclei behave similarly to nuclei in intact cells. First, the overall morphology of speckles, as assessed by fluorescence microscopy, was unaltered over extended periods of time in permeabilized cells compared with intact cells as long as ATP and Mg^{2+} ions were supplied. Also, using electron microscopy no significant changes in the fine structure of IGCs were detected over the course of the incubation. Second, in permeabilized cells after incubation with nuclear extract, transcription and DNA replication appeared to be normal, as measured by the incorporation of fluorescently labeled nucleotides. Third, just like intact cells, nuclei responded to the addition of specific inhibitors of RNA polymerase II by loss of transcriptional activity. Fourth, dynamic changes, such as the rounding up of speckles in response to inhibition of RNA polymerase II could be faithfully reproduced in permeabilized cells in the presence of nuclear extract and the resulting nuclear organization was indistinguishable from the *in vivo* situation.

Requirement for PP1 in the Redistribution of pre-mRNA Splicing Factors

Inhibition of RNA polymerase II transcription causes the rounding up of speckles *in vivo* (Sinclair and Brasch, 1978; Spector *et al.*, 1983) and, as we show here, in permeabilized cells in the presence of soluble nuclear factors, but not in their absence. The mechanism that causes this dramatic change is unclear. From electron microscopy, it is known that the redistribution observed with fluorescence microscopy is accompanied by the physical rounding up and fusion of IGCs (Spector *et al.*, 1983; O'Keefe *et al.*, 1994). The simplest explanation might be that the chromosomal architecture is dependent on the level of transcription and that by inhibiting RNA polymerase II the overall structure of chromosome domains and, therefore, the structure of the cell nucleus is altered (Haaf and Ward, 1996). However, our finding that in transcriptionally inactive permeabilized cells, the speckles were of normal morphology and only rounded up in the presence of soluble

Figure 8. PP1 is required for rounding up of the speckles. Permeabilized cells were incubated with α -amanitin and nuclear extract supplemented with the indicated concentration of ser/thr phosphatase inhibitor. The rounding up of the speckles was detected by IF microscopy using antibody against either SC35, U2-B', the m₃G, or the SM epitope. The percentage of round speckles was determined by visual inspection as described in MATERIALS AND METHODS. Results are mean values from three experiments $\pm$ SEM.

nuclear factors suggests that this interpretation may be oversimplified. It is thus clear that the redistribution, of at least pre-mRNA splicing factors, is not solely the consequence of overall nuclear changes in transcriptional activity, but that soluble nuclear factors mediate these redistributions.

Using permeabilized cells, one of the factors required for the redistribution was identified to be a member of the ser/thr protein phosphatase 1 family. The identity of the phosphatase was established by inhibitor titration experiments and confirmed by sensitivity to inhibitor 2, a cellular peptide which inhibits PP1 but not PP2A activity. PP1 activity is abundant in the cell nucleus (Kuret *et al.*, 1986), and the active enzyme typically consists of a generic PP1 catalytic domain and a regulatory subunit which determines subcellular localization and substrate specificity (Jagiello *et al.*, 1995). In the case of nuclear PP1 activity, two regulatory subunits of 41 and 111 kDa have been identified, the smaller one having homology to the U1-70K protein splicing factor (Jagiello *et al.*, 1995; Van Eynde *et al.*, 1995). Although nuclear PP1 activity has been implicated in nuclear functions such as alternative splice site selection in vitro (Cardinali *et al.*, 1994), chromatin structure (Dombradi and Cohen, 1992), or regulation of gene expression (Sheen, 1993; Alberts *et al.*, 1994), it is unclear what the substrates for nuclear PP1 activity are. We show that at least five SR proteins associated with speckles are dephosphorylated in the presence of the catalytic subunit of PP1. However, it is

not clear whether they are actual substrates for PP1. The same SR proteins have been shown to undergo dephosphorylation by PP1 in solution in nuclear extract (Mermoud *et al.*, 1994), and it remains to be determined whether the phosphorylation state of other pre-mRNA splicing factors are modulated by PP1 and what regulatory factors of nuclear phosphatase(s) are required for splicing factor dephosphorylation.

PP1 Activity in the Subnuclear Distribution of pre-mRNA Splicing Factors

Aside from its role in the redistribution of splicing factors after α -amanitin treatment, PP1 was also crucial for the proper distribution of splicing factors in the absence of α -amanitin. Increased PP1 activity resulted in the diffuse distribution of splicing factors in the nucleus, whereas decreased PP1 activity caused by addition of inhibitor 2 had the opposite effect. The requirement for PP1 in rounding up of speckles was not surprising, since recently two kinases, SRPK-1 and CLK/STY, have been identified which specifically phosphorylate SR protein-splicing factors and cause the diffuse distribution of splicing factors (Gui *et al.*, 1994a,b; Colwill *et al.*, 1996). However, unexpectedly,

Figure 9. Inhibitor 2 prevents rounding up of the speckles. (A and C) Permeabilized cells were incubated with α -amanitin and nuclear extract supplemented with inhibitor 2 and either the SC35 (A) or the SM epitope (C) was detected by indirect IF microscopy. Inhibitor 2 completely blocks the rounding up of speckles. (B and D) Permeabilized cells were incubated with buffer supplemented with the purified catalytic subunit of PP1 and the SC35 (B) or the SM epitope (D) was detected by indirect IF. Increased PP1 activity results in the diffuse distribution of the epitopes. Bar, 10 μ m.

the addition of the catalytic subunit of purified PP1 to permeabilized cells had the same effect on splicing factor distribution as overexpressed kinase activities *in vivo*.

It is possible that this behavior reflects the fact that an intermediate level of splicing factor phosphorylation is required for the structural integrity of speckled domains and that hyperphosphorylation or hypophosphorylation leads to the disruption of speckles. A likely prediction from this model is that excess kinase or phosphatase activities have opposite effects on the structure of speckles. However, this was not the case in our studies using inhibitors of PP1 or a purified catalytic subunit of PP1. An alternative model incorporating recent data on the topology of pre-mRNA splicing in the cell nucleus and the role of PP1 on pre-mRNA splicing *in vitro* is more consistent with our observations: pre-mRNA splicing occurs cotranscriptionally at, or very near, the site of transcription (Fakan *et al.*, 1986; Beyer and Osheim, 1988; Wu *et al.*, 1991; Zhang *et al.*, 1994; Huang and Spector, 1996). However, the majority of splicing factors are found in speckles, which in most cases do not colocalize with transcription and splicing sites (Wansink *et al.*, 1993). Therefore, it is likely that splicing factors are recruited to sites of transcription and splicing. Such recruitment has been demonstrated in virally infected cells where

Figure 10. Interference with PP1 activity modulates the nuclear distribution of pre-mRNA splicing factors. Permeabilized cells were incubated for 30 min at 37°C with nuclear extract supplemented with either inhibitor 2 (A and C) or the purified catalytic subunit of PP1 (B and D). Cells were fixed and processed for fluorescence microscopy using antibodies against SC35 (A and B) or SM (C and D). Bar, 10 μ m.

Figure 11. PP1 dephosphorylates SR proteins in nuclear speckles. Cells were permeabilized, washed, and incubated for 30 min at 37°C with either nuclear extract alone, nuclear extract supplemented with the catalytic subunit of PP1, or nuclear extract supplemented with the catalytic subunit of PP2A. The cells were extracted with detergent as described in MATERIALS AND METHODS, and proteins were separated by using SDS gel-electrophoresis, transferred onto nitrocellulose, and the blots probed with mAb104, which recognizes a phosphoepitope on SR proteins.

splicing factors leave the speckles and accumulate at sites of viral transcription (Jiménez-García and Spector, 1993). Similarly, accumulation of large amounts of splicing factors on nascent transcripts can be visualized in transiently transfected cells expressing constructs containing intron sequences (Jiménez-García and Spector, 1993; Huang and Spector, 1996). Given the fact that the two SR protein-specific kinases SRPK-1 and CLK/STY have the ability to displace splicing factors from speckles, it has been proposed that phosphorylation is a means to activate splicing factors and to release them from speckles (Colwill *et al.*, 1996). Release from these storage sites (IGCs) allows splicing factors to be incorporated into forming spliceosomes in PFs at the periphery of the speckles. Based on results from *in vitro* splicing assays (Mermoud *et al.*, 1992, 1994) and the results reported here, it is likely that PP1 activity is directly or indirectly required for the dissociation of splicing factors from the spliceosome and the return of splicing factors to IGCs. Therefore, the phosphorylation in the storage site (IGCs) which activates splicing factors is counteracted by dephosphorylation in PFs, establishing a cycle of phosphorylation and dephosphorylation which determines function and localization of pre-mRNA splicing factors.

The rounding up of speckles in response to α -amanitin occurs because in the absence of active transcription, no nascent RNAs are generated and the released, phosphorylated splicing factors cannot find a target. PP1 present in the nucleoplasm directly or indirectly dephosphorylates the free splicing factors, causing them to return to their storage sites where they accumulate in round speckles. On the other hand, excess PP1 activity results in the increased release of splicing factors from spliceosomes, and since spliceosomes are located predominantly at the periphery of the speckles (Xing *et al.*, 1995), this will result in a diffuse nucleoplasmic distribution of splicing factors.

plasmic distribution, as observed when the catalytic subunit of PP1 was added to permeabilized cells. Finally, inhibition of PP1 by inhibitor 2 results in the failure to resolve spliceosomes and as a consequence splicing factors accumulate in PFs at the periphery of speckles, giving rise to speckles which appear to be enlarged.

Several lines of evidence support this model. First, sites of transcription and splicing have been mapped to the periphery of speckled domains as observed with fluorescence microscopy (Wansink *et al.*, 1993; Xing *et al.*, 1995). Specifically, the site of splicing of actin RNA has been localized either entirely outside of the speckles or in close association with the periphery of the speckles (Zhang *et al.*, 1994; Xing *et al.*, 1995). As determined by electron microscopy, the majority of active transcription sites localize to PFs, which are predominantly found at the periphery of the speckles (Fakan and Bernhard, 1971; Fakan and Nobis, 1978; Fakan, 1994; Bachelierie *et al.*, 1975), suggesting that the sites of spliceosome formation are indeed the PFs. Second, phosphorylation has been demonstrated to be a prerequisite for incorporation of splicing factors into spliceosomes. Using an *in vitro* splicing assay, Mermoud *et al.* (1994) have shown that treatment of nuclear extracts with the catalytic subunit of PP1 abolishes incorporation of splicing factors and formation of spliceosomes *in vitro*, an effect which can be rescued by the readdition of excess amounts of SR proteins. Along with the observation that only phosphorylated SR proteins are functional in the transition from pre-spliceosome to spliceosome, this indicates that splicing factors, at least SR proteins, must be phosphorylated to be incorporated into the spliceosome (Mermoud *et al.*, 1992, 1994; Roscigno and Garcia-Blanco, 1995). SR proteins might not be an exception in this, since kinase activities have been identified which specifically phosphorylate U1-70K (Woppmann *et al.*, 1993) or the snRNP C protein (Meyrand *et al.*, 1993). Third, dephosphorylation by PP1 is required for the resolution of spliceosomes after completion of the splicing reaction. Inclusion of okadaic acid or tautomycin in the *in vitro* reaction allows the formation of spliceosomes, but the execution of the splicing reaction is blocked. When the purified catalytic subunit of PP1 is readded to these stalled spliceosomes, the reaction is completed, strongly suggesting that PP1 activity is required for one or both of the catalytic steps of splicing and, ultimately, for the resolution of the spliceosome (Mermoud *et al.*, 1992, 1994).

Taken together, we suggest that a cycle of phosphorylation and dephosphorylation, possibly involving SRPK1, CLK/STY, and PP1, plays a crucial role in the subnuclear localization of pre-mRNA splicing factors. The localization data shown here extend previously reported biochemical data on the effect of phosphorylation on spliceosome formation and resolution and

demonstrate that the state of phosphorylation of splicing factors does not only affect their function but is also critical for their localization within the cell nucleus.

ACKNOWLEDGMENTS

We thank Mathieu Bollen and Tim Haystead for supplying phosphatase reagents; Mark Roth for antibody mAb104, Larry Gerace for anti-A/C lamin antibody, Joe Kraft for SM antibody, Adrian Krainer for antibodies against m₃G and SF2/ASF; Mike Murray for providing nuclear extracts; Tamara Howard for excellent electron microscopy and help with photography; the members of the Spector lab for discussion and technical advice and Adrian Krainer, Nick Tonks, Mathieu Bollen, and members of the Spector lab for critical reading of this manuscript. T.M. was supported by a fellowship from the Swiss National Science Foundation and the Human Frontiers Science Program. This work was supported by National Institutes of Health grants GM-42694 and 5P30 CA-45508 to D.L.S.

REFERENCES

- Alberts, A.S., Montminy, M., Shenolikar, S., and Feramisco, J.R. (1994). Expression of a peptide inhibitor of protein phosphatase 1 increases phosphorylation and activity of CREB in NIH-3T3 fibroblasts. *Mol. Cell. Biol.* 14, 4398–4407.
- Bachelierie, J.P., Puvion, E., and Zalta, J.P. (1975). Ultrastructural organization and biochemical characterization of chromatin RNA protein complexes isolated from mammalian cell nuclei. *Eur. J. Biochem.* 58, 327–337.
- Bauren, G., and Wieslander, L. (1994). Splicing of Balbiani ring 1 gene pre-mRNA occurs simultaneously with transcription. *Cell* 76, 183–192.
- Bernhard, W. (1969). A new staining procedure for electron microscopical cytology. *J. Ultrastruct. Res.* 27, 250–265.
- Buellers, M., Van Eynde, A., Bollen, M., and Stalmans, W. (1993). Inactivation of nuclear inhibitory polypeptides of protein phosphatase-1 (NIPP-1) by protein kinase A. *J. Biol. Chem.* 268, 13172–13177.
- Beyer, A.L., and Osheim, Y.N. (1988). Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. *Genes Dev.* 2, 754–765.
- Beyer, A.L., and Osheim, Y.N. (1991). Visualization of RNA transcription and processing. *Semin. Cell Biol.* 2, 131–140.
- Bialojan, C., and Takai, A. (1988). Inhibitory effect of a marine sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J.* 256, 283–290.
- Birney, E., Kumar, S., and Krainer, A.R. (1993). Analysis of the RNA-recognition motif and RS- and RGG-domains: conservation in metazoan pre-mRNA splicing factors. *Nucleic Acids Res.* 21, 5803–5816.
- Bisotto, S., Lauriault, P., Duval, M., and Vincent, M. (1995). Colocalization of a high molecular mass phosphoprotein of the nuclear matrix (p255) with spliceosomes. *J. Cell Sci.* 108, 1873–1882.
- Brautigan, D.L., Sunwoo, J., Labbe, J.-C., Fernandez, A., and Lamb, N.J.C. (1990). Cell cycle oscillation of inhibitor-2 in rat fibroblasts coincides with p34cdc2 restriction. *Nature* 344, 74–78.
- Bravo, R., and Macdonald-Bravo, H. (1987). Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. *J. Cell Biol.* 105, 1549–1554.

- Cardinali, B., Cohen, P., and Lamond, A.I. (1994). Protein phosphatase 1 can modulate 5' splice site selection in a HeLa splicing extract. *FEBS Lett.* 352, 276–280.
- Cohen, P., Holmes, C.F.B., and Tsukitani, Y. (1990). Okadaic acid: a new probe for the study of cellular regulation. *Trends Biochem. Sci.* 15, 98–102.
- Colwill, K., Pawson, T., Andrews, B., Prasad, J., Manley, J.L., Bell, J.C., and Duncan, P.I. (1996). The Clk/Sty protein kinase phosphorylates splicing factors and regulates their intranuclear distribution. *EMBO J.* 15, 265–275.
- De Guzman, A., and Lee, E.Y.C. (1988). Preparation of low-molecular-weight forms of rabbit muscle protein phosphatases. *Methods Enzymol.* 159, 356–368.
- Dombradi, V., and Cohen, P.T. (1992). Protein phosphorylation is involved in the regulation of chromatin condensation during interphase. *FEBS Lett.* 312, 21–26.
- Fakan, S. (1994). Perichromatin fibrils are in situ forms of nascent transcripts. *Trends Cell Biol.* 4, 86–90.
- Fakan, S., and Bernhard, W. (1971). Localisation of rapidly and slowly labelled nuclear RNA as visualized by high resolution autoradiography. *Exp. Cell Res.* 67, 129–141.
- Fakan, S., Leser, G., and Martin, T.E. (1986). Immunoelectron microscope visualization of nuclear ribonucleoprotein antigens within spread transcription complexes. *J. Cell Biol.* 103, 1153–1157.
- Fakan, S., and Nobis, P. (1978). Ultrastructural localization of transcription sites and of RNA distribution during the cell cycle of synchronized CHO cells. *Exp. Cell Res.* 113, 327–337.
- Fey, E.G., Krochmalnic, G., and Penman, S. (1986). The nonchromatin substructures of the nucleus: the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. *J. Cell Biol.* 102, 1654–1665.
- Fu, X.D. (1995). The superfamily of arginine/serine-rich splicing factors. *RNA* 1, 663–680.
- Fu, X.D., and Maniatis, T. (1990). Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. *Nature* 343, 437–441.
- Gerace, L., Blum, A., and Blobel, G. (1978). Immunocytochemical localization of the major polypeptide of the nuclear pore complex-lamina fraction. *J. Cell Biol.* 79, 546–566.
- Green, M.R. (1991). Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. *Annu. Rev. Cell Biol.* 7, 559–599.
- Gui, J.F., Lane, W.S., and Fu, S.D. (1994a). A serine kinase regulates intracellular localization of splicing factors in the cell cycle. *Nature* 369, 678–682.
- Gui, J.F., Tronchere, H., Chandler, S.D., and Fu, S.D. (1994b). Purification and characterization of a kinase specific for the serine- and arginine-rich pre-mRNA splicing factors. *Proc. Natl. Acad. Sci. USA* 91, 10824–10828.
- Haaf, T., and Ward, D.C. (1996). Inhibition of RNA polymerase II transcription causes chromatin decondensation, loss of nucleolar structure, and dispersion of chromosomal domains. *Exp. Cell Res.* 224, 163–173.
- Habets, W.J., Hoet, M.H., DeJong, B.A.W., VanDer Kemp, A., and Van Venrooij, W.J. (1986). Mapping of B cell epitopes on small nuclear ribonucleoproteins that react with human autoantibodies as well as with experimentally induced mouse monoclonal antibodies. *J. Immunol.* 143, 2560–2566.
- Högner, D., Lepper, K., Seibold, G., and Jost, E. (1988). Lamin disassembly kinetics: a cell-free system with extracts from mitotic HeLa cells. *Exp. Cell Res.* 176, 281–296.
- Hozak, P., Hassan, A.B., Jackson, D.A., and Cook, P.R. (1993). Visualization of replication factories attached to a nucleoskeleton. *Cell* 73, 361–373.
- Huang, S., and Spector, D.L. (1996). Intron-dependent recruitment of pre-mRNA splicing factors to sites of transcription. *J. Cell Biol.* 131, 719–732.
- Hughes, T.A., Pombo, A., McManus, J., Hozak, P., Jackson, D.A., and Cook, P.R. (1995). On the structure of replication and transcription factories. *J. Cell Sci. Suppl.* 19, 59–65.
- Jackson, D.A., Hassan, A.B., Errington, R.J., and Cook, P.R. (1993). Visualization of focal sites of transcription within human nuclei. *EMBO J.* 12, 1059–1065.
- Jackson, D.A., Yuan, J., and Cook, P.R. (1988). A gentle method for preparing cyto- and nucleoskeletons and associated chromatin. *J. Cell Sci.* 90, 365–378.
- Jagiello, I., Beullens, M., Stalmans, W., and Bollen, M. (1995). Subunit structure and regulation of protein phosphatase-1 in rat liver nuclei. *J. Biol. Chem.* 270, 17257–17263.
- Jiménez-García, L.F., and Spector, D.L. (1993). In vivo evidence that transcription and splicing are coordinated by a recruiting mechanism. *Cell* 73, 47–59.
- Krainer, A.R. (1988). Pre-mRNA splicing by complementation with purified human U1, U2, U4/U6 and U5 snRNPs. *Nucleic Acids Res.* 16, 9415–9429.
- Krainer, A.R., Maniatis, T., Ruskin, B., and Green, M.R. (1984). Normal and mutant human β -globin pre-mRNAs are faithfully and efficiently spliced in vitro. *Cell* 36, 993–1005.
- Kuret, J., Bell, H., and Cohen, P. (1986). Identification of high levels of protein phosphatase-1 in rat liver nuclei. *FEBS Lett.* 203, 197–202.
- Lamm, G.M., and Lamond, A.I. (1993). Non-snRNP protein splicing factors. *Biochim. Biophys. Acta* 1173, 247–265.
- Lindell, T.J., Weinberg, F., Morris, P.W., Roeder, R.G., and Rutter, W.J. (1970). Specific inhibition of nuclear RNA polymerase II by α -amanitin. *Science* 170, 447–448.
- Lührmann, R., Kastner, B., and Bach, M. (1990). Structure of spliceosomal snRNPs and their role in pre-mRNA splicing. *Biochim. Biophys. Acta* 1087, 265–292.
- MacKintosh, C., and Klumpp, S. (1990). Tautomycin from the bacterium *Streptomyces verticillatus*: another potent and specific inhibitor of phosphatases 1 and 2A. *FEBS Lett.* 277, 137–140.
- Mermoud, J.E., Cohen, P.T.W., and Lamond, A.I. (1992). Ser/Thr-specific protein phosphatases are required for both catalytic steps of pre-mRNA splicing. *Nucleic Acids Res.* 20, 5263–5269.
- Mermoud, J.E., Cohen, P.T.W., and Lamond, A.I. (1994). Regulation of mammalian spliceosome assembly by a protein phosphorylation mechanism. *EMBO J.* 13, 5679–5688.
- Meyrand, S.H., Dwen, P., and Pederson, T. (1993). Serine/threonine phosphorylation regulates binding of C hnRNP proteins to pre-mRNA. *Proc. Natl. Acad. Sci. USA* 90, 7764–7768.
- Moore, J.M., Query, C.C., and Sharp, P.A. (1993). Splicing of precursors to mRNA by the spliceosome. In: *The RNA World*, ed. R.F. Gesteland and J.F. Atkins, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 303–358.
- Moroi, Y., Peebles, C., Fritzler, M.J., Steigerwald, J., and Tan, E.M. (1980). Autoantibody to the centromere (kinetochore) in scleroderma sera. *Proc. Natl. Acad. Sci. USA* 77, 1627–1631.
- Northway, J.D., and Tan, E.M. (1972). Differentiation of antinuclear antibodies giving speckled staining patterns in immunofluorescence. *Clin. Immunol. Immunopathol.* 1, 140–154.

- O'Keefe, R.T., Mayeda, A., Sadowski, C.L., Krainer, A.R., and Spector, D.L. (1994). Disruption of pre-mRNA splicing in-vivo results in reorganization of splicing factors. *J. Cell Biol.* 124, 249–260.
- Roscigno, R.F., and Garcia-Blanco, A. (1995). SR proteins escort the U4/U5/U6 tri-snRNP to the spliceosome. *RNA* 1, 692–706.
- Roth, M.B., Murphy, C., and Gall, J.G. (1990). A monoclonal antibody that recognizes a phosphorylated epitope stains lampbrush chromosomes and small granules in the amphibian germinal vesicle. *J. Cell Biol.* 111, 2217–2223.
- Sheen, J. (1993). Protein phosphatase activity is required for light-inducible gene expression in maize. *EMBO J.* 12, 3497–3505.
- Sinclair, G.D., and Brasch, K. (1978). The reversible action of α -amanitin on nuclear structure and molecular composition. *Exp. Cell Res.* 111, 1–14.
- Spector, D. (1993). Macromolecular domains within the cell nucleus. *Annu. Rev. Cell Biol.* 9, 265–315.
- Spector, D.L., Fu, X.-D., and Maniatis, T. (1991). Associations between distinct pre-mRNA splicing components and the cell nucleus. *EMBO J.* 10, 3467–3481.
- Spector, D.L., Schrier, W.H., and Busch, H. (1983). Immunoelectron microscopic localization of snRNPs. *Biol. Cell* 49, 1–10.
- Takai, A., and Mieskes, G. (1991). Inhibitory effect of okadaic acid on the p-nitrophenyl phosphate phosphatase activity of protein phosphatases. *Biochem. J.* 275, 233–239.
- Takai, A., Sasaki, K., Nagai, H., Mieskes, G., Isobe, M., Isono, K., and Yasumoto, T. (1995). Inhibition of specific binding of okadaic acid to protein phosphatase 2A by microcystin-LR, calyculin-A and tautomycin: method of analysis of interactions of tight-binding ligands with target protein. *Biochem. J.* 306, 657–665.
- Turner, B.M., and Franchi, L. (1987). Identification of protein antigens associated with the nuclear matrix and with clusters of interchromatin granules in both interphase and mitotic cells. *J. Cell Sci.* 87, 269–282.
- Van Eynde, A., Wera, S., Beullens, M., Torreken, S., Van Leuven, F., Stalmans, W., and Bollen, M. (1995). Molecular cloning of NIPP-1, a nuclear inhibitor of protein phosphatase 1, reveals homology with polypeptides involved in RNA processing. *J. Biol. Chem.* 270, 28068–28074.
- Verheijen, R., Kuijpers, H., Vooijs, P., Van Venrooij, W., and Ramaekers, F. (1986). Distribution of the 70K U1 RNA-associated protein during interphase and mitosis. Correlation with other snRNP particles and proteins of the nuclear matrix. *J. Cell Sci.* 86, 173–190.
- Wansink, D.G., Schul, W., van der Kraan, I., van Steensel, B., van Driel, R., and de Jong, L. (1993). Fluorescent labelling of nascent RNA reveals transcription by RNA polymerase II in domains scattered throughout the nucleus. *J. Cell Biol.* 122, 282–293.
- Woppmann, A., Will, C.L., Kornstadt, U., Zuo, P., Manley, J.L., and Lührmann, R. (1993). Identification of a snRNP-associated kinase activity that phosphorylates arginine/serine rich domains typical of splicing factors. *Nucleic Acids Res.* 21, 2815–2822.
- Wu, Z., Murphy, C., Callan, H.G., and Gall, J.G. (1991). Small nuclear ribonucleoproteins and heterogeneous nuclear ribonucleoproteins in the amphibian germinal vesicle: loops, spheres and snurposomes. *J. Cell Biol.* 113, 465–483.
- Xing, Y., Johnson, C.V., Moen, P.T., Jr., Mcneil, J.A., and Lawrence, J.B. (1995). Nonrandom gene organization: structural arrangements of specific pre-mRNA transcription and splicing with SC-35 domains. *J. Cell Biol.* 131, 1635–1647.
- Yang, S.D., Vandenheede, J.R., and Merlevede, W. (1981). A simplified procedure for the purification of the protein phosphatase modulator (inhibitor-2) from rabbit skeletal muscle. *FEBS Lett.* 132, 293–295.
- Zhang, G., Taneja, K.L., Singer, R.H., and Green, M.R. (1994). Localization of pre-mRNA splicing in mammalian nuclei. *Nature* 372, 809–812.