

Disruption of Pre-mRNA Splicing In Vivo Results in Reorganization of Splicing Factors

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Abstract. We have examined the functional significance of the organization of pre-mRNA splicing factors in a speckled distribution in the mammalian cell nucleus. Upon microinjection into living cells of oligonucleotides or antibodies that inhibit pre-mRNA splicing in vitro, we observed major changes in the organization of splicing factors in vivo. Interchromatin granule clusters became uniform in shape, decreased in number, and increased in both size and content of splicing factors, as measured by immunofluorescence.

These changes were transient and the organization of splicing factors returned to their normal distribution by 24 h following microinjection. Microinjection of these oligonucleotides or antibodies also resulted in a reduction of transcription in vivo, but the oligonucleotides did not inhibit transcription in vitro. Control oligonucleotides did not disrupt splicing or transcription in vivo. We propose that the reorganization of splicing factors we observed is the result of the inhibition of splicing in vivo.

TRANSSCRIPTION in mammalian cells by RNA polymerase II (Pol II)¹ results mostly in pre-messenger RNAs (pre-mRNAs) that contain intron sequences. These introns must be efficiently removed before the pre-mRNA is transported from the nucleus to the cytoplasm, where it is translated. The removal of introns and the ligation of the remaining exons is catalyzed by small nuclear ribonucleoprotein particles (snRNPs) and a number of non-snRNP protein splicing factors (reviewed in Green, 1991; Guthrie, 1991).

The pre-mRNAs produced by Pol II transcription contain conserved sequences at the 5' and 3' splice sites and a conserved region near the 3' splice site in the intron called the branchpoint. These conserved sequences are recognized by snRNPs and non-snRNP splicing factors as they assemble onto the pre-mRNA to form a spliceosome (reviewed in Green, 1991; Guthrie, 1991). The assembly of snRNPs onto pre-mRNA to form a spliceosome occurs in an ordered pathway that culminates in the removal of intron sequences and ligation of exon sequences. A large number of experiments in yeast and mammalian systems have led to a model for the assembly of snRNPs onto pre-mRNA (reviewed in McKeown, 1993). First, the U1 snRNP recognizes sequences at the 5' splice site in the pre-mRNA. Next, the U2 snRNP binds to

the branchpoint region. Following the binding of the U1 and U2 snRNPs to the pre-mRNA, a pre-assembled U4/U6/U5 particle associates with the bound snRNPs and the pre-mRNA, with U5 interacting with 5' and 3' exon sequences. The U1 association is then destabilized, U5 binds to the 5' end of the intron, and the U4/U6 helix becomes partially unpaired resulting in the association of U6 with U2 forming a U2/U4/U6 complex. Finally, the U4 snRNP exits the complex leaving a U2/U6 complex with U2 bound at the branchpoint and U6 associated with the 5' end of the intron. This U2/U6-pre-mRNA complex has been proposed to be a component of the spliceosomal active site (Madhani and Guthrie, 1992). It is this ordered interaction of RNAs and proteins that enables the cellular splicing machinery to remove introns from the pre-mRNA correctly.

In addition to the snRNPs, a number of protein factors have been identified which are required for spliceosome assembly and splicing. For example, the protein factors U2AF (Ruskin et al., 1988; Zamore and Green, 1989), SF1, and SF3 (Krämer and Utans, 1991) are required for the binding of the U2 snRNP to the intron branchpoint and assembly of the pre-splicing complex. Two other non-snRNP splicing factors, SF2/ASF (Krainer et al., 1990b) and SC-35 (Fu and Maniatis, 1990; Spector et al., 1991), are both required for the first step of splicing and spliceosome assembly. SF2/ASF and SC-35 are also involved in 5' splice site selection of alternatively spliced pre-mRNAs (Ge and Manley, 1990; Krainer et al., 1990a; Fu et al., 1992).

The snRNPs and some non-snRNP splicing factors have been localized in the mammalian cell nucleus. The snRNPs

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1. *Abbreviations used in this paper:* AdML, adenovirus major late; m₃G, 2,2,7-trimethylguanosine cap; Pol II, RNA polymerase II; snRNP, small nuclear ribonucleoprotein particle.

are organized in a specific distribution within the nucleus that has been termed the speckled pattern (Northway and Tan, 1972; Perraud et al., 1979; Lerner et al., 1981; Spector et al., 1983; Nyman et al., 1986; Verheijen et al., 1986; Habets et al., 1989). The speckled pattern is composed of 20–50 intensely stained and irregularly shaped regions in the nucleus that are connected in places and set against a diffuse nuclear labeling (Spector, 1990). The use of antibodies to the trimethylguanosine cap of some of the snRNAs (Reuter et al., 1984) and of antisense probes to the snRNAs (Carmo-Fonseca et al., 1992; Huang and Spector, 1992) revealed that the RNA components of the snRNPs are also organized in the nucleus as a speckled pattern. Additionally, the essential non-snRNP splicing factor SC-35 displays a speckled distribution in the nucleus that colocalizes with snRNPs except that it does not give diffuse nuclear labeling (Fu and Maniatis, 1990; Spector et al., 1991). In the nucleus snRNPs and U2AF are also concentrated in coiled bodies, but SC-35 is not (reviewed in Lamond and Carmo-Fonseca, 1993). However, the functional significance of the localization of some splicing factors in the coiled body remains to be elucidated.

Electron microscopy with antibodies to snRNPs and SC-35 showed that the speckled pattern seen by immunofluorescence corresponds to structures termed interchromatin granules and perichromatin fibrils (Spector et al., 1983; Fakan et al., 1984; Puvion et al., 1984; Spector, 1990; Spector et al., 1991). Interchromatin granules correspond to the larger intensely stained and irregularly shaped speckles seen by immunofluorescence; they have little to no [³H]uridine labeling in their interior (Fakan and Bernhard, 1971; Fakan and Nobis, 1978) and may represent the sites of splicing factor storage and/or assembly. Perichromatin fibrils are found on the surface of and between interchromatin granule clusters; they are rapidly labeled with [³H]uridine (Bachelier et al., 1975; Fakan et al., 1976) and are thought to represent nascent pre-mRNA transcripts. It was proposed that snRNPs from interchromatin granule clusters move to the sites of active transcription (perichromatin fibrils) to splice nascent pre-mRNA transcripts (Jiménez-García and Spector, 1993).

Numerous studies have used oligonucleotides and antibodies targeted to snRNAs to inhibit splicing *in vitro* (Padgett et al., 1983; Krämer et al., 1984; Black et al., 1985; Krainer and Maniatis, 1985; Berget and Roberson, 1986; Black and Steitz, 1986; Fabrizio et al., 1989; McPheeters et al., 1989; Fabrizio and Abelson, 1990; Krämer, 1990; McPheeters and Abelson, 1992). Oligonucleotides have also been microinjected into *Xenopus* oocytes to inhibit splicing *in vivo* (Pan and Prives, 1988, 1989; Hamm et al., 1989; Pan et al., 1989; Hamm et al., 1990; Vankan et al., 1990, 1992; Prives and Foukal, 1991; Tsvetkov et al., 1992). These *in vitro* and *in vivo* studies have contributed to the demonstration that snRNAs are essential for splicing and have helped to identify specific regions of snRNAs that are important for this nuclear function. The mechanism by which oligonucleotides inhibit splicing is via the degradation of the snRNAs through the ribonuclease H activity found both in splicing extracts and *in vivo* (Hélène and Toulmé, 1990). Antibodies probably inhibit splicing by binding to the snRNAs and thus preventing their interactions with the pre-mRNA and other snRNAs.

In this study, we have investigated the effects of disruption of splicing *in vivo* on the organization of splicing factors in the mammalian cell nucleus. We have microinjected into liv-

ing cells oligonucleotides or antibodies that inhibit splicing *in vitro* and then visualized the organization of splicing factors in the nucleus. Following microinjection, splicing factors were reorganized into interchromatin granule clusters that decreased in number and became rounder and larger in size. The reorganization of splicing factors observed in the oligonucleotide-injected cells was transient, and splicing factors returned to their normal distribution over time. The transcriptional activity of cells injected with oligonucleotides or antibodies that inhibit splicing *in vitro* was reduced. This did not result from a direct effect of the oligonucleotides on transcription, and appears to be a consequence of the disruption of splicing. Therefore, the disruption of splicing *in vivo* results in the reorganization of splicing factors to larger, rounder and fewer interchromatin granule clusters. The disruption of splicing also results in the reduction of transcription possibly due to the temporal and spatial coupling of transcription and pre-mRNA splicing *in vivo*.

Materials and Methods

Oligonucleotides and Antibodies for Microinjection

Oligodeoxynucleotides were synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Foster City, CA), dried down, and resuspended in 1 ml deionized water. The oligonucleotides were then isolated from this solution with a C₁₈ SEP-PAK cartridge (Millipore Corp., Bedford, MA), dried down, and resuspended in 100 μ l microinjection buffer (10 mM NaH₂PO₄, 70 mM KCl, pH 7.2). The concentration of each oligonucleotide solution was calculated by reading the OD₂₆₀ and using the extinction coefficient for each oligonucleotide. The following oligonucleotides were used: U1 Comp (5'-CTCCCCTGCCAGGTAAGTAT-3'), complementary to nucleotides 1–20 of U1 snRNA (Pan and Prives, 1988); U1-20 (5'-ATACTT-ACCTGGCAGGGGAG-3'), identical to nucleotides 1–20 of U1 snRNA; U6 Comp (5'-CCTTGCGCAGGGGCCATGCTAATC-3'), complementary to nucleotides 49–72 of U6 snRNA; Control (5'-TCCGGTACCACGACG-3') (Pan and Prives, 1988).

Purified antibodies to the 2,2,7-trimethylguanosine cap (m₃G) of snRNAs (Krainer, 1988) or goat anti-rat antibodies (Organon Teknica-Cappel, West Chester, PA) were applied to a G-25 column (PD-10; Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with microinjection buffer. Fractions containing antibodies were pooled and concentrated with a Centricon 30 microconcentrator (Amicon Corp., Danvers, MA).

Cell Culture and Microinjection

HeLa cells were grown on photoetched glass coverslips (Bellco Glass, Inc., Vineland, NJ) in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Gaithersburg, MD) containing 10% fetal bovine serum and antibiotic-antimycotic (GIBCO-BRL). Oligonucleotides in microinjection buffer were injected at a concentration of 150 μ M with 15 mg/ml lysine fixable, Texas red-conjugated 70-kD dextrans (Molecular Probes Inc., Junction City, OR) into the cytoplasm of cells. The oligonucleotides were free to move into the nucleus, whereas the dextrans were restricted to the cytoplasm by their size and thereby marked the cytoplasm of the injected cells. Antibodies in microinjection buffer were injected at a concentration of 1 mg/ml with 1.5 mg/ml dextrans into the nucleus of cells. The antibodies and dextrans both stayed in the nucleus because of their size, with the dextrans marking the injected nuclei.

The oligonucleotides and antibodies were loaded into microinjection needles (Femtotips, Eppendorf; Brinkman Instruments Inc., Westbury, NY) and injected into cells using an Eppendorf Microinjector 5442 system paired with a Zeiss Axiovert 405M inverted microscope. Following microinjection cells were washed three times with DMEM at 37°C and returned to the incubator for the specified amount of time.

Immunofluorescence and Light Microscopy

Cells were washed once with PBS, pH 7.4, and then fixed for 15 min at room temperature with 2% formaldehyde in PBS made fresh from paraformal-

hyde. Cells were then washed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 5 min on ice. Cells were washed with PBS and then incubated with either monoclonal SC-35 ascites fluid (Fu and Maniatis, 1990) diluted 1:1,000 in PBS or purified monoclonal m₃G antibody (Krainer, 1988) diluted 1:1,000 in PBS for 1 h at room temperature. Cells were washed with PBS and then incubated with FITC-conjugated goat anti-mouse secondary antibodies (Organon Teknika) diluted 1:50 in PBS for 1 h at room temperature. Cells were washed with PBS and the coverslips were mounted on glass slides with 9:1 glycerol/PBS containing 0.1% *p*-phenylenediamine buffered to pH 8.0 with 0.5 M carbonate/bicarbonate buffer (Johnson and Nogueira Araujo, 1981). Following microinjection of m₃G antibodies, cells were fixed and permeabilized as described above and then incubated with rat anti-mouse antibodies (Organon Teknika) diluted 1:3 in PBS for 1 h at room temperature to mask any mouse antibody epitopes that may be recognized by the FITC-conjugated goat anti-mouse secondary antibodies. The m₃G-injected cells were then labeled for SC-35 localization as described above. Cells prepared for immunofluorescence were photographed using either Kodak T-Max 3200 or Kodak Ektachrome 400 film using a Nikon Microphot-FXA epifluorescence microscope equipped with a 60X, 1.4 NA objective lens.

Cycloheximide Treatment

The culture medium of HeLa cells grown as described above was removed and replaced with culture medium containing 100 µg/ml cycloheximide (Sigma Immunochemicals, St. Louis, MO) for 2 h. Cells were then prepared for immunofluorescence as described above and for light microscopic autoradiography as follows.

Light Microscopic Autoradiography

5,6-[³H]Uridine (Amersham Corp.) was added to a final concentration of 50 µCi/ml in the culture medium of HeLa cells that were microinjected or treated with cycloheximide prior to fixation. Incorporation was carried out for 10 min at 37°C and the cells were washed two times for 2 min each with DMEM containing 1 mg/ml nonradioactive Uridine (Fluka AG, Buchs, Switzerland) at 37°C. Cells were then fixed and processed for immunofluorescence as described above. Prior to mounting, the coverslips were washed once with water, coated with Ilford K.5D autoradiographic emulsion, and allowed to dry. Coverslips were stored in the dark at 4°C for 7–21 d, and then the emulsion was developed (0.02 M methylaminophenol sulfate, 0.04 M sodium sulfite, 0.02 M potassium thiocyanate) for 7 min, washed twice with water for 2 min each, fixed for 5 min with Kodak fixer, and washed with water. The coverslips were then mounted as described above.

Electron Microscopy

For electron microscopy, HeLa cells were cultured and microinjected as described above. At 2 h after microinjection, cells were washed once with PBS and fixed in 2% formaldehyde/2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. Cells were then washed three times for 5 min each with 0.1 M cacodylate buffer and postfixed with 2% osmium tetroxide in 0.1 M cacodylate buffer. Next, cells were washed three times for 5 min each with water followed by en bloc staining for 1 h with 2% uranyl acetate. The cells were then washed again with water, dehydrated with increasing percentages of ethanol, and infiltrated overnight with a 50:50 mixture of 100% ethanol and Epon-Araldite. The coverslips were then embedded in 100% Epon-Araldite and polymerized at 60°C overnight. The glass coverslips were then removed with hydrofluoric acid and the injected areas were cut out and reembedded for ultramicrotomy. Thin sections were cut with a diamond knife (Diatome Ltd., Bienne, Switzerland) using a Reichert-Jung Ultracut E ultramicrotome. Sections ~90 nm in thickness were picked up on 300-mesh copper grids and stained with 2% uranyl acetate and lead citrate (Reynolds, 1963) and viewed with a Hitachi H-7000 transmission electron microscope operated at 75 kV.

In Vitro Splicing Assay

For the in vitro splicing assay, the oligonucleotides were purified by preparative electrophoresis on 20% acrylamide/7 M urea gels and the DNA was recovered from the excised and crushed gel slices by elution overnight in 1 ml deionized water. Gel fragments were removed with a 0.22 µm filter and the DNA in the remaining solution was isolated with a C₁₈ SEP-PAK cartridge, dried down, and resuspended in 100 µl diethyl pyrocarbonate treated water. The plasmid pSP64-HβΔ6 (Krainer et al., 1984), containing

the human β-globin gene, was linearized with BamHI. ³²P-labeled, capped pre-mRNA substrates were prepared by runoff transcription with SP6 RNA polymerase as described (Krainer and Maniatis, 1985; Mayeda and Ohshima, 1988). β-globin pre-mRNA (20 fmol), 10 µl HeLa cell nuclear extract, and each oligonucleotide (300 pmol, final concentration 12 µM) was pre-incubated in 17 µl with 20 mM Hepes-KOH (pH 7.3) at 30°C for 15 min prior to the splicing reaction. For the splicing reaction, ATP/creatine phosphate, MgCl₂, KCl, and polyvinyl alcohol were added to a final volume of 25 µl and incubated at 30°C for 3 h as described (Mayeda et al., 1990). RNA products were analyzed by electrophoresis on a 5.5% polyacrylamide/7 M urea gel followed by autoradiography with an intensifying screen at -70°C.

In Vitro Transcription Assay

For the in vitro transcription assay the oligonucleotides were purified in the same manner as was described for the in vitro splicing assay. A G-less cassette construct containing the adenovirus 2 major late promoter (Sawadogo and Roeder, 1985; Lobo et al., 1992) was used in a 25 µl transcription reaction containing the following components: 60 mM KCl, 20 mM Hepes (pH 7.9), 9 mM MgCl₂, 12% glycerol, 1 mM spermidine, 1 mM 3'-O-methyl-GTP, 0.5 µg of supercoiled template, 0.5 U of ribonuclease T1, and 4 µl (100 µg) of whole cell extract. To this was added either water, 2 µg/ml α-amanitin, or oligonucleotide (300 pmol, final concentration 12 µM). The reactions were incubated at 30°C for 1 h and terminated by the addition of stop mix and processed as described (Sadowski et al., 1993). Preincubation of the oligonucleotides with the extract before the addition of nucleotides made no difference in the levels of transcription observed.

Results

Oligonucleotides Targeted to snRNAs Inhibit Splicing In Vitro

The assembly of snRNPs onto a pre-mRNA to form a spliceosome, within which introns are removed, requires specific RNA/RNA interactions. One of the first RNA/RNA interactions involved in spliceosome assembly and splicing is the association of the U1 snRNP with the pre-mRNA. The U1 snRNA has been shown to bind specifically to the pre-mRNA conserved sequences at the 5' splice site and to the conserved AG at the 3' splice site to set up interactions between other snRNPs and the pre-mRNA (Reich et al., 1992; Wassarman and Steitz, 1992). To disrupt these interactions and inhibit splicing, an oligonucleotide was synthesized that was complementary to the 5' end of the U1 snRNA (U1 Comp). This oligonucleotide is similar to oligonucleotides used to inhibit splicing in amphibian oocytes (Pan and Prives, 1988; Pan et al., 1989; Prives and Foukal, 1991; Jantsch and Gall, 1992; Tsvetkov et al., 1992) and should bind to U1 snRNA and prevent its association with the pre-mRNA by causing cleavage at its 5' end. When this oligonucleotide was added to an in vitro splicing reaction, it inhibited the splicing of a β-globin pre-mRNA substrate whereas a control oligonucleotide (*Control*) did not (Fig. 1, compare lanes 3 and 2).

The next oligonucleotide we used to disrupt splicing was identical to the 20 nucleotides at the 5' end of the U1 snRNA (U1-20). This oligonucleotide should compete with U1 snRNPs for binding to the pre-mRNA and therefore disrupt splicing. This oligonucleotide is identical to the 5' end of the U1 snRNA so it may also compete with the U5 snRNP (Newman and Norman, 1992; Wassarman and Steitz, 1992) and U6 snRNP (Madhani and Guthrie, 1992; Sawa and Abelson, 1992; Sawa and Shimura, 1992; Wassarman and Steitz, 1992), which have also been shown to interact at, or near, the 5' and 3' splice sites. When this oligonucleotide was

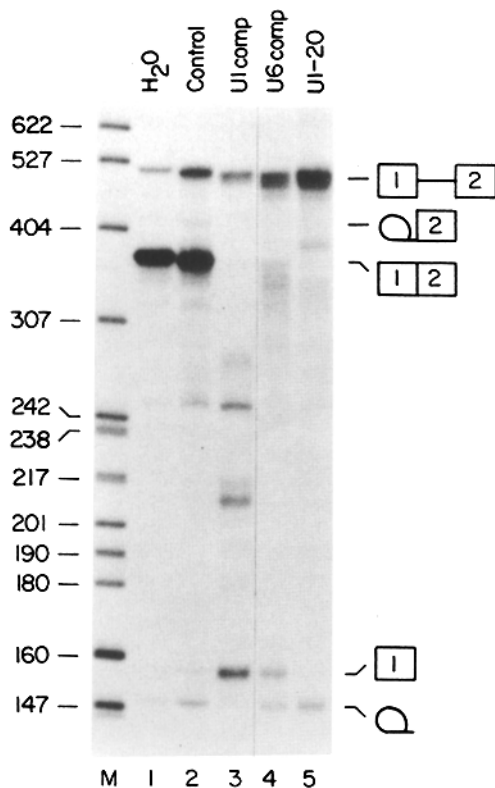


Figure 1. Oligonucleotides complementary to snRNAs or pre-mRNA inhibit splicing of a pre-mRNA substrate in vitro. In vitro splicing of a β -globin pre-mRNA containing the first two exons and first intron was analyzed following preincubation of the HeLa nuclear extract with oligonucleotides. The oligonucleotides that were complementary to snRNAs or pre-mRNA inhibited the splicing of the β -globin pre-mRNA substrate (lanes 3–5). A control oligonucleotide does not inhibit the splicing of the β -globin pre-mRNA (lane 2). The pre-mRNAs in lanes 3–5 are partially cleaved to various extents, most likely by endogenous ribonuclease H in combination with other ribonucleases. Some of the resulting fragments fortuitously co-migrate with exon 1 or excised intron RNAs. Lane 1, water added in same volume as oligonucleotides. Lane 2, Control oligonucleotide. Lane 3, U1 Comp oligonucleotide complementary to 5' end of U1 snRNA. Lane 4, U6 Comp oligonucleotide complementary to nucleotides 49–72 of U6 snRNA. Lane 5, U1-20 oligonucleotide identical to the 5' end of the U1 snRNA. Lane M, 32 P-labeled pBR322/HpaII DNA size markers with length in nucleotides. β -globin pre-mRNA and its splicing products are schematically represented on the right side.

added to an in vitro splicing reaction, it also inhibited the splicing of a β -globin pre-mRNA substrate (Fig. 1, compare lanes 5 and 2).

Another RNA/RNA interaction that is important for splicing is the association of U6 snRNA with U4 snRNA (Bringmann et al., 1984; Hashimoto and Steitz, 1984; Rinke et al., 1985) and U2 snRNA (Madhani and Guthrie, 1992; McPheeters and Abelson, 1992). To disrupt these interactions and inhibit splicing, we synthesized an oligonucleotide complementary to the region of U6 that binds to U4 and U2 snRNA (U6 Comp). As for the U1 Comp and U1-20 oligonucleotides, the U6 Comp oligonucleotide inhibited the splicing of a β -globin pre-mRNA substrate (Fig. 1, compare lanes 4 and 2). Oligonucleotides that target different regions of U6 snRNA for ribonuclease H degradation have previously been

used to inhibit splicing in *Xenopus* oocytes (Vankan et al., 1990, 1992). These results demonstrate that the oligonucleotides targeted to disrupt splicing can specifically inhibit the processing of a pre-mRNA substrate in vitro.

Microinjection of Oligonucleotides or Antibodies that Inhibit Splicing In Vitro Results in Reorganization of Splicing Factors In Vivo

To determine what effect the disruption of splicing in vivo has on the organization of splicing factors in the nucleus we microinjected the oligonucleotides that inhibited splicing in vitro into HeLa cells. The oligonucleotides were co-injected into the cytoplasm of living cells with fixable, Texas red-conjugated, 70-kD dextrans. The dextrans mark the cytoplasm of injected cells, whereas the small oligonucleotides are free to diffuse into the nucleus. At 2 h after microinjection, the cells were fixed and an antibody directed against the splicing factor SC-35 was used to examine the nuclear organization of this splicing factor. For each of the oligonucleotides that inhibited splicing in vitro, U1 Comp (Fig. 2 a), U1-20 (Fig. 2 b), and U6 Comp (Fig. 2 c), there was a reorganization of SC-35 compared to the noninjected cells in the same field. The irregularly shaped speckles became larger, rounded, and decreased in number with a concurrent increase in their fluorescent intensity. In addition, the connections that are normally observed between speckles were absent. Microinjection of the Control oligonucleotide that did not inhibit splicing in vitro had no effect on the organization of SC-35 (Fig. 2 e).

Since the oligonucleotides were microinjected into the cytoplasm of cells it is possible that the experimental oligonucleotides may exert their effects there as well. The snRNAs are known to assemble with their respective snRNP proteins in the cytoplasm (Zieve and Sauterer, 1990). Therefore, the U1 Comp oligonucleotide may prevent the import of new U1 snRNA into the nucleus because of the loss of the m_3G cap at its 5' end needed for nuclear import (Hamm et al., 1990; Jantsch and Gall, 1992).

The changes observed in splicing factor organization following microinjection of oligonucleotides targeted to disrupt splicing were transient. At 5–6 h following microinjection of the U1 Comp oligonucleotide the localization of SC-35 began to return to normal and by 24 h most of the microinjected cells had reverted to the typical nuclear distribution of SC-35 (Fig. 2 f). The return of SC-35 to its normal distribution over time was also observed following microinjection of the other oligonucleotides that inhibited splicing in vitro and reorganized SC-35 (data not shown). This reversion can be explained by the fact that microinjected oligonucleotides are eventually degraded in vivo (Pan and Prives, 1988) and that new snRNA synthesis and snRNP assembly will occur.

The reorganization of splicing factors is not restricted to microinjected oligonucleotides that inhibit splicing. Microinjection into the nucleus of cells of antibodies to the m_3G cap of snRNAs that inhibit splicing in vitro (Krämer et al., 1984) also resulted in the reorganization of SC-35 within the nucleus (Fig. 2 d) similar to that observed with the oligonucleotides. The speckles again rounded up, became larger, and decreased in number. In addition, there was a loss of connections between speckles that are normally observed. Microinjection of a control goat anti-rat antibody, however,

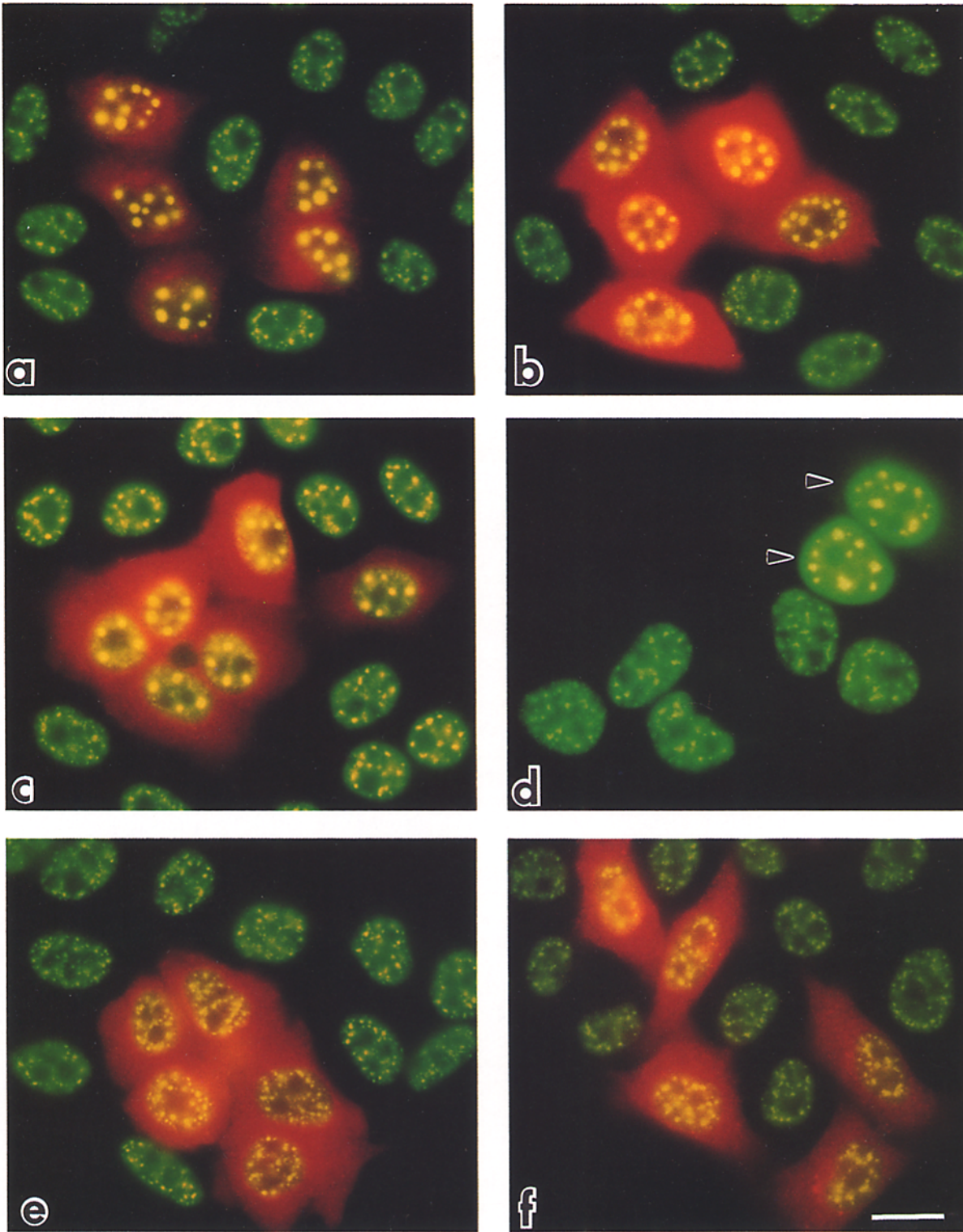


Figure 2. Microinjection of oligonucleotides or antibodies that inhibit splicing *in vitro* results in the reorganization of SC-35 *in vivo*. Oligonucleotides were microinjected into the cytoplasm of HeLa cells with a Texas red-labeled dextran to mark the cytoplasm of the injected cells. At 2 h after microinjection the cells were processed for immunofluorescence of the splicing factor SC-35 (green) as described in Materials and Methods. Cells microinjected with the oligonucleotides that inhibit splicing *in vitro*, U1 Comp (a), U1-20 (b), and U6 Comp (c), display a reorganization of splicing factors to large, rounded structures in the nucleus. The Control oligonucleotide (e) that does not inhibit splicing *in vitro*, did not change the organization of SC-35 when injected into cells. At 24 h after microinjection the reorganization seen with the U1 Comp oligonucleotides was no longer apparent in the injected cells (f) which display a normal distribution of SC-35. Nuclear microinjection of antibodies against the m³G cap of snRNAs and Texas red-labeled dextrans (d) also resulted in the reorganization of SC-35 similar to that observed with the oligonucleotides. In d only SC-35 labeling (green) is shown and the injected nuclei are indicated with arrows. Bar, 20 μ m.

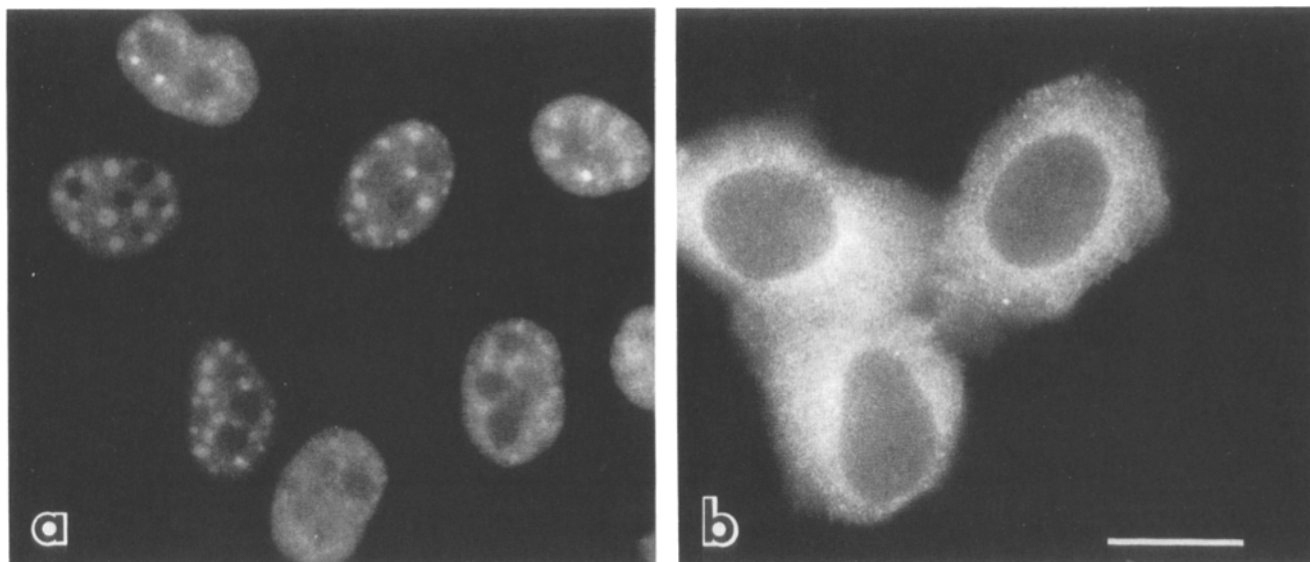


Figure 3. Microinjection of oligonucleotides that inhibit splicing *in vitro* results in the reorganization of snRNAs *in vivo*. The U1 Comp oligonucleotide was microinjected into the cytoplasm of HeLa cells with a Texas red-labeled dextran to mark the cytoplasm of the injected cells. At 2 h after microinjection the cells were processed for immunofluorescence to reveal the localization of snRNAs as described in Materials and Methods. snRNAs detected with antibodies to their m₃G cap (a) are reorganized to rounded structures within the nuclei of oligonucleotide injected cells that are marked in their cytoplasm with the Texas red-labeled dextrans (b). The oligonucleotide injected cells have a different nuclear organization of snRNAs than the noninjected cells in the same field. This reorganization is similar to that observed when oligonucleotides that inhibit splicing were microinjected into cells and labeled with antibodies to SC-35 (Fig. 2). Bar, 20 μ m.

did not affect the organization of splicing factors (see Fig. 6, b and f).

The effects observed within the nucleus following microinjection of oligonucleotides that inhibit splicing were not limited only to SC-35. If cells were microinjected with the U1 Comp oligonucleotide (Fig. 3) and then processed for immunofluorescence with an antibody specific to the m₃G cap of snRNAs, a reorganization of snRNAs was also observed (Fig. 3). This reorganization of snRNAs was similar to that observed with SC-35 following oligonucleotide injection. A reorganization of snRNAs was also observed with the other oligonucleotides that inhibit splicing, but not the Control oligonucleotide (data not shown).

To determine the nature of the enlarged and rounded speckles, we prepared cells microinjected with the U6 Comp oligonucleotide for electron microscopy. Visualization of oligonucleotide injected cells by electron microscopy revealed that these rounded speckles corresponded to enlarged interchromatin granule clusters (Fig. 4 a) as compared to the smaller and irregularly shaped interchromatin granule clusters observed in non-injected cells or cells microinjected with the Control oligonucleotide (Fig. 4 b). These results, and the immunofluorescent results described above, indicate that the disruption of splicing *in vivo* by two different methods caused a reorganization of splicing factors to larger and rounder interchromatin granule clusters.

Transcription is Reduced in Cells Microinjected with Oligonucleotides or Antibodies that Inhibit Splicing In Vitro

The reorganization of splicing factors seen following microinjection of oligonucleotides or antibodies that disrupt splicing is similar to the changes observed when cells are treated

with inhibitors of transcription (Spector et al., 1983; Carmo-Fonseca et al., 1992). To investigate what effects microinjection of the oligonucleotides or antibodies had on the transcriptional activity of cells, we added [³H]uridine to the culture medium prior to fixation. Cells were then processed for immunofluorescence of splicing factors, coated with an autoradiographic emulsion, and developed for light microscopic autoradiography at various times following coating. As seen in Fig. 5, microinjection of either the U6 Comp oligonucleotide (Fig. 5, a and b) or m₃G antibodies (Fig. 5, c and d) that disrupt splicing reduced the transcriptional activity of the injected cells (arrows). This was observed as fewer autoradiographic grains over the injected cells as compared to the non-injected cells in the same field. The U1 Comp and U1-20 oligonucleotides also reduced transcription in the injected cells (data not shown). The control oligonucleotides (Fig. 6, a, c, and e) or control goat anti-rat antibodies (Fig. 6, b, d, and f), however, did not affect the transcriptional activity of injected cells, as compared to non-injected cells in the same field.

The reduction in transcription could result either from a direct inhibitory effect of the oligonucleotides and antibodies, or from the disruption of splicing. To distinguish between these two possibilities, we added the oligonucleotides to an *in vitro* transcription reaction to determine whether they had any effect on transcription by Pol II. Addition of both specific and control oligonucleotides to an *in vitro* transcription reaction programmed with the adenovirus major late (AdML) promoter did not have any major effect on transcription (Fig. 7, lanes 3–6). There is no significant difference in the amount of transcription product made when the U1 Comp or the Control oligonucleotides were added to the reaction (Fig. 7, compare lanes 3 and 4). Although there

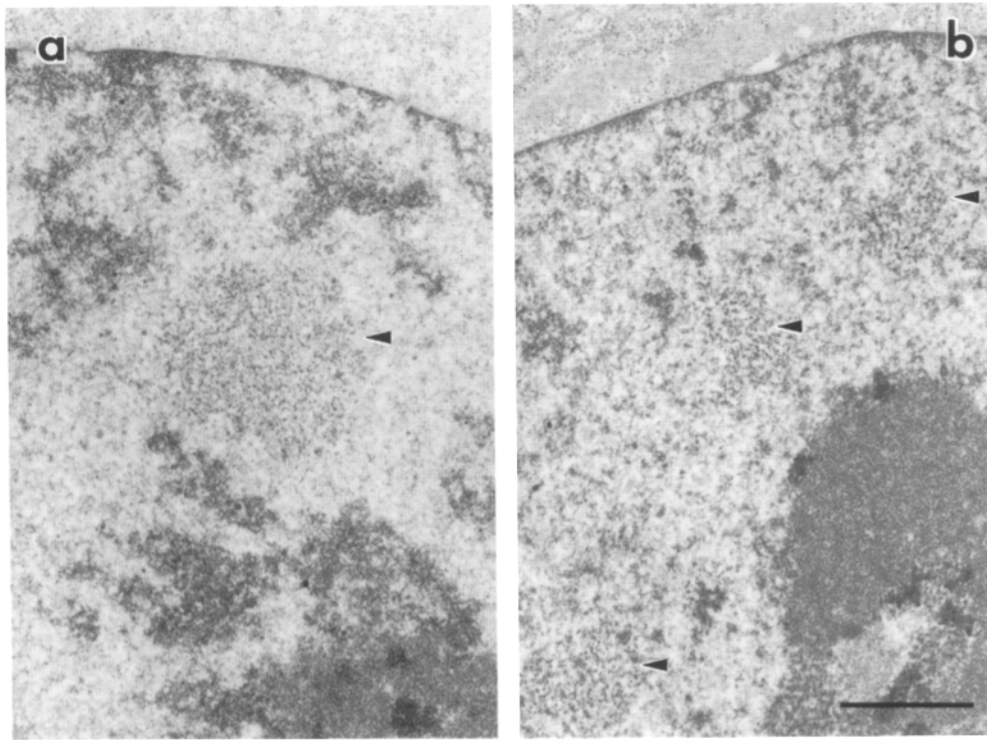


Figure 4. Interchromatin granule clusters become enlarged and uniform in shape following microinjection of oligonucleotides that inhibit splicing. Oligonucleotides that inhibit splicing in vitro were microinjected into cells, which were then processed for electron microscopy as described in Materials and Methods. Interchromatin granule clusters (*arrows*) became larger, rounded, and fewer in number following microinjection of the U6 Comp oligonucleotide (*a*) compared to interchromatin granule clusters in cells microinjected with the Control oligonucleotide (*b*). The enlarged interchromatin granule cluster observed in the U6 Comp injected cells (*a*) corresponds to the large, rounded, structures that were observed by immunofluorescence in the oligonucleotide injected cells (Figs. 2 and 3). Bar, 1 μ m.

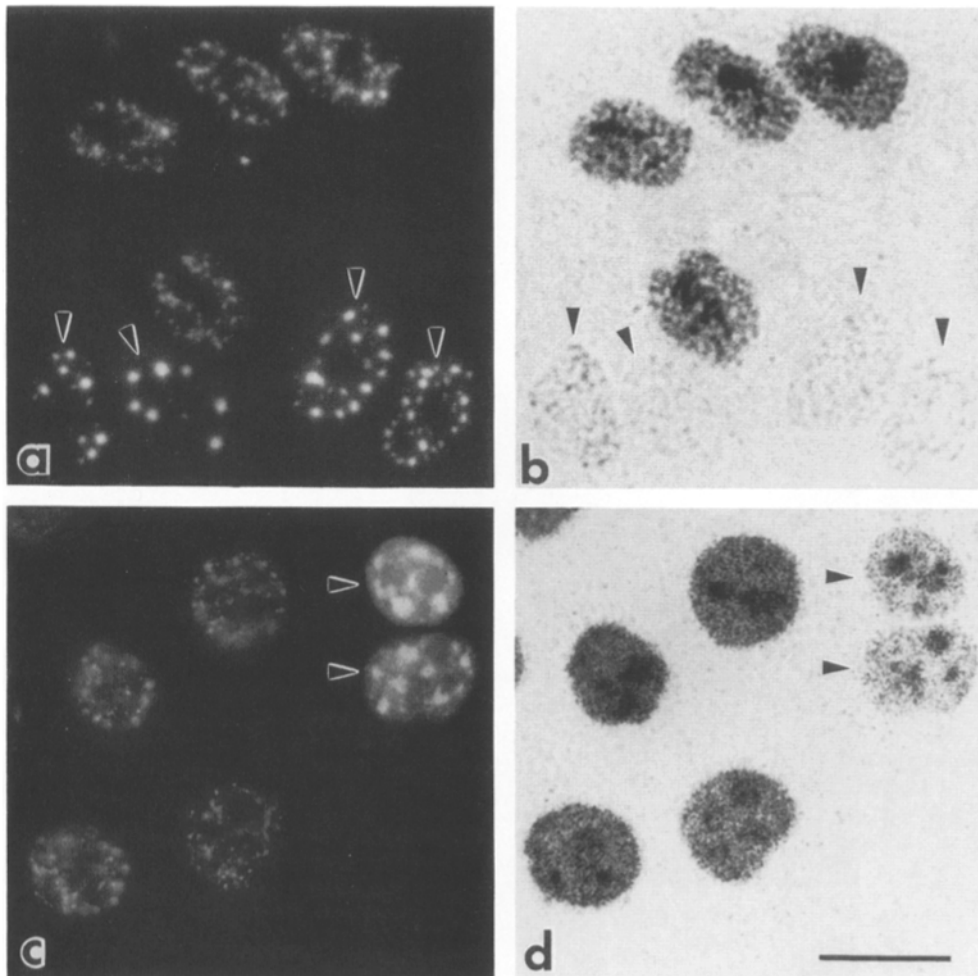


Figure 5. Microinjection of oligonucleotides or antibodies that inhibit splicing results in a reduction of the transcriptional activity of cells. HeLa cells were microinjected with the U6 Comp oligonucleotide (*a* and *b*) or antibodies against the m^3G cap of snRNAs (*c* and *d*). At 2 h after injection the cells were labeled with [3H]uridine to monitor their transcriptional activity (*b* and *d*) and the cells were processed for immunofluorescence of SC-35 (*a* and *c*) and light microscopic autoradiography as described in Materials and Methods. Autoradiography shows that both oligonucleotides (*b*) and antibodies (*d*) that inhibit splicing and caused a reorganization of splicing factors (*a* and *c*, *arrows*), also disrupt transcription in the injected cells (*b* and *d*, *arrows*). The cells shown in this figure are representative of many injected cells that all had decreased transcriptional activity. Bar, 20 μ m.

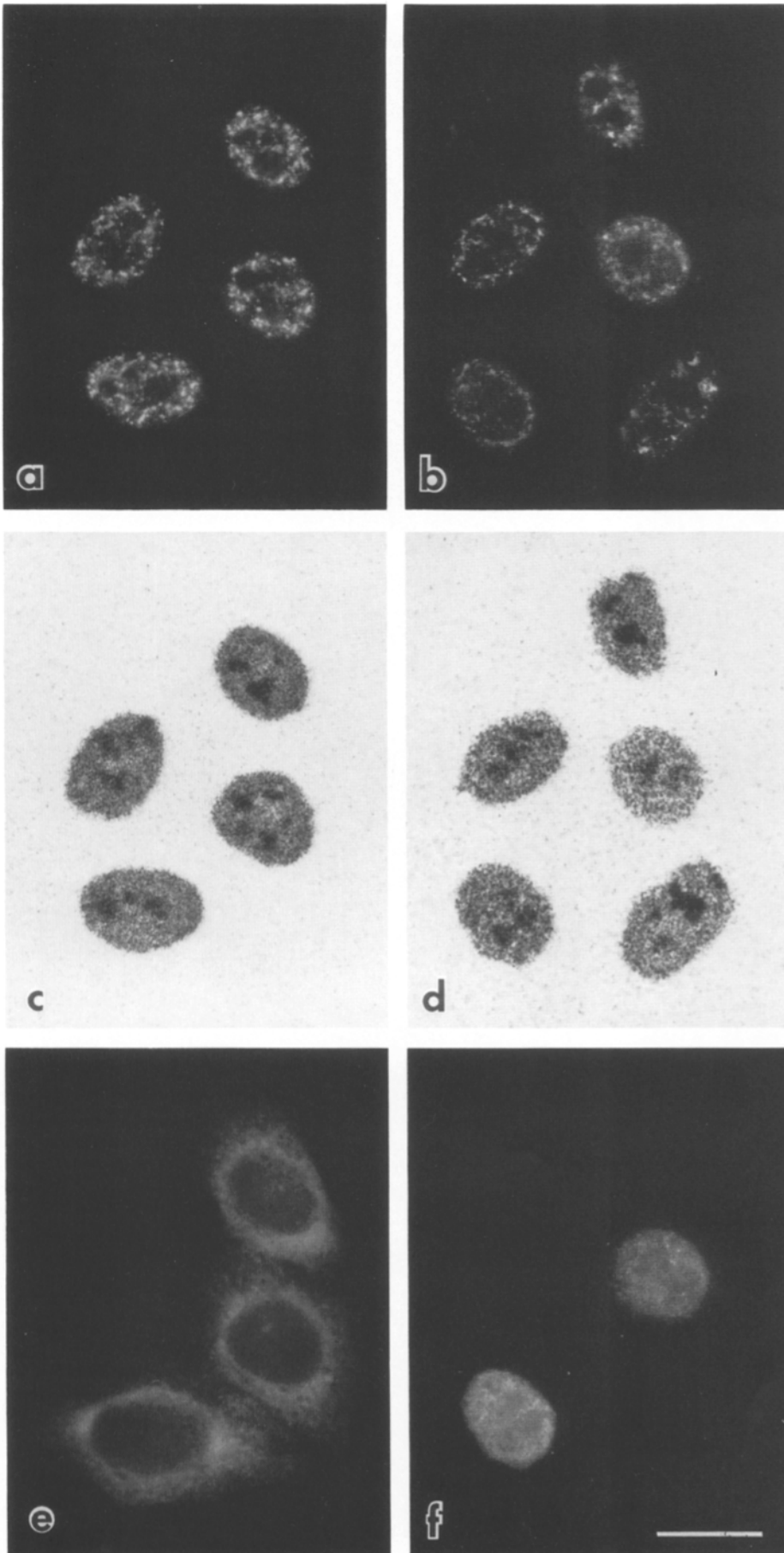


Figure 6. Microinjection of control oligonucleotides or antibodies does not change the transcriptional activity of cells. HeLa cells were microinjected in the cytoplasm with the Control oligonucleotide (*a*, *c*, and *e*) or in the nucleus with a control goat anti-rat antibody (*b*, *d*, and *f*). At 2 h after injection the cells were labeled with [³H]Juridine to monitor transcriptional activity (*c* and *d*) and processed for immunofluorescence of SC-35 (*a* and *b*) and light microscopic autoradiography as described in Materials and Methods. Microinjection of the Control oligonucleotide (*a*) or a control goat anti-rat antibody (*b*) does not change the localization of SC-35. The transcriptional activity of the Control oligonucleotide (*c*) or the control goat anti-rat antibody (*d*) injected cells is also unchanged. Cells were co-injected with Texas red-labeled dextrans to mark the oligonucleotide- (*e*) and antibody- (*f*) injected cells. Bar, 20 μ m.

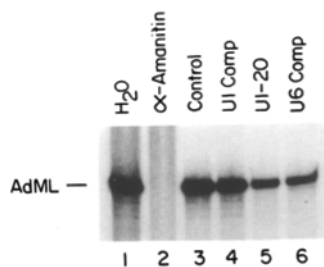


Figure 7 Oligonucleotides that inhibit splicing do not inhibit RNA polymerase II transcription *in vitro*. The transcription from the adenovirus 2 major late (*AdML*) promoter was monitored following addition of oligonucleotides. The oligonucleotides did not significantly affect the generation of transcription products from

the *AdML* promoter. In contrast, the addition of a known RNA Pol II inhibitor, α -amanitin, abolished all transcription from the *AdML* promoter. Lane 1, water added in same volume as oligonucleotides. Lane 2, α -amanitin (2 μ g/ml). Lane 3, Control oligonucleotide. Lane 4, U1 Comp oligonucleotide. Lane 5, U1-20 oligonucleotide. Lane 6, U6 Comp oligonucleotide.

does appear to be some decrease in transcription when the U1-20 and U6 Comp oligonucleotides are added (Fig. 7, lanes 5 and 6), this is a small decrease as compared to the addition of a known transcription inhibitor, α -amanitin, that resulted in no transcription products at all (Fig. 7, lane 2). However, when each of the experimental oligonucleotides were microinjected into cells there was a dramatic decrease in transcriptional activity. Therefore, to the extent that *in vitro* transcription faithfully mimics *in vivo* transcription, the inhibition of the latter by microinjected oligonucleotides was probably an indirect phenomenon.

Transcription and Splicing May Be Coordinated In Vivo

The results described above suggest that the disruption of splicing reduces transcription in the injected cells. There are at least two possible ways by which this may occur. One pos-

sibility is that splicing and transcription are directly linked *in vivo*. Therefore, the disruption of splicing might disrupt transcription. The other possibility is that the disruption of splicing results in the inability of the cell to synthesize one or more labile transcription factors from pre-mRNAs that must be spliced. The second possibility was tested by inhibiting translation in the cell to determine if labile proteins are required for transcription to occur. We therefore treated cells with cycloheximide for 2 h and then monitored the transcriptional activity of the cells by [3 H]uridine incorporation. Treatment of cells with cycloheximide did not affect the organization of splicing factors (Fig. 8 *a*) or transcription by RNA Pol II or Pol III (Fig. 8 *b*). This was revealed by no apparent decrease in the level of autoradiographic grains in the non-nucleolar regions of the nucleus. RNA Pol I transcription, which is restricted to the ribosomal RNA genes in the nucleoli, however, appeared to be reduced, as seen by the presence of fewer autoradiographic grains over the nucleoli (Fig. 8 *b*). These results indicate that a labile factor or factors are required for RNA Pol I transcription in the nucleoli. This is in accordance with previous biochemical studies that have shown that cycloheximide treatment reduces the transcription of ribosomal RNA in the nucleolus (Higashi et al., 1968; Willems et al., 1969). Pol II and Pol III transcription, however, are not dependent on highly labile factors. Therefore, transcription by these factors may be disrupted in the injected cells because splicing and transcription are tightly coupled.

Discussion

We have demonstrated that when oligonucleotides or antibodies that inhibit splicing *in vitro* are introduced into living cells by microinjection, there is a reorganization of splicing

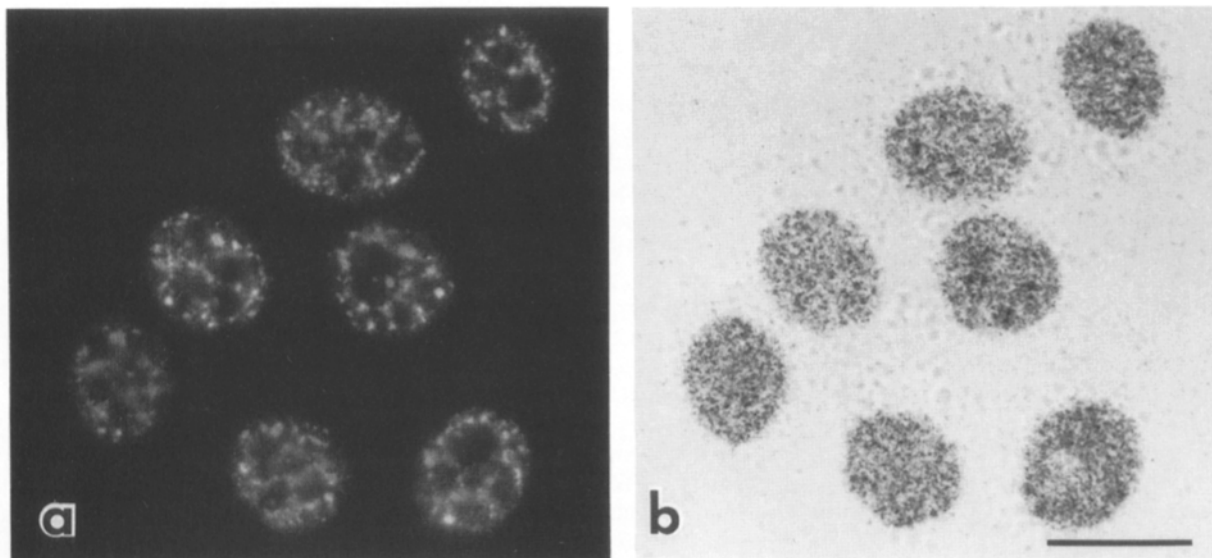


Figure 8 Cycloheximide treatment reveals that a labile factor(s) is not needed for RNA Pol II transcription. Cells were treated with cycloheximide for 2 h, labeled with [3 H]uridine to monitor transcriptional activity (*b*) and processed for immunofluorescence of SC-35 (*a*) and light microscopic autoradiography as described in Materials and Methods. The localization of SC-35 (*a*) does not appear to be changed by this treatment. The transcriptional activity of cycloheximide-treated cells (*b*) is unchanged in the non-nucleolar regions of the nucleus (Pol II and Pol III transcription). The nucleolar regions of the nucleus, however, have reduced transcriptional activity indicating that a labile factor(s) is needed for RNA Pol I transcription. Bar, 20 μ m.

factors within the nucleus. Splicing factors accumulated in large, rounded, structures in the nucleus that corresponded to enlarged interchromatin granule clusters. The reorganization of splicing factors following oligonucleotide microinjection was transient and splicing factors returned to their normal distribution over time. The transcriptional activity of cells microinjected with oligonucleotides or antibodies that inhibit splicing in vitro was reduced; however, this may not be a direct effect of the oligonucleotides or the antibodies on transcription. We propose that the reorganization of splicing factors to enlarged interchromatin granule clusters may represent the inhibition of pre-mRNA splicing in vivo.

Organization of Splicing Factors within the Nucleus

Studies on the organization of splicing factors by electron microscopy have pointed to a model in which splicing factors in the nucleus make up, or are associated with, three structural components of the nucleus. These components are the interchromatin granule clusters, perichromatin fibrils, and coiled bodies (reviewed in Spector, 1993). It is now thought that the interchromatin granule clusters are the sites of splicing factor storage and/or assembly, whereas the perichromatin fibrils represent the actual nascent pre-mRNA transcripts to which splicing factors from interchromatin granule clusters have moved to carry out splicing (reviewed in Spector, 1993). The role of coiled bodies in nuclear function is less clear because of their absence in some cells (Spector et al., 1992) and their protein composition (reviewed in Lamond and Carmo-Fonseca, 1993; Brasch and Ochs, 1992). We have shown that the reorganization of splicing factors, observed following microinjection of oligonucleotides or antibodies targeted to disrupt splicing, is to the interchromatin granule clusters. This may result from the loss of perichromatin fibrils (i.e., nascent transcripts) following the inhibition of splicing and transcription. These interchromatin granule clusters, which are irregularly shaped and fairly numerous in normal cells, become enlarged, rounded, and decrease in number. We propose that this morphological change of the interchromatin granule clusters may represent the return of splicing factors from perichromatin fibril regions (i.e., connections between speckles) to their sites of storage and/or assembly (i.e., interchromatin granule clusters), until splicing is resumed. The enlargement of interchromatin granule clusters could result from the accumulation of splicing factors while their reduction in number may result from the association of interchromatin granule clusters with one another. Visualization of splicing factors hours after injection of the oligonucleotides indicates that these enlarged interchromatin granule clusters are only transient structures. It appears that when splicing resumes, the interchromatin granule clusters are reduced in size, most likely reflecting the movement of splicing factors back to perichromatin fibrils, where they are needed to splice nascent pre-mRNAs. It is, therefore, likely that when splicing is disrupted in vivo, the cell responds by moving splicing factors back to the regions of storage and/or assembly until splicing is resumed. This suggests a dynamic organization of splicing factors in the nuclei of transcriptionally active cells.

A similar reorganization of splicing factors to larger interchromatin granule clusters in the nucleus has previously been observed following herpes simplex virus infection of cells (Martin et al., 1987) as well as in many pathological situations (reviewed in Fakan and Puvion, 1980). It was

speculated by Martin et al. (1987) that this reorganization in infected cells resulted from the partial inactivation of host pre-mRNA splicing. Our work is in concordance with this previous study and extends it by demonstrating that microinjection into cells of oligonucleotides which inhibit pre-mRNA splicing in vitro results in a reorganization of splicing factors to larger interchromatin granule clusters.

Associations Between Splicing and Transcription

When we monitored transcription in cells that were injected with oligonucleotides or antibodies that inhibit splicing in vitro, in addition to the reorganization of splicing factors we observed a reduction in the transcriptional activity of these cells. The control oligonucleotides or antibodies, however, did not affect transcription in the cells into which they were injected. In *Xenopus* oocytes, transcription of microinjected SV-40 DNA (Pan and Prives, 1988, 1989; Pan et al., 1989; Prives and Foukal, 1991) or DNA for mutant snRNAs (Hamm et al., 1989; Pan and Prives, 1989; Hamm et al., 1990; Vankan et al., 1990; Vankan et al., 1992) occurred following the inhibition of splicing with oligonucleotides targeted to snRNAs. In another study when any oligonucleotide was microinjected into *Xenopus* or *Notophthalmus* oocytes, cytological changes, disappearance of some or all of the B snurposomes, and shutdown of endogenous Pol II transcription were initially observed (Tsvetkov et al., 1992). However, at later times after microinjection, transcription resumed in these oocytes in the absence of U2 snRNA as evaluated by Northern analysis and in situ hybridization. From these results it was concluded that splicing was not required for transcription on lampbrush chromosome loops (Tsvetkov et al., 1992).

In HeLa cells we observed changes in the organization of splicing factors and in Pol II transcription with oligonucleotides that inhibit splicing but not with control oligonucleotides. In addition, in the cells injected with the oligonucleotides that inhibit splicing, we observed an increase in the size of the interchromatin granule clusters. We believe interchromatin granule clusters are the sites of splicing factor storage and/or assembly. Similarly, the B snurposomes present in amphibian oocyte germinal vesicles have been proposed to be the sites of splicing factor assembly, storage or recycling as well (Wu et al., 1991). However, the reduction in number or disappearance of the B snurposomes observed in the *Xenopus* and *Notophthalmus* oocytes following oligonucleotide injection is in contrast to our observation of an increase in size of interchromatin granule clusters. This contrast may reflect the fact that cultured mammalian somatic nuclei and amphibian oocyte nuclei are organized differently and that interchromatin granule clusters and B snurposomes may be structurally different although they contain many of the same constituents.

We found little effect of the oligonucleotides on the transcription of the AdML promoter, as compared to the transcription inhibitor α -amanitin, when we monitored transcription in vitro uncoupled from splicing. This suggests that the reduction of Pol II transcription we observed in vivo with oligonucleotides that inhibit splicing may be a consequence of the inhibition of splicing rather than the direct disruption of transcription. It is possible that when the cell detects that splicing is disrupted, splicing factors return to their sites of storage and/or assembly (i.e., interchromatin granule clusters) and transcription is shut down until splicing is restored.

In this way energy is not wasted generating transcripts that cannot be processed and would therefore accumulate in the nucleus. It is likely, therefore, that splicing and transcription may be coordinated within the nucleus with the status of one function affecting the other.

A number of researchers have proposed that splicing occurs cotranscriptionally and that splicing and transcription are coordinated (Sass and Pederson, 1984; Fakan et al., 1986; Beyer and Osheim, 1988; LeMaire and Thummel, 1990; Huang and Spector, 1991; Xing et al., 1993). The results we present here are in agreement with these proposals. Recent studies using adenovirus serotype 2 infection and transient transfection of a plasmid containing a portion of the β -tropomyosin gene into HeLa cells showed that splicing factors move to new transcription sites from storage and/or assembly sites (Jiménez-García and Spector, 1993). From these data a model has been proposed in which transcription and splicing are coordinated in the nucleus by a recruiting mechanism. In this model, splicing factors are associated with specific storage and/or assembly sites in the nucleus and are recruited to the active sites of transcription by another factor or chaperone molecule before, or at, the initiation of transcription. Our experiments corroborate this model. This idea is also supported by the splicing factor distribution observed when cells are treated with transcription inhibitors (Spector et al., 1983; Carmo-Fonseca et al., 1992). This distribution is very similar to the one we have observed following microinjection of oligonucleotides or antibodies that inhibit splicing. The inhibition of transcription can be thought of also as inhibiting splicing. Since inhibition of transcription results in the reduction of pre-mRNA transcripts, if there are no pre-mRNAs, splicing cannot occur. We believe this is why when transcription is inhibited, splicing factors move to interchromatin granule clusters in a manner similar to what we observed when we disrupted splicing. It is possible then that splicing and transcription might be linked within the nucleus. It will be interesting to determine how these processes are coordinated in vivo to splice transcripts efficiently.

In conclusion, we have shown that inhibition of splicing in vivo causes a reorganization of splicing factors which accumulate in enlarged interchromatin granule clusters that we believe are the sites of splicing factor storage and/or assembly. We propose that this reorganization of splicing factors, which is obtained by microinjection of either oligonucleotides or antibodies that inhibit splicing in vitro, is specific for cells that have reduced pre-mRNA splicing activities. The inhibition of splicing also affects the transcriptional activity of cells, indicating that splicing and transcription may be coordinated within the cell nucleus.

The authors thank Spencer Teplin for oligonucleotide synthesis, and Jim Duffy and Phil Renna for artwork. We also thank Joe Gall, Scott Henderson, Nouria Hernandez, Sui Huang, and Tokio Tani for helpful discussions and critical reading of the manuscript.

This study was supported by grants from the National Institutes of Health (GM42694 and 5P30CA45508-07) to D. L. Spector.

Received for publication 23 August 1993 and in revised form 3 November 1993.

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