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Splicing-independent recruitment of U1 snRNP to a transcription unit in living cells

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Summary

Numerous non-coding RNAs are known to be involved in the regulation of gene expression. In this work, we analyzed RNAs that co-immunoprecipitated with human RNA polymerase II from mitotic cell extracts and identified U1 small nuclear RNA (snRNA) as a major species. To investigate a possible splicing-independent recruitment of U1 snRNA to transcription units, we established cell lines having integrated a reporter gene containing a functional intron or a splicing-deficient construction. Recruitment of U snRNAs and some splicing factors to transcription sites was evaluated using fluorescence in situ hybridization (FISH) and immunofluorescence. To analyze imaging data, we developed a quantitative procedure, 'radial analysis', based on averaging data from multiple fluorescence images. The major splicing snRNAs (U2, U4 and U6 snRNAs) as well as the U2AF65 and SC35 splicing factors were found to be recruited only to transcription units containing a functional intron. By contrast, U1 snRNA, the U1-70K (also known as snRNP70) U1-associated protein as well as the ASF/SF2 (also known as SFRS1) serine/arginine-rich (SR) protein were efficiently recruited both to normally spliced and splicing-deficient transcription units. The constitutive association of U1 small nuclear ribonucleoprotein (snRNP) with the transcription machinery might play a role in coupling transcription with pre-mRNA maturation.

Key words: Nuclear imaging, RNA polymerase II, Splicing, Transcription, U1 snRNP, Coupling transcription to splicing

Introduction

This study was initiated to identify non-coding RNAs (ncRNAs) that might associate with and regulate RNA polymerase II (RNAPII). During the past decade, numerous ncRNAs have been identified as regulators of transcription (Prasanth and Spector, 2007; Wilusz et al., 2009). Some of them directly interact with and regulate components of the transcription machinery (Barrandon et al., 2008). Examples of such ncRNAs include 6S, B2, Alu RNAs and U small nuclear RNAs (snRNAs). 6S RNA was the first to be described; it directly targets bacterial RNA polymerases and probably binds within the active site (Wassarman, 2007). Binding of 6S RNA inhibits transcription from several but not all σ 70-dependent promoters and might contribute to modifying the gene expression profile when bacteria enter the stationary phase. Mouse B2 (Allen et al., 2004; Espinoza et al., 2004) and human Alu RNAs (Mariner et al., 2008) have been suggested to be the mammalian counterparts of the 6S RNA; they bind RNAPII and contribute to the transcriptional shut-off during stress.

A number of unrelated studies have reported that RNAPII co-purifies with U snRNAs. However, the results were quite variable. Some studies found that several splicing U snRNAs (U1, U2, U4) co-immunoprecipitated with RNAPII (Kim et al., 1997; Robert et al., 2002). Others identified the U1 snRNA as the major RNA associated with RNAPII (Das et al., 2007; Tian, 2001). One study did not even observe the U1 small nuclear ribonucleoprotein (snRNP)-RNAPII association (Listerman et al., 2006). All these experiments used cell extracts and it was unclear whether U snRNA would be associated to RNAPII in the absence of splicing in living cells. This issue is important given the coupling of transcription

and splicing (de Almeida and Carmo-Fonseca, 2008; Hirose and Manley, 2000; Moore and Proudfoot, 2009; Pandit et al., 2008; Perales and Bentley, 2009).

In the present work, we first re-examined the ncRNAs that might co-purify with RNAPII from unstressed cells in the absence of transcription, using mitotic cell extract. U1 snRNA was found as a major RNA co-immunoprecipitating with RNAPII. We next checked its co-recruitment along with RNAPII on an active transcription unit using an imaging approach. A quantitative image-processing procedure, 'radial analysis', was developed, based on averaging data from multiple fluorescence images. U1 snRNA was efficiently recruited to model active transcription units whether or not they were subjected to splicing. By contrast, recruitment of the other U snRNAs (U2, U4, U5, U6) was only found on transcription units subjected to splicing. This finding might partly account for the previously reported 5'-splice-site stimulation of transcription (Damgaard et al., 2008; Furger et al., 2002). Alternatively, having U1 snRNP preloaded on the transcription machinery might facilitate 5'-splice-site recognition on nascent RNAs.

Results

U1 snRNA is a major RNA associated with RNAPII

To look for RNAs that might associate with and regulate RNAPII, we investigated RNAs that co-immunoprecipitate with RNAPII from mitotic-enriched cell extract (mitotic index 80%). The choice of a mitotic cell lysate was based on two considerations: (1) there is virtually no transcription during mitosis (Gottesfeld and Forbes, 1997; Jiang et al., 2004), hence the amount of nascent transcripts binding to RNAPII is expected to be minimal; and (2) because the

nuclear membrane breaks down during mitosis, it is possible to achieve an efficient RNAPII extraction in low-salt non-denaturing buffers, preserving the salt-sensitive RNA-protein interactions. Indeed, most RNAPII was found in the clarified supernatant of a mitotic cell extract, whereas more than half of RNAPII remained in the nuclear pellet from an asynchronous cell extract (Fig. 1A).

The mitotic cell supernatant was next fractionated on a glycerol gradient and RNAPII was immunoprecipitated. RNAs retained by anti-Rpb1 (the largest subunit of RNA polymerase II) were isolated and 3'-labeled with 3',5'-cytidine diphosphate (pCp) and T4 RNA ligase. A major radioactive band of about 160 nucleotides was observed (data not shown). The size of this RNA prompted us to test for the presence of U1 snRNA (164 nucleotides) by northern blot. RNAPII was immunoprecipitated using two distinct antibodies against the largest subunit of RNAPII (Rpb1): a rabbit polyclonal against the largest subunit of RNAPII (Rpb1): a rabbit polyclonal against Rpb1 N-terminal domain (N20) and a mouse monoclonal against the C-terminal domain of Rpb1 (8WG16). A strong hybridization signal corresponding to U1 snRNA was detected by northern blot (Fig. 1B, lanes 3 and 6). Other RNAs present in the extract, such as 7SK snRNA or U2 snRNA, remained within background levels in both control and anti-Rpb1 immunoprecipitates.

To avoid artifacts that could originate from a reassociation between RNA and RNA-binding proteins in cell extracts (Mili and Steitz, 2004), the northern blot experiment was repeated on RNAs co-immunoprecipitated with Rpb1 from extracts of cells that had been crosslinked with formaldehyde prior to lysis in a dissociating buffer (Fig. 1C). Again, U1 but not U2 snRNA was markedly co-immunoprecipitated from crosslinked mitotic cells (Fig. 1C, lane 3).

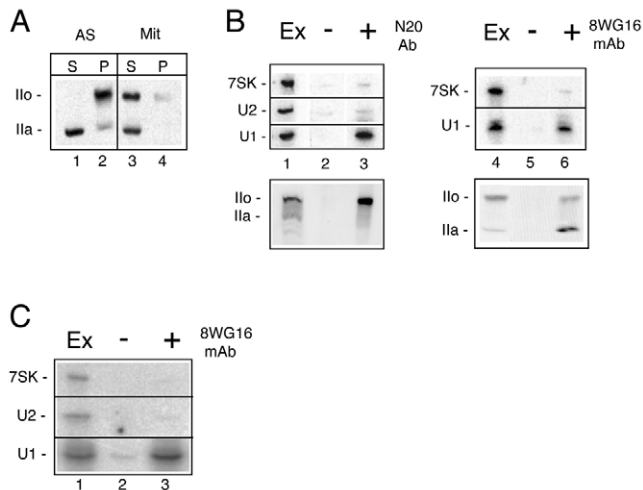


Fig. 1. Detection of U1 snRNA associated with RNAPII in mitotic cells. (A) Detection of RNAPII in asynchronous (AS) or mitotic-arrested (Mit) HeLa cells. Western blot using Pol3/3 mAb was performed on 10,000 *g* supernatants (S) (lanes 1 and 3) and nuclear pellets (P) (lanes 2 and 4) of cells lysed in a low-salt buffer (0.15 M NaCl). Hyperphosphorylated (Ilo) and hypophosphorylated (Ila) RNAPII forms are present. (B) Detection of U1 snRNA associated with RNAPII in mitotic cell extracts using different anti-Rpb1 antibodies. Control mock antibodies (lanes 2 and 5), anti-Rpb1 N20 (lane 3) or 8WG16 (lane 6) antibodies were added to a mitotic cell extract (Ex) and recovered using protein-A beads. RNAs were probed for U1, U2 or 7SK snRNAs by northern blot. Western blot for RNAPII was performed using Pol3/3 antibody (upper part). (C) Detection of U1 snRNA associated with RNAPII in formaldehyde crosslinked mitotic HeLa cells. RNA was immunoprecipitated with control mock (-) or anti-RNAPII 8WG16 (+) antibodies and further probed for U1, U2 or 7SK snRNAs by northern blot.

Hence, association of U1 snRNA with RNAPII molecules in a mitotic-enriched population of cells suggests that U1 snRNA interacts with RNAPII independently of transcription *in vivo*.

Transcription units with a functional or splicing-deficient β -globin intron

The constitutive association of U1 snRNA with RNAPII suggested that it might be recruited to a gene independently of splicing. To evaluate such recruitment, we chose to use microscopy techniques on artificial transcription units represented by gene cassettes inserted at a single locus (Misteli and Spector, 1999). We first constructed plasmid pTet-on-CFP-MS2wt, which drives the synthesis of a transcript carrying the β -globin exon 1 (including the translation start codon), intron 1 and the beginning of exon 2 fused to the cyan fluorescent protein (CFP) coding sequence followed by 24 MS2 repeats (Fig. 2A). The 5' and 3' splice sites of the β -globin intron were next mutated for the production of pTet-on-CFP-MS2mut plasmid deficient in splicing. U2OS cells were stably transfected with either plasmid pTet-on-CFP-MS2wt or pTet-on-CFP-MS2mut. Cells that integrated multiple copies of a plasmid at a single locus were independently isolated and cloned.

To evaluate the splicing profile of the β -globin transcripts produced from the plasmids, RNAs isolated from doxycycline-induced cells were analyzed by reverse transcriptase (RT)-PCR using a pair of primers located on each side of the β -globin intron (Fig. 2A). A single major PCR product compatible with the expected 829-bp size of spliced transcript was detected in BGwt cells containing constructs with a functional wild-type β -globin intron (Fig. 2B, lane 2). A single larger product compatible with the expected 958-bp size of unspliced transcript was detected in BGmut cells containing a mutant splicing-deficient β -globin construction (Fig. 2B, lane 3). The RT-PCR indicated a strong phenotype: there were very low amounts of unspliced RNAs in cells carrying the wild-type plasmid as well as no spliced RNAs in cells harboring the mutated plasmid. Quantification of the reporter RNA expression levels by real-time RT-quantitative (q)PCR showed that it was 2.5-fold less expressed in BGmut cells than in BGwt cells (data not shown). CFP proteins were localized to peroxisomes owing to a C-terminal peroxisome-targeting sequence (Fig. 2C).

To visualize transcription start sites, the doxycycline-induced reporter RNAs were analyzed by fluorescence *in situ* hybridization (FISH) with antisense probes labeled with different fluorophores (Darzacq et al., 2006; Darzacq et al., 2007; Femino et al., 1998; Shav-Tal et al., 2004). Exon 1 and MS2 probes stained cytoplasm and nuclei of both BGwt and BGmut cells (Fig. 2C). A unique bright spot was visible in the nucleoplasm of most cells, revealing accumulation of transcripts at the transcription site (Darzacq et al., 2007). Such a spot was not seen in cells that had not been exposed to doxycycline. It was the only structure labeled with the intron-1 probe in BGwt cells, reflecting the co-transcriptional splicing of this intron (Darzacq et al., 2007). By contrast, the cytoplasm of BGmut cells was stained by the intron probe, confirming a splicing deficiency induced by splice-site mutations.

Quantitative analysis of the recruitment of U snRNAs to transcription sites

To monitor the recruitment of U snRNAs to transcription units, oligonucleotide probes labeled with distinct colors were first hybridized to BGwt cells. Colocalization of U1 or U2 snRNA with the transcription sites was difficult to appreciate by eye as their respective labeling covered the whole nucleoplasm, with local

enrichment in numerous nuclear speckles (Fig. 3, top and enlarged nuclear fields #1 to #5). Their putative enrichment at transcription sites was therefore addressed statistically over many cells in order to average single-cell differences due to fortuitous proximity of a speckle, a nucleoli or the nuclear periphery. To this aim, FISH

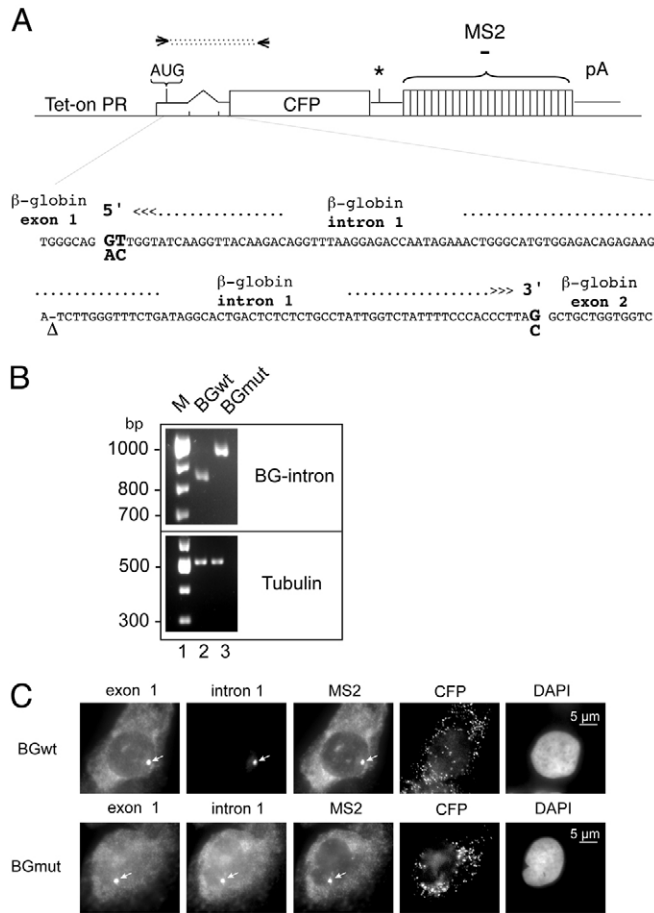


Fig. 2. Characterization of transcription units with a functional or splicing-deficient β -globin intron. (A) Scheme of plasmids used for the construction of β -globin gene cassettes. The plasmid used to make BGwt cell lines comprises a doxycycline-inducible promoter (Tet-on PR) that drives the synthesis of an mRNA carrying the β -globin exon 1 with AUG initiation codon, intron 1 and the beginning of exon 2 fused to the CFP coding sequence terminated with a peroxisome targeting signal. The translation stop codon (*) is followed by 24 repeats of the MS2 RNA hairpin and a polyadenylation signal (pA). To make BGmut cell lines, the 5' and 3' splice sites of the β -globin intron were mutated. Mutated nucleotides are indicated below their wild-type counterparts. A single nucleotide was deleted (Δ) in the β -globin intron-1 sequence to maintain the CFP translation frame in the case of splicing deficiency. Arrows in the top diagram indicate positions of RT-PCR primers framing the β -globin intron. (B) RT-PCR detection of gene reporter mRNA in BGwt and BGmut cells induced with doxycycline (primers are shown in A). M, 100-bp DNA ladder; BGwt and BGmut, RNA samples used for the reaction. PCR product sizes for mRNA containing (BGwt) or not containing (BGmut) the β -globin intron sequences were 958 and 829 bp, respectively. Tubulin α 3 was used as a housekeeping-gene reference (500-bp product size). (C) Detection of the gene reporter mRNA by FISH. Doxycycline-induced BGwt and BGmut cells were hybridized with fluorescent DNA probes of different colors for β -globin exon 1 and intron 1 as well as MS2 sequences. Both cells expressed CFP localized in peroxisomes; nuclei were stained with DAPI. Transcription sites are pointed with arrows.

images of U snRNA were obtained for a collection of cells (50 cells), centered on the MS2 spot (transcription site; TS) and summed up. The averaged U1 and U2 images (Σ) provided central bright spots, broader than the MS2 ones (Fig. 3, bottom). By contrast, averaged images (Σ) taken at random within the nuclei of the same cells (random site; RS) provided a rather homogeneous image. Single random sites also could present strong signals because the distribution of U snRNA has a speckled pattern (like U1 RS #5 on Fig. 3B); however, averaging erased signals from positions that were uncorrelated with the center of the windows (see U1 and U2 RS Σ on Fig. 3B). A small decrease in light intensity seen on the border of the averaged windows was probably due to the contribution of nucleoli and cytoplasm that are not stained and have an increased probability to contribute with increasing distance from the center of the windows.

A quantitative statistical procedure was next developed. Light intensities provided by the U1 FISH were determined as a function of distance from the transcription-site center: rings were centered on the transcription site (Fig. 4A) and the light intensity at a given distance was defined as the ratio of the sum of pixel intensities in an annulus between two consecutive rings to the area of the same annulus (Fig. 4B). To define a background reference, the same analysis was repeated on a site chosen at random in the nucleoplasm (RS). Data were plotted as the relative enrichment (the difference

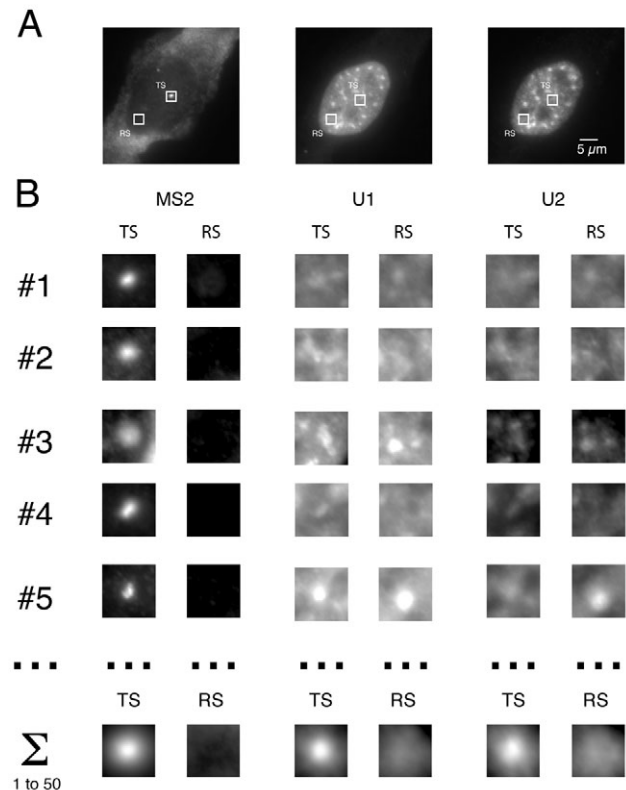


Fig. 3. Imaging of snRNA distribution around a transcription site. (A) Doxycycline-induced BGwt cells were hybridized with fluorescent DNA probes of different colors for U1 snRNA, U2 snRNA or MS2 sequences. The transcription site (TS) and a randomly selected site (RS) in the nuclei are framed by squares. (B) Enlarged pictures ($3.25 \mu\text{m} \times 3.25 \mu\text{m}$ squares) of the transcription (TS) and random (RS) sites in the nuclei of five different cells (numbered #1 to #5). The bottom line shows images resulting from the summation (Σ) of images taken for 50 different cells.

between light intensity at a given distance and the light intensity at 1.6 μm (Fig. 4C). There was a trend to observe a higher U1 intensity at the center of transcription sites (Fig. 4C, black symbols) than at the center of the random sites (Fig. 4C, white symbols). To increase the reliability of these conclusions, relative enrichment data were averaged from a collection of cells and found to be higher at the center of transcription sites (TS) than at the random site (RS) average (Fig. 4D). Highly similar data were obtained for two clonal BGwt cell lines studied, confirming that enrichment of U1 snRNA is unlikely to be influenced by the plasmid insertion locus (Fig. 4D). The shape of U1 snRNA enrichment curve was very similar to that of the averaged transcript intensity detected with the MS2 probe (Fig. 4E). As a negative control, the distribution of a nuclear-localized β -galactosidase (Bonnerot et al., 1987) was

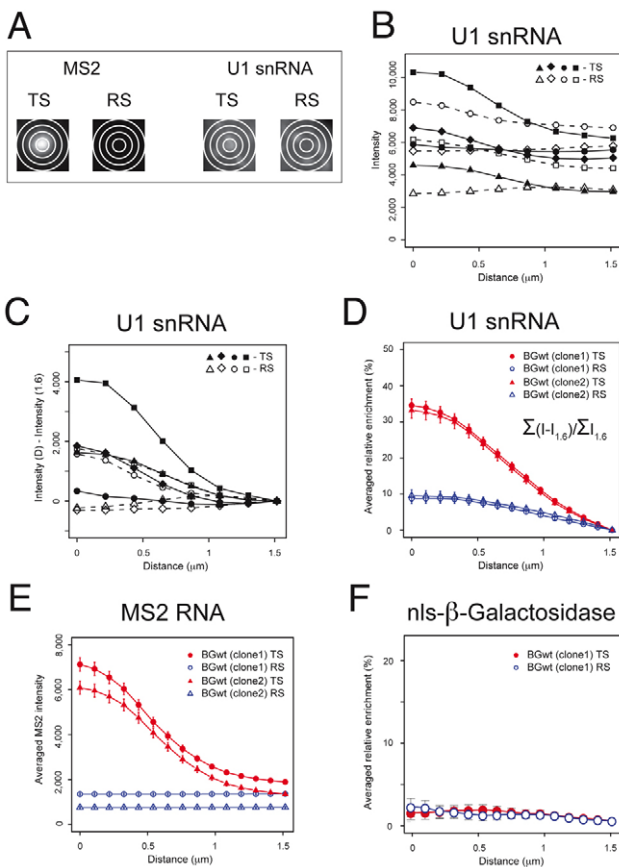


Fig. 4. Radial analysis of snRNA distribution around a transcription site. BGwt cells were hybridized with probes labeled with different colors for MS2 and for U1 snRNA. Rings separated by 1 pixel (107.5 nm) were centered on the transcription site (TS) determined with an MS2 probe image or centered on a random site (RS). (A) Illustration of ring centering for measuring light intensity. (B) Light intensities [$I(d)$] in annuli between rings were determined for individual cells and plotted as a function of distance to the center for four different cells (triangles, diamonds, circles and squares). (C) The difference [$I(d) - I(1.6 \mu\text{m})$] was plotted as in B for the same cells. (D) Averaged data for U1 snRNA signal enrichment in TS for 92 BGwt cells (clone 1, WTA9B41; circles) and 95 BGwt cells (clone 2, WTP2; triangles). The equation $\frac{\sum [I(d) - I(1.6 \mu\text{m})]}{\sum I(1.6 \mu\text{m})}$ was used. (E) Averaged data for MS2 signal enrichment for the same set of cells as in D. Error bars represent standard errors. (F) Recruitment of nuclear-localized β -galactosidase to TS was evaluated by immunofluorescence as an independent negative control. BGwt cells were transfected with NLS- β -galactosidase expression plasmid.

analyzed by immunofluorescence in BGwt cells transiently transfected with an expression plasmid (Fig. 4F). This time, the transcription site and random site curves were indistinguishable.

Recruitment of U1 but not U2, U4 and U6 snRNAs to transcription sites is splicing independent

U1, U2, U4 and U6 snRNAs were all clearly enriched above background (in the 30% range) at the transcription site (TS) in doxycycline-induced BGwt cells (Fig. 5). However, in BGmut cells, U1 snRNA was the only U snRNA clearly enriched at transcription sites (22%) (Fig. 5A). By contrast, the U2, U4 and U6 snRNA curves were slightly below the background random-site curve (RS) and displayed a characteristic S shape (Fig. 5B-D). This result was reproducibly observed when averaging a distinct set of cell images, and suggested that U2, U4 and U6 snRNA concentrations at a transcription site lacking a functional intron are lower than in the average nucleoplasm. The same observations were made with another BGmut clonal cell line (data not shown). Thus, U1 enrichment and U2, U4 and U6 snRNA absence at a splicing-deficient transcription site were not associated with the integration locus.

To further confirm these results, we performed the same experiments with a distinct gene-cassette model using another promoter, another intron model and another reporter gene. U2OS cells were stably transfected with a plasmid in which the HIV-LTR promoter drives the Tat-activated synthesis of an mRNA including the TAR hairpin fused to the MINX sequence [an artificial derivative from the adenovirus genome (Zillmann et al., 1988)]

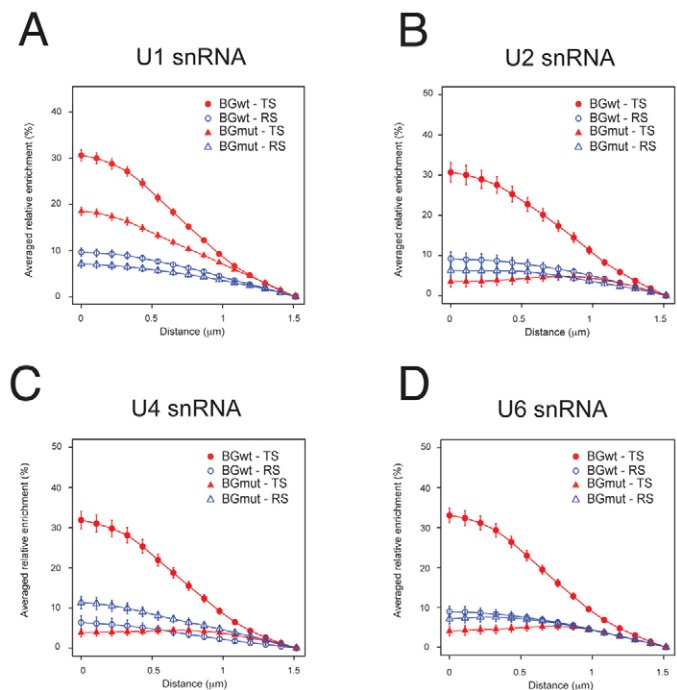


Fig. 5. Splicing-independent recruitment of U1 snRNA to doxycycline-induced transcription sites. Transcription of the reporter gene in BGwt and BGmut cells was induced by doxycycline. Transcription sites were determined by FISH using an MS2 probe. (A) Averaged U1 data from 146 BGwt cells (circles) and from 129 BGmut cells. (B) Averaged U2 snRNA FISH data from 132 BGwt cells (circles) and 67 BGmut cells (triangles). (C) Averaged U4 snRNA FISH data from 136 BGwt cells (circles) and 157 BGmut cells (triangles). (D) Averaged U6 snRNA FISH data from 185 BGwt cells (circles) and 166 BGmut cells (triangles).

and followed by the β -galactosidase coding sequence (Fig. 6A). The MINX sequence contains two exons flanking an intron. Both U1 and U2 snRNAs were recruited to transcription sites of the cells containing a functional MINX intron (Fig. 6B). When the cells were transfected with a splicing-deficient construction lacking the whole MINX intron, U1 snRNA but not U2 snRNA was recruited to transcription sites (Fig. 6C).

Thus, transcription units comprising distinct promoters driving unrelated sequences recruit U1 snRNA even when they lack functional introns. It is concluded that U1 recruitment and U2 snRNA exclusion at transcription sites is a characteristic of splicing-deficient gene cassettes.

Recruitment of RNAPII, U1-70K and ASF/SF2 SR protein to transcription sites is splicing independent

Following the working hypothesis of constitutive RNAPII-U1-snRNA association, we investigated the recruitment of Rpb1, the largest subunit of RNAPII, and U1-snRNP-associated proteins on the same transcription unit. The radial-analysis method could be applied either to FISH or immunofluorescence images. However, some antibodies were incompatible with FISH procedures. In this case, positions of the transcription sites were determined using transfection with a plasmid driving the expression of the MS2-YFP protein that binds the MS2 RNA repeats in vivo (Bertrand et al.,

1998). U1 FISH distributions obtained using transcription-site determination by either YFP fluorescence or MS2 FISH were identical (data not shown).

Recruitment of Rpb1 to transcription units in splicing-deficient BGmut cells was nearly as efficient as in splicing-positive BGwt cells (Fig. 7A). U1 snRNA is associated with several proteins, such as U1-70K (also known as snRNP70), that form the U1 snRNP (Hochleitner et al., 2005; Will and Lührmann, 2001). Similar to U1 snRNA, U1-70K was also enriched on both splicing-positive and splicing-deficient transcription units (Fig. 7B). The weaker efficiencies in U1 snRNA, U1-70K and RNAPII recruitments to splicing-deficient BGmut transcription units compared with splicing-positive BGwt transcription units were consistent with the hypothesis of RNAPII-U1-snRNP co-recruitment to transcription sites.

We next investigated the recruitment of serine/arginine-rich (SR) proteins that are known to interact with both the transcription

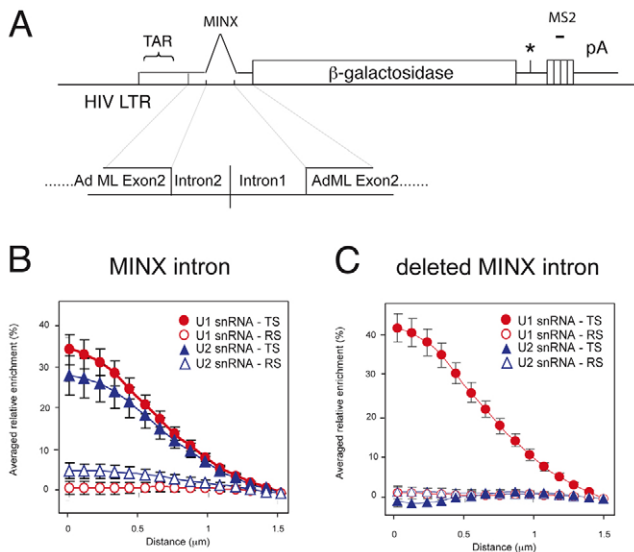


Fig. 6. Splicing-independent recruitment of U1 snRNA to Tat-induced transcription sites. Relative enrichment of U1 and U2 snRNA at transcription (TS) or random (RS) sites was evaluated in cells that integrated the plasmid containing a functional MINX intron (MINXwt) or no MINX intron (MINX Δ in). Transcription was induced by transfection with a Flag-Tat expression plasmid. (A) Scheme of plasmid used for construction of MINX gene cassettes. The plasmid comprises the HIV-LTR promoter, which drives the synthesis of an mRNA that includes the TAR hairpin followed by the MINX sequence fused to the β -galactosidase coding sequence. The translation stop codon (*) is followed by four repeats of the MS2 RNA hairpin and a polyadenylation signal (pA). The MINX intron sequence consists of adenovirus major late gene sequences (AdML): an exon-2/intron-2 sequence fragment is fused to a fragment of intron-1/exon-2 sequence (Zillmann et al., 1988). To prepare the plasmid without MINX intron, the fused intron-2/intron-1 AdML sequences were excised. (B) Averaged U1 snRNA data from 54 MINXwt cells and U2 snRNA from 60 MINXwt cells. (C) Averaged U1 snRNA data from 46 MINX Δ in cells and U2 snRNA from 57 MINX Δ in cells.

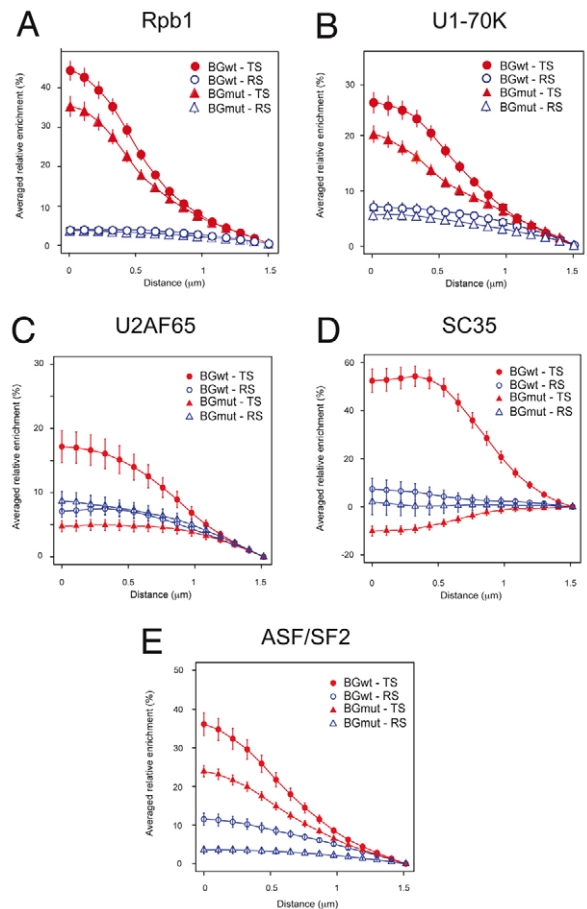


Fig. 7. Splicing-dependent recruitment of proteins to doxycycline-induced transcription sites. Relative enrichment of proteins at transcription (TS) or random (RS) sites was evaluated by radial analysis of immunofluorescence images in BGwt cells and BGmut cells. (A) Averaged Rpb1 (CTD4H8 Ab) immunofluorescence data from 85 BGwt cells (circles) and 100 BGmut cells (triangles). (B) Averaged U1-70K immunofluorescence data from 84 BGwt cells (circles) and 104 BGmut cells (triangles). (C) Averaged U2AF65 data from 195 BGwt cells (circles) and 217 BGmut cells (triangles). (D) Averaged SC35 data from 185 BGwt cells (circles) and 154 BGmut cells (triangles). (E) Averaged ASF/SF2 data from 109 BGwt cells (circles) and 110 BGmut cells (triangles). Transcription sites (TS) were determined using transient transfection with MS2-YFP plasmid.

and splicing machineries (Long and Caceres, 2009; Zhong et al., 2009). The U2 snRNP particle auxiliary factor of 65 kDa, U2AF65, was chosen because it binds the intronic polypyrimidine tract sequence and has been reported to promote U1 snRNP recruitment to 5' splice sites (Förch et al., 2003; Hoffman and Grabowski, 1992). Although the polypyrimidine tract sequence had not been modified in our splicing-deficient β -globin construct, recruitment of U2AF65 was found to be splicing dependent (Fig. 7C). ASF/SF2 (also known as SFRS1) and SC35 proteins were investigated next because both have been reported to interact with U1-70K of the U1 snRNP (Cao and Garcia-Blanco, 1998; Ellis et al., 2008; Golovkin and Reddy, 1999). In agreement with a previous study (Huang and Spector, 1996), SC35 was only recruited to transcription units containing a functional intron (Fig. 7D). By contrast, ASF/SF2 was found to be recruited to both functional-intron-containing and splicing-deficient transcription units (Fig. 7E). Similar to U2, U4 and U6 snRNA, both U2AF65 and SC35 densities were lower than average at splicing-deficient transcription sites. ASF/SF2 was the only SR protein found to be recruited on splicing-deficient transcription units, as were the U1 snRNA and the U1-70K protein.

Discussion

U1 snRNA is a major ncRNA associated with non-transcribing RNAPII

Using a mitotic cell extract, the present study shows a highly significant U1 snRNA association with RNAPII, whereas the other splicing U snRNAs were not detected. There have been conflicting reports on U-snRNA–RNAPII association. Some teams found all the splicing U snRNA associated with RNAPII (Kim et al., 1997; Robert et al., 2002) and others did not detect significant amounts of U snRNA (Listerman et al., 2006). Our observations are consistent with those from Tian (Tian, 2001) and from the Reed laboratory (Das et al., 2007) who only found U1 snRNP. So much U1 snRNA was immunoprecipitated by the Reed laboratory that it was stained by ethidium bromide. Furthermore, for his part, Tian isolated RNA aptamers that inhibited transcription initiation in a HeLa nuclear extract and found that four out of eight of these aptamers contained an 11-nucleotide sequence identical to the 5' splice site. Furthermore, depletion of U1 snRNA from the extracts abrogated the aptamer inhibition. It might be speculated that, if U1 snRNP sits close to the transcript exit pore, the linker sequence of these aptamers bound to U1 snRNP might enter the RNAPII cleft and inhibit transcription, similar to the FC aptamer (Kettenberger et al., 2006).

The studies cited above used nuclear extracts. We now find that U1-snRNA–RNAPII association is maintained in a dissociating buffer when the mitotic cells were crosslinked prior to lysis. The *in vivo* crosslinking technique eliminates a possible reassortment between RNA and RNA-binding proteins that might occur in cell extracts. Because there is very little, if any, transcription during mitosis (Gottesfeld and Forbes, 1997; Jiang et al., 2004), our finding supports the notion that U1 snRNA is constitutively associated with non-transcribing RNAPII.

Radial analysis of microscopic images: a sensitive assay to study colocalizations

Assuming that U1-snRNA–RNAPII association is constitutively permanent implied that U1 snRNA might be recruited to genes in the absence of splicing. To verify this prediction, we set up a quantitative analysis of fluorescence images from cells containing

inserted gene cassettes at a single locus. It was based on averaging data from many cells. Simple verification of colocalization of spliceosomal snRNAs with transcription sites was not fully convincing because of the punctated distribution of U snRNAs. The variations of averaged light densities as a function of distance to the transcription center were compared with the variations of light densities in averaged images chosen at random in the nucleoplasm of the same cells. This radial-analysis method proved to be robust, because different clonal cell lines provided identical results. It was highly reliable and sensitive, because distinct probes provided distinct results although individual images were hardly indistinguishable. The radial analysis was also efficient in processing data from immunofluorescence. It is to be compared with statistical scoring methods. A major advantage resides in the lack of observer decision to score a colocalization or not. Furthermore, the reliability of the enrichments at transcription sites versus random sites might easily be challenged by statistical tests based on distribution profiles, bootstrapping and standard errors.

Nucleoplasm heterogeneity

Molecules evenly distributed across the nucleus such as nuclear localization signal (NLS)– β -galactosidase or NLS-YFP (not shown) were neither enriched nor excluded from the transcription sites. By contrast, U1 snRNA was recruited to both splicing-positive and splicing-deficient gene transcription units, and U2, U4 and U6 snRNAs were only recruited to splicing-positive ones. Furthermore, in the vicinity of splicing-deficient transcription sites, the density of these RNAs was lower than in the average nucleoplasm. Some proteins (Rpb1, U1-70K, ASF/SF2) were found to be recruited to both splicing-positive and splicing-deficient transcription units and others were only recruited to splicing-positive ones (SC35, U2AF65). The radial analysis reveals the heterogeneity of the nucleoplasm, because some molecules are recruited to active transcription units whereas others seem to be excluded from their vicinity. The cause of this exclusion remains to be established. Yet, it might be a size effect because structures such as spliceosomes or speckles are much larger than β -galactosidase.

Splicing-independent U1 snRNP recruitment

U1 snRNA and at least one U1-associated protein (U1-70K) were found to be recruited to a splicing-deficient gene cassette almost as efficiently as to its splicing-positive counterpart. Importantly, U1 recruitment was observed using two totally distinct model genes. One construct used a Tet-on promoter derived from the *Cytomegalovirus* early immediate promoter whereas the other construct used a promoter derived from the HIV-LTR promoter. One construct used the β -globin first intron and the other one used the MINX intron derived from the adenovirus genome. One used the β -galactosidase cDNA and the other one used the *CFP* cDNA. Recruitment of U1 snRNA to splicing-deficient genes is consistent with previous reports that U1 snRNP is recruited to the intronless *Drosophila* heat-shock puffs (Martin et al., 1987; Matunis et al., 1993). By contrast, previously reported chromatin immunoprecipitation experiments using antibodies directed against either U1-70K or the Smith (Sm) antigen did not detect these proteins on the intronless heat-shock genes (Listerman et al., 2006; Metz et al., 2004). The discrepancy between these results and our observations might rely in the techniques. Efficiencies in chromatin immunoprecipitation decrease when the target protein is 'far' from DNA. Imaging methods are much less sensitive to the distance between the protein or RNA probe and its gene target.

U1 snRNP interacts with multiple components of the transcription and transcript-maturation machineries

How is U1 snRNP recruited to transcription sites? U snRNPs have previously been reported to bind transcripts not subjected to splicing. For example, U2 snRNP binds an RNA sequence in the intronless histone transcripts (Friend et al., 2007). An assay based on injection of fluorescently labeled snRNAs into amphibian oocytes showed that U1 snRNA targets nascent transcripts independently of splicing (Patel et al., 2007). A central argument in that study was that a U1 snRNA lacking the 5'-splice-site recognition sequence still targeted the nascent transcripts. However, in discrepancy with our findings, a U2 snRNA lacking the branch-point recognition sequence was also targeted to nascent transcripts. An interaction of U1 snRNP with either the transcription or the post-transcription machineries is more likely to account for our observations.

The co-immunoprecipitation experiments suggest a direct interaction of U1 snRNA with RNAPII. Also, a number of specific factors might bridge U1 snRNP to the transcription machinery. The human homologue of Prp40 yeast splicing factor, associated with U1 snRNP (Kao and Siliciano, 1995), binds the phosphorylated C-terminal domain (Morris and Greenleaf, 2000). Alternatively, U1 snRNA has also been reported to interact with general transcription factors such as TFIID (Kwek et al., 2002) and the TBP-associated factor TAF15 (Jobert et al., 2009). However, it should be mentioned that, in both examples, U1 snRNA was devoid of its companion proteins such as U1-70K.

Several SR proteins that are involved in splicing have been reported to associate to RNAPII and U1 snRNP (Long and Caceres, 2009; Zhong et al., 2009). The splicing-associated factor SC35 plays an active role in the elongation of transcription (Lin et al., 2008) and interacts with U1 snRNP (Golovkin and Reddy, 1999). U2AF65 interacts with U1 snRNP (Ellis et al., 2008) and binds nascent transcripts in an *in vitro* assay (Ujvári and Luse, 2004). However, recruitment of both SC35 and U2AF65 factors was strictly dependent on splicing. By contrast, recruitment of ASF/SF2, similar to U1 snRNA and U1-70K, was splicing independent. ASF/SF2 has been found to interact with U1-70K of the U1 snRNP *in vitro* and *in vivo* independently of transcription (Cao and Garcia-Blanco, 1998; Ellis et al., 2008; Golovkin and Reddy, 1999). It is noteworthy that ASF/SF2 also displays a crucial splicing-independent function. It prevents the formation of RNA-DNA hybrids between transcripts and the template DNA strand that provoke the appearance of DNA double-stranded breaks (Li and Manley, 2005). In agreement with our observations, the Reed group found ASF/SF2 but neither SC35 nor U2AF65 associated with RNAPII (Das et al., 2007). Protein-RNA interactions contribute to ASF/SF2 recruitment at transcription sites (Mabon and Misteli, 2005). It remains to be solved whether ASF/SF2 and U1 snRNP are co-recruited with RNAPII or whether the nascent transcript binds ASF/SF2 first and next recruits U1 snRNP.

U1 snRNP has also been shown to interact with pre-mRNA maturation machineries. The nuclear cap-binding complex facilitates association of U1 snRNP with the cap-proximal 5' splice site (Lewis et al., 1996). U1-70K binds the 25-kD cleavage factor I subunit CF-I m (Awasthi and Alwine, 2003). The U1A protein interacts with the 160-kD subunit of the cleavage-polyadenylation specificity factor (CPSF) (Lutz et al., 1996) and CPSF forms a complex with TFIID of the basal transcription machinery (Dantonel et al., 1997). Thus, the multiple interactions of U1 snRNP with transcription and transcript-maturation machineries might contribute to their coordinated assembly at transcription sites.

U1 snRNP recruitment: a possible role in coupling transcription to splicing?

The splicing-independent recruitment of U1 snRNP to transcription units might be understood in the broader context of coupling between transcription and post-transcriptional maturation of transcripts (Bentley, 2005; de Almeida and Carmo-Fonseca, 2008; Hirose and Manley, 2000; Moore and Proudfoot, 2009). Interruption of transcribed sequences by introns can enhance mRNA expression of recombinant genes (Lacy-Hulbert et al., 2001; Palmiter et al., 1991). Some splicing-machinery components seem to stimulate transcription. Indeed, the splicing-associated factor SC35 has been shown to play an active role in the elongation of transcription (Lin et al., 2008). In addition, Tat-SF1, being a U2-snRNA-associated protein involved in prespliceosome assembly (Perriman et al., 2003; Yan et al., 1998), cooperates with DSIF to facilitate elongation (Chen et al., 2009; Li and Green, 1998; Zhou and Sharp, 1996).

The involvement of U1 snRNA in the regulation of transcription is suggested by a number of findings. Removal of proximal 5' splice sites from yeast or mammalian genes decreases their transcription (Furger et al., 2002). Binding of U1 snRNA stem loop II to the 5' splice site stimulates transcription from an HIV-LTR promoter (Alexander et al., 2010). The 5' proximal splice site can enhance promoter docking of basal transcription factors (Damgaard et al., 2008). U1 snRNA binding was found to stimulate the formation of the first phosphodiester bond in an abortive transcription assay using only purified factors (Kwek et al., 2002). Furthermore, U1 snRNA stimulated reinitiation of transcription in this assay. Because U1 snRNP is the key element of the splicing machinery and it is also associated with other transcript-maturation mechanisms, one might conceive that it could be an important player in the coupling of the transcription and transcript-processing mechanisms. An interaction of U1 snRNP with RNAPII and/or TFIID and/or CPSF among multiple other components could favor the activity of transcription and/or transcript-maturation complexes. It might also be speculated that having U1 snRNP preloaded on the transcription complex facilitates the scanning and detection of 5' splice sites on the nascent transcript.

To summarize, our data confirm the constitutive association of U1 snRNP with the transcription machinery and show that U1 snRNP is recruited to a transcription unit independently of splicing. In view of recent reports about the possible role of U1 snRNA in the regulation of transcription and its association to transcript-maturation machineries other than spliceosome, our findings are in accordance with the hypothesis that U1 snRNP could be an important player in the coupling of different processes related to gene expression.

Materials and Methods

Plasmids

MS2-YFP protein was expressed from pMS2-YFP plasmid (Fusco et al., 2003). pTet-on-CFP-MS2wt was generated by sequentially inserting PCR fragments into pTet-globin-CFP-18MS2-2 (Darzacq et al., 2006). One nucleotide within the β -globin intron-1 sequence was deleted to maintain the translational frame in the absence of splicing; exon 2 from the β -globin cassette was fused to CFP cDNA followed by MS2 repeats that were brought up to 24. pTet-on-CFP-MS2mut was derived from pTet-on-CFP-MS2wt by targeted point mutagenesis of 5' and 3' splice sites (Stratagene mutagenesis kit); both plasmids comprised a puromycin resistance gene. To obtain pCMV-lacZ-MS2 \times 24, 24 MS2 repeats were ligated into pRSV- β gal (Kislauskis et al., 1993) using *Sma*I and *Xba*I enzymes. Subsequently, lacZ-MS2 \times 24 was inserted into pcDNA3.1 (Invitrogen) as a *Hind*III-*Not*I fragment. To generate pHIV-LTR MINX-lacZ-MS2 \times 4, the HIV-LTR (nucleotides 1-734) from plasmid pEXO-MS2 \times 24 (Boireau et al., 2007) was fused to the MINX sequence (Zillmann et al., 1988) by PCR and subsequently ligated into *Nru*I-*Hind*III-digested CMV-lacZ-MS2 \times 24 as a blunt-end *Hind*III fragment. Reduction of the MS2 repeats from

24 to 4 occurred spontaneously during cloning. The MINX construct with deleted intron (pHIV-LTR MINX Δ in-lacZ-MS2 \times 4) was obtained by stepwise fusion of MINX exonic sequences with the HIV-LTR by PCR and ligation into pHIV-LTR MINX-lacZ-MS2 \times 4 as a *Hind*III fragment replacing the MINX intron.

Cells

U2OS Tet-on cells (Clontech) and HeLa cells were propagated in DMEM (Invitrogen) with 1 g/l and 4.5 g/l glucose, respectively, supplemented with 10% fetal calf serum. HeLa cells arrested in mitosis after exposure to nocodazole (50 ng/ml) for 18 hours were detached by shaking (mitotic index higher than 80%) and pelleted by centrifugation at 200 g.

To obtain BGwt and BGmut cell lines, U2OS cells were stably co-transfected with pBABE-puro (Addgene) and pTet-on-CFP-MS2wt or pTet-on-CFP-MS2mut, respectively. Individual colonies were selected in culture medium containing puromycin (10 μ g/ml), screened for CFP expression after doxycycline induction and subcloned. Expression of reporter gene was induced using 5 μ g/ml of doxycycline overnight. FuGene6 (Roche) was used for transient transfection assays. To obtain cells containing constructs with functional MINX intron or deleted MINX intron, U2OS cells were transfected with either pHIV-LTR MINX-lacZ-MS2 \times 4 or pHIV-LTR MINX Δ in-lacZ-MS2 \times 4 plasmids as previously described (Boireau et al., 2007). Expression of the MINX-fused *lacZ* gene was induced by transfection with pcDNA3-Tat(1-101)Flag, a plasmid encoding for a Flag-Tat protein.

Crosslinking, immunoprecipitation and northern blot assays

For protein-RNA crosslinking, formaldehyde (1% final concentration) was added to the culture medium for 10 minutes and then neutralized with addition of glycine (125 mM, pH 7) for 5 minutes. Cells were washed in PBS, lysed in RIPA buffer [50 mM Tris (pH 7.9), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl] and sonicated. The lysate was clarified by centrifugation at 10,000 g. Protein-RNA complexes were immunoprecipitated from supernatants and the crosslink was reversed by incubation for 2 hours at 65°C in a mixture of 50 mM Tris (pH 7.9), 5 mM EDTA, 10 mM dithiothreitol and 1% SDS. RNAs were isolated with the RNeasy micro kit (Qiagen). In the experiment without crosslinking, the immunoprecipitation was performed directly on a clarified extract from mitotic cell extracts in 50 mM HEPES (pH 7.9), KCl 10 mM, 150 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5% Nonidet P40 supplemented with protease inhibitors (Sigma) and Ribolock (Fermentas).

RNAs were electrophoresed in polyacrylamide gels, transferred onto Hybond N (GE Healthcare) and hybridized to nick-end-translated ³²P-labeled DNA probes. The membranes were washed in 0.1 \times SSC buffer (0.015 M NaCl, 0.015 M sodium citrate) at 65°C. Radioactivity was quantified with a Fuji FLA 3000 phosphorimager using the Image Gauge software.

RT-PCR

Reverse transcription was performed with an oligo-dT primer using Superscript III reverse transcriptase (Invitrogen). End-point PCR for verification of cryptic splice sites was performed at 35 cycles using Taq polymerase (Invitrogen). Products were separated by electrophoresis in 3% agarose gels, stained with ethidium bromide. The expression levels of reporter RNA for β -globin plasmids were evaluated by real-time RT-qPCR using SYBR Green I (Qiagen) and normalized to the tubulin α 3 as a reference gene.

Antibodies

The following antibodies were used and were supplied from the indicated sources: 8WG16 (Nancy E. Thompson, McArdle Lab., Madison, WI), Pol3/3 (Ekkhard K. Bautz, Heidelberg, Germany), N20 (sc-899, Santa Cruz Biotechnology), CTD4H8 (Upstate 05-623), U1-70K (Douglas Blake, UCLA, CA), U1A (ab55751, Abcam), U2AF65 (ab37530, Abcam), ASF/SF2 (clone 103, Zymed Laboratories), SC35 (sc-53518, Santa Cruz Biotechnology). Primary antibodies were detected with horseradish-peroxidase-labeled (Promega) or Cy3- or Cy5-conjugated (GE Healthcare) secondary antibodies.

FISH and immunofluorescence

Immunofluorescence and FISH were performed as described previously on paraformaldehyde-fixed cells (Darzacq et al., 2006). Oligodeoxynucleotides (below) containing 5-amino-allyl thymidines (Eurogentec) were labeled with Cy3 or Cy5 mono-reactive dyes (GE Healthcare) or with Alexa Fluor 488 (Molecular Probes). In some cases, an immunostaining was performed after FISH using the standard protocol. The sequences of oligodeoxynucleotides used for FISH are as follows (shown 5' to 3'; 5-amino-allyl thymidines are noted 't' in the sequences): U1-1, CtGGGAAAACCACCTCGTGATCAIGGTATCTCCCTGCCAGGTAAGAT; U1-2, CGAACGCAGTCCCCACACCCACAAAATTAIGCAGTCGAGITCCACAT; U2, AGtGGACGGAGCAAGCtCCTATTCACtCCCTGCtCCAAAATCCATt; U4-1, tAGCAATAAtCGCGCTCGGAtAAACCTCAItGGCTACGATACtGCC- ACT; U4-2, AGACtGTCAAAAAtGCCAATGCGGACAtATTGCAAGtCGTCA- CGGCGGtA; U6, CgItGCATCCTtGCGCAGGGGCCAtGCTAATCTTtCTGT- ATCGTtCCAA; beta-glob-E1, AGGAGtCAGGTGCACCAItGGTGTCTGTtTGAG- GTTGCTAGtGACACAGtA; beta-glob-II, tCAGTGCCTAtCAGAAACCCAA- GAGtCTTCTGTtCtCCACATGCCAGtA; MS2 NBX, CtAGGCAAT-

AGGTACCTtAGGATCTAAAtGAACCCGGGAATActGCAGA. U2, U6, beta-glob-E1, beta-glob-II and MS2 NBX were designed as previously published (Shav-Tal et al. 2005; Darzacq et al. 2006).

Radial analysis of site images

Images were acquired on an Olympus IX 81 epifluorescence microscope equipped with plan apo 60 \times 1.4 NA objective and a CoolSNAP HQ camera (Roper Scientific). Three-dimensional imaging was performed on successive planes separated by 200 nm in the z-axis. Images were processed using the ImageJ software (<http://rsb.info.nih.gov/ij/>). MS2 FISH or MS2-YFP fluorescence images were used to accurately define the transcription-site center. Images in the transcription-site focal plane were summed up with images in planes immediately above and below (a stack of 400 nm z depth). For a given probe, averaged intensities $[AI(d)]$ at a distance 'd' from the transcription-site center (TS) were defined as the sum of pixel intensities in an annulus between rings centered on the transcription site and separated by 1 pixel (107.5 nm) divided by the area of the same annulus. Assuming that transcription sites are much smaller than 1.6 μ m (Shav-Tal et al., 2004), we arbitrarily chose this distance as representative of the intensity outside the transcription site. Thus, the difference $[AI(d) - AI(1.6)]$ represents the enrichment of a given factor at the transcription site. $[AI(d) - AI(1.6)]/[AI(1.6)]$ is the relative enrichment. To test the significance of the observed enrichments, the same analysis was repeated with a site (RS) that was selected at random by the ImageJ software within a rectangle of 20 μ m² positioned manually within the nucleoplasm but excluding nucleoli. To average data from different cells on various slides prepared independently and take into account variations in signal outside the transcription site, we used an averaged relative enrichments defined as: $\Sigma[AI(d) - AI(1.6)]/\Sigma[AI(1.6)]$. Almost identical results were obtained when the averaged relative enrichments for n cells were defined as: $1/n \times \Sigma[AI(d) - AI(1.6)]/[AI(1.6)]$.

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References

- Alexander, M. R., Wheatley, A. K., Center, R. J. and Purcell, D. F. (2010). Efficient transcription through an intron requires the binding of an Sm-type U1 snRNP with intact stem loop II to the splice donor. *Nucleic Acids Res.* [Epub ahead of print] doi:10.1093/nar/gkp1224.
- Allen, T. A., Von Kaenel, S., Goodrich, J. A. and Kugel, J. F. (2004). The SINE-encoded mouse B2 RNA represses mRNA transcription in response to heat shock. *Nat. Struct. Mol. Biol.* **11**, 816-821.
- Awasthi, S. and Alwine, J. C. (2003). Association of polyadenylation cleavage factor I with U1 snRNP. *RNA* **9**, 1400-1409.
- Barrandon, C., Spiluttini, B. and Bensaude, O. (2008). Non-coding RNAs regulating the transcriptional machinery. *Biol. Cell* **100**, 83-95.
- Bentley, D. L. (2005). Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors. *Curr. Opin. Cell Biol.* **17**, 251-256.
- Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S. M., Singer, R. H. and Long, R. M. (1998). Localization of ASH1 mRNA Particles in Living Yeast. *Mol. Cell* **2**, 437-445.
- Boireau, S., Maiuri, P., Basyuk, E., de la Mata, M., Knezevich, A., Pradet-Balade, B., Bäcker, V., Kornblihtt, A., Marcello, A. and Bertrand, E. (2007). The transcriptional cycle of HIV-1 in real-time and live cells. *J. Cell Biol.* **179**, 291-304.
- Bonnerot, C., Rocancourt, D., Briand, P., Grimmer, G. and Nicolas, J. F. (1987). A beta-galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. *Proc. Natl. Acad. Sci. USA* **84**, 6795-6799.
- Cao, W. and Garcia-Blanco, M. A. (1998). A serine/arginine-rich domain in the human U1 70k protein is necessary and sufficient for ASF/SF2 binding. *J. Biol. Chem.* **273**, 20629-20635.
- Chen, Y., Yamaguchi, Y., Tsugeno, Y., Yamamoto, J., Yamada, T., Nakamura, M., Hisatake, K. and Handa, H. (2009). DSIF, the Paf1 complex, and Tat-SF1 have non redundant, cooperative roles in RNA polymerase II elongation. *Genes Dev.* **23**, 2765-2777.
- Damgaard, C. K., Kahns, S., Lykke-Andersen, S., Nielsen, A. L., Jensen, T. H. and Kjems, J. (2008). A 5' splice site enhances the recruitment of basal transcription initiation factors in vivo. *Mol. Cell* **29**, 271-278.
- Dantonel, J. C., Murthy, K. G., Manley, J. L. and Tora, L. (1997). Transcription factor TFIID recruits factor CPSF for formation of 3' end of mRNA. *Nature* **389**, 399-402.
- Darzacq, X., Kittur, N., Roy, S., Shav-Tal, Y., Singer, R. H. and Meier, U. T. (2006). Stepwise RNP assembly at the site of H/ACA RNA transcription in human cells. *J. Cell Biol.* **173**, 207-218.
- Darzacq, X., Shav-Tal, Y., de Turris, V., Brody, Y., Shenoy, S. M., Phair, R. D. and Singer, R. H. (2007). In vivo dynamics of RNA polymerase II transcription. *Nat. Struct. Mol. Biol.* **14**, 796-806.

- Das, R., Yu, J., Zhang, Z., Gygi, M. P., Krainer, A. R., Gygi, S. P. and Reed, R. (2007). SR proteins function in coupling RNAP II transcription to pre-mRNA splicing. *Mol. Cell* **26**, 867-881.
- de Almeida, S. F. and Carmo-Fonseca, M. (2008). The CTD role in cotranscriptional RNA processing and surveillance. *FEBS Lett.* **582**, 1971-1976.
- Ellis, J. D., Llères, D., Denegri, M., Lamond, A. I. and Cáceres, J. F. (2008). Spatial mapping of splicing factor complexes involved in exon and intron definition. *J. Cell Biol.* **181**, 921-934.
- Espinoza, C. A., Allen, T. A., Hieb, A. R., Kugel, J. F. and Goodrich, J. A. (2004). B2 RNA binds directly to RNA polymerase II to repress transcript synthesis. *Nat. Struct. Mol. Biol.* **11**, 822-829.
- Femino, A. M., Fay, F. S., Fogarty, K. and Singer, R. H. (1998). Visualization of single RNA transcripts in situ. *Science* **280**, 585-590.
- Förch, P., Merendino, L., Martínez, C. and Valcarcel, J. (2003). U2 small nuclear ribonucleoprotein particle (snRNP) auxiliary factor of 65 kDa, U2AF65, can promote U1 snRNP recruitment to 5' splice sites. *Biochem. J.* **372**, 235-242.
- Friend, K., Lovejoy, A. F. and Steitz, J. A. (2007). U2 snRNP binds intronless histone pre-mRNAs to facilitate U7-snRNP-dependent 3' end formation. *Mol. Cell* **28**, 240-252.
- Furger, A., O'Sullivan, J. M., Binnie, A., Lee, B. A. and Proudfoot, N. J. (2002). Promoter proximal splice sites enhance transcription. *Genes Dev.* **16**, 2792-2799.
- Fusco, D., Accornero, N., Lavoie, B., Shenoy, S. M., Blanchard, J. M., Singer, R. H. and Bertrand, E. (2003). Single mRNA molecules demonstrate probabilistic movement in living mammalian cells. *Curr. Biol.* **13**, 161-167.
- Golovkin, M. and Reddy, A. S. (1999). An SC35-like protein and a novel serine/arginine-rich protein interact with Arabidopsis U1-70K protein. *J. Biol. Chem.* **274**, 36428-36438.
- Gottesfeld, J. M. and Forbes, D. J. (1997). Mitotic repression of the transcriptional machinery. *Trends Biochem. Sci.* **22**, 197-202.
- Hirose, Y. and Manley, J. L. (2000). RNA polymerase II and the integration of nuclear events. *Genes Dev.* **14**, 1415-1429.
- Hochleitner, E. O., Kastner, B., Fröhlich, T., Schmidt, A., Lührmann, R., Arnold, G. and Lottspeich, F. (2005). Protein stoichiometry of a multiprotein complex, the human spliceosomal U1 small nuclear ribonucleoprotein: absolute quantification using isotope-coded tags and mass spectrometry. *J. Biol. Chem.* **280**, 2536-2542.
- Hoffman, B. E. and Grabowski, P. J. (1992). U1 snRNP targets an essential splicing factor, U2AF65, to the 3' splice site by a network of interactions spanning the exon. *Genes Dev.* **6**, 2554-2568.
- Huang, S. and Spector, D. L. (1996). Intron-dependent recruitment of pre-mRNA splicing factors to sites of transcription. *J. Cell Biol.* **133**, 719-732.
- Jiang, Y., Liu, M., Spencer, C. A. and Price, D. H. (2004). Involvement of transcription termination factor 2 in mitotic repression of transcription elongation. *Mol. Cell* **14**, 375-385.
- Jobert, L., Pinzón, N., Van Herreweghe, E., Jány, B. E., Guialis, A., Kiss, T. and Tora, L. (2009). Human U1 snRNA forms a new chromatin-associated snRNP with TAF15. *EMBO Rep.* **10**, 494-500.
- Kao, H. Y. and Siliciano, P. G. (1995). Identification of Prp40, a novel essential yeast splicing factor associated with the U1 small nuclear ribonucleoprotein particle. *Mol. Cell Biol.* **16**, 960-967.
- Kettenberger, H., Eisenfuhr, A., Brueckner, F., Theis, M., Famulok, M. and Cramer, P. (2006). Structure of an RNA polymerase II-RNA inhibitor complex elucidates transcription regulation by noncoding RNAs. *Nat. Struct. Mol. Biol.* **13**, 44-48.
- Kim, E., Du, L., Bregman, D. B. and Warren, S. L. (1997). Splicing factors associate with hyperphosphorylated RNA polymerase II in the absence of pre-mRNA. *J. Cell Biol.* **136**, 19-28.
- Kislauskis, E. H., Li, Z., Singer, R. H. and Taneja, K. L. (1993). Isoform-specific 3'-untranslated sequences sort alpha-cardiac and beta-cytoplasmic actin messenger RNAs to different cytoplasmic compartments. *J. Cell Biol.* **123**, 165-172.
- Kwek, K. Y., Murphy, S., Furger, A., Thomas, B., O'Gorman, W., Kimura, H., Proudfoot, N. J. and Akoulitchev, A. (2002). U1 snRNA associates with TFIIF and regulates transcriptional initiation. *Nat. Struct. Mol. Biol.* **9**, 800-805.
- Lacy-Hulbert, A., Thomas, R., Li, X. P., Lilley, C. E., Coffin, R. S. and Roes, J. (2001). Interruption of coding sequences by heterologous introns can enhance the functional expression of recombinant genes. *Gene Ther.* **8**, 649-653.
- Lewis, J. D., Izaurralde, E., Jarmolowski, A., McGuigan, C. and Mattaj, J. W. (1996). A nuclear cap-binding complex facilitates association of U1 snRNP with the cap-proximal 5' splice site. *Genes Dev.* **10**, 1683-1698.
- Li, X. and Manley, J. L. (2005). Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. *Cell* **122**, 365-378.
- Li, X.-Y. and Green, M. R. (1998). The HIV-1 Tat cellular coactivator is a general transcription elongation factor. *Genes Dev.* **12**, 2992-2996.
- Lin, S., Coutinho-Mansfield, G., Wang, D., Pandit, S. and Fu, X.-D. (2008). The splicing factor SC35 has an active role in transcriptional elongation. *Nat. Struct. Mol. Biol.* **15**, 819-826.
- Listerman, I., Sapra, A. K. and Neugebauer, K. M. (2006). Cotranscriptional coupling of splicing factor recruitment and precursor messenger RNA splicing in mammalian cells. *Nat. Struct. Mol. Biol.* **13**, 815-822.
- Long, J. C. and Cáceres, J. F. (2009). The SR protein family of splicing factors: master regulators of gene expression. *Biochem. J.* **417**, 15-27.
- Lutz, C. S., Murthy, K. G., Schek, N., O'Connor, J. P., Manley, J. L. and Alwine, J. C. (1996). Interaction between the U1 snRNP-A protein and the 160-kD subunit of cleavage-polyadenylation specificity factor increases polyadenylation efficiency in vitro. *Genes Dev.* **10**, 325-337.
- Mabon, S. A. and Misteli, T. (2005). Differential recruitment of pre-mRNA splicing factors to alternatively spliced transcripts in vivo. *PLoS Biol.* **3**, e374.
- Mariner, P. D., Walters, R. D., Espinoza, C. A., Drullinger, L. F., Wagner, S. D., Kugel, J. F. and Goodrich, J. A. (2008). Human Alu RNA is a modular transacting repressor of mRNA transcription during heat shock. *Mol. Cell* **29**, 499-509.
- Martin, T. E., Monsma, S. A., Romac, J. M. and Leser, G. P. (1987). The intranuclear states of snRNP complexes. *Mol. Biol. Rep.* **12**, 180-181.
- Matunis, E. L., Matunis, M. J. and Dreyfuss, G. (1993). Association of individual hnRNP proteins and snRNPs with nascent transcripts. *J. Cell Biol.* **121**, 219-228.
- Metz, A., Soret, J., Vourc'h, C., Tazi, J. and Jolly, C. (2004). A key role for stress-induced satellite III transcripts in the relocalization of splicing factors into nuclear stress granules. *J. Cell Sci.* **117**, 4551-4558.
- Mili, S. and Steitz, J. A. (2004). Evidence for reassociation of RNA-binding proteins after cell lysis: implications for the interpretation of immunoprecipitation analyses. *RNA* **10**, 1692-1694.
- Misteli, T. and Spector, D. L. (1999). RNA polymerase II targets pre-mRNA splicing factors to transcription sites in vivo. *Mol. Cell* **3**, 697-705.
- Moore, M. J. and Proudfoot, N. J. (2009). Pre-mRNA processing reaches back to transcription and ahead to translation. *Cell* **136**, 688-700.
- Morris, D. P. and Greenleaf, A. L. (2000). The splicing factor, Prp40, binds the phosphorylated carboxyl-terminal domain of RNA polymerase II. *J. Biol. Chem.* **275**, 39935-39943.
- Palmiter, R. D., Sandgren, E. P., Avarbock, M. R., Allen, D. D. and Brinster, R. L. (1991). Heterologous introns can enhance expression of transgenes in mice. *Proc. Natl. Acad. Sci. USA* **88**, 478-482.
- Pandit, S., Wang, D. and Fu, X. D. (2008). Functional integration of transcriptional and RNA processing machineries. *Curr. Opin. Cell Biol.* **20**, 819-826.
- Patel, S. B., Novikova, N. and Bellini, M. (2007). Splicing-independent recruitment of spliceosomal small nuclear RNPs to nascent RNA polymerase II transcripts. *J. Cell Biol.* **10**, 937-949.
- Perales, R. and Bentley, D. (2009). "Cotranscriptionality": the transcription elongation complex as a nexus for nuclear transactions. *Mol. Cell* **36**, 178-191.
- Perriman, R., Barta, I., Voeltz, G. K., Abelson, J. and Ares, M. (2003). ATP requirement for Prp5 function is determined by Cus2p and the structure of U2 small nuclear RNA. *Proc. Natl. Acad. Sci. USA* **100**, 13857-13862.
- Prasanth, K. V. and Spector, D. L. (2007). Eukaryotic regulatory RNAs: an answer to the 'genome complexity' conundrum. *Genes Dev.* **21**, 11-42.
- Robert, F., Blanchette, M., Maes, O., Chabot, B. and Coulombe, B. (2002). A human RNA polymerase II-containing complex associated with factors necessary for spliceosome assembly. *J. Biol. Chem.* **277**, 9302-9306.
- Shav-Tal, Y., Darzacq, X., Shenoy, S. M., Fusco, D., Janicki, S. M., Spector, D. L. and Singer, R. H. (2004). Dynamics of single mRNPs in nuclei of living cells. *Science* **304**, 1797-1800.
- Shav-Tal, Y., Blechman, J., Darzacq, X., Montagna, C., Dye, B. T., Patton, J. G., Singer, R. H. and Zipori, D. (2005) Dynamic sorting of nuclear components into distinct nucleolar caps during transcriptional inhibition. *Mol. Biol. Cell* **16**, 2395-2413.
- Tian, H. (2001). RNA ligands generated against complex nuclear targets indicate a role for U1 snRNP in co-ordinating transcription and RNA splicing. *FEBS Lett.* **509**, 282-286.
- Ujvári, A. and Luse, D. S. (2004). Newly initiated RNA encounters a factor involved in splicing immediately upon emerging from within RNA polymerase II. *J. Biol. Chem.* **279**, 49773-49779.
- Wassarman, K. M. (2007). 6S RNA: a regulator of transcription. *Mol. Microbiol.* **65**, 1425-1431.
- Will, C. L. and Lührmann, R. (2001). Spliceosomal UsnRNP biogenesis, structure and function. *Curr. Opin. Cell Biol.* **13**, 290-301.
- Wilusz, J. E., Sunwoo, H. and Spector, D. L. (2009). Long noncoding RNAs: functional surprises from the RNA world. *Genes Dev.* **23**, 1494-1504.
- Yan, D., Perriman, R., Igel, H., Howe, K. J., Neville, M. and Ares, M., Jr (1998). CUS2, a yeast homolog of human Tat-SF1, rescues function of misfolded U2 through an unusual RNA recognition motif. *Mol. Cell Biol.* **18**, 5000-5009.
- Zhong, X. Y., Wang, P., Han, J., Rosenfeld, M. G. and Fu, X. D. (2009). SR proteins in vertical integration of gene expression from transcription to RNA processing to translation. *Mol. Cell* **35**, 1-10.
- Zhou, Q. and Sharp, P. A. (1996). Tat-SF1: cofactor for stimulation of transcriptional elongation by HIV-1 Tat. *Science* **274**, 605-610.
- Zillmann, M., Zapp, M. L. and Berget, S. M. (1988). Gel electrophoretic isolation of splicing complexes containing U1 small nuclear ribonucleoprotein particles. *Mol. Cell Biol.* **8**, 814-821.