Splicing-independent recruitment of spliceosomal small nuclear RNPs to nascent RNA polymerase II transcripts

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In amphibian oocytes, most lateral loops of the lampbrush chromosomes correspond to active transcriptional sites for RNA polymerase II. We show that newly assembled small nuclear ribonucleoprotein (RNP [snRNP]) particles, which are formed upon cytoplasmic injection of fluorescently labeled spliceosomal small nuclear RNAs (snRNAs), target the nascent transcripts of the chromosomal loops. With this new targeting assay, we demonstrate that nonfunctional forms of U1 and U2 snRNAs still associate with the active transcriptional units. In particular, we find that their association with nascent RNP fibrils is independent of their base pairing with pre–messenger RNAs. Additionally, stem loop I of the U1 snRNA is identified as a discrete domain that is both necessary and sufficient for association with nascent transcripts. Finally, in oocytes deficient in splicing, the recruitment of U1, U4, and U5 snRNPs to transcriptional units is not affected. Collectively, these data indicate that the recruitment of snRNPs to nascent transcripts and the assembly of the spliceosome are uncoupled events.

Introduction

In eukaryotes, the removal of introns from pre-mRNAs requires the five phylogenetically conserved small nuclear RNP (snRNP) particles (U1, U2, U4, U5, and U6 snRNPs; for reviews see Hastings and Krainer, 2001; Patel and Steitz, 2003). The formation of functional spliceosomal snRNPs is a complex event (for reviews see Will and Luhrmann, 2001; Kiss, 2004; Matera and Shpargel, 2006), and several discrete nuclear domains, such as Cajal bodies (CBs), interchromatin granule clusters (IGCs), and nucleoli have been implicated in their maturation and/or storage (Gall, 2003). The snRNPs, along with >100 other splicing factors, assemble onto pre-mRNA to form the spliceosome, and it is this dynamic macromolecular machine that orchestrates the excision of introns and the ligation of exons through two successive trans-esterification reactions (for review see Patel and Steitz, 2003). Spliceosomal assembly and splicing itself, which are key events in the maturation of pre-mRNAs, are tightly coupled to RNA transcription (for reviews see Neugebauer, 2002; Bentley, 2005). Accordingly, nascent RNA polymerase (RNAP) II transcripts were previously shown to recruit splicing factors, such as the snRNPs and SR (serine-arginine rich) proteins (Fu and Maniatis, 1990; Wu et al., 1991; Huang and Spector, 1996; Gall et al., 1999) and, more recently, the exon junction complexes (EJCs), which mark the ultimate products of splicing, exon–exon junctions (for review see Aguilera, 2005).

Although data on the spatial and temporal recruitment of splicing factors onto a template pre-mRNA abound, very little is still known about the essential characteristics of a spliceosomal snRNP that contribute in vivo to its association with nascent transcripts. Previous work on U1 and U2 snRNPs highlighted the importance of the base pairing of their RNA moieties to cis-acting sequences on pre-mRNAs, the intronic 5′ splice site (SS) and the branch point sequence (BPS), respectively (Krämer et al., 1984; Parker et al., 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989). In the case of the U1 snRNP, however, it was shown that the base pairing of its 5′ end with the 5′ SS is only one of several interactions that contribute to the formation of a U1 snRNP–pre-mRNA complex (Du and Rosbash, 2001) and occurs after an initial recruitment of the U1 snRNP (Lacadie and Rosbash, 2005). Interestingly, cleavage of the 5′ end of the U1 small nuclear RNA (snRNA) has no effect on the rate of association of the U1 snRNP with a consensus 5′ SS RNA oligonucleotide in vitro (Rossi et al., 1996). Rather, recognition of the 5′ SS by the U1 snRNP appears to be driven by its overall protein complement. Which of the several U1 snRNP proteins and which sequence elements of the U1 snRNA are critical for its targeting

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Abbreviations used in this paper: BPS, branch point sequence; CB, Cajal body; DIC, differential interference contrast; EJC, exon junction complex; IGC, interchromatin granule cluster; LBC, lampbrush chromosome; RNAP, RNA polymerase; snRNA, small nuclear RNA; snRNP, small nuclear RNP; SS, splice site.

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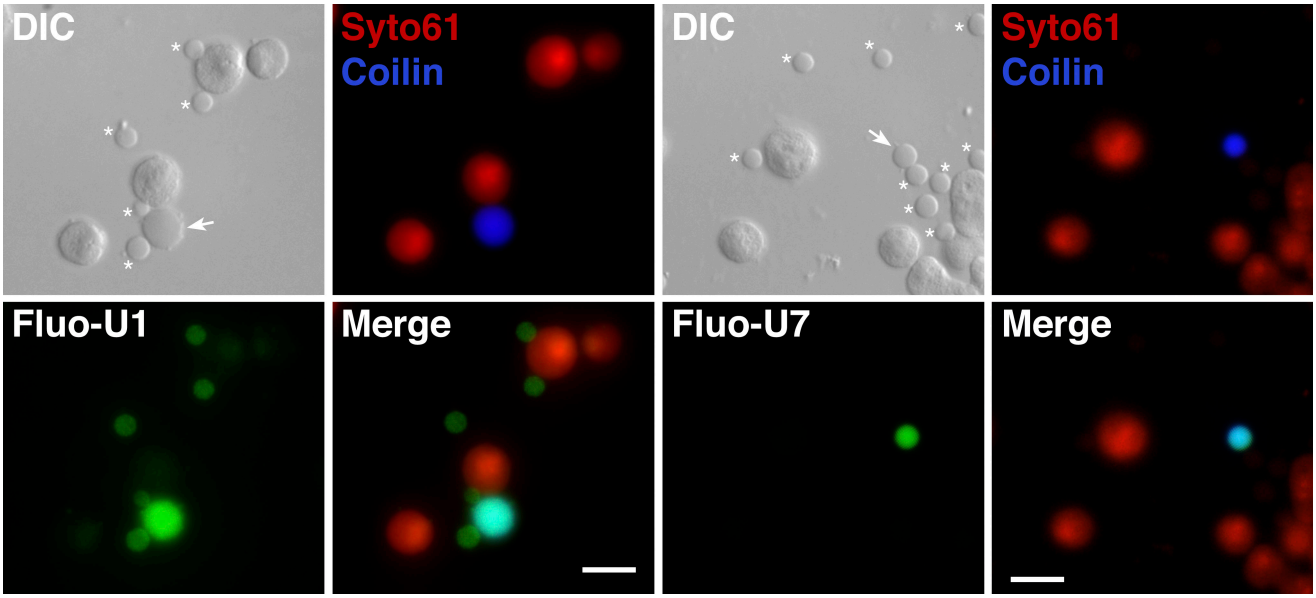

Figure 1. **Newly assembled spliceosomal snRNPs associate rapidly with CBs and IGCs (B-snurposomes).** Differential interference contrast (DIC) and corresponding fluorescent micrographs of nuclear spreads from oocytes injected with fluorescent U1 or U7 snRNAs, respectively (green). Organelles are readily distinguished by their morphology with DIC and specific probes. Here, the DNA-specific dye Syto61 was used to labeled nucleoli (red), whereas the anti-coilin antibody (mAb H1) was used to label CBs (blue; arrows). Newly made fluorescent U1 snRNP is detected in both CBs and IGCs (asterisks) as early as 1 h after cytoplasmic injection of fluorescent U1 snRNA. In contrast, a newly assembled fluorescent U7 snRNP, which is not involved in splicing, accumulates exclusively within CBs. In both cases, nucleoli are negative. Bars, 5 μm.

to nascent transcripts is still unclear, however. The same question also remains unanswered for the other spliceosomal snRNPs, and, in light of their complex intranuclear trafficking before engaging pre-mRNA splicing (for review see Kiss, 2004), it cannot be addressed directly using in vitro systems.

The lampbrush chromosomes (LBCs) of amphibian oocytes exhibit unique structural characteristics that make it possible to study the recruitment of snRNPs to nascent transcripts in vivo. In particular, these extended diplotene bivalent chromosomes display numerous lateral loops of chromatin that correspond to regions of intense transcriptional activity by RNAPII (for review see Morgan, 2002). The chromosomal loops are composed of two distinct domains: the first domain corresponds to a decondensed euchromatin axis that can be demonstrated using antibodies against the RNAPII transcriptional machinery or various chromatin components (Gall et al., 1999). The second domain corresponds to nascent RNP fibrils, which are formed from nascent pre-mRNAs associated with a cortege of factors involved in their maturation. These RNP fibrils create a dense RNP matrix around the loop axis that is readily observable by phase contrast or differential interference contrast (DIC) microscopy. Indeed, the elongation of transcripts along the axis is reflected in a characteristic thin to thick morphology of the loops (Gall et al., 1999; Beenders et al., 2003; for review see Morgan, 2002). We show here for the first time that newly assembled snRNPs, which are formed upon cytoplasmic injection of fluorescein-labeled snRNAs, associate rapidly with nascent transcripts, and we used this novel cytological assay to begin dissecting the molecular mechanisms that regulate the association of splicing snRNPs with active transcriptional units. In particular, we demonstrate that U1 and U2 snRNPs need not be functional for their association with elongating transcripts. We also characterize the first stem loop domain of the U1 snRNA as a structure that is both necessary and sufficient for targeting the U1 snRNP to nascent pre-mRNAs. Finally, we present evidence that pre-mRNA splicing occurs on the LBC loops and that recruitment of the splicing snRNPs to active transcriptional units is independent of their integration into a spliceosome.

Results

Newly assembled fluorescent snRNPs target chromosomal loops

It was previously established that in vitro–synthesized spliceosomal snRNAs injected into the cytoplasm of amphibian oocytes assemble into functional snRNP particles (Mattaj, 1988) that are competent to splice pre-mRNA. Curiously, in these experiments, the subnuclear localization of the newly formed snRNPs does not correspond to the steady-state distribution of the endogenous snRNPs, which are associated with CBs, IGCs (B-snurposomes in the oocyte), and chromosomal loops. Notably, although the newly formed snRNPs accumulate within CBs, their association with IGCs is very weak, and their targeting to chromosomal loops was never documented (Gall et al., 1999).

Chromosomal loops are likely sites of pre-mRNA processing, however, and because injected synthetic spliceosomal RNAs can rescue splicing in oocytes depleted of the corresponding endogenous snRNA (Pan and Prives, 1988; Yu et al., 1998), our hypothesis was that injected fluorescent snRNAs do associate with chromosomal loops but at a concentration too low to be detected without amplification. To test that idea, fluorescein-conjugated U1, U2, U4, and U5 snRNAs were synthesized and injected into

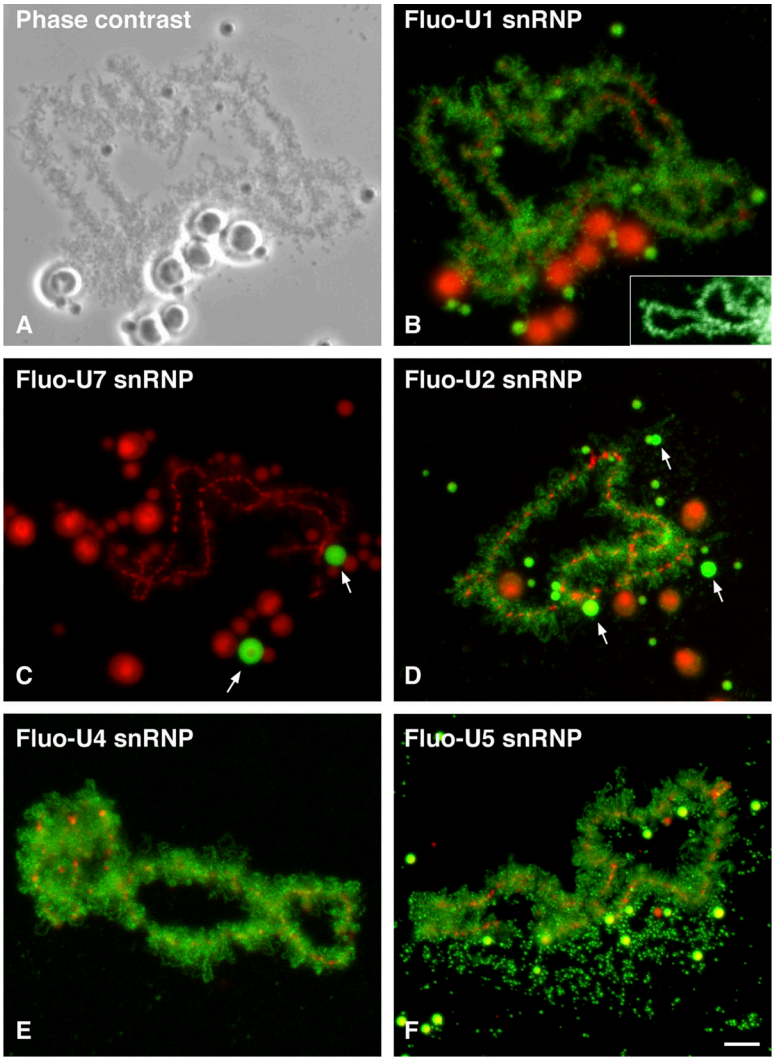

Figure 2. **Association of newly made fluorescent spliceosomal snRNPs with active transcriptional units.** In vitro transcribed snRNAs were injected into the cytoplasm of stage V oocytes, and nuclear spreads were prepared 18 h later. In all preparations, the DNA was counterstained with DAPI, which is pseudocolored here in red. The fluorescent snRNA signal is shown in green. (A and B) A phase-contrast image and its corresponding fluorescent image are presented for the U1 snRNP. The inset in B corresponds to a laser-scanning confocal image of several chromosomal loops showing association of the U1 snRNP with the nascent RNP fibrils. (C–F) Fluorescent images are shown for U7, U2, U4, and U5 snRNPs. Consistent with the distribution of the endogenous splicing snRNPs, the newly assembled U1, U2, U4, and U5 snRNPs were detected in IGCs, CBs (arrows), and on the loops of LBCs. (C) The nonspliceosomal U7 snRNP accumulated in CBs but was absent from IGCs and the chromosomal loops. Note that DAPI labels well the nucleoli and, to a lesser extent, IGCs (most likely because of their high content in RNAs), which are structures of ~1 μm in diameter. DAPI also labels well the chromosomal axes, which correspond to transcriptionally inactive domains. Bar, 10 μm.

the cytoplasm of *Xenopus laevis* oocytes, and their was fate monitored over time on fixed nuclear spreads. A two-antibody detection system was used to enhance the fluorescent signals, and, as expected, all four snRNAs entered the nucleus and associated with CBs. Fig. 1 shows the targeting of the U1 snRNP to both CBs and IGCs only 1 h after cytoplasmic injection. The same result was obtained with U2, U4, and U5 snRNAs. In contrast, the nonspliceosomal U7 snRNP (discussed in the next paragraph), which was used here as a negative control, did not associate with IGCs but strongly targeted CBs.

Also, for the first time, we were able to demonstrate their association with the active transcriptional units (Fig. 2). Unlike a previous study (Gall et al., 1999), we found that fluorescent snRNPs target the chromosomal loops rapidly after injection because a weak but specific signal was also detected in these nuclear domains as soon as 1 h after injection. Detailed analyses of the loop staining using laser-scanning confocal microscopy revealed an association of the fluorescent snRNPs with the nascent RNPs rather than with the axial chromatin (Fig. 2 B, inset). This loop distribution is identical to that of the endogenous snRNPs as previously determined by in situ hybridization (Wu et al., 1991). Importantly, labeling of the loops cannot be attributed to an incorporation of free fluorescent UTP (possibly produced by degradation of the injected snRNAs) into nascent transcripts because the injection of 200 pmol of fluorescent UTP fails to generate any detectable signal (unpublished data). Instead, staining of the loops is most likely caused by association of the snRNAs in their snRNP conformation.

The U7 snRNP is not recruited to chromosomal loops

To test whether the presence of the fluorescent snRNPs on chromosomes was the result of a genuine recruitment rather than that of random binding, we analyzed the subnuclear distribution of a synthetic fluorescent U7 snRNA after cytoplasmic injections. Just like the spliceosomal snRNAs, the U7 snRNA assembles into snRNP that is subsequently recruited to the nucleus (Grimm et al., 1993; Stefanovic et al., 1995; Wu et al., 1996; Bellini and Gall, 1998). The nuclear U7 snRNP comprises part of the processing machinery responsible for the maturation of histone pre-mRNAs (Birchmeier et al., 1984; Mowry and Steitz, 1987; Birnstiel and Schaufele, 1988; Scharl and Steitz, 1994; Dominski et al., 2002), and it was previously shown by in situ hybridization that >90% of the nuclear U7 snRNA associates with CBs

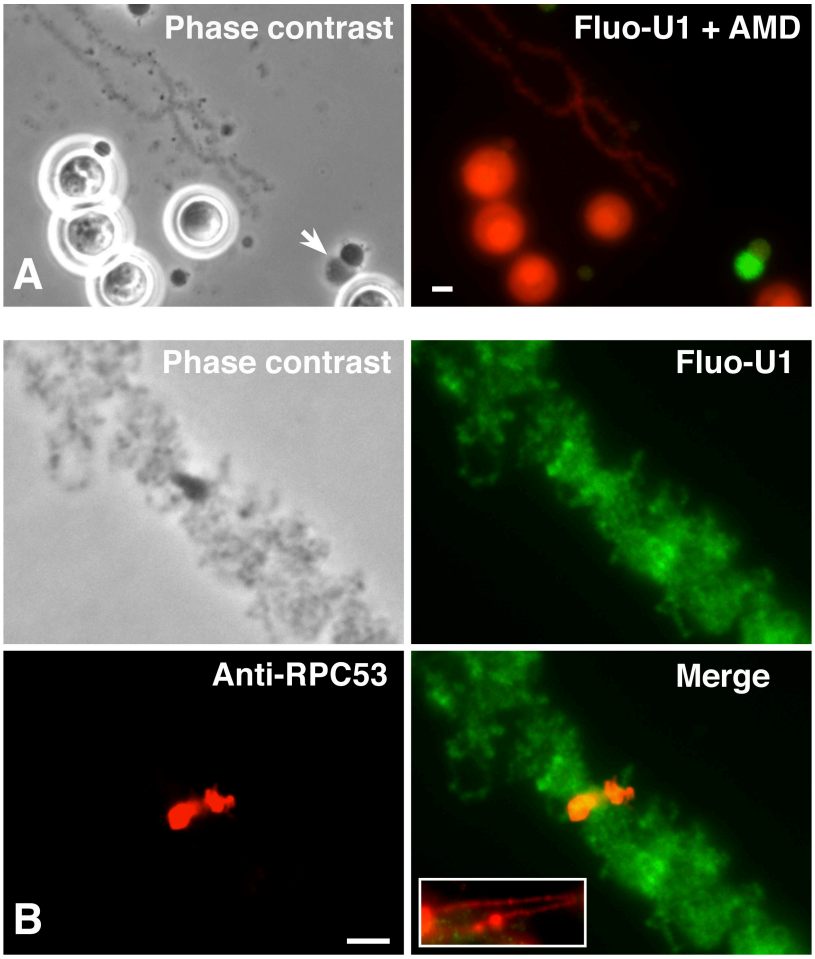

Figure 3. **Newly assembled snRNPs associate with RNAPII but not RNAPIII nascent transcripts.** Phase-contrast and corresponding fluorescent micrographs of nuclear spreads from oocytes injected 18 h earlier with fluorescent U1 snRNA (green). (A) Oocytes were treated with actinomycin D (AMD) for 1 h before nuclear spread preparation. Phase contrast shows one of the 18 LBCs, which are devoid of lateral loops as a result of transcription inhibition. Fluorescent U1 snRNP associates with CBs (arrow) and IGCs but fails to target chromosomes. The chromosomal axis and nucleoli are counterstained with DAPI (pseudocolored in red). (B) An anti-RPC53 antibody was used to identify the ~90 RNAPIII transcriptional sites. One such RNAPIII locus is shown here (red) to illustrate the fact that newly assembled fluorescent U1 snRNPs are not recruited there. Note that this locus is not visible by phase contrast. If many RNAPIII loci appear as rather amorphous structures like the one presented here, several others tend to display long lateral loops. One such loop is presented in the inset at the same magnification. Notice that no green signal is associated with the loop. Bars, 5 μm.

and is absent from chromosomes (Wu et al., 1996; Bellini and Gall, 1998). Thus, the U7 snRNP is not expected to interact with chromosomal loops. Figs. 1 and 2 C show that the newly made fluorescent U7 snRNP was efficiently targeted to CBs but not to chromosomes.

The chromosomal targeting of fluorescent snRNPs requires RNAPII transcripts

To further test whether snRNPs are recruited to active sites of transcription, oocytes were treated with the transcription inhibitor actinomycin D before nuclear spread preparation. Such treatment results in a complete loss of chromosomal signal, as shown in Fig. 3 A for the U1 snRNP. These data further support the conclusion that association of the fluorescent snRNPs with chromosomes depends on the presence of nascent transcripts. Although there is no RNAPI activity on LBCs, both RNAPII and RNAPIII are actively engaged in transcription. The sites of RNAPIII transcription have been mapped to ~90 distinct chromosomal loci (Murphy et al., 2002). These sites are not visible by light microscopy because they lack the density of an RNP matrix but are readily detected by immunofluorescence using anti-RNAPIII antibodies (Murphy et al., 2002). An antibody directed against one of the specific subunits of RNAPIII, RPC53, was used in Fig. 3 B to show that a newly assembled fluorescent U1 snRNP does not associate with RNAPIII transcriptional units. This result is in agreement with the fact that RNAPIII transcripts are not substrates of the spliceosome. Identical results were obtained with the U2 snRNP (unpublished data).

Collectively, these data demonstrate that the association of newly made U1, U2, U4, and U5 snRNPs with chromosomal loops reflects physiologically relevant interactions between these snRNPs and the elongating RNAPII transcripts. They also establish a new cytological system to determine in vivo which characteristics of a spliceosomal snRNP is essential to regulate its recruitment to the active RNAPII transcriptional units of the amphibian oocyte.

Nonfunctional U1 and U2 snRNPs are still recruited to nascent transcripts

U1 and U2 snRNPs are thought to be involved early in the stepwise formation of the spliceosome onto a target pre-mRNA, and they both display a short sequence that hybridizes to the 5′ SS or BPS of an intron, respectively (for review see Patel and Steitz, 2003). In the case of the U1 snRNA, the 5′ SS recognition sequence lies within its first 20 nucleotides. To test whether the recruitment of the U1 snRNP to transcriptional units requires its hybridization with pre-mRNAs, a fluorescent U1 snRNA truncated from its first 20 residues, U1(ΔSS) snRNA, was synthesized and injected into the cytoplasm of *Xenopus* oocytes. It was established that removal of these residues of the U1 snRNA

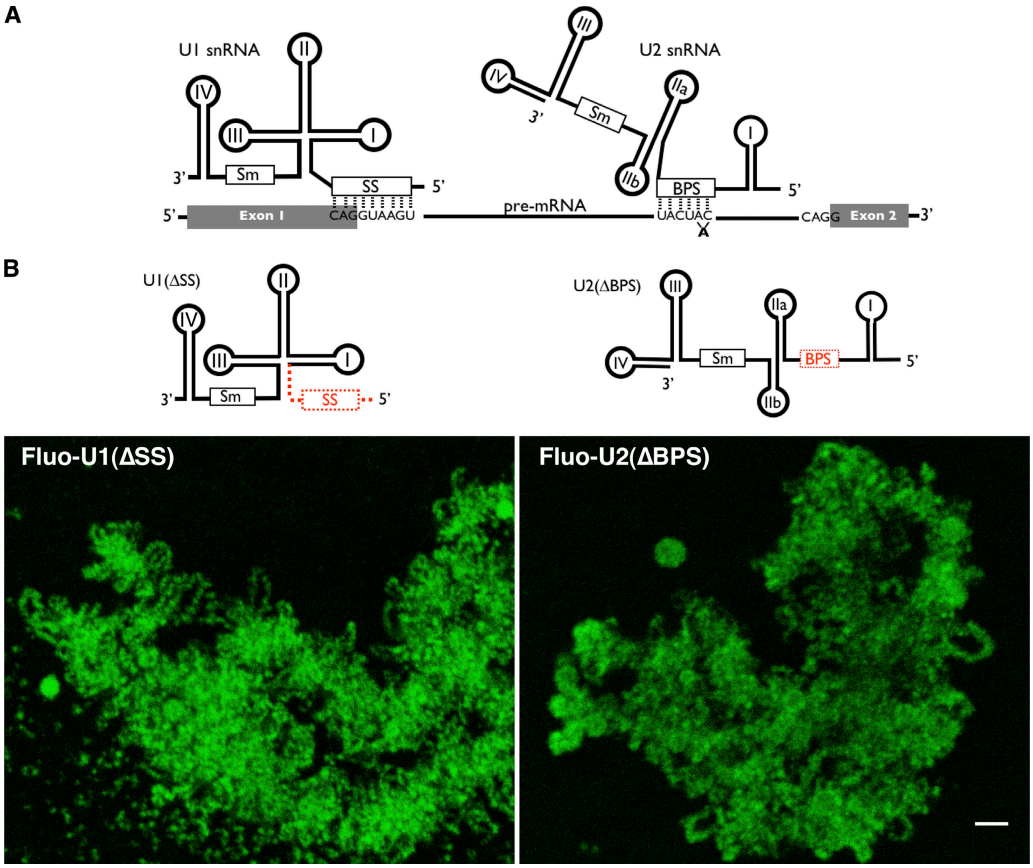

Figure 4. **Mutant U1 and U2 snRNAs that cannot engage splicing are still recruited to active transcriptional units.** U1(ΔSS) and U2(ΔBPS) snRNAs (green) were injected into the cytoplasm of stage V oocytes, and nuclear spreads were prepared 18 h later. (A) Diagram shows the regions of the U1 and U2 snRNAs involved in interacting with pre-mRNA. (B) Diagram shows the regions deleted (red dashed lines) in the mutant U1(ΔSS) and U2(ΔBPS) snRNAs. Laser-scanning confocal images showing the association of U1(ΔSS) and U2(ΔBPS) snRNPs with the nascent transcripts of the chromosomal loops. Bar, 5 μm.

does not prevent the assembly of a U1(ΔSS) snRNP with its full protein complement (Rossi et al., 1996; Du and Rosbash, 2001), and, as expected, the newly made U1(ΔSS) snRNP was rapidly recruited to the nucleus. Interestingly, in addition to accumulating within CBs and IGCs, the U1(ΔSS) snRNP targeted the chromosomal loops just as well as the full-length U1 snRNP (Fig. 4). A similar deletion analysis was performed for the U2 snRNP in which the BPS recognition sequence was removed. Such a U2(ΔBPS) snRNA can no longer engage splicing by hybridizing with an intronic BPS, yet the newly formed U2(ΔBPS) snRNP associates with chromosomal loops as well as with CBs and IGCs, identical to wild-type U2 snRNP (Fig. 4). Together, these data demonstrate that the recruitment of U1 and U2 snRNPs to nascent transcripts is not directed by hybridization of their snRNA moieties to cis-acting signals on pre-mRNAs. Importantly, they also highlight the fact that the association of U1 and U2 snRNPs with elongating transcripts can be uncoupled from their function in splicing.

In the case of the U2 snRNP, its splicing activity depends greatly on modification of the U2 snRNA by 2′-*O*-methylation and pseudouridylation (Yu et al., 1998; Donmez et al., 2004). In particular, the modification of several residues within the first 29 nucleotides of the U2 snRNA is critical for the formation of a mature 17S snRNP particle (Yu et al., 1998). Thus, we produced a fluorescently labeled U2 snRNA deleted of these residues (U2(Δ29) snRNA), injected it into the cytoplasm of stage V oocytes, and analyzed its nuclear distribution 18 h later on nuclear spreads. Fig. 5 shows that the newly assembled U2(Δ29) snRNP associates well with the chromosomal loops, further supporting the idea that the association of snRNPs with active RNAPII transcriptional units is independent of their ability to engage splicing. In addition, IGCs are brightly labeled, but, surprisingly, CBs appear to be only weakly stained (Fig. 5, arrow), especially when the fluorescent signal is compared with that of the full-length U2 snRNP (Fig. 2). Because CBs are implicated in the internal modification of the spliceosomal snRNAs, one possible explanation is that lack of the first 29 residues, among which many are modified, renders the U2(Δ29) snRNA a poor substrate for the modification machinery and, as a result, reduces its overall residence time within CBs.

Targeting of U1 snRNP to nascent transcripts and IGCs is directed by stem loop I

Three proteins are known to be specific for the U1 snRNP: U1A, U1C, and U1-70K. Both U1-70K and U1A bind directly to the U1 snRNA through stem loop I and stem loop II, respectively, whereas U1C interacts with U1-70K (Surowy et al., 1989;

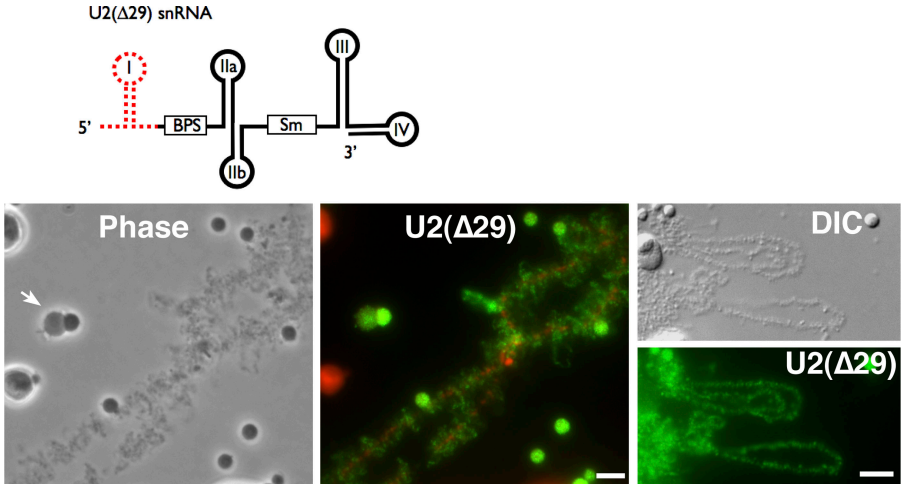

Figure 5. **A nonfunctional U2 snRNP targets nascent RNP fibrils.** Phase contrast or DIC and corresponding fluorescent micrographs of nuclear spreads from an oocyte injected 18 h earlier with fluorescent U2(Δ29) snRNA (green). The newly assembled U2(Δ29) snRNP is recruited to CBs (arrow), IGCs, and the chromosomal loops. Note that the labeling of CBs is dramatically reduced when compared with full-length U2 snRNA (Fig. 2). Chromosomal axes and nucleoli were counterstained with DAPI (pseudocolored in red). A diagram shows the deleted region of U2 snRNAs (dashed red line). Bars, 5 μm.

Nelissen et al., 1994). Because U1C was previously implicated in association of the U1 snRNP with pre-mRNAs in vitro (Du and Rosbash, 2002), we tested whether the deletion of stem loop I would impact the subnuclear distribution of the U1 snRNP. The first 47 residues of U1 were deleted, and the resulting mutant U1(Δ47) snRNA was injected into the cytoplasm of stage V oocytes. Nuclear spreads were prepared 18 h later. Fig. 6 A shows that U1(Δ47) snRNP accumulates in CBs but fails to associate with both IGCs and the nascent transcripts on chromosomal loops. Because the removal of the first 20 residues of the U1 snRNA does not disrupt its chromosomal targeting (Fig. 4), we concluded that stem loop I is the structure present within the first 47 nucleotides that is critical for the association of the U1 snRNP with nascent transcripts. To test that idea, we constructed a chimeric RNA by fusing stem loop I to the 3′ end of the U7 snRNA, which is exclusively found associated with CBs (Figs. 1 and 2). The resulting U7/U1(I) snRNA was injected into stage V oocytes, and its subnuclear distribution was analyzed on nuclear spreads (Fig. 6 B). Remarkably, stem loop I alone is sufficient to promote targeting of the U7 snRNP to chromosomal loops and IGCs. Surprisingly, CBs are only weakly labeled (Fig. 6, A and B; arrows). This result was unexpected, as the U7 snRNP is

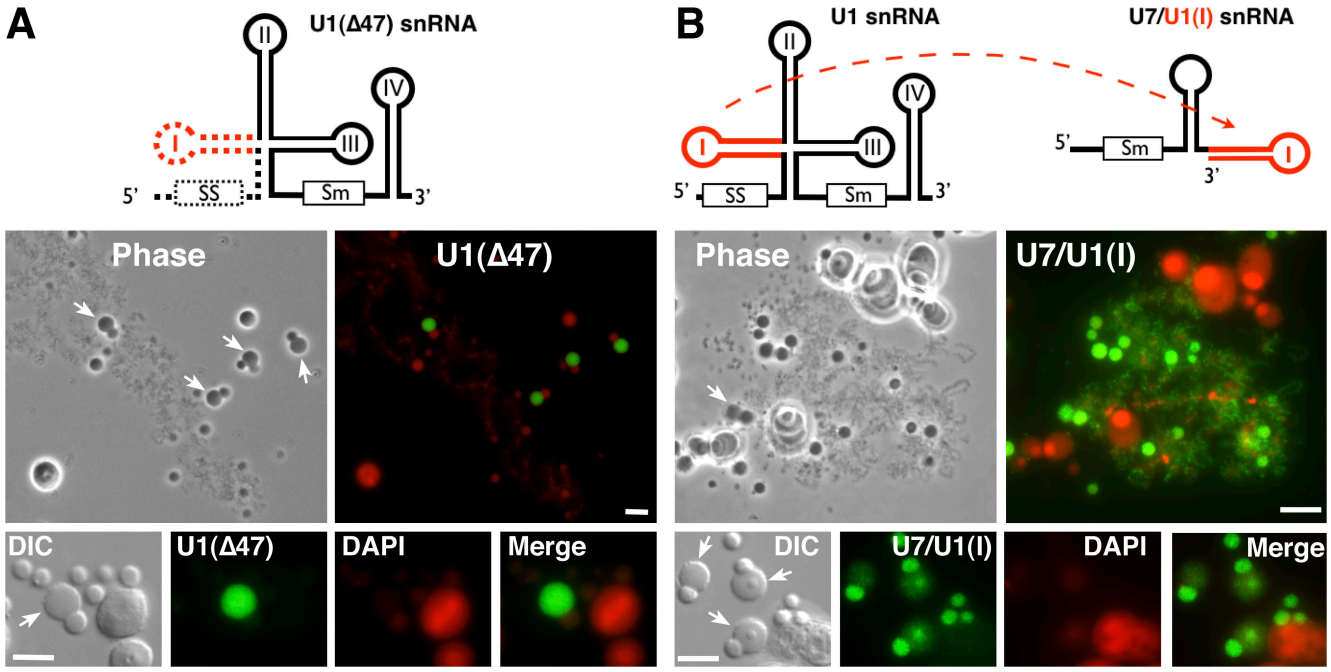

Figure 6. **The first stem loop of the U1 snRNA is necessary and sufficient for its association with IGCs and nascent RNP fibrils.** (A and B) Phase contrast or DIC and corresponding fluorescent micrographs of nuclear spreads from oocytes injected 18 h earlier with either mutant U1(Δ47) RNA (A) or chimeric U7/U1(I) RNA (green; B). The diagram above each panel indicates the structure of the corresponding RNAs. The deleted residues in U1(Δ47) are indicated by a dashed line. Stem loop I is colored in red. The newly assembled U1(Δ47) snRNP targets CBs (arrows) but fails to associate with chromosomal loops and IGCs. In contrast, the U7/U1(I) snRNP associates with nascent RNP fibrils and IGCs in addition to CBs. A group of CBs, IGCs, and nucleoli are presented at a higher magnification in both cases. Note that the signal resulting from the association of U7/U1(I) snRNP with CBs is very weak. DAPI (pseudocolored in red) was used here to counterstain nucleoli and chromosomal axes. Note that IGCs are weakly labeled because of their high RNA content. Bars, 10 μm.

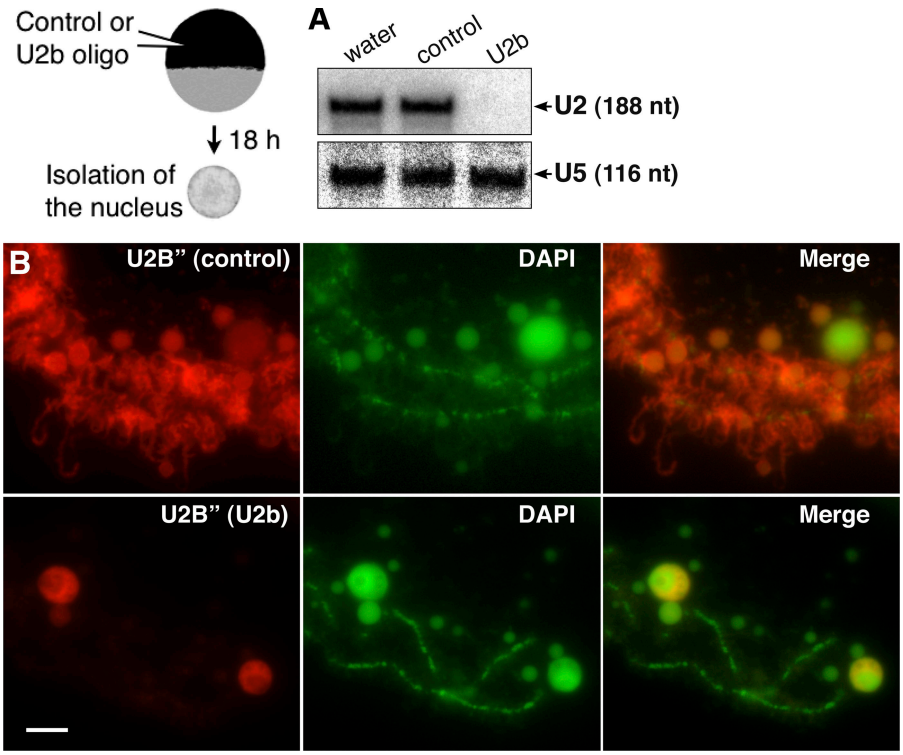

Figure 7. **Depletion of the U2 snRNA inhibits U2B′′ targeting to chromosomal loops.** (A) Northern blot analysis indicates that U2 snRNA is completely depleted in U2b oligonucleotide–injected oocytes but is unaffected in control oocytes that were injected with the control oligonucleotide or just water. Each lane was loaded with the total RNA fraction of one nucleus isolated 18 h after injection. U5 snRNA was used here as a loading control. (B) Fluorescent micrographs of nuclear spreads prepared 18 h after injection with either the U2b or C oligonucleotide. The U2-specific protein U2B′′ was detected using mAb 4G8 (red). In control oocytes, U2B′′ is found associated with the nascent transcripts of the chromosomal loops as well as with IGCs. Nucleoli are also weakly stained. In U2b-injected oocytes, U2B′′ is no longer detected on the chromosomal loops or the IGCs. Instead the granular region of nucleoli is brightly labeled. DAPI is pseudocolored in green. Bar, 5 μm.

known to accumulate in CBs at very high concentrations (Wu and Gall, 1993; Bellini and Gall, 1998), and suggests that stem loop I is important to regulate the kinetics of U1 snRNP exchange between CBs and the nucleoplasm.

Spliceosomal assembly on nascent transcripts is not required to recruit snRNPs

The observation that several mutant U1 and U2 snRNPs, which cannot participate in the assembly of the spliceosome, still target chromosomal loops prompted us to ask whether the association of snRNPs with active transcriptional units could be uncoupled from the splicing reaction itself. An efficient way to inhibit pre-mRNA splicing is to deplete the oocyte of U2 snRNAs using an antisense oligonucleotide–RNase H degradation strategy (Tsvetkov et al., 1992; Yu et al., 1998). In the absence of U2 snRNP, formation of the A complex (a spliceosomal intermediate containing both U1 and U2 snRNPs) and, thus, splicing itself is inhibited (Pan et al., 1989; Ségault et al., 1995; Yu et al., 1998). Importantly, splicing can be rescued by a cytoplasmic injection of in vitro–made U2 snRNAs (Pan and Prives, 1988; Yu et al., 1998).

We first showed that depletion of the U2 snRNA results in the loss of the U2 snRNP–specific protein U2B′′ from the chromosomal loops (Fig. 7). Interestingly, U2B′′ was found to relocalize from chromosomes, IGCs, and CBs to nucleoli. The significance of U2B′′ relocalization is not known, but we subsequently used it in all of our experiments as a cytological indicator of successful U2 snRNA depletions. Because microinjected DNA oligonucleotides are short lived, we were able to show that newly injected U2 snRNA could reestablish the normal distribution pattern of U2B′′ in U2-depleted oocytes (Fig. 8). This result further validates our previous conclusion that association of fluorescent snRNAs with the chromosomal loops reflects the targeting of fully mature snRNPs.

We then asked whether pre-mRNA splicing occurs on the chromosomal loops and whether it is prevented by depletion of the U2 snRNA. During pre-mRNA splicing, the spliceosome stably deposits a large proteinaceous complex named the EJC ~20 nucleotides upstream of exon–exon junctions (for review see Aguilera, 2005). Such EJCs influence the cellular fate of spliced mRNAs, with which they remain associated during nuclear export and until they are displaced by translating ribosomes. One of the EJC core subunits, Y14 (Bono et al., 2006; Stroupe et al., 2006), is deposited after exon–exon ligation (Kataoka and Dreyfuss, 2004). Importantly, then, deposition of Y14 on nascent transcripts is a reliable indication of splicing activity. To test whether EJCs are present on chromosomal loops, Y14 was expressed in fusion with an HA tag, and its subcellular distribution was analyzed using the anti-HA antibody mAb 3F10. Fig. 9 A shows that upon injection of HA-Y14 transcripts into the cytoplasm of stage V oocytes, a protein with the expected molecular mass of ~24 kD is synthesized and efficiently recruited to the nucleus. There, it associates with CBs, IGCs as previously reported in somatic nuclei (Degot et al., 2004), and, to a lesser extent, with nucleoli. In addition and in agreement with the fact that pre-mRNA splicing occurs cotranscriptionally, Y14 also associates with nascent transcripts. Remarkably, the depletion of U2 snRNA results in a complete loss of Y14 from chromosomal loops (Fig. 9 B), indicating a lack of spliceosomal activity on nascent RNP fibrils. Finally, a cytoplasmic injection of fluorescently labeled U2 snRNAs restores the presence of the U2 snRNP and Y14 on chromosomal loops (Figs. 8 and 9 B). Together, these data show that pre-mRNA splicing occurs on the chromosomal loops in the presence but not in the absence of U2 snRNP.

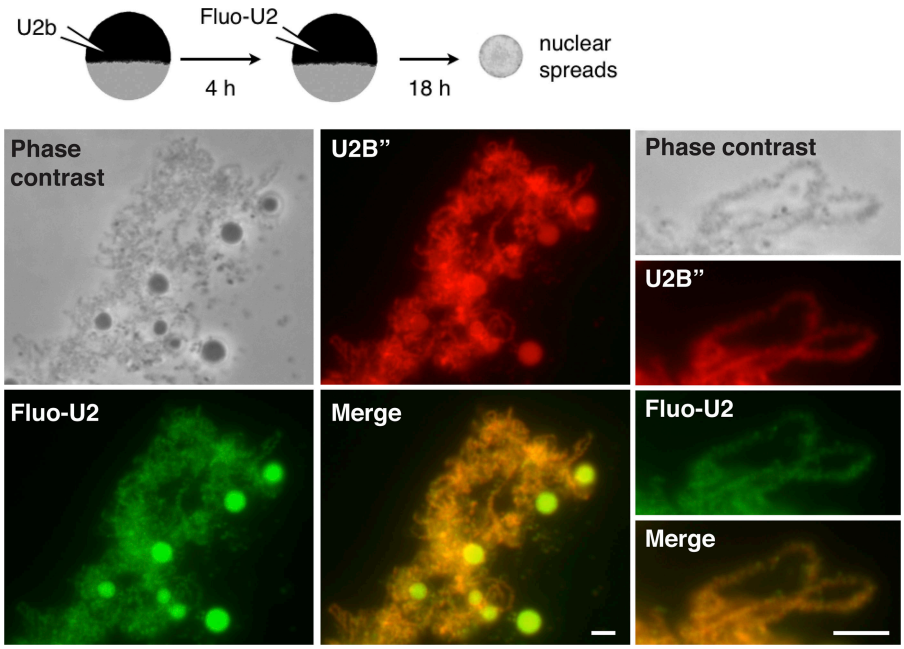

Figure 8. **Newly formed fluorescent U2 snRNP rescues the association of U2B″ with nascent transcripts and IGCs in U2-depleted oocytes.** Phase contrast and corresponding fluorescent micrographs of nuclear spreads from oocytes injected with U2b and fluorescent U2 snRNA as indicated in the diagram. U2B′′ was detected using mAb 4G8 (red) and displays an extensive colocalization with fluorescent U2 snRNA (green) on both chromosomal loops and IGCs. Bars, 2 μm.

Finally, we tested whether U1, U4, and U5 snRNPs could still be recruited to transcriptional sites in the absence of any splicing activity. It was shown previously that the presence of a fully functional U1 snRNP is critical to transcription and, thus, to the maintenance of chromosomal loops in amphibian oocytes (Tsvetkov et al., 1992). As expected, we found that the U1 snRNP still associates with the nascent transcripts of the chromosomal loops in U2 snRNA–depleted oocytes (Fig. 10). This result is also consistent with an early recruitment of the U1 snRNP to the pre-mRNA template, as it would be in the canonical model of splicing, which proposes a stepwise assembly of the spliceosome. In such a model, the U4/U6.U5 tri-snRNP is recruited only after the formation of the A complex. Although the U5 snRNP is commonly used as a representative member of the U4/U6.U5 tri-snRNP, it is present in both the major (U2 type) and minor (U12 type) spliceosomes. Thus, the U4 snRNP, a specific member of the U2-type spliceosome, was also used here as a marker of the U4/U6.U5 tri-snRNP. Surprisingly, in the absence of U2 snRNP, the U4/U6.U5 tri-snRNP is still recruited to nascent transcripts (Fig. 10), indicating that the A complex is not required. Together, these data demonstrate that the splicing activity present on the chromosomal loops does not direct the association of snRNPs with nascent RNP fibrils.

Discussion

The recruitment of U1 and U2 snRNPs to nascent RNP fibrils is independent of their base pairing with pre-mRNAs

The removal of most introns requires a conserved 5′ SS, a BPS followed by a polypyrimidine tract, and a 3′ SS. Although current models propose that the spliceosome assembles onto the target pre-mRNA in an ordered process, it is still unclear which early intermediate complexes form in vivo and in which order. The establishment of one of these intermediates, the A complex, involves base pairing of the U1 and U2 snRNAs to the 5′ SS and BPS, respectively. Whether removal of the 5′ SS recognition sequence on U1 snRNA results in a nonfunctional U1 snRNP is difficult to assess, as the requirement of U1 snRNA itself for intron removal in vitro depends on the pre-mRNA template as well as the concentration of SR proteins in the chosen splicing extract (Crispino et al., 1994, 1996). In addition, although one study indicates a strict requirement of the 5′ end of the U1 snRNA for intron removal (Krämer et al., 1984), others present the hybridization of U1 snRNA 5′ end to pre-mRNA as a nonessential stabilizing force (Du and Rosbash, 2001) that might influence the transition between spliceosomal intermediate complexes (Lund and Kjems, 2002). In the case of the U2 snRNA, however, the requirement of the BPS recognition sequence for efficient pre-mRNA splicing has been well established (Parker et al., 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989).

Interestingly, we have shown here that U1(ΔSS) and U2(ΔBPS) snRNAs, which cannot hybridize to introns, are assembled into snRNPs and target the nascent transcripts on chromosomal loops. One interpretation is that the respective base pairing of U1 and U2 snRNAs with the 5′ SS and BPS is not essential for their association with nascent transcripts in vivo. This is in agreement with previous work showing that initial recruitment of the U1 snRNP to pre-mRNAs appears to be mediated by U1 snRNP proteins in a 5′ SS–independent manner (Wilk et al., 1991; Rossi et al., 1996; Du and Rosbash, 2001, 2002; Lacadie and Rosbash, 2005). In addition, an in vitro study showed that hybridization of the U1 snRNA to target pre-mRNAs is dispensable for early intermediate formation and intron removal (Wilk et al., 1991). In particular, the U1 snRNP was recently shown to be cotranscriptionally recruited to pre-mRNAs with mutations in the 5′ SS that abolish hybridization with the U1 snRNA (Lacadie and Rosbash, 2005), suggesting that the 5′ SS/U1 snRNA base pairing occurs after an initial recruitment phase (Du and Rosbash, 2001; Lund and Kjems, 2002).

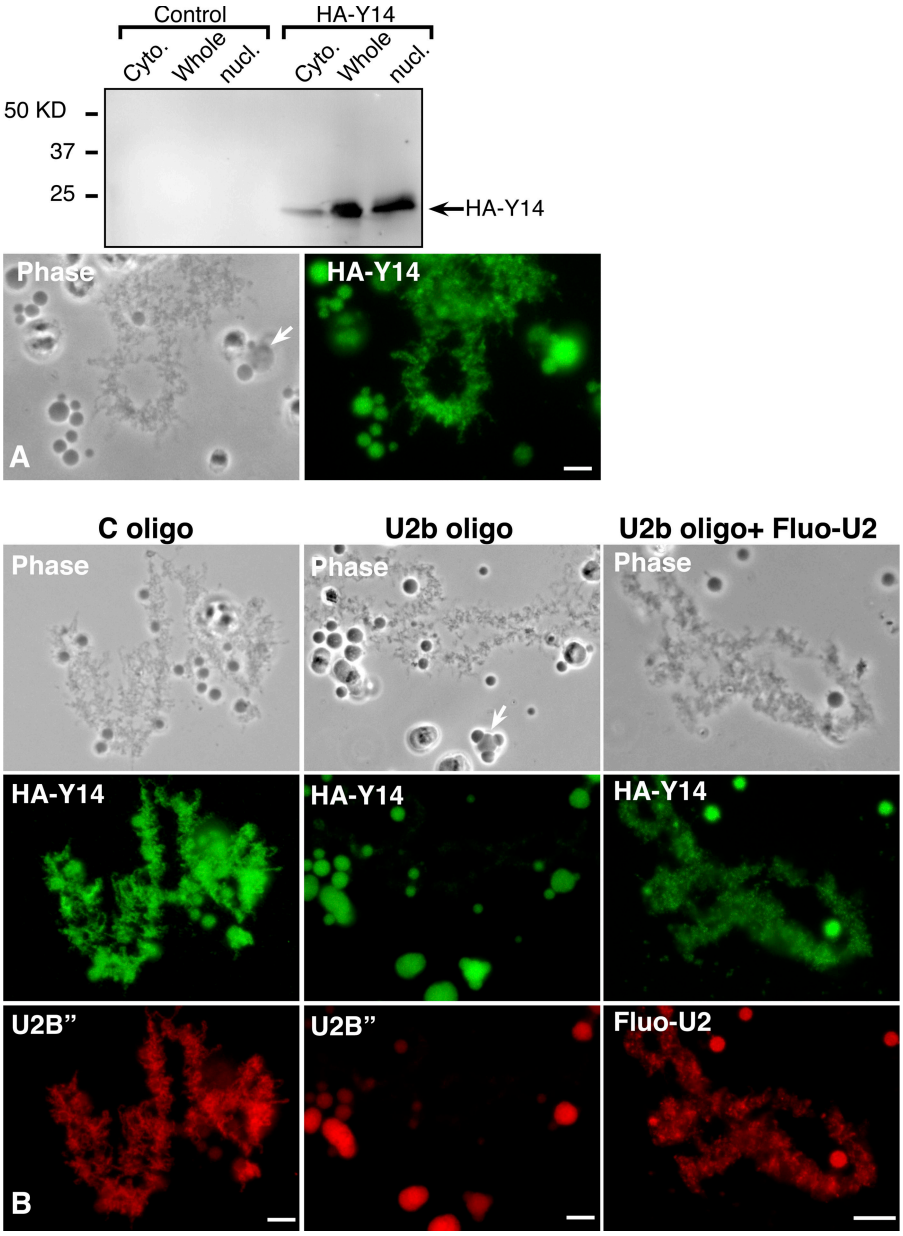

Figure 9. **Y14 is not recruited to nascent transcripts in the absence of the U2 snRNA.** (A) The fate of newly expressed HA-Y14 was followed in stage V oocytes using the anti-HA antibody mAb 3F10 48 h after the injection of its corresponding transcript. A single band of ~24 kD, which is primarily nuclear, is detected on immunoblots. On nuclear spreads, HA-Y14 (green) associates strongly with CBs (arrow) as well as with the nascent transcripts on chromosomal loops. IGCs and the dense fibrillar region of nucleoli are weakly stained. (B) Phase contrast and corresponding fluorescent micrographs of nuclear spreads from oocytes coinjected with HA-Y14 transcripts and either the C or the U2b oligonucleotide. In the rescue experiment, fluorescent U2 snRNA was injected 18 h later. All nuclear spreads were prepared 48 h after the initial injections. The distributions of HA-Y14 (green) and U2B'' (red) were defined using mAb 3F10 and mAb 4G8, respectively. In U2 snRNA–depleted oocytes (U2b injected), HA-Y14 is still found within CBs (arrow), IGCs, and nucleoli, but it is absent from chromosomal loops. In these oocytes, U2B'' accumulates within nucleoli. Remarkably, the chromosomal association of HA-Y14 is rescued by the fluorescent U2 snRNA (pseudocolored in red). Bars, 5 μm.

Another possibility stems from the structural organization of the chromosomal loops. In amphibian oocytes, the RNAPII loops are readily visible by light microscopy because of their dense RNP matrix, which is composed of the nascent RNAPII transcripts and associated maturation factors. Surprisingly, some of these factors, such as the 3′ end processing factor CstF77, are only involved in the late steps of pre-mRNA maturation (Takagaki and Manley, 1994). Therefore, the presence of CstF77 over the entire length of the loops (Gall et al., 1999) suggests that some pre-mRNA processing factors might associate with nascent RNP particles but remain inactive until the occurrence of their corresponding cis-acting RNA elements. Thus, the efficient recruitment of U1(ΔSS) and U2(ΔBPS) snRNPs to nascent transcripts could be the result of a staging event in which snRNPs would first be recruited to the nascent RNP fibrils and be maintained there until spliceosomal assembly could occur. In this model, the initial recruitment of snRNPs would rely, in part, on already associated heterogeneous nuclear RNPs, such as the SR proteins, whose presence was previously shown to require intronic sequences on the pre-mRNA (Huang and Spector, 1996).

U1 snRNP intranuclear trafficking depends on stem loop I

We show here that deletion of the first 47 nucleotides of U1 snRNA, which contain both the 5′ SS recognition sequence and stem loop I, has a dramatic effect on its subnuclear distribution. The resulting U1(Δ47) snRNP still accumulates strongly within CBs, but it fails to target IGCs and the chromosomal loops. Although these data demonstrate that a discrete region of U1 snRNA is critical for its intranuclear trafficking, it also raises the question of how stem loop I regulates the association of the U1 snRNP with two subnuclear domains that are distinct in structure and functions. The lack of association of the U1(Δ47) snRNP with nascent RNP fibrils could to be caused, in part, by

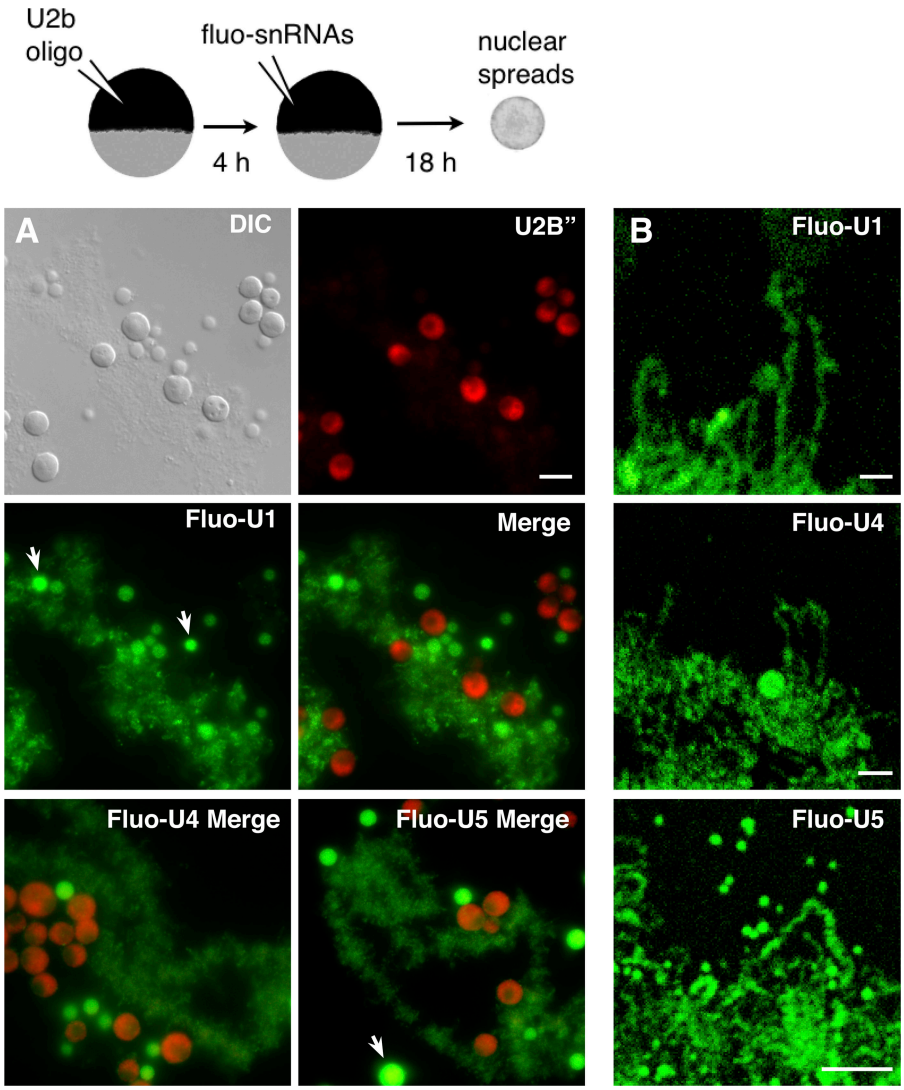

Figure 10. **Spliceosomal U1, U4, and U5 snRNPs target LBC loops in the absence of splicing.** Fluorescent U1, U4, or U5 snRNAs were injected into the cytoplasm of stage V oocytes previously depleted of their endogenous U2 snRNA. Nuclear spreads were prepared 18 h later, and the distribution of the newly assembled snRNPs (green) was determined by fluorescence microscopy. (A) In all three cases, a signal was associated with the chromosomal loops as well as with CBs (arrows) and IGCs. Thus, targeting of the U1 snRNP and the U4/U6.U5 tri-snRNP to nascent transcripts does not require the presence of the U2 snRNP. U2B′′ (red) is detected using mAb 4G8 and is found accumulated in the granular region of nucleoli, which is indicative of an efficient U2 snRNA depletion. (B) Magnified views (laser-scanning microscopy) of particularly extended chromosomal loops that illustrate the association of newly assembled snRNPs with nascent transcripts. Bars (A), 10 μm; (B) 1 μm.

the fact that the U1C protein cannot associate with the U1(Δ47) snRNA in the absence of stem loop I (Du and Rosbash, 2002). U1C was previously implicated in the binding of pre-mRNAs by the U1 snRNP (Rossi et al., 1996; Chen et al., 2001; Förch et al., 2002), and, thus, its absence from a U1(Δ47) snRNP could result in the loss of chromosomal targeting. There is no pre-mRNA splicing activity occurring in IGCs, however. Instead, one demonstrated function of these nuclear bodies is to serve as reservoirs for RNAPII maturation factors, which are subsequently recruited to active transcriptional sites (Saitoh et al., 2004; for review see Kiss, 2004). In light of the current model in which newly assembled snRNPs transit through CBs for modification and assembly before their association with IGCs (Gall et al., 1999; Stanek and Neugebauer, 2006; for review see Kiss, 2004), an attractive possibility is that stem loop I is essential to regulate kinetic exchanges of the U1 snRNP between CBs and the nucleoplasm. In particular, stem loop I might be essential for U1 snRNP to exit CBs. Interestingly, we showed that stem loop I is not only sufficient to direct the association of the nonspliceosomal U7 snRNP to nascent transcripts and IGCs, but it also modifies the association of the U7 snRNP with CBs. Indeed, although the normal fluorescent U7 snRNP accumulates greatly in CBs, this association is dramatically reduced by its fusion with stem loop I. Importantly, the chromosomal association of chimeric U7/U1(I) snRNP demonstrates that a snRNP, which cannot participate in splicing, can be targeted to nascent transcripts. In agreement with this idea, we find that both U2(ΔBPS) and U2(Δ29) snRNPs, which are nonfunctional (Parker et al., 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989; Yu et al., 1998), are recruited efficiently to nascent transcripts.

The recruitment of snRNPs onto nascent transcripts is splicing independent

A model in which the recruitment of snRNPs and spliceosomal assembly are uncoupled in vivo is further supported by our finding that the U1 snRNP and U4/U6.U5 tri-snRNP still associate with the chromosomal loops when pre-mRNA splicing is inhibited by depletion of the U2 snRNA. Because current paradigms for spliceosome assembly command a stable binding of the U2 snRNP to the BPS before engagement of the U4/U6.U5 tri-snRNP, a likely explanation for these data is that the U4/U6.U5 tri-snRNP targets the nascent transcripts but does not engage in

splicing even when the cis-acting RNA elements become available during transcription elongation.

However, one cannot exclude two other interesting possibilities. The first one is that the U4/U6.U5 tri-snRNP was previously shown to recognize the 5′ SS in the absence of the U2 snRNP in vitro (Konforti and Konarska, 1994). In addition, the U5 snRNP was demonstrated to interact with the 5′ SS before the start of splicing (Wyatt et al., 1992), and, more recently, the U4/U6.U5 tri-snRNP together with the U1 snRNP was proposed to comprise part of a very early intermediate that presumably plays an important role in defining the 5′ SS (Maroney et al., 2000). Thus, the observed recruitment of the U1 snRNP and tri-snRNP to chromosomal loops in the absence of the U2 snRNP might reflect the formation and stalling of this early intermediate form on nascent transcripts.

The second possibility comes from the development over the last decade of a different model for spliceosome assembly. A large RNP complex named the penta-snRNP containing all five splicing snRNPs in equal stoichiometric abundance and at least 13 other proteins was purified in yeast (Stevens et al., 2002), and a similar complex was found in mammals (Malca et al., 2003). The penta-snRNP forms in the absence of a pre-mRNA template and, thus, challenges the canonical view of stepwise assembly of the spliceosome. Importantly, when supplemented with an snRNP-depleted extract, the penta-snRNP was competent to splice synthetic substrates as a unitary particle, providing evidence for a preassembly model of splicing wherein all five snRNPs engage the pre-mRNA in one step as a single complex (Stevens et al., 2002). Therefore, another interpretation of the U1 snRNP and tri-snRNP association with chromosomal loops in the absence of splicing is that snRNPs are recruited to the nascent transcripts as part of a preassembled complex. In that case, however, one would have to assume that such a complex could be formed and recruited to the transcriptional units without the U2 snRNP.

Interestingly, a model in which the splicing machinery is staged directly on the transcriptional unit implies some level of preassembly of the splicing machinery before or directly onto the nascent RNP fibrils. Importantly, such a paradigm does not antagonize the canonical view of an ordered assembly process of the spliceosome onto intronic sequences, as the various spliceosomal intermediates could form by recruitment of the splicing factors already associated with nascent transcripts onto the cis-acting RNA elements during transcription elongation.

EJCs associate with the active RNAPII transcriptional units of the LBCs

In the course of our study, we used the deposition of EJCs onto nascent transcripts as an indication of splicing, as it allows the simultaneous monitoring on nuclear spreads of all RNAPII transcriptional units in the same oocyte. EJCs are recruited cotranscriptionally by the spliceosome to mark exon–exon junctions after intron removal (for review see Aguilera, 2005), and, accordingly, we demonstrate here that Y14, a subunit of the EJC, targets the numerous LBC lateral loops. In the absence of the U2 snRNA, spliceosomal assembly and, thus, pre-mRNA splicing is inhibited (Pan et al., 1989; Ségault et al., 1995; Yu et al., 1998), which is illustrated on nuclear spreads by the loss of Y14 from LBCs. Interestingly, we recently obtained evidence that Magoh, another EJC subunit, distributes similarly to Y14 in the oocyte. This result was expected, as Y14 and Magoh were shown previously to interact (Bono et al., 2006; Stroupe et al., 2006). We are now currently using the advantageous spatial resolution offered by LBCs together with the fact that these chromosomes can now be visualized in in vivo–like conditions (unpublished data) to characterize the kinetics of the association of Y14, Magoh, and splicing factors with the active transcriptional units.

Materials and methods

RNA transcription and labeling

The DNA templates U1, U2, U4, U5, U7/U1(l), U1(Δ47), U1(ΔSS), U2(Δ29), and U2(ΔBPS) used for the transcription of fluorescein-labeled RNA were amplified by PCR using the high fidelity Deep Vent$_R$ DNA Polymerase (New England Biolabs, Inc.) from corresponding human U1 (Wu et al., 1991), amphibian U2 and U5 (Gall et al., 1999), amphibian U7 (Wu and Gall, 1993), or chicken U4B (Hoffman et al., 1986; Gerbi et al., 2003) clones.

The templates for producing P^{32}-labeled riboprobes (anti-U2 and -U5) for Northern blotting were amplified with GoTaq DNA Polymerase (Promega). In all cases, the 5′ primer used for amplification contains the T3 or T7 promoter for direct transcription with the respective polymerase. Amplified DNA fragments were gel purified using 0.45-mm cellulose acetate spin-X plastic centrifuge tube filters (Corning Inc.). The U2 DNA template was produced by a two-step PCR in which the first step deletes the BPS recognition sequence and two residues on each side (residues 31–40 of the U2 snRNA) and the second step introduces the T3 promoter. The DNA templates for the transcription of fluorescein–U7 snRNA and HA-Y14 mRNA were prepared by linearizing the pUC9/T7-X.l.U7 snRNA vector with PvuII (Invitrogen) and of pcDNA3.1/HA-tagged human Y14 vector with XbaI (Invitrogen). All DNA templates were phenol extracted, ethanol precipitated, and washed with 70% ethanol before transcription.

The DNA primers (Integrated DNA Technologies) used were as follows (T3 and T7 promoters are underlined): U1 (5′-GCAATTAACCCTCACTAAAGGGATACTTACCTGGCAGGGGAG and 3′-CAGGGGAAAGCGCGAACGCAGTCCCCCAC), U1(ΔSS) (5′-GCAATTAACCCTCACTAAAGGGATACCATGATCATGAAG and 3′-CAGGGGAAAGCGCGAACGCAGTCCCCCAC), U1(Δ47) (5′-CGTAATACGACTCACTATAGGCAGGGCCAGGCTCAGCC and 3′-CAGGGGAAAGCGCGAACGCAGTCCCCCAC), U2 (5′-GCAATTAACCCTCACTAAAGGGATCCTTTCGCCTTTGC and 3′-AAGTGCACCGGTCCTGGAGGT ACTGC), U2(ΔBPS) first step (5′-ATCGCTTCTCGGCCTTTTGGCTAAGATCAATGTTCTTATCAGTTTAATATCTG and 3′-AAGTGCACCGGTCCTGGAGGTACTGC), U2(ΔBPS) second step (5′-GCAATTAACCCTCACTAAAGGGATCCTTTCGCCTTTGC and 3′-AAGTGCACCGGTCCTGGAGGTACTGC), U2(Δ29) (5′-CGAATTAACCCTCACTAAAGGGAGTGTAGTATCTGTTCTTATC and 3′-AAGTGCACCGGTCCTGGAGGTACTGC), U4 (5′-TAATACGACTCACTATAGGGAGCTTTGCGCAGTGGCAGTATC and 3′-CAGTCTCCGTAGAGACTGTCA), U5 (5′-CGGAATTCAATTAACCCTCACTAAAGGG and 3′-ATACCTGGTGTGAACCAGGCTTC), U7/U1(l) (5′-ATACCATGATCATGAAGGTGGTTCTCCAAGTGTTACAGCTC and 3′-TGTGGCTCCTACAGAG), anti-U2 (5′-CGTAATACGACTCACTATAGGGAAGTGCACCGGTCCTGGAG and 3′-ATCGCTTCTCGGCCTTTTGG), and anti-U5 (5′-CGTAATACGACTCACTATAGGGTACCTGGTGTGAACCAGGCTT and 3′ ATACTCTGGTTTCTCTTCAAATTC and 3′-TGTGGCTCCTACAGAG).

Fluorescently labeled snRNA and mutants were transcribed with T3 or T7 RNA polymerases (Stratagene) in the presence of 125 nM ATP, 62.5 nM GTP, 125 nM CTP, 62.5 nM UTP, 25 nM ChromaTide fluorescein-12–UTP (Invitrogen), and 125 nM $m^7G(5')ppp(5')G$ cap analogue (GE Healthcare). P^{32}-labeled riboprobes were transcribed with T7 RNA polymerase (see previous two paragraphs) except that the cap analogue was omitted in the reaction and the fluorescein-coupled UTP was replaced by 50 μCi α-[^{32}P]UTP (GE Healthcare). The HA-Y14 mRNA was transcribed with T7 RNA polymerase similarly except that UTP was present at 125 nM and fluorescein-12–UTP was omitted. Recombinant RNasin Ribonuclease Inhibitor (Promega) was present in all transcription reactions. After 2 h of incubation at 37°C, transcription reactions were treated with RQ1 RNase-free DNase (Promega) for 15 min at 37°C. All labeled RNAs were purified using NucAway Spin columns (Ambion) equilibrated with water. The HA-Y14 mRNA was phenol extracted, ethanol precipitated, washed, and resuspended in water.

Oocytes and microinjection

Female adult *Xenopus* was anesthetized in 0.15% tricaine methane sulfonate (MS222; Sigma-Aldrich), and fragments of ovary were surgically removed. Oocytes were defolliculated for 2 h at room temperature in saline buffer OR2 (Wallace et al., 1973) containing 0.2% collagenase (type II; Sigma-Aldrich). Stage IV–V oocytes were isolated and maintained in OR2 at 18°C. Oocytes were always injected into the cytoplasm with volumes of 20–30 nL. Glass needles were prepared using a horizontal pipette puller (P-97; Sutter Instrument Co.). All injections were performed under a dissecting microscope (S; Leica) using an injector (nanojet II; Drummond). For snRNP targeting assays, ~10–20 fmol of the respective in vitro–made fluorescent snRNAs were injected per oocyte. For the U2 depletion experiments, 50 ng of the following DNA oligonucleotides (Integrated DNA Technologies) were used per oocyte: U2b oligonucleotide (complimentary to residues 28–42 of the U2 snRNA) CAGATACTACACTTG and C oligonucleotide (sequence unrelated to any *Xenopus* RNA) TCCGGTACCACGACG.

It is noteworthy to mention that the injection of any DNA oligonucleotide into amphibian oocytes has several reversible nonspecific effects, including a transient inhibition of transcription as visualized by a loss of the lateral chromosomal loops and their reformation over time (Tsvetkov et al., 1992). In all experiments, oocytes were thus incubated for a minimum of 18 h after DNA oligonucleotide injection before preparing nuclear spreads to allow for the recovery of their transcriptional activity. When actinomycin D treatment was required to inhibit transcription, oocytes were incubated in OR2 medium containing 10 μg/ml actinomycin D (Sigma-Aldrich).

RNA electrophoresis and Northern blotting

Single nuclei were isolated and homogenized in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.2% SDS. Total nuclear RNAs were phenol extracted, ethanol precipitated, and fractionated on an 8 M urea, 1× Tris borate EDTA, and 8% polyacrylamide gel in 1× Tris borate EDTA electrophoresis buffer using an electrophoresis system (Mini-PROTEAN 3; Bio-Rad Laboratories). The RNA was electrophoretically transferred to a Zeta probe membrane (Bio-Rad Laboratories) in 1× Tris acetate EDTA transfer buffer using the Mini Trans-Blot cell (Bio-Rad Laboratories). The RNA was UV cross-linked (12 kJ/cm^2) to the membrane using a cross-linker (Spectrolinker; Spectronics Corp.). The membrane was blocked for 10 min with hybridization buffer (171 mM Na_2HPO_4, 79 mM NaH_2PO_4, 1 mM EDTA, pH 8.0, and 7% SDS), probed overnight with ^{32}P-labeled antisense U2 and U5 snRNA probes at 10^6 cpm/ml in hybridization buffer, and washed twice for 30 min with wash buffer (13.7 mM Na_2HPO_4, 6.3 mM NaH_2PO_4, 1 mM EDTA, pH 8.0, and 1% SDS). Blocking, hybridization, and washing were performed at 65°C with rotation in an incubator (Isotemp Hybridization; Fisher Scientific). A phosphorscreen was exposed for 1 h and scanned with the Cyclone Storage Phosphor system (PerkinElmer).

Protein expression and Western blotting

To express HA-tagged Y14, 25 nL (0.5 ng/nL) HA-Y14 mRNA was injected into the cytoplasm of stage V oocytes. After a 50-h incubation, 10 oocytes, cytoplasms, or nuclei were hand isolated using jeweler's forceps and homogenized in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.2% SDS. The crude extract was centrifuged at 22,000 *g* at 4°C for 10 min. The clarified extract was collected and fractionated on a 12% polyacrylimide gel using an electrophoresis system (Mini-PROTEAN 3; Bio-Rad Laboratories). Immunoblotting was then performed as described previously (Beenders et al., 2003) with the anti-HA antibody mAb 3F10 (Hoffmann-La Roche, Inc.) used at 50 ng/ml.

Nuclear spreads and immunofluorescence

Nuclear spreads were prepared as described previously (Bellini and Gall, 1998) and fixed with 2% PFA in PBS + 1 mM $MgCl_2$ for 1 h at room temperature. After fixation, nuclear spreads were rinsed in PBS and blocked with 0.5% BSA (Sigma-Aldrich) + 0.5% gelatin (from cold water fish) in PBS (blocking buffer) for 10 min. Spreads were incubated with primary antibody for 1 h at room temperature, washed for 30 min with two changes of PBS, incubated with secondary antibody for 1 h at room temperature, and washed again for 30 min with two changes of PBS before they were mounted in 50% glycerol/PBS containing 1 mg/ml phenylenediamine and 10 pg/ml DAPI. When a red fluorescent DNA counterstain was desired, spread preparations were incubated with 1 μM Syto61 (Invitrogen) in PBS for 20 min at room temperature and briefly rinsed in PBS before mounting. In these preparations, DAPI was omitted from the mounting medium.

Standard fluorescence microscopy was performed using a microscope (DMR; Leica), a PL Fluotar 40× NA 1.0 oil objective (Leica), and a Fluotar 100× NA 1.30 oil objective (Leica). Either a Spot RT monochrome CCD camera (Diagnostic Instruments) or a Retiga EXI monochrome CCD camera (QImaging) was used to capture images with Spot RT software (Diagnostic Instruments) or In Vivo software (version 3.2.0; Media Cybernetics), respectively. Confocal microscopy was performed on a laser-scanning microscope (Axiovert 100M 510; Carl Zeiss MicroImaging, Inc.) and its associated software system. The scans were obtained using a plan Apochromat 63× NA 1.40 oil objective (Carl Zeiss MicroImaging, Inc.). All images were captured at room temperature. Figures were processed using Photoshop CS version 8.0 (Adobe) and assembled with InDesign CS version 3.0 (Adobe).

The antibodies used in this study were all diluted in the blocking buffer. The following AlexaFluor-conjugated antibodies (Invitrogen) were used at a concentration of 2.5 μg/ml: AlexaFluor488 rabbit antifluorescein/Oregon green, AlexaFluor488 goat anti–rabbit IgG, AlexaFluor594 goat anti–mouse IgG, AlexaFluor350 goat anti–mouse IgG, and AlexaFluor488 goat anti–mouse IgG. The AlexaFluor488 mouse antifluorescein IgG (Millipore) was used at 1 μg/ml. The anticoilin antibody mAb H1 was used at 500 ng/ml. The anti-U2B′′ antibody mAb 4G8 is a cell supernate and was used at a 1:50 dilution. Anti-RPC53 is a purified rabbit polyclonal serum and was used at a dilution of 1:50,000.

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