Molecular Cell
Article

L30 Binds the Nascent *RPL30* Transcript to Repress U2 snRNP Recruitment

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DOI 10.1016/j.molcel.2008.05.002

SUMMARY

The mechanisms of pre-mRNA splicing regulation are poorly understood. Here we dissect how the *Saccharomyces cerevisiae* ribosomal L30 protein blocks splicing of its pre-mRNA upon binding a kink-turn structure including the 5′ splice site. We show that L30 binds the nascent *RPL30* transcript without preventing recognition of the 5′ splice site by U1 snRNP but blocking U2 snRNP association with the branch site. Interaction of the factors BBP and Mud2 with the intron, relevant for U2 snRNP recruitment, is not affected by L30. Furthermore, the functions of neither the DEAD-box protein Sub2 in the incipient spliceosome nor the U2 snRNP factor Cus2 on branch site recognition are required for L30 inhibition. These findings contrast with the effects caused by binding a heterologous protein to the same region, completely blocking intron recognition. Collectively, our data suggest that L30 represses a spliceosomal rearrangement required for U2 snRNP association with the transcript.

INTRODUCTION

The current model of spliceosome assembly proposes a stepwise recognition of the intron (reviewed in Will and Lührmann, 2006). In *Saccharomyces cerevisiae* extracts, the intron is committed to splicing by the recognition of U1 snRNP, producing the commitment complex or CC (Seraphin and Rosbash, 1989). Recognition of the 5′ splice site (SS) by U1 generates the CC1, while the further detection of the branch site (BS) and its downstream region, by the factors BBP (SF1 in metazoans) (Berglund et al., 1997) and Mud2 (putative homolog of the metazoan U2AF65) (Abovich et al., 1994; Abovich and Rosbash, 1997), respectively, renders the CC2. The mammalian (and *S. pombe*) counterpart of CC is the "E" complex (Huang et al., 2002; Michaud and Reed, 1991), which further incorporates U2AF35 and U2 snRNP (Donmez et al., 2004), even in the absence of the BS (Das et al., 2000).

There is a poorly understood network of interactions stabilizing the CC and E complexes. They include contacts of U1 snRNP proteins, such as Prp40, with BBP (Abovich and Rosbash, 1997; Rutz and Seraphin, 1999), and a close spatial proximity of the 5′SS and BS (Kent and MacMillan, 2002). These interactions need to be remodeled to allow stable base pairing of the BS with the U2 snRNP (Donmez et al., 2007) and subsequent engagement of U4/U6.U5 tri-snRNP (Staley and Guthrie, 1999). This remodeling is likely to be mediated by DExH/D RNA helicases (Cordin et al., 2006), and the factors Sub2 and Prp5 are known to act during U2 engagement. Prp5 is involved in the BS recognition by U2 (Xu et al., 2004) and interacts with some U2 snRNP components (Perriman and Ares, 2000; Perriman and Ares, 2007). Sub2 has been proposed to act removing Mud2 from its intronic binding site (Kistler and Guthrie, 2001; Rutz and Seraphin, 1999). Little is known on how these processes can be regulated.

Recent developments in budding yeast, based on chromatin immunoprecipitation (ChIP) techniques, indicate that in this system spliceosome assembly starts cotranscriptionally with U1 snRNP recognition of the 5′SS (Gornemann et al., 2005; Lacadie and Rosbash, 2005). However, full spliceosome assembly is usually completed after transcription, depending on the length of the downstream exon (Tardiff et al., 2006). How this may affect control of splicing has yet to be investigated.

In general, regulated splicing requires the presence of particular sequences in the pre-mRNA and specific factors that bind to them (reviewed in Black, 2003). Two important families of metazoan factors are involved in the regulation of splicing, the SR and hnRNP proteins (Hertel and Graveley, 2005). There are no clear homologs of SR splicing factors in *S. cerevisiae*. However, splicing in yeast can be regulated when a transcript folds in a specific structure, as is the case of *RPL30* (Eng and Warner, 1991), or contains specific sequence elements, as the meiosis-specific gene *MER2* (Nandabalan and Roeder, 1995) or *YRA1* (Dong et al., 2007; Preker and Guthrie, 2006).

The essential gene *RPL30* encodes L30, a ribosomal protein that, when in excess, binds to its transcript and inhibits its splicing (Vilardell and Warner, 1994). The *RPL30* transcript folds in a particular structure that mimics the L30 rRNA binding site (Vilardell et al., 2000b). This structure involves the boundaries of the first exon and the initial nucleotides of the intron, folding in a kink-turn that is stabilized by binding to L30 (Chao and

Williamson, 2004; Klein et al., 2001). In vitro studies indicated that L30 blocks spliceosome assembly on *RPL30*, with U1 snRNP present in the stalled complex ("inhibited complex," IC). However, the extent of the interaction between U1 and the intron was unknown (Vilardell and Warner, 1994). Therefore, binding of L30 to the transcript might be interfering with one or more of the following steps: (1) proper recognition of the 5′SS by U1 snRNP, (2) recognition of the BS by BBP or the downstream region by Mud2, (3) crossintron interactions during intron recognition, or (4) the necessary rearrangements in this complex before stable U2 snRNP incorporation. Information on how this regulation occurs will provide valuable insights on the molecular basis of these processes. In addition, the *RPL30* transcript is short enough so that spliceosome assembly is likely to be completed posttranscriptionally (Tardiff et al., 2006). This raises the possibility that L30 is able to regulate splicing only after release of the transcript. Since the L30 system of splicing control can be engineered on longer downstream exons, where assembly will be cotranscriptional, this question can be addressed in vivo.

In this paper we use the *RPL30*/L30 system to undertake a detailed molecular analysis, both in vitro and in vivo, of an early spliceosome assembly event and its control, showing that the engagement of the BS by U2 snRNP is blocked after intron recognition. We also monitor cotranscriptional splicing regulation, revealing that spliceosome assembly on a transcript can be subjected to control from the onset of transcription.

RESULTS

L30 Represses *RPL30* Splicing Even When the 5′ Splice Site Is Outside Its Binding Site

RPL30 5′SS is included in the binding site for L30 protein (Figure 1A) (Chao and Williamson, 2004; Eng and Warner, 1991). To understand whether this location is required for regulation, a series of mutants were constructed that shifted the relative position between the 5′SS and the L30 binding site without affecting L30 binding (see the Experimental Procedures). Increasing this distance between 3 and 6 nt did not prevent regulation (Figure 1B, lanes 3–6). This result indicates that the 5′SS does not need to be in a double-stranded region for L30 to inhibit splicing. On the other hand, inhibition was abolished when the 5′SS was shifted by 12 nt (lanes 7 and 8), suggesting that distance constraints exist as well, between the intron and the L30 binding site, for inhibition. This synthetic substrate, hereafter "+12," was used in subsequent experiments as a control of lack of repression in the presence of L30 binding.

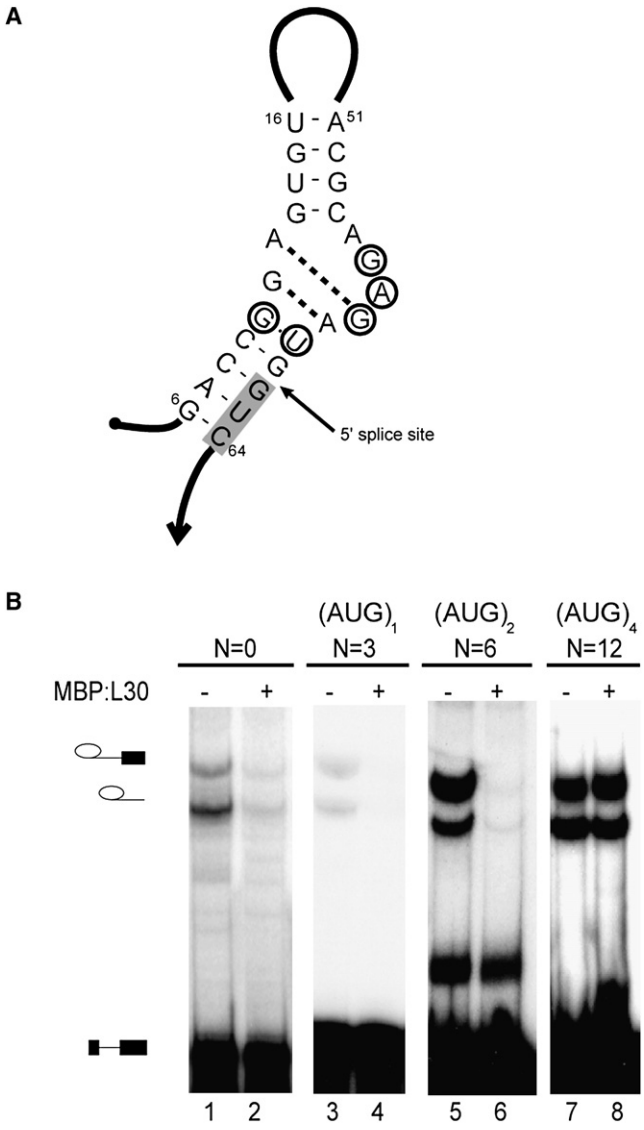

Figure 1. Inhibition of *RPL30* Splicing Depends on the Distance between L30 Binding Site and the 5′SS

(A) Schematic representation of the *RPL30* kink-turn. It includes exon 1 sequences and intron first nucleotides (gray box). Depicted interactions in the purine loop are based on the X-ray structure, with positions contacting L30 encircled (Chao and Williamson, 2004). Numbers are relative to the start of transcription.

(B) Effect on splicing inhibition of displacing the intron of *RPL30* from the kink-turn fold by N nucleotides inserted (sequence on top) at the end of the first exon while keeping strand complementarity. In vitro splicing was assessed in the absence (odd lanes) or presence (even lanes) of MBP-L30 fusion protein in the extracts. In all experiments, the same amount of substrate, extracts, and fusion protein were used (see the Experimental Procedures).

Recognition of the *RPL30* Intron and Inhibition by L30

To dissect L30 inhibition, we investigated the composition of the IC as well as the effects on regulation of mutations in splicing signals or depletion of spliceosomal components. Thus, we tested whether U snRNAs were coimmunoprecipitated with L30 using WT and +12 substrates. Only U1 snRNA could be detected under conditions of repression (Figure 2A). This interaction is stabilized by the presence of the BS sequence (compare lanes 3 and 5 or 7 and 9), suggesting that L30 does not prevent formation of the CC2 complex. To test to what extent U1 function is impaired, we next asked whether base pairing between U1 and the intron occurs during regulation. Psoralen-induced crosslinkings are detected between the *RPL30* 5′SS and U1 snRNA (Figure 2B, lane 3), as seen in other systems (Du et al., 2004). Interestingly, crosslinking is maintained in L30-supplemented extracts (Figure 2B, lanes 4 and 6). The identity of the crosslinks was verified by digestion with RNase H and a specific anti-U1