

A Simple Whole Cell Lysate System for *in Vitro* Splicing Reveals a Stepwise Assembly of the Exon-Exon Junction Complex*

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Pre-mRNA splicing removes introns and leaves in its wake a multiprotein complex near the exon-exon junctions of mRNAs. This complex, termed the exon-exon junction complex (EJC), contains at least seven proteins and provides a link between pre-mRNA splicing and downstream events, including transport, localization, and nonsense-mediated mRNA decay. Using a simple whole cell lysate system we developed for *in vitro* splicing, we prepared lysates from cells transfected with tagged EJC proteins and studied the association of these proteins with pre-mRNA, splicing intermediates, and mRNA, as well as formation of the EJC during splicing. Three of the EJC components, Aly/REF, RNPS1, and SRm160, are found on pre-mRNA by the time the spliceosome is formed, whereas Upf3b associates with splicing intermediates during or immediately after the first catalytic step of the splicing reaction (cleavage of exon 1 and intron-lariat formation). In contrast, Y14 and magoh, which remain stably associated with mRNA after export to the cytoplasm, join the EJC during or after completion of exon-exon ligation. These findings indicate that EJC formation is an ordered pathway that involves stepwise association of components and is coupled to specific intermediates of the splicing reaction.

In eukaryotic cells, removal of introns from pre-mRNAs by pre-mRNA splicing is an essential process for gene expression (1, 2). It has recently become apparent that pre-mRNA splicing serves not only to remove introns but also to enhance the utilization of the spliced mRNA in subsequent steps of the gene expression pathway (3–5). Several lines of evidence converged to demonstrate that pre-mRNA splicing alters the composition of the proteins that are associated with the mRNA (6–9). Pre-mRNA splicing recruits specific proteins to the mRNA, and these assemble into a discrete complex near the exon-exon junction (8). This complex, termed the exon-exon junction complex (EJC),¹ contains at least seven proteins, including Y14 (7), magoh (10, 11), Aly/REF (12), RNPS1 (13), SRm160 (14), Upf3 (15, 16), and possibly also DEK (17).

The components of the EJC function in post-splicing events (for example, Aly/REF) enhance the export of mRNAs, likely by

recruiting the mRNA export factor TAP (12, 18, 33). Y14, magoh, and RNPS1 can also interact with TAP (10, 19), suggesting they may help recruit additional TAP molecules to mRNAs and play a role in mRNA transport. Components of the EJC also appear to function in nonsense-mediated mRNA decay (NMD) (20–25). NMD is a quality control process that surveys and selectively degrades mRNAs that contain premature termination codons, because the translation of such mRNAs may result in production of potentially harmful carboxyl-terminal-truncated proteins. Factors required for NMD have been previously isolated from various organisms and termed Upf1, -2, and -3. Among these factors, Upf3 has been shown to interact with Y14 and be a component of the exon-exon junction complex (16). These observations strongly suggest that the EJC proteins have the capacity to communicate critical information on mRNA from the nucleus to the cytoplasm where the translation-dependent process of NMD occurs (in addition, there may be nuclear NMD) (26–31). There is further supporting evidence for the role of the EJC in NMD. When tethered downstream of a legitimate stop codon, RNPS1, and to a lesser extent Y14, can elicit NMD (19). RNPS1 can also be co-immunoprecipitated with Upf proteins (19), suggesting that it can recruit Upf proteins onto mRNA.

Another EJC protein is magoh, the human homologue of the *Drosophila* mago nashi protein. Magoh binds avidly and specifically to Y14. These two proteins remain stably associated with newly exported mRNAs in the cytoplasm in the same position near the exon-exon junction, whereas most of the EJC components rapidly dissociate during or immediately after mRNA export (7, 10, 32, 33). Translation of the mRNA is required for removal of Y14 and magoh (34). Both Y14 and magoh (also referred to as Tsunagi and Mago nashi in *Drosophila*, respectively) are essential for cell viability in *Drosophila*, and mutants of Y14 and mago nashi show mislocalization of oskar mRNA in *Drosophila* oocytes (35, 36). These findings suggest that cytoplasmic remnants of the EJC, comprising at least Y14 and magoh, also have important roles in cytoplasmic events such as mRNA localization.

Although the formation of the EJC is splicing-dependent, little is known about the process of assembly of the EJC. The splicing factor UAP56, a DEXD-box-type RNA helicase, has been shown to interact with Aly/REF; this interaction is evolutionarily conserved among *Saccharomyces cerevisiae*, *Drosophila*, and human (37–40). This provides an explanation for why Aly/REF is enriched in spliced mRNA. However, it is not known where and how the EJC is formed from its individual components during or after splicing. Here we show that EJC formation is an ordered stepwise process and that its known components can be separated into three groups based on when they begin to associate with the pre-mRNA/mRNA substrate. Aly/REF, RNPS1, and SRm160 make up the first, early group.

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¹ The abbreviations used are: EJC, exon-exon junction complex; RNP, ribonucleoprotein; NMD, nonsense-mediated mRNA decay.

These three proteins are found enriched on pre-mRNA prior to the first catalytic step of splicing and remain bound to the mRNA. Upf3b, on the other hand, mainly associates with the splicing intermediates 5' exon and intron-lariat with 3' exon, prior to exon ligation, and therefore forms a second group. Interestingly, Y14 and magoh, which persist on mRNAs in the cytoplasm after other EJC proteins have been removed, join the EJC at a later step of splicing after several of the other EJC proteins have already been added and form the third group.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Aly/REF, RNPS1, DEK, Srm160, and Y14 were PCR-amplified and cloned into FLAG-pcDNA3 using BamH1 and XhoI (16). FLAG-magoh and FLAG-hUpf3b were described previously (10, 16).

Cell Culture and Transfection—HEK293T cell culture was done as described previously (41). Calphos™ mammalian transfection kit (Clontech) was used according to the manufacturer's protocol.

Whole Cell Extract Preparation—24 h post-transfection, HEK293T cells were harvested and whole extracts were prepared. Briefly, the cells were washed three times with 10 ml of ice-cold phosphate buffered saline (PBS) each time. The cells were scraped, resuspended in 10 ml of PBS, centrifuged at $3,000 \times g$ for 3 min at 4 °C, and the PBS was completely removed. The resulting pellet was resuspended in ice-cold buffer E (20 mM Hepes-KCl, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 10% glycerol, and 1 mM dithiothreitol). 200 μ l of buffer E was used per 10-cm plate. The resuspended cells were disrupted by sonication with an Ultrasonic processor Model VC130PB (Sonics & Materials Inc., Newtown, PA). The cells were sonicated at 30% continuous setting for 5 s three times with a 30-s incubation on ice between bursts and then centrifuged at $15,000 \times g$ for 20 min at 4 °C. The supernatant was saved as whole cell extracts, which were quickly frozen in small aliquots in liquid nitrogen and stored at -80 °C.

Western Blotting—Western blotting was performed as described previously (41). Anti-FLAG tag polyclonal antibody (Sigma) was used at a 1:500 dilution as the manufacturer recommends.

In Vitro Splicing and Immunoprecipitation—The template plasmids for *in vitro* transcription were generous gifts from Dr. Christopher W. J. Smith (University of Cambridge). *In vitro* transcription and splicing were performed as described previously (42). Briefly, a typical 40- μ l splicing reaction contained 4 μ l of 10 \times SP (42), 24 μ l of HEK293T whole cell extract, and 32 P-labeled transcript. Following a 3-h incubation at 30 °C, immunoprecipitation with anti-FLAG antibody M2 beads was carried out as described previously (10, 16).

RESULTS

To study the interaction of proteins with pre-mRNAs and splicing intermediates and assess their roles in splicing, we have previously described a system that allows for the rapid expression of proteins and preparation of active splicing extracts. To do so, we expressed tagged proteins by transfection in HEK293T cells, which allow efficient transient protein expression; we produced extracts from these cells and combined them with standard HeLa nuclear extracts for *in vitro* splicing. The tagged proteins could then be isolated by immunoprecipitation with anti-tag antibodies, and the RNAs with which they associate could be identified (10, 16). We have now improved and simplified this system further. Production of active HeLa nuclear extracts is time-consuming and labor intensive and involves multiple centrifugation and dialysis steps, resulting in batch-to-batch variation. To simplify this system and combine it with the high transfection efficiencies of HEK293T cells, we produced HEK293T whole cell extract, made by simply sonicating the cells in the splicing buffer followed by centrifugation and collection of the resulting supernatant for *in vitro* splicing. The HEK293T cells were first transfected with FLAG-tagged cDNA under the control of a mammalian promoter that allowed the use of anti-FLAG affinity beads for subsequent immunoprecipitations after the *in vitro* splicing reaction in the lysate of these cells. As shown in Fig. 1, HEK293T whole cell lysate maintains a splicing activity. The splicing efficiency of the whole cell lysates is almost similar with several different

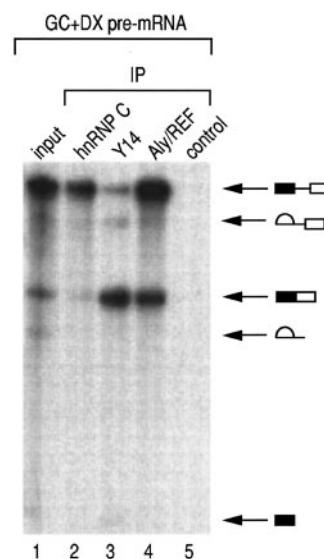


FIG. 1. Immunoprecipitation of RNAs from *in vitro* splicing reactions in HEK293T whole cell lysates. 32 P-labeled GC+DX pre-mRNA was incubated with HEK293T whole cell lysates under splicing conditions, followed by immunoprecipitation with the anti-heterogeneous nuclear ribonucleoprotein C (4F4), anti-Y14 (4C4), anti-Aly/REF (11G5), and control nonspecific (SP2/0) antibodies. Recovered RNAs were resolved by electrophoresis on 5% denaturing polyacrylamide gels. The identity of the precipitated RNAs is shown at the right side of the panel. Boxes and lines represent exons and introns, respectively. The lane marked *input* contains 10% of input RNA.

pre-mRNAs (data not shown). To further verify the validity of this system, antibodies against heterogeneous nuclear ribonucleoprotein C1/2, Y14, and Aly/REF were used to immunoprecipitate 32 P-labeled RNA from the splicing reaction. As reported previously, anti-heterogeneous nuclear ribonucleoprotein C1/2 antibody preferentially immunoprecipitated pre-mRNA (Fig. 1, lane 2) and anti-Y14 antibody predominantly immunoprecipitated mRNA (Fig. 1, lane 3) (7), whereas anti-Aly/REF immunoprecipitated both species (Fig. 1, lane 4) (38). Thus, HEK293T whole cell lysates are active in splicing and give consistent results when compared with HeLa nuclear extracts. Furthermore, transfected FLAG-tagged proteins work efficiently at immunoprecipitation of *in vitro* spliced pre-mRNA (10, 16). We therefore used whole cell lysates of HEK293T cells that were transiently transfected with FLAG-tagged cDNAs for *in vitro* splicing and immunoprecipitation. This system allowed us to set up the assays using only anti-FLAG affinity beads; this avoided the need to produce the proteins separately, purify them, and obtain antibodies specific to each protein for immunoprecipitations. Moreover, the use of the same anti-FLAG antibodies for all the proteins studied allowed a better comparison of the differences between the various proteins.

To study the association of the various EJC proteins with pre-mRNAs, splicing intermediates, and mRNAs, we produced whole cell lysates that express FLAG-tagged EJC components. The level of expression of each of the FLAG-tagged proteins was determined by Western blotting using anti-FLAG antibody. As shown in Fig. 2A, all FLAG-tagged proteins were expressed as full-length and the expression level was comparable, except for magoh, which consistently gave a lower signal (10). This may be the result of less efficient expression of magoh, but it is also possible that the lower signals are simply because of poor transfer or lower retention of magoh on the membrane because of this protein's small size. We also checked the efficiency of immunoprecipitation for each protein from splicing reaction to ensure that the epitope used for the immu-

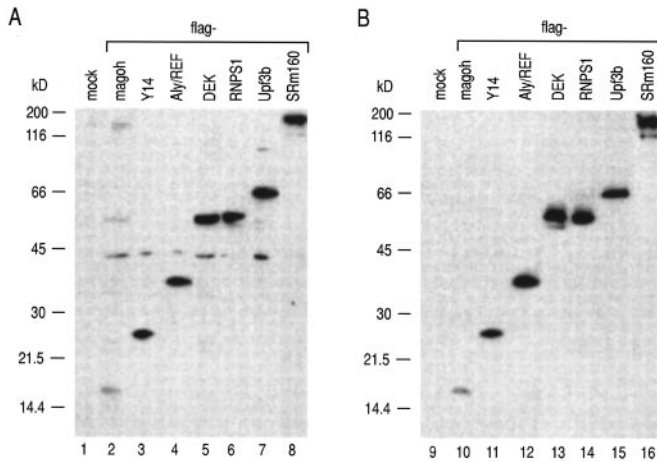


FIG. 2. *A*, immunoblotting (Western blotting) of the splicing reactions in whole cell lysates of HEK293T cells transfected with control plasmid (*mock*) or with plasmids containing the indicated FLAG-tagged cDNAs encoding the individual EJC proteins. The FLAG-tagged proteins were detected using anti-FLAG polyclonal antibody (Sigma). 10% of the input protein used in the immunoprecipitation was analyzed. *B*, immunoblotting of the proteins from the splicing reactions by immunoprecipitation with the anti-FLAG monoclonal antibody M2 (Sigma). The immunoprecipitated FLAG-tagged proteins were detected with polyclonal anti-FLAG antibody (Sigma).

noprecipitation (FLAG tag) is accessible in all of them. The results presented in Fig. 2*B* show that each protein was immunoprecipitated with very similar efficiency (10–15%). These results indicate that this system allows us to compare the association of these EJC proteins with RNA.

The lysates were then used for *in vitro* splicing of a pre-mRNA construct, designated GC + DX, derived from α -tropomyosin gene pre-mRNA (43). We chose to study this pre-mRNA because of the availability of a derivative of this pre-mRNA that contains a stem-loop structure in the intron that blocks splicing between the first and second catalytic steps (see below). There was no detectable difference in splicing efficiency among the extracts produced from the cells transfected with the various constructs (data not shown). Immunoprecipitation following splicing was then carried out using anti-FLAG affinity beads. Aly/REF, RNPS1, and SRm160 efficiently co-immunoprecipitated pre-mRNA (Fig. 3*A*, lanes 2, 3, and 5), whereas Y14 and magoh pulled down mainly mRNA (lanes 6 and 7). Interestingly, splicing intermediates such as the 5' exon and the intron-lariat with 3' exon were efficiently immunoprecipitated by Upf3b (lane 8). There was no detectable immunoprecipitation of RNA from either the DEK- or mock-transfected extracts (lanes 4 and 9). The observed differences in the intermediates that were immunoprecipitated from the various extracts suggest that the EJC is not pre-assembled and does not assemble on the mRNA all at once but rather that there is an order in which the EJC components assemble on spliced mRNA. For instance, because Upf3b associates with the 5' exon and intron-lariat intermediates, this protein most likely interacts with the RNA substrate during or immediately after the first catalytic step of splicing, whereas Aly/REF, RNPS1, and SRm160 associate with pre-mRNA prior to the first catalytic step. Y14 and magoh, on the other hand, only significantly associate with the mRNA and therefore probably interact only at a late stage of splicing.

To further investigate the stepwise assembly of the EJC we used HP1 pre-mRNA as the splicing substrate. HP1 pre-mRNA is derived from the GC + DX pre-mRNA, but it contains a stable stem-loop structure that blocks the second catalytic step of splicing and thus results in the accumulation of splicing intermediates (Fig. 3*B*, lane 10) (43). As before, the pre-mRNA

substrate was used in an *in vitro* splicing reaction and interacting RNA intermediates were immunoprecipitated using anti-FLAG affinity beads and analyzed by gel electrophoresis. Aly/REF, RNPS1, and SRm160 again predominantly immunoprecipitated pre-mRNA (Fig. 3*B*, lanes 11, 12, and 14). Upf3b, on the other hand, efficiently immunoprecipitates the splicing intermediates 5' exon and intron-lariat with 3' exon from the splicing reaction (Fig. 3*B*, lane 17). In contrast, Y14 and magoh showed no significant interaction with either the pre-mRNA or the splicing intermediates (Fig. 3*B*, lanes 15 and 16). No co-immunoprecipitation of RNA was observed for DEK- or mock-transfected lysates (Fig. 3*B*, lanes 13 and 18). Taken together, Aly/REF, RNPS1, and SRm160 associate with the pre-mRNA prior to or during spliceosome assembly, whereas Upf3b associates during or immediately after the first catalytic step. Y14 and magoh, however, are only enriched on spliced mRNA and thus associate during or after the second catalytic step. These findings indicate that EJC formation is a stepwise process and is splicing-dependent.

DISCUSSION

Using a simplified whole cell lysate system for *in vitro* splicing in conjunction with transfection of tagged proteins as a way to obviate the need for separate production of proteins or the need for protein-specific antibodies, we investigated the assembly of the EJC. We expressed the EJC proteins individually as FLAG-tagged fusions from the corresponding cDNAs and identified the splicing precursors, intermediates, and products with which they associate by co-immunoprecipitation with anti-FLAG antibodies. This strategy also eliminated differences that may result from different efficiencies of the various antibodies against the individual EJC components and thus allowed for a better direct comparison among them. We tested the seven known components of the EJC and found they could be divided into three groups based on when in the course of the splicing reaction they become associated with the RNA. A schematic representation of these observations is provided in Fig. 4.

Aly/REF, RNPS1, and SRm160 associate with pre-mRNAs before the first catalytic step and comprise the first group (Fig. 3). Aly/REF was shown to interact with the splicing factor UAP56, a DEXD-box helicase, and this interaction is conserved from human to yeast (37–40). UAP56 is an essential splicing factor that is required for stable association of the U2 small nuclear ribonucleoprotein with the branch point and for spliceosome assembly (44–47). These results strongly suggest that UAP56 promotes the binding of Aly/REF to pre-mRNA during splicing at an early step, consistent with our observation that Aly/REF stably bound to pre-mRNA before the first catalytic reaction. RNPS1 also associates with pre-mRNA (13). SRm160 interacts with SR proteins, factors that have essential roles in splicing during early spliceosome assembly, and it cannot associate with pre-mRNA in the absence of SR proteins (14, 48). It is therefore likely that SR proteins recruit or stabilize the interaction of SRm160 with the pre-mRNA during this early stage. Upf3b, which associates with the splicing intermediates, comprises the second group in the EJC assembly pathway (Fig. 3). Upf3b is a nuclear protein that has a role in NMD (15, 49) and is a component of the EJC (16, 33). Upf3b can interact with RNPS1 (19), and it is therefore possible that RNPS1 facilitates the association of Upf3b with the spliceosome once it associates with the pre-mRNA. Y14 and magoh can be classified as the third group. These two proteins remain bound to the mRNA even after export (7, 10, 11, 32, 33) and require translation of the mRNA to which they are bound for removal from the mRNA in the cytoplasm (34). The association of Y14 and magoh with the mRNA at a late step of splicing may serve as a mark that splicing is complete (Fig. 3). Because ALY/REF, RNPS1, and

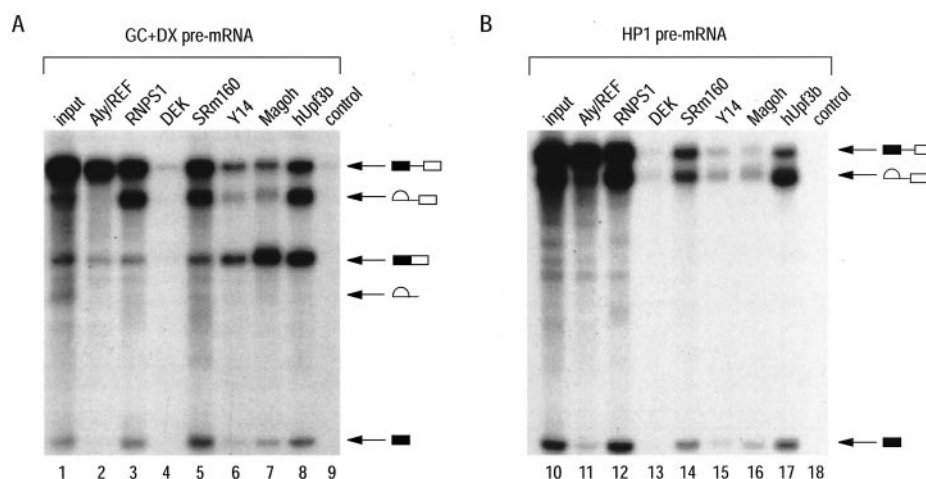


FIG. 3. Immunoprecipitation of RNAs from *in vitro* splicing reactions in HEK293T whole cell lysates transfected with plasmids encoding FLAG-tagged cDNAs of the indicated EJC proteins. Immunoprecipitations and RNA analyses were performed as described in the legend to Fig. 1. A, 32 P-labeled GC+DX pre-mRNAs were incubated under splicing condition, and RNAs were immunoprecipitated by anti-FLAG antibody M2. B, 32 P-labeled HP1 pre-mRNAs were incubated under splicing condition, followed by immunoprecipitation with the anti-FLAG monoclonal antibody M2.

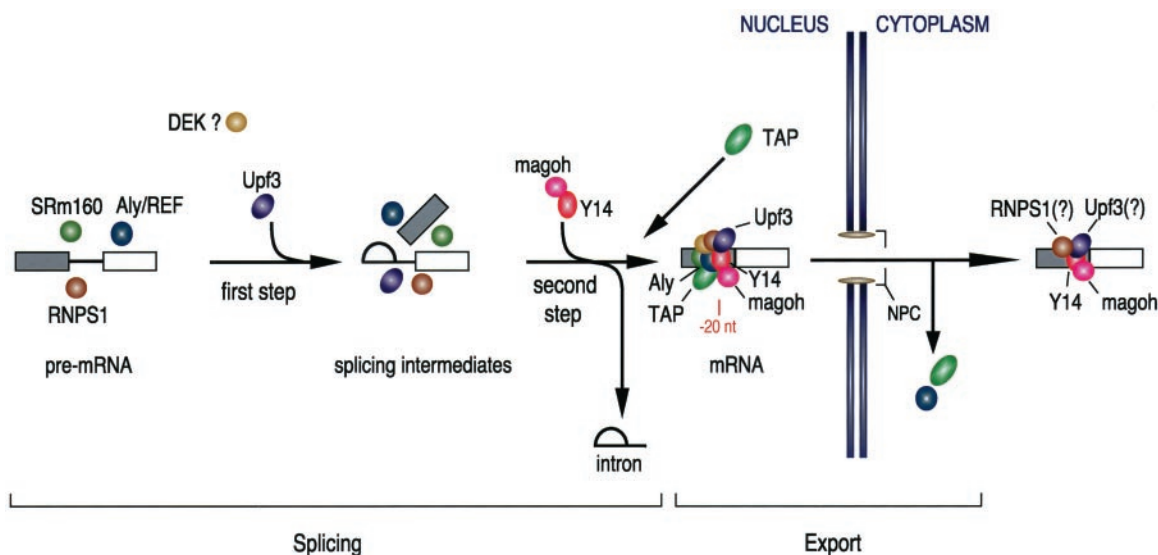


FIG. 4. Schematic representation of the assembly of the exon-exon junction complex. See "Discussion" for details.

Upf3 all have the capacity to interact with Y14 (10, 16), one or all of these proteins may recruit the Y14-magoh complex into the EJC at the late stage of splicing. We note that we did not observe an association between DEK, another reported component of the EJC (8, 17), with any of the RNAs from the splicing reaction used in this report or with other pre-mRNAs that we have studied.

Recently, while this manuscript was in preparation, Reichert *et al.* (50) reported on the EJC assembly in HeLa nuclear extract. They suggest that Aly/REF is bound to the pre-mRNA prior to spliceosome assembly but becomes enriched during splicing, whereas Y14, magoh, RNPS1, and SRm160 enter the spliceosome prior to exon ligation. Upf3b was not examined in this study, but the general conclusions are in partial agreement with our results, with some exceptions. First, we do not observe significant amounts of Y14 and magoh prior to exon ligation; second, we do detect RNPS1 and SRm160 interacting strongly with the pre-mRNA prior to intron-lariat and 5' exon formation. Upf3b is the only protein we see becoming enriched on the 5' exon and intron-lariat. It is possible that some of the differences are because of differences in the experimental approaches and perhaps also to transcript-specific differences. In

either case, both sets of data point to EJC formation being a stepwise process that is in concert with splicing. How the multiple proteins of the EJC all bind to a small region of the RNA and how this occurs specifically at \sim -20 nucleotides upstream of the exon-exon junction in a position-specific but sequence-independent manner remains to be clarified. Finally, the simple whole cell lysate system we described here should facilitate rapid and small-scale preparation of active splicing lysates from many different cell types and tissues.

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