Flexibility in the site of exon junction complex deposition revealed by functional group and RNA secondary structure alterations in the splicing substrate

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ABSTRACT

The exon junction complex (EJC) is critical for mammalian nonsense-mediated mRNA decay and translational regulation, but the mechanism of its stable deposition on mRNA is unknown. To examine requirements for EJC deposition, we created splicing substrates containing either DNA nucleotides or RNA secondary structure in the 5' exon. Using RNase H protection, toeprinting, and coimmunoprecipitation assays, we found that EJC location shifts upstream when a stretch of DNA or RNA secondary structure appears at the canonical deposition site. These upstream shifts occur prior to exon ligation and are often accompanied by decreases in deposition efficiency. Although the EJC core protein eIF4AIII contacts four ribose 2'OH groups in crystal structures, we demonstrate that three 2'OH groups are sufficient for deposition. Thus, the site of EJC deposition is more flexible than previously appreciated and efficient deposition appears spatially limited.

Keywords: EJC; pre-mRNA splicing; eIF4AIII; NMD; secondary structure

INTRODUCTION

To become mature mRNAs, metazoan precursor mRNAs (pre-mRNAs) undergo a number of processing steps. One of these is the excision of introns, which is catalyzed by the spliceosome and occurs in the cell nucleus. The splicing process shapes several aspects of a mature mRNA's subsequent life, including localization, translational yield, and stability in response to a surveillance process known as nonsense-mediated mRNA decay (NMD) (Le Hir et al. 2003; Chang et al. 2007). Splicing influences these later events by depositing a set of proteins known as the exon junction complex (EJC) on the spliced mRNA (Tange et al. 2004; Lejeune and Maquat 2005; Le Hir and Seraphin 2008). The EJC is composed of four core proteins, eIF4AIII, Magoh, Y14, and MLN51 (also known as Barentsz), as well as secondary proteins that interact transiently with the core (Ballut et al. 2005; Tange et al. 2005; Le Hir and Andersen 2008).

Deposition of the EJC core proteins on the 5' exon occurs sometime after the first step of splicing (Reichert

et al. 2002; Kataoka and Dreyfuss 2004). RNase protection mapping, cross-linking, and coimmunoprecipitation experiments with several splicing substrates revealed that the EJC protects ~8 nucleotides (nt) from RNase digestion and that the location of deposition, which spans nucleotides –20 to –24 upstream of the splice junction, is sequence independent (Le Hir et al. 2000). Other studies have shown that although mRNAs with truncated 5' exons of only 17 nt do not assemble an EJC (Le Hir et al. 2001), the core proteins still associate with spliceosomal complexes (Shibuya et al. 2006; Ideue et al. 2007; Merz et al. 2007) and interact with at least one intron-binding protein, IBP160 (Ideue et al. 2007). These results have generated the impression that the site of deposition of the EJC on the 5' exon is rather rigidly dictated by the architecture of the spliceosome.

The EJC core has been reconstituted using the four purified recombinant core proteins, single-stranded RNA, and an ATP analog, AMP-PNP (Ballut et al. 2005). Two crystal structures of this complex reveal that both MLN51 and eIF4AIII contact the RNA (Andersen et al. 2006; Bono et al. 2006). While MLN51 interacts with only a single RNA nucleotide, eIF4AIII contacts 6 nt by forming hydrogen bonds or salt bridges with the phosphates and hydrogen bonds with four 2'OH groups. These structures explain how the EJC binds the 5' exon in a sequence-independent

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manner, but do not address which or how many interactions are critical for deposition during pre-mRNA splicing.

eIF4AIII is a DEAD-box protein that is homologous to, but functionally distinct from, eIF4AI and eIF4AII, which function in translation initiation (Li et al. 1999). eIF4AIII's sequence-independent interaction with RNA is consistent with the known RNA-binding properties of DEAD-box proteins (Cordin et al. 2006). eIF4AIII exhibits *in vitro* ATPase activity that is inhibited by Magoh and Y14 (Ballut et al. 2005); this inhibition is proposed to enable stable binding of the EJC core to RNA (Ballut et al. 2005). The ATPase activity of eIF4AIII is stimulated by MLN51 and accompanied by *in vitro* helicase activity (Noble and Song 2007), but its *in vivo* significance is uncertain. Mutations within the Walker A and Walker B motifs as well as within motif III of eIF4AIII, which would be expected to abolish or reduce these activities (Cordin et al. 2006), do not affect EJC deposition, suggesting that eIF4AIII's ATPase and helicase activities are not required (Shibuya et al. 2006; Zhang and Krainer 2007).

Here, we address several questions regarding EJC deposition. We use RNase H protection, toeprinting, and coimmunoprecipitation assays to show that EJC deposition can occur upstream of position -24 relative to the exon-exon junction. This shifted interaction is triggered by replacing the nucleotides between positions -20 and -24 with DNA, can be observed on the 5' exon intermediate, and decreases the efficiency of deposition as the size of the DNA stretch increases. We also determine that three 2'OH groups are sufficient for EJC deposition during pre-mRNA splicing. By examining two different RNA stem-loops, we find that secondary structures within the 5' exon can also alter the location of the EJC. These results suggest that the sequence of a nascent mRNA may influence downstream processes by affecting both the site and efficiency of EJC deposition.

RESULTS

EJC binding shifts upstream when DNA is present at the normal site of deposition

To investigate how the site of EJC deposition is defined during splicing in

HeLa cell nuclear extract, we created chimeric RNA-DNA splicing substrates using three-piece ligation (Moore and Sharp 1992; Szewczak et al. 2002). These constructs contain a single 32 P-label 4 nt upstream of the stretch of DNA within the 5' exon, allowing detection of both pre-mRNA and spliced product (Fig. 1A). We first compared a splicing substrate containing DNA in positions -20 to -27 upstream of the 5' splice site, called D8, to an All-RNA pre-mRNA. This length and location for the substitution were chosen based on previous reports showing that the EJC protects 8 nt from RNase digestion, including positions -20 to -24 (Le Hir et al. 2000; Reichert et al. 2002; Ballut et al. 2005). In the DNA segment, both U to T and

FIGURE 1. (Legend on next page)

2'OH to 2'H substitutions were made because splicing substrates containing 2'deoxyuridine were unstable in nuclear extract (data not shown).

To define the position of the EJC on spliced RNA, we first subjected the products of the splicing reaction to an RNase H protection assay (Le Hir et al. 2000). After a 90-min incubation, complementary DNA oligonucleotides (most 12 nt long) (Fig. 1B) were individually added to the reaction. An intronless body-labeled control RNA, also incubated in extract for 90 min, showed RNase H cleavage products in the presence of all eight oligonucleotides complementary to the 5' exon (Fig. 1C). Similarly, the unspliced All-RNA AdML substrate remaining in the splicing reaction was cleaved by endogenous RNase H activity along the entire 5' exon (Fig. 1D); Oligo 6 is an exception since it spans the splice junction. In contrast, for the spliced product, specific protection of the 5' exon was seen with oligonucleotides 4, 12, and 16, which are all complementary to positions -20 to -28 (Fig. 1D), consistent with previously published results (Le Hir et al. 2000). The 5' exon splicing intermediate also showed protection along the 5' exon from ~-30 to -1 (oligonucleotides 4, 5, 12, and 16), as previously reported (Reichert et al. 2002).

The D8 construct exhibited a different pattern (Fig. 1E) of protection from RNase H cleavage. Protection of spliced D8 was observed in the presence of oligonucleotides 2 and 3, between residues -28 and -39, compared to unspliced D8. Note that positions -20 to -27 (covered by oligonucleotides 4 and 12) could not be cleaved by RNase H because these positions are DNA (Fig. 1F). In contrast to the protection of spliced D8 in the presence of oligonucleotides 2 and 3, protection of intronless D8 from RNase H cleavage after incubation in extract (data not shown) mirrored the

protection pattern for intronless All-RNA (Fig. 1C), as expected, since the sequence had not been altered. Although some protection in the presence of oligonucleotides 2 and 3 was observed, the protection of spliced D8 was considerably higher.

Quantitative comparison of the data for the All-RNA and D8 substrates (Fig. 1F) revealed that the protection between -28 and -39 for spliced D8 was accompanied by a decrease of protection when oligonucleotide 16, which is complementary to residues -15 to -30, was present. Together, these results indicate that EJC deposition on spliced D8 was shifted ~8 nt upstream relative to its position on the All-RNA construct, matching the length of the DNA substitution. Interestingly, this upstream shift in protection was also seen on the D8 5' exon intermediate (Fig. 1, cf. D and E); the protection extended to ~-40, while the protection of the 5' exon farther downstream remained, suggesting that the site of EJC assembly is identified prior to exon-exon ligation.

To ask if further EJC shifts could be obtained in the presence of different-length stretches of DNA, we constructed AdML splicing substrates D4, D12, and D16, with the 3'-most DNA nucleotide located at -20 in all constructs (Fig. 2A). The pattern of protection from RNase H cleavage for D4 was nearly identical to that for All-RNA, while the D12 and D16 substrates exhibited lower amounts of shifted, splicing-specific protection (data not shown, but also subjected to toeprint assay below).

The upstream shift of the EJC can be visualized by a toeprint assay

To locate the position of the EJC more precisely, a toeprint assay (Ringquist and Gold 1998) was employed (protocol kindly provided by T. Nilsen, Case Western University).

First, the splicing reaction was fractionated on a 10%–40% glycerol gradient (Fig. 2B). Pooled Spliced and Unspliced fractions were then subjected to primer extension using a 5'-labeled DNA primer (Fig. 2C). When the spliced fractions from an All-RNA reaction were treated with proteinase K to eliminate bound proteins prior to primer extension, the product was elongated to the 5' end without significant premature termination (Fig. 2C, Sp, lane 1). The untreated spliced fraction (Fig. 2C, lane 2) showed a second prominent band at -19. This toeprint likely identifies the 3' edge of the EJC bound to the spliced All-RNA substrate: Its position is consistent with the RNase H protection data, as well as with previous reports of EJC deposition spanning positions -20 to -24 (Le Hir et al. 2000). To confirm that the -19 band did not result from aborted primer

FIGURE 1. Splicing-dependent EJC assembly shifts upstream when DNA is present at the usual site of deposition. (A) Schematic of AdML splicing substrate created by three-piece ligation. The 5' exon and 3' exon are black and gray, respectively. The white box within the 5' exon depicts the 8 nt of DNA in positions -27 to -20 relative to the 5' splice site. A single ³²P label is located 4 nt upstream of the 8 nt of DNA. The lengths of each segment are shown below. (B) Schematic of DNA oligonucleotides complementary to the AdML RNA used in the RNase H protection assays. DNA oligonucleotides are 12 nt long, except for oligonucleotide 16, which is 16 nt long. (C–E) RNase H cleavage of the intronless control RNA (C), All-RNA (D), and D8 (E) after splicing. Following a 90-min incubation in HeLa nuclear extract, the complementary DNA oligonucleotides indicated above each lane were added and incubated for an additional 15 min to elicit cleavage by endogenous RNase H activity. RNAs were separated by 8% denaturing PAGE. Splicing substrates contained a single ³²P label, while intronless RNA was body labeled. Gels were visualized using a PhosphorImager. Input RNA substrates are shown in the I lanes. Unspliced, spliced, and 5' exon intermediate RNAs are indicated. Assignment of RNA species was determined by size and RNase H cleavage (not shown). The * indicates pre-mRNA with a truncated 3' exon generated in the extract. The smear near the top of each gel lane is nonspecific, appearing only upon incubation of radiolabeled RNA in extract. DNA size markers are on the left in panel D. (F) Quantitation of unspliced pre-mRNA and spliced mRNA protected from RNase H activity in panels D and E. The 5' exon is schematized to show the position of EJC deposition deduced from the protection profiles of the DNA oligonucleotides complementary to the 5' exon. Quantitation was based upon at least four experiments and represents the fraction of either unspliced or spliced RNA relative to total RNA in the lane that survived in the presence of each DNA oligonucleotide relative to the fractions in the corresponding no oligonucleotide lane.

FIGURE 2. Shifts in EJC position can be visualized by a toeprint assay. (A) Schematic of the spliced DNA-containing AdML constructs. DNA nucleotides are shown in white within the 5' exon (black); the 3' exon is gray. The 3'-most position of the DNA was always -20 . (B) Glycerol gradient fractionation of a splicing reaction containing body-labeled AdML pre-mRNA. RNA profiles before and after the reaction are shown in lanes I and T. RNAs recovered from each fraction were separated by 8% denaturing PAGE. Spliced and Unspliced indicate the fractions that were pooled for toeprint analysis. (C–E) Toeprint analyses of the Spliced and Unspliced fractions of All-RNA and D8 (C), of intronless All-RNA and D8 (D), and of the additional DNA-containing constructs schematized in panel A (E). Prot. K indicates treatment with proteinase K prior to reverse transcription. Arrows show locations on the Spliced RNA of the major reverse transcriptase stops (toeprints) that are sensitive to prior treatment with proteinase K (even versus odd lanes). Full-length extension on the Unspliced and Spliced RNA is indicated by Un and Sp, respectively. DNA products were separated by 8% denaturing PAGE. Gels are representative of at least three experiments. (C) Toeprints are shown for the All-RNA (lanes 1,2,5,6) and D8 (lanes 3,4,7,8) substrates using gradient-fractionated Spliced RNA (lanes 1–4) or Unspliced RNA (lanes 5–8) from panel B. Lane P shows the 32 P-labeled DNA primer. Intron-specific stops are indicated with *. (D) Toeprint analyses comparing intronless All-RNA (C, lanes 1,2) and intronless D8 (C, lanes 5,6) to spliced All-RNA (lanes 3,4) and spliced D8 (lanes 7,8). (E) Toeprint analyses for the intronless control RNA (C, lanes 1,2) and the DNA-containing constructs shown in panel A (lanes 3–12) using gradient fractions containing Spliced RNA. To the right are sequencing ladders generated using dideoxy nucleotides.

extension on the unspliced RNA present in the spliced fraction, primer extension was also performed on gradient fractions predominantly containing unspliced RNA (Fig. 2B, Unspliced). The intensity of the -19 reverse transcrip-

tase stop for this fraction was about threefold lower, and a cluster of additional bands appeared, indicating termination immediately downstream of the branch point sequence within the intron (Fig. 2C, * in lanes 6,8).

Having determined the position of the EJC assembled on All-RNA AdML, we performed toeprint analyses on D8. Primer extension of the spliced D8 fraction revealed a proteinase K-sensitive reverse transcriptase stop at position -26 (Fig. 2C, lane 4), indicating that the 3' edge of the EJC shifted upstream ~ 7 nt relative to All-RNA (Fig. 2C, lane 2). When an unspliced D8 fraction was used instead, the intensity of the -26 band decreased (Fig. 2C, lane 8), indicating that the -26 band is specific to spliced D8. To confirm that the primer extension stops at -19 on All-RNA and -26 on D8 are splicing specific, the toeprint reaction was performed on intronless All-RNA and D8, which showed no proteinase K-sensitive stop at position -19 or -26 (Fig. 2D, cf. lanes 2,6 and 4,8). We conclude that the position of the EJC monitored by the toeprint assay is consistent with the observed shift in RNase H protection (Fig. 1F) and the length of the DNA substitution.

Toeprint experiments using the additional DNA-containing substrates diagrammed in Figure 2A exploited the superior resolution of this assay for locating the sites of EJC deposition on the spliced products of D4, D12, and D16 (Fig. 2E). Using the intronless AdML control (C), no splicing-independent, proteinase K-sensitive stops were detected between position -17 and the 5' end (Fig. 2E, lane 2). The predominant toeprint for spliced All-RNA was at -19 , as expected, while the spliced D4 substrate exhibited a toeprint at -21 (Fig. 2E, lanes 4,6). For D8, the predominant toeprint occurred at -27 with a fainter band at -26 and two other bands at -29 and -32 (Fig. 2E, lane 8). For D12, there were two equally strong bands, representing stops at positions -28 and -31 (Fig. 2E, lane 10). The -31 toeprint corresponds to the expected location of the 3' edge of the EJC based on the results in Figure 2B, which showed the shift in EJC position correlating with the length of the DNA

substitution; the –28 stop was unexpected. For D16, there were two faint bands, representing stops at positions –35 and –37 (Fig. 2E, lane 12). Although there is a reduction in the amount of RNA present after proteinase K treatment and purification, as seen by the differences in the intensity of the Sp bands, the differences in the toeprint profiles presented here are also seen in other gels where the reductions are less pronounced (including those in Fig. 2C,D, as well as in Figs. 4, 5, below). These data demonstrate that the site of EJC deposition shifts upstream to the nearest 2'OH when DNA is present starting at –20, arguing that the site of EJC deposition can be altered in response to features of the 5' exon. The observed multiple toeprints for some of the substrates may represent EJC binding at distinct positions along the spliced RNA or could reflect an inherent limitation of the assay.

The efficiency of EJC deposition decreases as the location shifts upstream

Coimmunoprecipitation (co-IP) experiments were carried out to determine the efficiency of EJC deposition on the DNA-containing substrates D8, D12, and D16 (Fig. 2A). Nuclear extracts were prepared from cells that had been transiently transfected with a plasmid expressing a FLAG-tagged EJC protein (Lykke-Andersen et al. 2001), namely, eIF4AIII or Magoh. Upon completion of splicing, immunoprecipitation with anti-FLAG antibody confirmed that EJC core proteins specifically associate with the splicing intermediates and spliced mRNA (Lykke-Andersen et al. 2001; Hirose et al. 2004) relative to the unspliced RNA (Fig. 3, cf. lane 7 and lanes 5,6). Moreover, spliced All-RNA AdML was preferentially coimmunoprecipitated in the presence of FLAG-eIF4AIII, not of FLAG peptide (Fig. 3, cf. lane 7 and lane 4). Spliced D8, D12, and D16 were also preferentially coimmunoprecipitated relative to their unspliced counterparts, although at different efficiencies (Fig. 3, lanes 11,15,19). To control for nonspecific precipitation, the efficiency of spliced RNA co-IP relative to that of unspliced RNA was determined by dividing the fraction of spliced RNA coimmunoprecipitated by the fraction of unspliced RNA coimmunoprecipitated. The spliced All-RNA co-IP efficiency was then set to 1 to control for variability between experiments, and the relative spliced co-IP efficiencies for D8, D12, and D16 were determined to be 0.93, 0.71, and 0.52, respectively (Fig. 3, bottom). These results suggest that EJC deposition efficiency decreases as the length of the DNA substitution increases. Similar results were obtained when FLAG-Magoh was present in the extracts used for co-IP (data not shown).

Three ribose 2'OH groups are both necessary and sufficient for EJC deposition

Two crystal structures of the EJC core bound to oligo(U) exhibit contacts between four of the RNA 2'OH groups and eIF4AIII, with the 5'-most contacted ribonucleotide sepa-

FIGURE 3. Efficiency of EJC deposition decreases as the EJC shifts upstream. Co-IP of DNA-containing AdML substrates with anti-FLAG antibody upon completion of splicing. Splicing reactions used nuclear extract containing FLAG peptide (lanes 2–4) or FLAG-tagged eIF4AIII (lanes 5–19). Lanes I show 5% of the input, S show 10% of the supernatant, and P show 100% of the pellet. Lanes 1,8,12,16 contain splicing substrates prior to splicing. Unspliced, spliced, and 5' exon intermediate RNAs are indicated on the left. Quantitations at the bottom of the gel are the average of four experiments with standard deviations given below. The fraction of spliced RNA coimmunoprecipitated [P lane/(P lane + S lane)] was divided by the fraction of unspliced RNA precipitated to control for background levels of coimmunoprecipitation. Values for the DNA-containing RNAs were then normalized to the All-RNA co-IP efficiency, which was set to 1 to control for variability between experiments. The amount of variability between experiments and the levels of background were similar to those previously reported (Hirose et al. 2004).

rated by a single nucleotide from the other three consecutive ribonucleotides (Andersen et al. 2006; Bono et al. 2006). The only nonbackbone interaction of the EJC is the observed stacking of the base of the 5'-most contacted ribonucleotide with Phe 188 of MLN51; the lack of base interactions in these structures (Andersen et al. 2006; Bono et al. 2006) is in accord with sequence-independent binding of eIF4AIII to RNA and argues that the thymine residues in our DNA-containing constructs should not significantly alter the results. We asked whether the 2'OH contacts are functionally relevant for the assembly of the EJC during splicing by constructing splicing substrates containing a limited number of DNA substitutions between positions –20 and –27. The constructs were designed to possess the same spacing of 2'OH groups as seen contacting eIF4AIII in the crystal structures (Andersen et al. 2006; Bono et al. 2006), but we successively shifted the position of the four 2'OH groups upstream 1 nt at a time (Fig. 4A, black squares; white squares are DNA).

The data in Figure 4B show that the EJC can be deposited during pre-mRNA splicing in the presence of

FIGURE 4. Four ribose 2'OH groups direct EJC deposition. (A) Schematic of the splicing constructs used in panel B. Residues with 2'OH or 2'H groups between -27 and -20 are indicated by black or white boxes, respectively. The sequence of the All-RNA substrate from positions -27 to -20 relative to the 5' splice site is shown. (B) Toeprint analyses of the constructs in panel A. Gradient fractions containing spliced RNA were examined and presented as described in Figure 2B–E.

four appropriately positioned 2'OH functional groups, demonstrating that the contacts deduced from the crystal structures are functionally relevant for EJC deposition. When subjected to the toeprint assay, the profiles for R20-22,24 and R21-23,25 resembled the All-RNA profile (Fig. 4B, lanes 2,4,6), with the predominant proteinase K-sensitive toeprint at -19 and lighter bands at -17, -21, and -27. For R22-24,26, the -19 toeprint appeared, but there were equally strong bands at -21, -23, and -27 (Fig. 4B, lane 8). R23-25,27 and R24-26,28 had strong toeprints at position -21 relative to the + proteinase K lane, with less frequent stops at -23 and -20 (Fig. 4B, lanes 10,12). The fact that the expected 1-nt shift between constructs R20-22,24 and R21-23,25 and constructs R23-25,27 and R24-26,28 was not observed indicates that the toeprint assay does not have single nucleotide resolution.

We next investigated the minimum number of ribose 2'OH groups required for EJC deposition during pre-mRNA splicing. Toeprinting assays were performed on

constructs containing four (R20-23), two (R22-23), or three (R21-23, R20-22, R22-24) consecutive 2'OH groups between -20 and -24 (Fig. 5A). Construct R20-23 yielded a toeprint profile that mirrors the All-RNA profile (Fig. 5B, lanes 2,4). R22-23, which contains only two 2'OH groups between -20 to -24, did not produce a -19 toeprint; instead the EJC toeprint was shifted upstream to position -26 (Fig. 5B, lane 11). The intermediate construct R21-23, which differs from R22-23 in containing one additional 2'OH group, exhibited both the -19 and -26 toeprint (Fig. 5B, lane 12). Two additional substrates, R20-22 and R22-24, with three 2'OH groups displaced by 1 nt either downstream or upstream relative to R21-23, had toeprint profiles similar to that of R21-23 (Fig. 5B, cf. lane 12 and lanes 16,18). R20-22 showed enrichment of a -17 stop, perhaps suggesting a downstream shift in EJC deposition on this construct. Together, these data verify the importance of the ribose 2'OH groups for EJC deposition, indicating that three adjacent 2'OH groups are sufficient and likely necessary for determining the site of stable EJC deposition during pre-mRNA splicing. However, we cannot rule out the possibility that some arrangement of only two

FIGURE 5. Three ribose 2'OH groups are necessary and sufficient for EJC deposition. (A) Schematic of the constructs analyzed in panel B. Boxes (black for 2'OH and white for 2'H) and labeling are as in Figure 4A. (B) Toeprint analyses of the constructs in panel A. Procedures and labeling are the same as in Figure 4B.

2'OH groups other than the one tested in Figure 5A might allow EJC deposition during pre-mRNA splicing.

RNA secondary structure can affect the location and the efficiency of EJC deposition

Having established that the site of EJC deposition can be altered by RNA functional group substitution, we investigated the effect of RNA secondary structure on the location of EJC assembly. An Altered All-RNA AdML substrate containing two point mutations, G-26C and G-23C (Fig. 6A), exhibited splicing-specific protection from RNase H cleavage in the presence of DNA oligonucleotides 2 and 3 (Fig. 6B). In contrast, intronless Altered AdML exhibited no protection from RNase H cleavage in the presence of these DNA oligonucleotides (Fig. 6C), suggesting that EJC assembly on Altered AdML had shifted upstream (Fig. 6D). This surprising result was illuminated when secondary structure predictions

by mfold (Zuker 2003) of the Altered RNA 5' exon sequence suggested formation of a stem-loop including nucleotides -27 to -13 (Fig. 6E). The positioning of this stem-loop partially occludes oligonucleotides 4 (positions -20 to -31) and 12 (positions -17 to -28) from hybridizing, explaining the protection seen in Figure 6C. Point mutations within the putative stem resulted in loss of the upstream shift in RNase H protection, while complete substitution of the loop sequence with its complement did not affect the shift (Table 1). These results indicated that the two original mutations produced an RNA secondary structure within the 5' exon that affected EJC deposition.

To explore further the effect of RNA secondary structure on EJC deposition, we positioned a well-characterized RNA stem-loop derived from the 3' end of a histone mRNA (HSL) (Battle and Doudna 2001) into the 5' exon of the All-RNA substrate at position -13. The histone mRNA sequence spans from positions -31 to -10 (Fig. 7A). Secondary

structure predictions by mfold (Zuker 2003) of this substrate and all additional substrates containing stem-loops (see below) indicated formation of the HSL. No alternative structures that would interfere with the formation of the HSL were found in any of the sequences (data not shown). Intronless HSL-13 showed protection from RNase H cleavage only in the presence of DNA oligonucleotides 4.1, 4.2, 4.5, and 5.0 (Fig. 7B), which are complementary to the HSL, demonstrating that the stem-loop has formed. Splicing-specific protection of HSL-13 from RNase H cleavage occurred upstream of the stem-loop from nucleotides -44 to -33 (Fig. 7C, Oligos 4, 12, and 4.0). Although oligonucleotide 4.0 is partially complementary to the stem-loop, most of its complementarity resides immediately upstream of the stem-loop (Oligo 4.0 is complementary to positions -35 to -24; see Materials and Methods). Thus, this DNA oligonucleotide targeted the unspliced mRNA for cleavage by RNase H, suggesting that protection of the spliced RNA was due to EJC deposition. Moreover, HSL-13's toeprint, with stops at -29 and -31, was strikingly different from the parent All-RNA profile (Fig. 7D), confirming an upstream shift in EJC position.

We then asked whether three additional HSL substrates derived from HSL-13, which contain elongated stems but are inserted at the same site in the 5' exon (Fig. 7A), exhibit altered EJC

FIGURE 6. RNA secondary structure can alter EJC location. (A) Sequence of the 5' exon of All-RNA AdML and Altered AdML between -38 and -1 relative to the 5' splice site. Point mutations at positions -26 and -23 are in open letters. (B-C) RNase H protection analyses of Altered AdML (B) and intronless Altered AdML (C) after incubation in HeLa nuclear extract. Procedures and labeling are as in Figure 1C-E, except body-labeled splicing substrates were used; splicing intermediates and products are indicated between the two panels. (D) Quantitation of the unspliced pre-mRNA and spliced mRNA protected from oligonucleotide-directed RNase H activity in panel B. Analysis and presentation are as in Figure 1F. The two point mutations from panel A are represented by gray bands. (E) Predicted secondary structure of the Altered AdML RNA and the deduced position of EJC deposition, immediately upstream of the stem-loop.

TABLE 1. Location of RNase H protection on Altered AdML

Point mutation	Position	EJC position by RNase H mapping
G-14C	Stem	–20 to –28
G-17C	Stem	–20 to –28
C-23G	Stem	–20 to –28
C-23A	Stem	–20 to –28
C-23U	Stem	–20 to –28
U-24A	Stem	–20 to –28
UCUGC –22—18 AGACG	Loop	–28 to –39

Substrates containing point mutations predicted to diminish base-pairing within the putative stem of Altered AdML have normal EJC deposition. The table gives the point mutations, their positions within the predicted stem-loop, and the deduced sites of EJC deposition. The predicted Altered AdML secondary structure is shown in Figure 6E.

deposition. All three ([HSL+3]–13, [HSL+8]–13, and [HSL+13]–13) showed splicing-specific protection in the presence of oligonucleotides 4 and 12, just upstream of the stem-loop (Fig. 7E). This extended protection (beyond position –28, Oligos 4 and 12) was also seen on the 5' exon intermediate (Fig. 1D, cf. Oligos 2 and 3, which are complementary to positions upstream of –28 on the All-RNA substrate). Co-IP experiments in the presence of FLAG-eIF4AIII determined that the efficiency of deposition on spliced HSL-13 and on the constructs containing either three or eight additional base pairs in the stem were each comparable to that of spliced All-RNA (Fig. 7F). However, when the stem was elongated by 13 bp ([HSL+13]–13), the co-IP efficiency fell to 0.48 relative to that of spliced All-RNA (Fig. 7F). These data reveal that EJC formation on an RNA undergoing splicing can be influenced by secondary structure, potentially altering both the site and the efficiency of deposition.

Finally, to assess how changes in the position of secondary structure within the 5' exon might affect EJC assembly, three further HSL-containing substrates were generated. In all cases, mfold (Zuker 2003) predicted that the HSL would form. With the HSL inserted at –19 (HSL-19; Fig. 8A), the spliced relative to unspliced RNA was specifically protected from RNase H at positions –25 to –20 (Fig. 8A, Oligo 4.6), suggesting that EJC deposition had unfolded the stem-loop; no other splicing-specific protection was seen. However, when the HSL was positioned at –17 or –15 (HSL-17 and HSL-15), splicing-specific protection was observed upstream of the stem-loop, that is, in the presence of oligonucleotides 4, 12, and 4.0, which encompass nucleotides –39 to –33 for HSL-17 and nucleotides –37 to –31 for HSL-15 (Fig. 8C,D, Oligos 4, 12, and 4.0). Consistent with these shifts, protection of the 5' exon intermediate was also seen upstream of the stem-loop for HSL-17 and HSL-15, but not for HSL-19 (Fig. 8, cf. B,C and A), again suggesting that the site of EJC deposition was identified prior to exon–exon ligation.

DISCUSSION

EJC deposition during pre-mRNA splicing is critical to many aspects of the subsequent life of an mRNA (Tange et al. 2004). Previously, deposition had been studied for only a handful of splicing substrates and was reported to occur ~20 nt upstream of the exon–exon junction (Le Hir et al. 2000; Kataoka et al. 2001; Hirose et al. 2004; Shibuya et al. 2004). Here, we have studied chimeric RNA–DNA splicing substrates and RNA substrates containing secondary structure. Our data clearly show that EJC deposition can occur at positions other than the canonical site, although sometimes with decreased efficiency. We also demonstrate that three ribose 2'OH groups are sufficient and likely necessary for EJC deposition and that the site of deposition is identified prior to the second catalytic step of splicing. Because of the EJC's multiple interactions, its precise positioning could modulate numerous cellular processes, such as mammalian NMD (Lejeune and Maquat 2005; Chang et al. 2007; Giorgi et al. 2007), translational yield (Wiegand et al. 2003; Nott et al. 2004; Diem et al. 2007), translational regulation in response to stress (Ma et al. 2008), and mRNA localization (Hachet and Ephrussi 2004; Palacios et al. 2004).

Three ribose 2'OH groups are sufficient for EJC deposition

Our data argue that three consecutive 2'OH groups, between positions –20 and –27 of the 5' exon, are sufficient for EJC deposition when it occurs coupled to pre-mRNA splicing. In crystal structures, eIF4AIII contacts four ribose 2'OH groups in the bound oligo(U) (Andersen et al. 2006; Bono et al. 2006). Our results verify the functional importance of these contacts. While the 5'-most nucleotide interacts with domain 2 of the protein, the three 2'OH groups that we find to be necessary and sufficient for EJC deposition appear to represent the three ribose 2'OH groups that contact domain 1. We speculate that for All-RNA AdML, the 2'OH groups at positions –21, –22, –23, and –25 are involved because the toeprint profile closely resembles those of R21-23,25 and R21-23 (Fig. 4). The lack of toeprints at –19 for DNA-containing constructs with shifted RNase H protection profiles (Fig. 2) and D8's susceptibility to RNase H cleavage between –15 and –30 (Fig. 1F, Oligo 16) argue that our results are not due to non-EJC proteins binding to the inserted DNA nucleotides. Surprisingly, positioning the three critical 2'OH groups between nucleotides –20 and –24 all gave similar toeprint profiles (Fig. 5); the expected 1-nt shift was not observed (Figs. 4 and 5). This may reflect a limitation of the toeprinting assay, since the ability of the reverse transcriptase to add the last nucleotide before an obstacle could be sequence specific.

FIGURE 7. RNA stem-loops can alter the site of EJC deposition. (A) Schematic of the 5' exon of the HSL-13 construct. Shown is the sequence and secondary structure of the 5' exon between -36 and -1 relative to the 5' splice site; nucleotides -31 to -10 were derived from the 3' end of the mouse histone H2A-614 pre-mRNA (Battle and Doudna 2001). Arrows indicate the positions of nucleotide insertions, which created 3, 8, or 13 additional base pairs in the stem (see panel E). (B–C) RNase H protection analyses of intronless HSL-13 (B) or HSL-13 (C) after incubation in HeLa nuclear extract for 90 min. Procedures and labeling are the same as in Figure 1C–E. DNA oligonucleotides 4.0–4.5 and 5.0 are complementary to part or all of the HSL sequence (see Materials and Methods). Gels are representative of at least three experiments. (D) Toeprint analyses of All-RNA and HSL-13 spliced products. Procedures and labeling are the same as in Figure 4B. (E) RNase H protection analyses after splicing HSL-containing RNAs with lengthened stems. Procedures and labeling are the same as in panel B. Substrates are named based on the number of additional base pairs introduced into the stem at the arrows shown in A and the names also indicate insertion at position -13 of the 5' exon. For the sequences of [HSL+3]-13, [HSL+8]-13, and [HSL+13]-13, see Materials and Methods. (F) Co-IP of HSL-containing AdML substrates with anti-FLAG antibody upon completion of splicing. Splicing reactions in nuclear extract containing FLAG-tagged eIF4AIII were performed and analyzed as in Figure 3. S lanes contained 10% of the supernatant and P lanes contained 100% of the pellet. The migration of splicing intermediates and products are indicated on the right. The * indicates pre-mRNA with a truncated 3' exon. The gel is representative of three experiments. Quantitations were carried out and are presented as in Figure 3.

RNA secondary structure affects EJC deposition

We have presented evidence that RNA secondary structure within the 5' exon can also alter the site of EJC deposition. Surprisingly, two point mutations in the 5' exon of All-RNA AdML were sufficient to shift the EJC position by 8–10 nt, apparently because they allow formation of an interfering stem-loop structure (Fig. 6). The influence of RNA secondary structure was confirmed by inserting a well-characterized stem-loop from the 3' end of histone mRNAs, the HSL (Battle and Doudna 2001), at the same position to generate substrate HSL-13. None of the proteins that have been reported to bind specifically to the HSL were found cross-linked to this construct compared to other splicing substrates, and point mutations within HSL-13 that alter the loop and its ability to bind these proteins (Battle and Doudna 2001), while maintaining the stem, resulted in the same shifted site of EJC deposition (data not shown).

When the HSL was present at positions -13, -15, or -17 relative to the splice junction, EJC deposition shifted upstream, but when positioned at -19, the stem-loop apparently melted to allow deposition at -20 (Fig. 8). Thus, the process of EJC deposition can potentially melt RNA secondary structures depending on their position and stability. A model emerging from our data is that the initial site contacted by eIF4AIII in the 5' exon may include positions -18 and -17, which if single-stranded could interact via their 2'OHs and facilitate melting of the stem-loop at -19. As the estimated ΔG of the inserted HSL is -9.7 kcal/mol from mfold (Zuker 2003) and the estimated ΔG for eIF4AIII binding to RNA is $\sim$ -11 kcal/mol, based upon the $\sim$ 10 nM K_d (Noble and Song 2007), the ATPase and helicase activity of eIF4AIII would not necessarily be required. The involvement of positions -18 and -17 would be consistent with specific enrichment of the -17 toeprint for substrate R20-22 (Fig. 5B, lane 16).

FIGURE 8. Ability of an RNA stem-loop to affect EJC deposition depends on its location in the 5' exon. (A–C) RNase H protection analyses after splicing HSL-19 (A), HSL-17 (B), and HSL-15 (C) substrates in nuclear extract. Procedures and labeling are the same as in Figure 7B. HSL-19, HSL-17, and HSL-15 contain the HSL in Figure 7A positioned at –19, –17, and –15 within the 5' exon (see Materials and Methods). (D) Deduced locations of EJC deposition for HSL-19 (left) versus HSL-17 and HSL-15 (right) are schematized. Sequences of the complementary DNA oligonucleotides are given in Materials and Methods. Gels are representative of three experiments.

We attempted to utilize eIF4AIII mutants to ask whether shifted deposition requires ATPase or helicase activity by following the immunodepletion procedure described by Zhang and Krainer (2007). Unfortunately, the results were inconclusive because of our inability to deplete fully WT eIF4AIII from splicing extracts (data not shown). A bioinformatics approach may be able to identify candidate mRNAs with secondary structures that influence EJC deposition, if such structures are underrepresented within 5' exons or are present or enriched in known disease-causing genes.

The site of EJC deposition is identified prior to exon ligation

The EJC core appears to be at least partially assembled prior to the second catalytic step of splicing, a time at which spliceosome components make extensive contacts with nucleotides downstream of position –27 in the 5' exon (Reichert et al. 2002). Our observation of an upstream shift in the RNase H protection profiles of several 5' exon splicing intermediates (cf. Figs. 1D, 8A and Figs. 1E, 6B, 7C,D, 8B,C) argues that the protection previously reported

along the 5' exon intermediate (Reichert et al. 2002) is at least partially EJC related. This conclusion is consistent with the presence of EJC proteins in spliceosomal B/C complexes (Reichert et al. 2002; Deckert et al. 2006). Previously, co-IP of the 5' exon and lariat-3' exon intermediates was seen for both wild-type eIF4AIII and eIF4AIII mutants that cannot stably associate with spliced mRNA (Shibuya et al. 2004, 2006), suggesting that prior association of EJC proteins with the spliceosome has requirements that are distinct from those for actual EJC deposition on the 5' exon. In contrast, a more recent study (Zhang and Krainer 2007) reported no co-IP of splicing intermediates with eIF4AIII and argued that EJC assembly may not occur or may not be completed until after exon ligation. Our studies agree with and extend the earlier studies (Shibuya et al. 2004, 2006) both by confirming the presence of EJC components and by indicating that choice of the EJC deposition site occurs on the 5' exon intermediate.

The spatial limitation we observe for deposition of the EJC is manifested as a loss in efficiency when the EJC forms at a site displaced from its normal location. Gudikote et al. (2005) previously suggested that EJC deposition may be

influenced by splicing efficiency based on the direct correlation they observed between splicing efficiency and mRNA susceptibility to NMD. In our studies, the splicing efficiency of a particular substrate is not related to the EJC deposition efficiency for that substrate (Table 2). Ideue et al. (2007) observed that depletion of IBP160 reduced both NMD activity and EJC formation on the RNA and thus postulated that interaction between intron-associated proteins and EJC components is critical for stable EJC deposition. Accordingly, the geometry of the spliceosome at the initiation of EJC deposition probably determines where eIF4AIII binds. If appropriately spaced ribose 2'OH groups are not present, as with DNA-containing substrates, the efficiency would drop as the protein searches for stable interactions, perhaps while still bound to intron-associated proteins. This hypothesis is supported by data from the HSL-containing substrates: Although EJC deposition was shifted by ~15 nt for [HSL+3]–13 and ~25 nt for [HSL+8]–13 (Fig. 7E), the deposition efficiency was not decreased, as anticipated from the expected base-pairing of the inserted nucleotides. However, deposition efficiency did decrease for [HSL+13]–13 with a shift of ~35 nt. Perhaps

TABLE 2. Splicing efficiency does not correlate with co-IP of spliced mRNA with FLAG-eIF4AIII

Splicing substrate	Relative splicing efficiency	Standard deviation	Relative co-IP efficiency
All-RNA	1	—	1
D8	1.01	± 0.02	0.93
D12	0.83	± 0.04	0.71
D16	0.94	± 0.03	0.52

The relative splicing efficiencies for the All-RNA, D8, D12, and D16 AdML pre-mRNAs are presented with the standard deviation from four experiments. The values were calculated from the same samples used to determine relative EJC deposition efficiencies in Figure 3, which are presented to the right. Splicing efficiency was determined by taking the amount of spliced RNA and dividing it by the amount of spliced, unspliced, and splicing intermediate RNAs. The values were then normalized to the All-RNA splicing efficiency to control for variation between experiments.

the longer stem-loop caused a steric clash that altered the architecture of the spliceosome during EJC deposition. Alternatively, fortuitous protein binding to the elongated stem might exacerbate any disruption, resulting in decreased deposition. Our results are consistent with the idea that interaction of EJC components with IBP160 precedes or facilitates EJC deposition. Future studies of the biological function of the EJC should include consideration of how 5' exon sequences, secondary structures, or protein-binding sites might alter EJC deposition, composition, and secondary interactions in an mRNA species-specific context.

MATERIALS AND METHODS

RNA splicing substrate sequences

Sequence of the AdML splicing substrate

Exons are shown in uppercase and the intron is shown in lowercase:

5'-GAATACAAGCTGATCCGTTTCGTCCTCACTCTCTTCCGCA
TCGCTGTCTGCGAGGGCCAGCTGTTGGGgtgagtactccctctcaa
aagcgggcatgacttctgcgctaagattgtcagtttccaaaaacgaggaggttgatattca
cctggcccggtgatgcctttgaggggtggcgcgtccatctggtcagaaaagacaattttt
tgtgtgaagcttgacctgcacgtctaggcgcagtagtccagggttcttgatgatgcata
cttatcctgtccctttttttccacagCTCGCGGTTGAGGACAAACTCTTC
GCGGTCTTCCAGTACTCTTGGATCGGAAACCGTCGCGC
CTCCGAACGGTAAGAGCCTAGCATGTAGAAGTGGTT-3'.

Sequences of the HSL-13 elongated stems

Inserted nucleotides are underlined: the unchanged 4-nt loop and unchanged nucleotides within the HSL stem are shown, but not underlined:

[HSL+3]–13: 5'-GGCUCUCAGUUUCCUGAGAGCC-3';
[HSL+8]–13: 5'-GGCUCUCAGAUCAGUUUCCUGAUCUGAG
AGCC-3'; and

[HSL+13]–13: 5'-GGCUCUCAGAUCAGGCAACUUUCCUGU
CCUGAUCUGAGAGCC-3'.

Creation of splicing constructs

Substrates were derived from an AdML splicing substrate (Yu et al. 1998). Constructs containing DNA were created using three-piece ligation (Szewczak et al. 2002). Briefly, the upstream (30 pmol) and downstream (15 pmol) RNAs were combined with 15 pmol of the synthesized 5'-labeled DNA-containing middle piece (Dharmacon) and 15 pmol of the DNA oligonucleotide bridge (IDT) complementary to the middle piece and parts of the upstream and downstream RNAs, and then precipitated with ethanol. The pellet was brought up in 132 mM Tris (pH 7.6) buffer containing 13.2 mM MgCl₂ and heated at 94°C for 2 min. After cooling, a 20 µL reaction containing 66 mM Tris (pH 7.6), 6.6 mM MgCl₂, 5 mM DTT, 1 mM ATP, and 10 U T4 DNA ligase (Roche) was incubated at room temperature for 4 h, followed by purification by 8 M urea polyacrylamide gel electrophoresis (PAGE). The upstream and downstream RNA were transcribed in vitro (Milligan et al. 1987) and purified by denaturing PAGE. The middle piece was 5'-end labeled as previously described (Szewczak et al. 2002).

Constructs containing RNA secondary structures were created using a pSP64-AdML plasmid and PCR primers containing insertions or mutations. PCR products were transformed into *Escherichia coli* DH5α cells to produce plasmid DNA, which was sequenced to confirm the presence of the desired insertions or mutations. The HSL-19 substrate was created by inserting the HSL sequence into the 5' exon between positions –16 and –15. The HSL-17, HSL-15, and HSL-13 substrates were derived from the HSL-19 substrate by removing nucleotides –15 and –14, nucleotides –15 to –12, or nucleotides –15 to –10, respectively. This process generated the three substrates, whose names indicate the starting position of the stem. RNA splicing substrates were transcribed in vitro (Milligan et al. 1987) using HincII-digested DNA templates in the presence of [α-³²P]UTP.

In vitro splicing in nuclear extract

Preparation of HeLa nuclear extract and in vitro splicing were essentially carried out as described (Tarn and Steitz 1994). Briefly, reactions containing 60% nuclear extract, 0.5 mM ATP, 20 mM creatine phosphate, 2.4 mM MgCl₂, and ~10 fmol of pre-mRNA were incubated at 30°C for 90 min. HEK splicing extracts containing FLAG-tagged proteins were made as previously described (Hirose et al. 2004). Cells were harvested 36–48 h after transfection. Thirty percent HEK extract and 30% HeLa extract were used in place of 60% HeLa nuclear extract to maintain splicing efficiency.

RNase H protection assays

As described (Le Hir et al. 2000; Hirose et al. 2004), after 90 min of splicing at 30°C, DNA oligonucleotides complementary to each splicing substrate were added to splicing reactions to a final concentration of 20 µM and incubated at 30°C for 15 min. Reactions were terminated by adding 1 µg proteinase K in the presence of 0.5% SDS, 5 mM EDTA, 0.05 mM CaCl₂ and incubating for 15 min at 30°C followed by phenol-chloroform extraction. RNAs were then precipitated with ethanol and separated

by 8% denaturing PAGE. Oligonucleotide sequences complementary to the 5' exon are given at the end of Materials and Methods. Mfold (Zuker 2003) was used for structural predictions of splicing substrates.

Toeprint assay

The toeprint assay (Ringquist and Gold 1998) was based upon a procedure kindly provided by Tim Nilsen (Case Western Reserve University). Upon completion of a 200 μ L splicing reaction with 1 pmol of pre-mRNA, 3 μ L were removed to serve as total (T in Fig. 2B) and 195 μ L were layered on a 5 mL 10%–40% glycerol gradient containing 150 mM NaCl, 1.5 mM MgCl₂, 20 mM Tris (pH 8.0), and 0.1% NP-40 alternative (Calbiochem). The splicing reaction was fractionated at 50k rpm for 285 min and 300 μ L fractions were successively removed from the top. Thirty-five-microliter aliquots from a fraction containing predominantly spliced RNA were either directly added to a reverse transcription reaction or treated with proteinase K followed by phenol-chloroform extraction and ethanol precipitation, and then brought up in gradient buffer before reverse transcription. Reverse transcription was carried out for 30 min at 37°C in 50 μ L containing 1 $\times$ FS buffer (Invitrogen), 1.6 mM dNTPs, 40 U RNase Inhibitor (Roche), 8 mM DTT, $\sim 1 \times 10^5$ cpm of radiolabeled DNA primer complementary to the 3' exon, and 200 U SS II reverse transcriptase (Invitrogen). Incubation with RNase A was followed by proteinase K, each for 10 min. The DNA product was isolated by phenol-chloroform extraction and ethanol precipitation with carrier RNA. The DNA product was separated by 8% denaturing PAGE.

Coimmunoprecipitation assay

The co-IP assay was based upon a previously reported method (Hirose et al. 2004). Briefly, after conducting a 60 μ L splicing reaction using HEK and HeLa extract, anti-FLAG antibody-agarose conjugate was added. After 2 h of gentle vortexing at 4°C, the beads were washed five times with 1 mL of NET2 (50 mM Tris at pH 7.5, 150 mM NaCl, 0.05% NP-40 alternative) buffer. RNA was recovered after addition of carrier RNA and proteinase K digestion, followed by phenol-chloroform extraction. The RNA was then precipitated with ethanol and separated by 8% PAGE.

DNA oligonucleotides used in RNase H protection assays

For All-RNA

DNA oligonucleotide	Sequence	Complementary position
1	5'-GGACGAACGGAT-3'	(-44 to -55)
2	5'-GGAAGAGAGTGA-3'	(-32 to -43)
3	5'-ATGCGGAAGAGA-3'	(-28 to -39)
4	5'-AGACAGCGATGC-3'	(-20 to -31)
5	5'-GCTGGCCCTCGC-3'	(-8 to -19)
6	5'-GCCAGCCCAACA-3'	(+5 to -7)
12	5'-CGCAGACAGCGA-3'	(-17 to -28)
16	5'-CTCGCAGACAGGATC-3'	(-15 to -30)

For altered RNA

Oligos 4, 12, and 16 are specific to altered RNA. The others are unchanged:

DNA oligonucleotide	Sequence	Complementary position
4	5'-AGAGAGGGATGC-3'	(-20 to -31)
12	5'-CGCAGAGAGGGA-3'	(-17 to -28)
16	5'-CTCGCAGAGAGGGATC-3'	(-15 to -30)

For HSL-containing RNAs

Oligos 2, 4, and 12 are identical to those previously used for All-RNA. They are presented below to indicate their complementary positions. Oligos 4.0, 4.1, 4.2, 4.5, 4.6, and 5.0 are specific to the inserted HSL. Oligos 4.0–4.5 are complementary to all of the HSL-containing substrates, but their positions are slightly shifted, based upon the position of the stem-loop (see Creation of splicing constructs, above). 5.0 is specific to each splicing substrate and 4.6 is specific to HSL-19.

For HSL-13

DNA oligonucleotide	Sequence	Complementary position
2	5'-GGAAGAGAGTGA-3'	(-48 to -59)
4	5'-AGACAGCGATGC-3'	(-36 to -47)
12	5'-CGCAGACAGCGA-3'	(-33 to -44)
4.0	5'-GAGCCTTTTCGC-3'	(-24 to -35)
4.1	5'-AAAAGAGCCTTT-3'	(-20 to -31)
4.2	5'-TCTGAAAAGAGC-3'	(-16 to -27)
4.5	5'-TGGCTCTGAAAA-3'	(-12 to -23)
5.0	5'-GCCGTGGCTCTG-3'	(-8 to -19)

For HSL-15

DNA oligonucleotide	Sequence	Complementary position
5.0 ¹⁵	5'-GCTGGGTGGCTC-3'	(-8 to -19)

For HSL-17

DNA oligonucleotide	Sequence	Complementary position
5.0 ¹⁷	5'-GCTGGCGGTGGC-3'	(-8 to -19)

For HSL-19

DNA oligonucleotide	Sequence	Complementary position
4.0	5'-GAGCCTTTTCGC-3'	(-30 to -41)
4.1	5'-AAAAGAGCCTTT-3'	(-26 to -37)
4.2	5'-TCTGAAAAGAGC-3'	(-22 to -33)
4.5	5'-TGGCTCTGAAAA-3'	(-18 to -29)
4.6	5'-CCGGTGGCTCTG-3'	(-14 to -25)
5.0 ¹⁹	5'-GCTGGCCCGGTG-3'	(-8 to -19)

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