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Antisense oligonucleotide binding to U5 snRNP induces a conformational change that exposes the conserved loop of U5 snRNA

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ABSTRACT

Conformational rearrangements of the spliceosomal small nuclear RNAs (U snRNAs) are essential for proper assembly of the active site prior to the first catalytic step of splicing. We have previously shown that conformational changes caused by binding of an antisense 2′-*O*-methyl RNA oligonucleotide (BU5Ae) to U5 snRNA nt 68–88 disrupted the U4/U5/U6 complex and induced formation of the U1/U4/U5 and U2/U6 complexes. Here we show that the conformational change induced by BU5Ae exposes the invariant loop of U5 that binds the 5′ exon and also reorganizes internal loop 1 (IL1) and the top of stem 2. Interestingly, we have also previously found that the U1/U4/U5 complex induced by BU5Ae brings the invariant loop of U5 into close proximity with the 5′-end of U1. Taken together, these data suggest that U1 and U5 may both contribute to the ability of the U1/U4/U5 complex to bind the 5′ splice site.

INTRODUCTION

Introns are removed from eukaryotic mRNA precursors (pre-mRNA) by a process known as mRNA splicing. This process is carried out by the spliceosome, a complex of five snRNPs (small nuclear ribonucleoprotein particles) each containing a single small nuclear RNA (U1, U2, U4, U5 or U6 snRNA). Spliceosome assembly occurs by ordered stepwise addition of the snRNPs to the pre-mRNA and splicing itself is a dynamic process involving a changing network of snRNP–snRNP, snRNP–protein and protein–protein interactions (1–3). Four conserved sequences in the pre-mRNA direct the components of the spliceosome to the correct exon/intron boundaries: the 5′ splice site (5′ SS), the 3′ splice site (3′ SS), the branch site sequence just upstream of the 3′ SS and (in metazoans) the polypyrimidine tract located between the branch site and the 3′ SS. Initially, U1 snRNP base pairs with the 5′ SS (4–6), followed by association of the 5′ SS with the polypyrimidine tract in a ‘commitment complex’ (7–10). U2 snRNP then base pairs with the branch site (11–13) and the pre-assembled U4/U5/U6 tri-snRNP complex enters the spliceosome, perhaps by associating with bound U2 snRNP (14–19).

Following assembly of the five spliceosomal snRNPs onto the pre-mRNA, the snRNAs (and possibly the pre-mRNA) undergo conformational rearrangements that select the actual 5′ SS, assemble the active site for the first step of splicing and initiate catalysis. Specifically, base pairing between the U4 and U6 snRNPs is disrupted after U4/U5/U6 tri-snRNP enters the spliceosome but before the first catalytic step (20–23). This disruption leads to U2/U6 base pairing through helix II (15,24,25), helix I (26,27) and helix III (28). In addition, base pairing of the 5′ SS with U1 snRNP is disrupted before the first catalytic step and replaced by two different pairing interactions: one between the conserved ACA motif of U6 and intron positions +4 to +6 immediately downstream from the 5′ SS (17,19,29–33) and the other between the invariant loop of U5 (34) and the last 2 or possibly 4 nt of the 5′ exon (35–40). Interestingly, the invariant loop I of U5 snRNA is dispensable for the first catalytic step of splicing in yeast (41), although U5 snRNP is essential for step I in both yeast and mammals (42–46). U5 snRNP may play a major role in juxtaposing the 5′ SS, 3′ SS and branch site (37,47–50). In fact, the U5 snRNP-specific protein p220 and its yeast homolog Prp8 can be crosslinked to the 5′ SS, the 3′ SS and the branch site (37,47–52).

We found previously that a 2′-*O*-methyl RNA oligonucleotide complementary to U5 snRNA nt 68–88 (BU5Ae) can disrupt the pre-assembled U4/U5/U6 tri-snRNP complex and induce a novel U1/U4/U5 snRNP complex which interacts specifically with an RNA oligonucleotide containing the 5′ SS sequence (53). We also found that the invariant loop of U5 snRNA could be crosslinked by psoralen to the 5′-end of U1 snRNA within the U1/U4/U5 complex induced by BU5Ae (54). Moreover, the same (or a very similar) crosslink between U1 and U5 snRNA could also be detected early during a normal mRNA splicing reaction in the absence of the antisense oligonucleotide. Taken together, these data indicated that the U1/U4/U5 complex brings together the 5′-end of U1 and the invariant loop of U5 snRNA in the vicinity of the 5′ SS. We therefore proposed that the U1/U4/U5 complex represents a transient interaction between U1 and U5 during the displacement of U1 from the 5′ SS, although other interpretations could not be excluded (54).

Here we ask whether the ability of BU5Ae to disrupt the U4/U5/U6 complex and to induce a U1/U4/U5 complex that

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specifically binds the 5′ SS (53) can be explained by a conformational change caused by binding of the antisense oligonucleotide to U5 snRNP. We show that binding of BU5Ae does indeed induce a major conformational change that exposes the invariant loop and the 5′-end of U5 snRNA and also reorganizes internal loop 1 (IL1) and the top of stem 2. The conformational change in the 5′-end of U5 snRNA was expected because BU5Ae binds the pairing partner of this sequence in the secondary structure (34,55), however, the invariant loop of U5 is a separate structural and functional domain (34) remote from the BU5Ae binding site in the U5 secondary structure (34,55). We had previously found that the 5′-end of U1 and the invariant loop of U5, two regions that are known to interact sequentially with the 5′ SS (4,5,6,35–41), can be crosslinked to each other within the U1/U4/U5 complex (54). The new data therefore suggest that the U5 conformational change caused by BU5Ae not only disrupts the U4/U5/U6 complex and induces the U1/U4/U5 complex, but also brings the 5′-end of U1, the U5 invariant loop and the bound 5′ SS into close proximity within the U1/U4/U5 complex.

MATERIALS AND METHODS

Chemical modification

Chemical modification was carried out as previously described (56) except that 25 µl of a typical binding reaction contained 15 µl HeLa nuclear extract (57), 0.5 mM ATP, 20 mM creatine phosphate, 2.2 mM $MgCl_2$ and 4 pmol/µl BU5Ae as indicated. The binding reaction was preincubated at 4°C for 20 min, followed by incubation for 10–20 min at 30°C. Binding reactions were incubated with the modifying reagents for 12 min at room temperature. Stop buffer (300 µl) was then added (56) and the reactions were immediately phenol extracted and ethanol precipitated.

Enzymatic digestion

Standard binding reactions (25 µl) were incubated in the absence or presence of BU5Ae (4 pmol/µl) for 20 min on ice followed by 10 min at 30°C. Four units of the indicated enzymes (Pharmacia) were added and incubation continued at 30°C for 12 min. The RNA was purified by proteinase K treatment (2 mg/ml), phenol extraction and ethanol precipitation.

RESULTS

To examine the effect of BU5Ae binding on the secondary structure of U5 snRNP, we used AMT psoralen to probe U5 snRNA structure. Psoralen is a bifunctional molecule that reacts primarily with uridine and to a lesser extent with cytosine. Upon long wavelength irradiation (365 nm) psoralen generates mostly monoadducts, but also crosslinks between adjacent pyrimidines on opposite strands. Although psoralen reacts mainly with double-stranded structures, it also reacts to a lesser extent with single strands (reviewed in 58,59). The binding of psoralen to DNA or RNA is sensitive to protein association, alternative conformations and (in the case of DNA) superhelical density. Monoadduct formation appears to have relatively little effect on mobility in denaturing PAGE (15) but blocks primer extension by reverse transcriptase (17).

Nuclear extract was preincubated in the presence or absence of BU5Ae for 20 min on ice, incubated for 10 min at 30°C and then reacted with psoralen (Fig. 1). The sites on U5 snRNA that

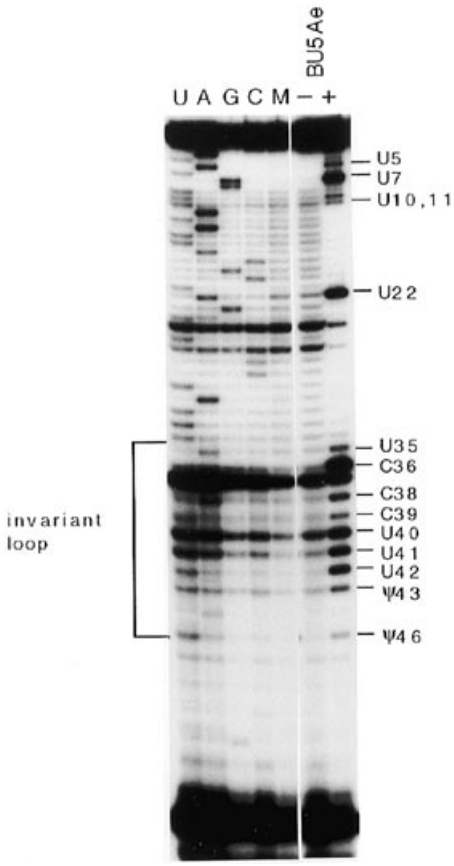

Figure 1. BU5Ae induces a conformational change that exposes the invariant loop of U5 snRNA in U5 snRNP. Sites accessible to psoralen monoadduct formation and/or crosslinking in U5 snRNA were identified by primer extension. Nuclear extract was incubated in the presence (+) or absence (–) of BU5Ae for 20 min at 30°C, AMT psoralen was added and the reaction irradiated at 365 nm for 10 min on ice. After deproteinization, primer extension products were generated using a 5′-end-labeled primer complementary to U5 nt 53–67 and crosslinked nuclear extract RNA incubated in the presence or absence of BU5Ae (+ or – BU5Ae) as template. A sequence ladder corresponding to unmodified U5 snRNA was generated using the same labeled primer, total nuclear extract RNA as template and appropriate ddNTPs (lanes U, A, G and C); as a control, primer extension was also performed with all four rNTPs (lane M or 'mock'). Products were resolved by denaturing 10% PAGE.

reacted with psoralen were then mapped as blocks to primer extension. Although psoralen treatment can generate both monoadducts and crosslinks, no internal U5 crosslinks were detected (54) and thus all psoralen-dependent blocks to primer extension must be due to monoadduct formation. Binding of BU5Ae affects the psoralen reactivity of two regions in U5 snRNA (Fig. 1, compare lanes + and –): the invariant loop (nt U35, C36, C38, C39, U40, U41, U42, ψ43 and ψ46) and the 5′-end of U5 snRNA (nt U2, U5, U7, U10, U11 and U22; the change in nucleotide U2 can only be seen on a shorter exposure). As summarized in Figure 4, these data indicate that BU5Ae induces a significant conformational change which exposes the invariant loop of U5. In addition, BU5Ae induces a strong block to primer extension at position U22 within an apparently unstructured bulged loop. The failure of U22 to react with

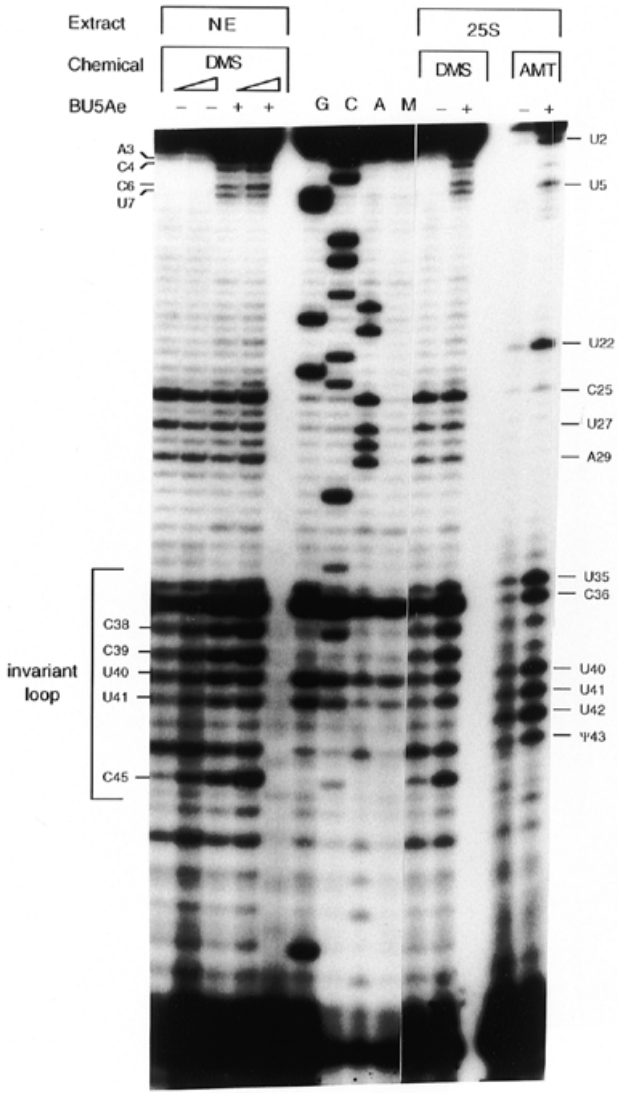

Figure 2. Identification of the conformational change induced by BU5Ae by chemical modification with DMS. Sites accessible to chemical modification in U5 snRNA were identified by primer extension. Nuclear extract (NE), partially purified U4/U5/U6 complex (25S –) or partially purified U1/U4/U5 complex (25S +) was incubated in the presence (+) or absence (–) of BU5Ae, chemically modified by DMS or psoralen (AMT) and then deproteinized before primer extension analysis as in Figure 1.

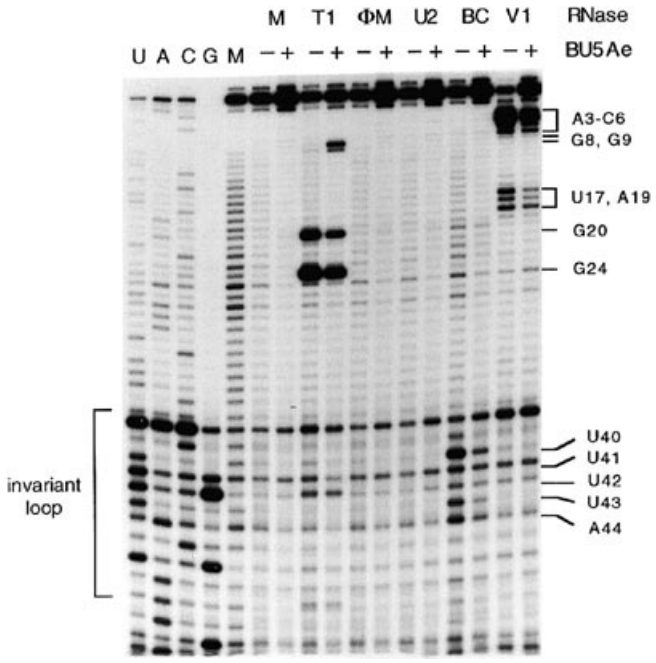

Figure 3. Identification of the U5 conformational change induced by BU5Ae using limited enzymatic digestion. Sites accessible to limited enzymatic digestion were mapped by primer extension. Nuclear extract was incubated in the absence (–) or presence (+) of BU5Ae for 20 min at 4°C and then treated with one of five endonucleases (RNase T1,ΦM, U2, BC or V1) or subjected to an equivalent incubation without RNase (lane M or 'mock'). The extract was then deproteinized and analyzed by primer extension as in Figure 1.

psoralen in the absence of BU5Ae suggests that this loop is structured or protected by protein(s) in the native snRNP (60) and that the change in psoralen reactivity reflects a reorganization of this region induced by the antisense oligonucleotide (see Fig. 3 for additional evidence).

To further examine the U5 conformational change induced by BU5Ae, we used the chemical footprinting reagent dimethyl sulfate (DMS) to selectively modify single-stranded nucleotides unprotected by proteins. Reverse transcriptase cannot read through the chemically modified base and the resulting block to primer extension generates a band in the primer extension ladder (56,61). As seen in Figure 2 and summarized in Figure 4, the interaction of BU5Ae with U5 snRNP exposes two regions on U5 snRNP to chemical modification: the 5′-end of U5 snRNA (nt A3, C4, C6 and U7) and the invariant loop (nt C38, C39, U40, U41 and C45; compare lanes NE + and – at low DMS concentration). Although other nucleotides in the invariant loop of U5 are also affected by BU5Ae, these changes appear to reflect a general increase in single-strandedness due to chemical modification. Increased accessibility of the 5′-end of U5 snRNA was expected because BU5Ae interacts with the normal pairing partner of this region in the acccepted secondary structure (34,55). However, modification of nucleotides in the U5 invariant loop, a region distant from the BU5Ae–U5 interaction, suggests that BU5Ae also induces a significant conformational change in U5 snRNA.

To determine whether exposure of the invariant loop correlates with formation of the U1/U4/U5 complex, we examined the effect of chemical modification and psoralen treatment on partially purified U1/U4/U5 and U4/U5/U6 complexes (Fig. 2). Nuclear extract was preincubated in the absence or presence of BU5Ae, fractionated by velocity sedimentation through a glycerol gradient and the 25S gradient fractions containing all five spliceosomal snRNPs were affinity selected with BU5Ae as described previously (53). Since conversion of the U4/U5/U6 complex to the U1/U4/U5 complex by BU5Ae requires soluble factors that can be separated from the U4/U5/U5 complex by velocity sedimentation (53), this protocol selects the U4/U5/U6 complex from untreated extract and the U1/U4/U5 complex from extract treated with BU5Ae. The 25S gradient fractions were then dialyzed into buffer D (57), subjected to chemical modification using DMS or psoralen and analyzed by primer extension. The

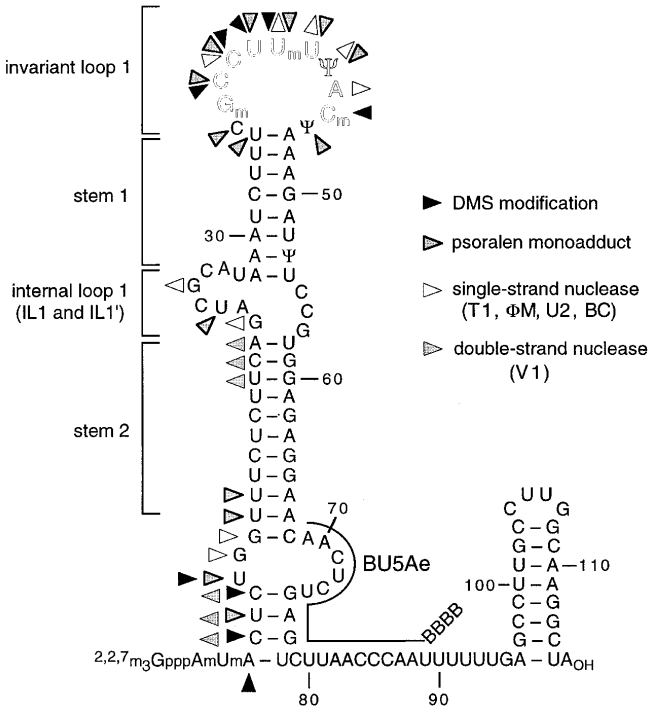

Figure 4. Location on the U5 snRNA secondary structure of the observed sites of DMS and psoralen modification and limited RNase digestion. The U5 invariant loop is denoted by an outline font and the region complementary to BU5Ae by an overbar. B, the biotin monomer unit. The significance of the arrowheads is indicated in the key. Arrowheads pointing toward the RNA denote increased effect, those pointing away decreased effect. Nomenclature as in Frank *et al*. (34).

data indicate that the U5 invariant loop is more accessible to modification by DMS and psoralen in the U1/U4/U5 complex (lanes 25S +) than in the U4/U5/U6 complex (lanes 25S –). Thus conversion of the U4/U5/U6 complex to a U1/U4/U5 complex involves a U5 conformational change that exposes the U5 invariant loop. Moreover, addition of BU5Ae to the partially purified U4/U5/U6 complex does not expose the invariant loop, indicating that the U5 conformational change, like conversion of the U4/U5/U6 complex to a U1/U4/U5 complex (53), requires at least one soluble factor in addition to the U4/U5/U6 complex and the U1 snRNP (data not shown).

An additional line of evidence supporting a BU5Ae-induced U5 conformational change was obtained by limited enzymatic digestion (Fig. 3). Nuclear extract was incubated in the presence or absence of BU5Ae, then digested with the single-strand-specific nucleases RNase T1, ΦM, U2 and *Bacillus cereus* RNase (BC) or with double-strand-specific RNase V1. Primer extension mapping revealed two BU5Ae-dependent sites of limited digestion with RNase T1 (position G8 and a minor site at G9) and two BU5Ae-independent sites (positions G20 and G24). All four sites lie within or immediately adjacent to single-stranded regions in the U5 secondary structure (34,55). The T1 sensitivity of the BU5Ae-dependent sites is easily explained, because these sequences would be rendered entirely single-stranded by binding of BU5Ae to the pairing partner (nt 68–88) in the U5 secondary structure (Fig. 4). However, T1 sensitivity of the BU5Ae-independent sites is more difficult to understand, because positions G20–U27 were unable to react with small molecules such as DMS yet are readily accessible to psoralen and enzymes as large as RNase T1 (40 kDa). These discrepancies presumably reflect multiple constraints; the ability of a probe to react with U5 snRNP is determined not just by the molecular size of the probe, but by stereochemistry, charge distribution and the chemical or enzymatic reactivity of the exposed sites. Indeed, the failure of DMS to modify IL1 in the presence or absence of BU5Ae could reflect RNA structure and/or protein binding, as previously suggested (34,60; see also Discussion below).

Although BU5Ae renders the U5 invariant loop more accessible to chemical modification and reaction with psoralen, 4 nt in the loop (U40–ψ43) actually become less accessible to BC digestion in the presence of BU5Ae (Fig. 3). Since U40 can be crosslinked to the 5′-end of U1 snRNA in the U1/U4/U5 complex induced by BU5Ae (54), decreased BC digestion of positions U40–ψ43 may indicate that the U5 invariant loop is protected from BC digestion by proximity to the 5′-end of U1 in the U1/U4/U5 complex. A similar scenario may explain why the T1 sensitivity of U41 decreases slightly in the presence of BU5Ae (Fig. 3).

Two regions in U5 are accessible to double-strand-specific RNase V1 and both lie within double-stranded elements of secondary structure: nt A3–C6 and U17–A19 (Fig. 3). Consistent with previous observations that only half of the U5 snRNA in the extract is accessible to BU5Ae (53), these regions become 2-fold less sensitive to RNase V1 digestion in the presence of BU5Ae. Nucleotides A3–C6 are undoubtedly rendered single-stranded when BU5Ae anneals with the complementary sequence, however, the decreased RNase V1 sensitivity of U17–A19 is likely to reflect the same conformational change that causes the psoralen-induced block to primer extension at U22 (Fig. 1) and the 2-fold decrease in T1 sensitivity of G20 and G24. Thus the apparently unstructured IL1 and the top of stem 2 (see Fig. 4) undergo a reorganization induced by BU5Ae. We conclude, provisionally, that IL1 and the top of stem 2 may function as a hinge or elbow to articulate the invariant loop relative to the body of the U5 snRNP.

DISCUSSION

We have shown by psoralen reactivity, chemical modification and limited enzymatic digestion that binding of the antisense RNA oligonucleotide BU5Ae induces a conformational change in U5 snRNP that exposes the conserved loop and the 5′-end of U5 snRNA and reorganizes IL1 and the top of stem 2. We had anticipated a conformational change in the 5′-end of U5 snRNA, because BU5Ae binds to the pairing partner of this sequence in the accepted secondary structure (34,55). However, the conformational change in the invariant loop of U5 (positions 36–46) was surprising because the invariant loop apparently constitutes a separate structural and functional domain (55) far removed from both the 5′-end of U5 and the BU5Ae binding site in the U5 secondary structure (34,55). We therefore conclude that exposure of the U5 invariant loop reflects a substantial conformational change caused by binding of BU5Ae to U5 snRNP.

The effect of BU5Ae on the invariant loop could be interpreted in several ways. One attractive possibility is that the invariant loop interacts (directly or indirectly) with the BU5Ae binding site on U5 snRNA within the U5 snRNP (positions 68–88); binding

of BU5Ae to U5 snRNP would then abolish this interaction. In fact, as seen in Figure 4, the invariant loop is separated from the BU5Ae binding site by an 18 bp stem interrupted about midway by asymmetric bulged loops (62; IL1 and IL1′). These bulged loops could function as a flexible hinge or elbow, allowing the invariant loop to interact with positions 68–88. Consistent with this scenario, the conformational change induced by BU5Ae appears to reorganize IL1 and the top of stem 2. The psoralen reactivity of U22 increases (Fig. 1) and the T1 sensitivity of G20 and G24 decreases within IL2 (Fig. 3), while U17–A19 within stem 2 become less sensitive to double-strand-specific RNase V1 (Fig. 3).

Interestingly, Bach and Lührmann (60) have also suggested, based on DMS modification and nuclease digestion of purified U5 snRNP, that IL1 and IL1′ may bind U5-specific proteins. Our results generally agree with theirs, but there are also discrepancies. In particular, Bach and Lührmann (60) find that the 'intact' 20S U5 snRNP is insensitive to RNase V1 digestion, whereas the stripped 10S form of the U5 snRNP is sensitive to RNase V1 digestion on the 3′-strand above and below IL1′. In contrast, we observe RNase V1 sensitivity on the 5′-strand below IL1 but no sensitivity on the 3′-strand near IL1′. Some of these discrepancies may be due to minor differences in the experimental protocols, but we suspect the main cause is the source of U5 snRNPs [purified U5 snRNP in Bach and Lührmann (60) but crude or partially purified nuclear extract in our work]. Thus the 3′-strand above and below IL1′ is likely to be protected in crude extract either by U5-specific proteins or by other proteins associated with the U4/U5/U6 and U1/U4/U5 snRNP complexes. The importance of internal loops IL1 and IL1′ for U5 function is underscored by the observation of Frank *et al*. (34) that a minimal U5, consisting solely of stem 2, IL1, stem 1 and the invariant loop, supports viability in yeast.

Although IL1 and IL1′ could function as a hinge to articulate the invariant loop relative to the rest of the U5 snRNP, binding of BU5Ae could also cause a local conformational change which induces a progressive reorganization of U5 snRNA structure that ultimately exposes the invariant loop. Alternatively, the local conformational change could affect interactions with other snRNP(s) or factors which in turn expose the invariant loop. In fact, binding of BU5Ae might even mimic or replace binding of spliceosomal factors that normally trigger conversion of U4/U5/U6 to U1/U4/U5 by binding in the vicinity of positions 68–88 of U5 snRNA (53).

The U5 conformational change induced by BU5Ae correlates well with conversion of the U4/U5/U6 complex into a U1/U4/U5 complex which can bind the 5′ SS (53). Indeed, although BU5Ae can bind to U5 within the U4/U5/U6 complex, exposure of the U5 invariant loop and conversion of the U4/U5/U6 complex to a U1/U4/U5 complex both require at least one soluble factor in addition to U1 snRNP (Fig. 2; 53). These observations suggest that the invariant loop of U5 is exposed by the same conformational change that converts the initial U4/U5/U6 complex into the early U1/U4/U5 complex that can bind the 5′ SS (53). Moreover, although both the U1/U4/U5 and U2/U6 complexes induced by biotinylated BU5Ae can be affinity selected on streptavidin–agarose, the U2/U6 complex can be released from the U1/U4/U5 complex by 250 mM salt (53). The U1, U4 and U5 snRNPs in the U1/U4/U5 complex must therefore have high affinity for each other and the 5′ SS but low affinity for U2 and U6. The yeast U1/U5 complex recently identified by Ruby (63) may be functionally homologous to the U1/U5 interaction within the mammalian U1/U4/U5 complex. Although U1 dissociates from the spliceosome upon native gel electrophoresis (21,22), the U1/U4/U5 and U2/U6 complexes induced by BU5Ae may exist within a very early spliceosomal complex containing all five spliceosomal snRNPs that can be isolated by the much gentler technique of gel filtration (7,64). Thus, early in spliceosome assembly the individual snRNPs in the U1/U4/U5 complex have high affinity for each other (53), the invariant loop of U5 is exposed (this paper), and the 5′-end of U1 binds the 5′ SS (53) and is brought into close proximity with the U5 invariant loop (54). Taken together, these data support our hypothesis that the U1/U4/U5 complex represents a transitional stage when responsibility for binding the 5′ exon passes from U1 to U5 (and also to U2 snRNP; Ast and Weiner, submitted for publication).

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