Impact of base pair identity 5' to the spliceosomal branch site adenosine on branch site conformation

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ABSTRACT

The branch site helix from *Saccharomyces cerevisiae* with pseudouridine (ψ) incorporated in a phylogenetically conserved position of U2 snRNA features an extrahelical branch site adenosine (A) that forms a base triple interaction with the minor groove edge of a widely conserved purine_{U2 strand}-pyrimidine_{intron strand} (R_{U2} - Y_{intron}) base pair two positions upstream. In these studies, NMR spectra of a duplex in which 2-aminopurine (2ap), a fluorescent analog of adenine lacking the proposed hydrogen bond donor, was substituted for the branch site A, indicated that the substitution does not alter the extrahelical position of the branch site residue; thus, it appears that a hydrogen bond between the adenine amino group and the R-Y pair is not obligatory for stabilization of the extrahelical conformation. In contrast, reversal of the orientation of A_{U2} - U_{intron} to U_{U2} - A_{intron} resulted in an intrahelical position for the branch site A or 2ap. Fluorescence intensity of 2ap substituted for the branch site A with the original R_{U2} - Y_{intron} orientation (AU or GC) was high, consistent with an extrahelical position, whereas fluorescence in helices with the reversed R-Y orientation, or with a mismatched pair ($A-U \rightarrow G \cdot A$ or $U \cdot C$), was markedly quenched, implying that the residue was stacked in the helix. The A 5' to the branch site residue was not extrahelical in any of the duplexes. These findings suggest that the R_{U2} - Y_{intron} base pair orientation in the ψ -dependent branch site helix plays an important role in positioning the branch site A for recognition and/or function.

Keywords: base triple; branch site; NMR; pseudouridine; 2-aminopurine fluorescence spectroscopy; RNA

INTRODUCTION

As a critical step in the maturation of precursor messenger (pre-m)RNA molecules, intervening sequences, introns, are excised and flanking regions, exons, are ligated to form a contiguous transcript. The spliceosome, which comprises small nuclear (sn)RNAs (U1, U2, U4, U5, U6) and a large number of proteins, is the biomolecular machinery responsible for catalysis of pre-mRNA splicing in eukaryotic nuclei. The first of two transesterification reactions occurs as a result of the nucleophilic attack by the 2' hydroxyl (2'OH) of a conserved adenosine residue of the intron, called the branch site because of the branched intermediate formed by the intron at the 5' splice site. The bulged branch site adenosine (A) is positioned by pairing of a consensus region of the intron with a short segment of U2 snRNA.

Because of the crucial role played by RNA components of the branch site region in recognition and catalytic events in splicing, determination of structural features of RNA components of the active site of the spliceosome is important. Solution structures of a short RNA duplex representing the branch site helix from the yeast *Saccharomyces cerevisiae* were previously solved by NMR in the presence and absence of a post-transcriptional base modification in a phylogenetically conserved location of U2 snRNA (Newby and Greenbaum 2001, 2002a). A duplex that included the conserved pseudouridine (ψ) residue in position 35 of the U2 snRNA strand was characterized by a kinked backbone of the intron strand and an extrahelical conformation of the branch site A, with its 2'OH exposed in the widened major groove. Calculation of electrostatic surface potentials by a nonlinear Poisson-Boltzmann approach identified a region of significant negative potential in the major groove surrounding the 2'OH of the branch site A (Xu et al. 2005) that may contribute to the recognition of the branch site A by other spliceosomal components or its activity in the first step of splicing. In contrast, the branch site

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A in the unmodified counterpart (U in place of the naturally occurring ψ in the U2 snRNA strand) adopted an intrahelical conformation, and both strands had A-type helical parameters throughout.

Identity of the branch site A is conserved in yeast and mammals (Langford and Gallwitz 1983; Pikielny et al. 1983; Gao et al. 2008). Although the flanking sequence is only loosely conserved in higher eukaryotes (YUNAY, where Y represents a pyrimidine, N is any nucleotide, and the underlined A represents the invariant branch site adenosine) (Gao et al. 2008), the A_{U2}-U_{intron} base pair associated with the observed base triple is found in ~75% of the U2-dependent spliceosomes studied to date, whereas a G-C base pair is found in ~25% of the studied spliceosomes (Lim and Burge 2001; Kol et al. 2005; Corvelo et al. 2010); however, an A-U base pair is found in 100% of the U12-dependent spliceosomes (Padgett and Burge 2003, 2005). Thus, the data suggest that a purine_{U2}-pyrimidine_{intron} (R_{U2}-Y_{intron}) base pair orientation is strictly conserved in yeast, widely conserved in mammals, and likely in all eukaryotes. Splicing assays in which the highly conserved A-U base pair was reversed (i.e., A-U $\rightarrow$ U-A) resulted in a significant decrease in splicing efficiency (McPheeters and Abelson 1992).

The structure of the branch site duplex suggested formation of a base triple involving the branch site adenosine (A24) (see numbering in Fig. 1) and the A7-U22 base pair (Newby and Greenbaum 2002a). A number of the nine converged structural models imply a hydrogen bond between the exocyclic amino N6H₂ of A24 and one or more acceptors in the minor groove of the A7-U22 pair, leading to the conclusion that the extrahelical branch site A may be stabilized by a hydrogen bond. In this study, we examine the need for formation of the postulated hydrogen bond by analysis of duplexes in which 2ap replaces the branch site A, as well as the role of the identity and orientation of the base pair involved in formation of the base triple (Fig. 2). Our results suggest that the exocyclic amino group in the 6 position of adenine may not be essential for stabilization of the extrahelical conformation of the branch site A but that a R_{U2}-Y_{intron} base pair is required. These findings support a model whereby the orientation of the R_{U2}-Y_{intron} base pair in the ψ -dependent branch site maintains a role in positioning and/or stabilizing the branch site nucleophile prior to the first step of splicing.

RESULTS

Effect of 2ap substitution on the ψ -dependent branch site duplex

In order to assess the requirement for a hydrogen bond between the amino group of the branch site A and the minor groove edge of the conserved A_{U2}-U_{intron} pair to stabilize its extrahelical conformation, we examined the conformation of the branch site residue in a duplex in

FIGURE 1. (A) Axial view of the base triple and the adjacent ψ 6 and A23 residues in the ψ -modified branch site duplex (ψ BP) as seen in the solution structure determined by Newby and Greenbaum (2002a) (1LPW). A7 and U22 participate in a canonical Watson-Crick base pair, while A24, the branch site adenosine residue, adopts an extrahelical conformation and forms a base triple with two hydrogen bonding interactions in the minor group of the A7-U22 pair (with A7 N3 and A7 2'O). (B) Stick structure of adenine and 2-aminapurine (2ap).

which 2ap replaced the branch site A (ψ BP_{2ap}). 2ap, which has an exocyclic amino group in position 2 instead of position 6 as found in adenine, forms hydrogen bonds with uracil that are comparable to a typical A-U Watson-Crick base pair but cannot form a hydrogen bond involving the N6H₂ exocyclic amino group. We collected one- and two-dimensional NOESY spectra of exchangeable and nonexchangeable protons of ψ BP_{2ap} and compared them with those of ψ BP, the ψ -containing duplex. Exchangeable protons were assigned from NMR spectra acquired at 7°C in 90% H₂O. Assignments of nonexchangeable proton resonances were made from a combination of NOESY and TOCSY experiments acquired in 99.96% D₂O at 20°C.

Spectra of ψ BP were essentially identical to those acquired previously (Newby and Greenbaum 2001). Proton (¹H) resonance chemical shifts and NOEs of ψ BP_{2ap} were very similar to corresponding resonances and NOEs in the unsubstituted counterpart (Fig. 3). As was the case for ψ BP, no resonance peak was observed for ψ 6 H3 resonance under conditions used for these spectra. However, when both temperature and pH were decreased (2°C, pH 5.5, absence of phosphate buffer), we observed a broad peak at ~10.8 ppm, similar to that observed for ψ H3 of

pseudouridine monophosphate or ψ 6 H3 of ψ BP at -18°C (Schroeder et al. 2005), suggesting that ψ 6 H3 in ψ BP is not involved in a Watson-Crick base pair. These observations are in contrast to the chemical shift of a Watson-Crick paired ψ 6 H3 at ~ 13.1 ppm assigned in a fully complementary RNA helix at temperatures up to at least 20°C (Durant and Davis 1999; Newby and Greenbaum 2002b; Schroeder et al. 2005). The absence of a visible peak for ψ 6 H3, therefore, led to the conclusion that, as in ψ BP, this proton is exchange-broadened beyond detection. Of the exchangeable protons, only the imino ^1H of G8 (see Fig. 1 for numbering scheme) displays a slight downfield shift (0.07 ppm) in the spectra of ψ BP_{2ap} as compared to the corresponding resonance in ψ BP, implying similar base-pairing patterns and helical parameters.

Nonexchangeable resonances exhibiting differences in chemical shift >0.10 ppm are 2ap24 H8 and C25 H5, with changes of -0.11 and -0.13 ppm, respectively (where the negative sign represents an upfield shift relative to ψ BP). Other resonances with lesser but detectable chemical shift changes (<0.10 ppm) are ψ 6 H6 and H1', A7 H8 and H1', A23 H8, H1' and H2, and G8 H1' and U22 H1' (Fig. 3). These changes in the chemical shifts were attributed to the altered distribution of electronegative groups on the Watson-Crick face of 2ap24 and, therefore, to differences in shielding effects in the 2ap-substituted duplex ψ BP_{2ap} or minor differences in configuration (Figs. 3, 4A). Importantly, the absence of significant chemical shift changes of residues in the immediate vicinity of 2ap24 in ψ BP_{2ap} suggests similar chemical environments and is, therefore, consistent with similar conformation of the branch site 2ap to that observed for the branch site A in ψ BP.

We detected no cross-strand NOEs involving A24 H2 in spectra of ψ BP that would have been expected if the branch site residue adopted an intrahelical position (e.g., for uBP, the branch site helix with U in place of ψ) (Newby and Greenbaum 2001). We were unable to confirm the A23 H2-C25 H1' NOE, a marker of the extrahelical A24-conformation, because of chemical shift overlap of C25 H1' with C28 H5 and A23 H2 with C28 H6. However, in both ψ BP and ψ BP_{2ap}, A23 H2 exhibited an NOE to A7 H1', as well as an unusual cross-strand NOE to ψ 6 H1', similarly to the NOEs previously observed in ψ BP. These NOEs were not observed in spectra of uBP, in which the branch site A is stacked intrahelically. Also clearly absent were NOEs between A7 H2 and A23 H1' and between A7 H2 and A24 H2, consistent with extrahelical A in both ψ BP and ψ BP_{2ap} (Fig. 4A). Moreover, we detected similar patterns of ribose H1'-H2' correlations in TOCSY spectra of ψ BP and ψ BP_{2ap} in the current study, as previously observed. Specifically, detection of medium intensity H1'-H2' TOCSY cross-peaks for riboses of U22, A23, and A24, as well as for terminal residues, implied non-C3'-endo ribose puckers analogous to the pattern observed for ψ BP. Chemical shifts of resonances corresponding to these TOCSY cross-peaks

were essentially the same for A10, U22, and U19 as noted for ψ BP and very similar for A23 and A(2ap)24.

Similarity of chemical shifts, NOE patterns, and ribose H1'-H2' correlations between ψ BP_{ap} and ψ BP suggest the same extrahelical conformation of the branch site duplex in ψ BP_{ap} as previously observed for ψ BP. Given these findings, we also conclude that the hydrogen bond(s) observed between an amino ^1H in position 6 of A in ψ BP is not an essential component of stabilization of the extrahelical branch site A.

Conformational changes of the branch site duplex associated with the reversed U_{U2}-A_{intron} base pair

In order to test the hypothesis that the widely conserved R_{U2}-Y_{intron} orientation is important for stabilization of the extrahelical conformation of the branch site A, we acquired and analyzed NMR spectra of a ψ -modified duplex with reversed orientation of the base pair associated with formation of the observed base triple ($m\psi$ BP) (shown in Fig. 2). Imino protons were assigned from one- and two-dimensional NMR spectra of $m\psi$ BP acquired at 7°C in 90% H₂O, 10% D₂O added for lock, and by similarity to corresponding resonances of ψ BP (this report) and uBP (Newby and Greenbaum 2001). The chemical shifts for ψ 6 H3 were 12.83 and 13.09 ppm in $m\psi$ BP and in $m\psi$ BP_{2ap} (the 2ap-substituted analogous duplex), respectively, similar to the value 13.1 ppm noted for a pseudouridine involved in Watson-Crick pairing (Durant and Davis 1999; Newby and Greenbaum 2002b; Schroeder et al. 2005).

Assignments of nonexchangeable proton resonances were made from a combination of NOESY (Fig. 4B) and TOCSY experiments in 99.96% D₂O at 20°C , 350- and 70-msec mixing times, respectively. Chemical shifts for resonances attributed to the helical stem region flanking the branch site region were very similar to those of corresponding protons of uBP and ψ BP, although chemical shifts of aromatic/anomeric resonances of U7 and A22 (the pair with reversed orientation) were unique to $m\psi$ BP (Figs. 4B, 5A). Significantly shifted nonexchangeable proton resonances ($|\Delta\delta| > 0.10$ ppm) in spectra of $m\psi$ BP relative to corresponding resonances in the spectrum of ψ BP were: ψ 6 H6, G8 H8, U9 H1', C21 H1', A23 H8, H1', and H2, as well as A24 H8, with the largest difference in chemical shift observed for G8 H8 (0.55 ppm) (Fig. 5A), suggesting significant structural differences between the two helices.

NOEs throughout the stem of $m\psi$ BP were typical of the stacked intrahelical conformation of residues. In particular, NOEs involving the A H2 protons suggested a stacked orientation for A24 and A23, including sequential A H2-H1'_{i+1} and cross-strand A H2-H1'_{i-1} NOEs for both A23 and A24. A24 H2 of $m\psi$ BP displayed NOESY cross-peaks to A23 H2 and C25 H1', as well as a long-range cross-strand $i \rightarrow i-2$ NOE to H1' of U7, consistent with an intrahelical and stacked conformation of A24. A24 H8-C25 H6 NOE is an

FIGURE 2. RNA duplexes and naming schemes used in these studies. The duplex ψ BP, with strands representing the wild-type U2 snRNA (*top* oligomer) and intron (*bottom* oligomer) sequences from *S. cerevisiae*, includes two additional complementary nucleotides added to the 5' side of the U2 strand and 3' side of the intron strand to increase thermal stability. Other duplexes include modifications in the base pair A7-U22 that participates in the base triple identified in the solution structure of ψ BP; in each case, the mutation from the native yeast sequence is the rectangle. Adenosine residues substituted by the fluorescent analog 2-aminopurine (2ap) are circled. The rationale for testing each of the mutated pairs is explained in the text.

additional indicator of the stacked conformation of A24 in $m\psi$ BP. The NOE between A23 H2 and U7 H1', as well as long-range G8 H1' in $m\psi$ BP are consistent with a stacked intrahelical position of A23 (Fig. 4B).

In contrast to ψ BP or ψ BP_{2ap}, all riboses of $m\psi$ BP, with the exception of those of the terminal residues A10 and U19, had very small H1'-H2' scalar couplings ($^3J_{H1'-H2'}$), as evidenced from the absence of TOCSY cross-peaks, and were thus attributed to a C3'-*endo* conformation. A phosphorous spectrum of $m\psi$ BP acquired at 20°C indicated a narrow chemical shift range of -0.11 to -1.04 ppm, typical of A-form helical parameters (data not shown). Thus, all spectral evidence supports the conclusion that the reversal of A_{U2}-U_{intron} to U_{U2}-A_{intron} orientation results in a stacked branch site A, a finding that supports the importance of the conserved purine_{U2}-pyrimidine_{intron} orientation in stabilization of the extrahelical branch site conformation.

We also analyzed spectra of an analogous duplex ($m\psi$ BP_{2ap}), with 2ap replacing the branch site residue. Chemical shifts of $m\psi$ BP_{2ap} were very similar to those of $m\psi$ BP (Fig. 5B); the small changes in or near 2ap are attributed to electrostatic differences between 2ap and A. Such changes were seen for ψ 6 H1' and H6, 2ap24 H8, and A23 H5 and H8. The chemical shift changes of ψ 6 H1' and H6, which are likely to pair either with A23 or 2ap24 (such an interaction was observed in the structural model for uBP, the unmodified branch site duplex) (Newby and Greenbaum 2002a), also imply an intrahelical orientation of the 2ap24. Observed NOEs support the conclusion that 2ap of $m\psi$ BP_{2ap}, like A of $m\psi$ BP, is in a stacked intrahelical conformation. The observed variations in $m\psi$ BP_{2ap} are fully consistent with altered electron shielding patterns of protons in the vicinity of the altered groups of 2ap24.

Relative position of adenosines in branch site helices by fluorescence spectroscopy using 2-aminopurine

Based upon similar structural features for duplexes in which the 2ap substituted for the branch site adenosine, we exploited the fluorescent properties of 2ap as a probe for conformation of the branch site residue and the 5' neighboring adenosine in a series of fluorescence experiments. 2ap is considered a useful reporter of conformation of an adenosine

because its fluorescence intensity is quenched when 2ap is stacked between neighboring nucleotides but not when involved in a hydrogen bond alone (Rachofsky et al. 2001) and is enhanced considerably when the 2ap fluorophore is exposed to solvent (Millar 1996; Jean and Hall 2001).

As controls, we measured 2ap fluorescence in RNA helices or strands of known, stacked conformation, including the native helix without the ψ modification (uBP_{2ap}), a complementary helix (10 + 10_{2ap}), and a single-stranded intron (10_{2ap}). Fluorescence signals from the sequences were normalized against that of a duplex containing the conserved ψ modification (ψ BP_{2ap}, where 2ap was shown by NMR studies to adopt an extrahelical conformation), which was given an arbitrary value of 1. Sequences are shown in Figure 2.

Relative fluorescence intensity for all samples is shown in Figure 6. Consistent with previous results (Newby and Greenbaum 2002a), fluorescence of 2ap substituted for

FIGURE 3. Changes in chemical shifts of nonexchangeable protons of the ψ -dependent branch site duplexes (ψ BP and ψ BP_{2ap}). Positive values for differences [$\delta(\psi$ BP_{2ap}) – $\delta(\psi$ BP)] are associated with downfield shifts and the negative values with upfield shifts. Differences are shown for the anomeric protons H1', aromatic protons H6/8 (for pyrimidines/purines), and H2/5 (for adenosine/pyrimidines). (A) Nucleotides of the U2 snRNA strand; (B) nucleotides of the intron strand. The slight chemical shift changes in ψ BP_{2ap} are consistent with a similar helical structure to the branch site helix ψ BP.

A24 in ψ BP_{2ap} is high, whereas 2ap fluorescence in a complementary helix (10 + 10_{2ap}) is only ~15% of the intensity of ψ BP_{2ap}. By comparison, fluorescence in the unmodified branch site duplex, uBP_{2ap}, or single-stranded (ss) RNA, 10_{2ap}, was ~53% or ~57% that of ψ BP_{2ap}, respectively, suggesting that A24 was predominantly stacked but (as shown by NMR studies) likely to be conformationally heterogeneous or flexible.

In the first set of experiments with altered duplexes, we tested the effects of a base pair switch A_{U2}-U_{intron} → G_{U2}-C_{intron}, which maintains the purine-pyrimidine orientation and is present in ~25% of the studied eukaryotes. Sequences tested in these experiments included a G7-C22 base pair with a ψ -modified and unmodified U2 strand, (ψ GCBP_{2ap} and uGCBP_{2ap}), respectively. Fluorescence of ψ GCBP_{2ap} was 9% greater than that of ψ BP_{2ap}, suggesting that the extrahelical position of A24 was stabilized even more than in the original duplex, whereas fluorescence of uGCBP_{2ap} was ~53% of the value for ψ BP_{2ap}, essentially the same as seen for uBP_{2ap} or the single-stranded intron 10_{2ap}, suggesting an intrahelical branch site A.

We then measured fluorescence in a duplex for which we reversed orientation of the native A_{U2}-U_{intron} base pair to U_{U2}-A_{intron} (m ψ BP_{2ap}) (Fig. 2), which is expected to result in a different geometry in the minor groove. We found that 2ap fluorescence of m ψ BP_{2ap} and of an analogous duplex

without the ψ modification (muBP_{2ap}) had emission intensities ~42% and ~33%, respectively, that of ψ BP_{2ap}, suggesting that the branch site residue was in a predominantly intrahelical conformation in these mutated sequences.

FIGURE 4. (A) NMR spectrum of the aromatic-anomeric (base-H1') ¹H region of a two-dimensional spectrum of the mutated branch site duplex ψ BP_{2ap}. Both spectra shown in A and B were acquired on 0.3 mM RNA, in 99.96% D₂O in 50 mM NaCl at 20°C and at 600 MHz with a mixing time of 350 msec. Sequential connectivities between protons H6/H8 and H1' are marked by lines. Boxed NOEs are those indicating contacts also observed in spectra of ψ BP but not in those of uBP (the unmodified duplex in which the branch site A was in an intrahelical conformation) (Newby and Greenbaum 2002a). (B) NMR spectrum of the aromatic-anomeric (base-H1') ¹H region of a two-dimensional spectrum of the mutated branch site duplex (m ψ BP duplex; 0.3 mM RNA, the same sample conditions as in A). Connectivities between sequential NOEs between protons H6/H8 to H1' are shown. NOEs involving A24 and A23 H2 protons that confirmed the stacked intrahelical conformation of those residues are marked by rectangles and identified.

FIGURE 5. (A) Effects of the reverse U_{U2} - A_{intron} base pair on the chemical shifts of the modified branch site duplex (m ψ BP and ψ BP). Positive values for differences δ (m ψ BP) – δ (ψ BP) are associated with downfield shifts of resonances in m ψ BP spectra as compared to those in ψ BP spectra, whereas the negative values are associated with upfield shifts of respective resonances (U2 snRNA oligomer) (upper panel) and the intron oligomer (lower panel). Large changes in chemical shifts of m ψ BP relative to ψ BP suggest perturbed helical parameters of the branch site region of m ψ BP. (B) Changes in chemical shifts of the modified ψ -dependent branch site duplexes as compared to the 2ap-substituted duplex (m ψ BP and m ψ BP_{2ap}). Notation and orientation of respective strands are the same as in Figure 3. Changes in chemical shifts of nonexchangeable protons of ψ_6 , which may form a hydrogen bond with A23 or A24, are consistent with an intrahelical stacked conformation of 2ap24 in m ψ BP_{2ap}.

In order to test the structural impact of disrupting the highly conserved Watson-Crick base pair in this position, we replaced the A7-U22 pair with purine-purine (A-U → G•A, ψ GABP_{2ap}) and pyrimidine-pyrimidine (A-U → U•C, ψ UCBP_{2ap}) pairs, and assessed the fluorescence. We observed that both ψ GABP_{2ap} and ψ UCBP_{2ap} displayed low fluorescence intensity, comparable to that of the complementary duplex (10 + 10_{2ap}; ~23% of ψ BP_{2ap}).

Conformation of adenosine 5' to the branch site

Biochemical assays have shown that the adenosine adjacent to the branch site (known as 5' A or, in the numbering scheme used here, A23) may pose as the nucleophile in the first step of splicing in higher organisms like humans, although not in yeast (Query et al. 1994). NMR models of the native yeast sequence indicated that the 5' A was intrahelical, thus lowering accessibility (Newby and Greenbaum 2002a). In order to determine the relative conformation of the neighboring A for the native and several of the mutated sequences, we detected 2ap fluorescence emission intensities in *S. cerevisiae* branch site duplexes by site-specific substitution of the neighboring A (2ap23) (Fig. 2) with 2ap

in the intron strand. 2ap23 in all duplexes tested, native (A_{U2} - U_{intron}) ψ -modified (ψ BP_a) and unmodified (u BP_a), as well as in reversed A-U base pair (U_{U2} - A_{intron}) ψ -modified m ψ BP_a and unmodified mu BP_a, exhibited low fluorescence intensities, exhibiting ~30% and ~27%, and ~40% and ~30% the intensity of ψ BP_a, respectively, indicating that the branch site neighboring residues, 2ap(A)23 were generally stacked in the helix (Fig. 6). It is, therefore, unlikely that the 5' A would readily substitute for the branch site A in the first step of splicing catalysis for these sequences.

DISCUSSION

The coplanar base triple observed in the branch site helix of *S. cerevisiae* involving the extrahelical branch site A and the minor groove edge of an A_{U2} - U_{intron} base pair two positions upstream suggested formation of one or two hydrogen bonds between the exocyclic amino group and acceptors on the base and ribose of the purine of the base pair (Newby and Greenbaum 2002a). This conformation was dependent upon incorporation of a modification conserved in the U2 snRNA strand. It still is not clear how presence of the ψ modification contributes to the extrahelical confor-

FIGURE 6. Relative fluorescence intensity profiles of 2ap incorporated in native and mutated branch site constructs. (A) 2ap was incorporated in place of the canonical branch site residue (A24). The native ψ -modified construct (ψ BP_{2ap}), single-stranded intron (10_{2ap}) and complementary duplex (10 + 10_{2ap}) were used as standards; these standards exhibited comparable relative intensities as previously published (Newby and Greenbaum 2002a). Naming conventions are explained in the text (duplex sequences shown in Fig. 2). (B) With the exception of the ψ BP_{2ap} control against which fluorescence of all samples was normalized (2ap in the A24 position), 2ap replaced the adenosine residue 5' to the branch site A (A23) to test for its intra- vs. extrahelical conformation.

mation. Although ψ substitutions in Watson-Crick helices and in multiple sites in tRNA molecules are associated with enhanced thermal stability (Davis and Poulter 1991; Hall and McLaughlin 1992; Arnez and Steitz 1994; Durant and Davis 1999; Yarian et al. 1999) and reduced flexibility in ribosomal RNA (Sakakibara and Chow 2012), inclusion of ψ in certain RNA loops has been shown to be destabilizing (Meroueh et al. 2000). Presence of two conserved pseudouridines at the terminus of a hairpin loop of telomerase RNA provide a stabilizing effect relative to the unmodified molecule but induce markedly different structures in the two hairpins (Kim et al. 2010), similar to the observations by Newby and Greenbaum (2001, 2002a). Formation of a water-mediated hydrogen bond between ψ 6 H1 and a phosphate oxygen atom has been proposed to be an important component of pseudouridine-mediated structure stabilization (Davis and Poulter 1991; Hall and McLaughlin 1992); demonstration of hydrogen bond formation was shown in both ψ -substituted complementary helices and the branch site helix (Newby and Greenbaum 2002b). X-ray crystallo-

graphic structures of several mammalian branch site duplexes have revealed interactions with tightly bound water molecules involving the ψ and branch site A that support the role of ψ in stabilizing the extrahelical conformation (Lin and Kielkopf 2008).

Based on geometry implying a hydrogen bond between the amino group of the branch site A (A24) and the N3 of the adenine of the base pair in most of the converged structures of ψ BP (A7 in our numbering scheme), we hypothesized that hydrogen bond formation was important for stabilization of extrahelical conformation of the branch site A. We tested this premise by NMR-based structural studies of a duplex in which 2ap, an adenine analog with the exocyclic amino group attached to the carbon atom in position 2 instead of position 6. All spectral features supported the conclusion that moving the amino group (the hydrogen bond donor) to a position in which it is not likely to participate in a hydrogen bond did not alter the extrahelical position of the branch site residue, suggesting that, although hydrogen bond formation appears to occur in solution, it is not necessary for stabilization of the extrahelical conformation. Thus, although we cannot rule out the possibility that N2H₂ of 2ap could substitute as a direct or water-mediated hydrogen bond donor with acceptors in the minor groove of the R_{U2}-Y_{intron} base pair, it appears that the conformation is at least partially stabilized by other factors, such as energetically preferred stacking interactions.

We next probed the importance of the identity and orientation of the A_{U2}-U_{intron} base pair with which the branch site residue interacts. Mapping of U2 snRNA and intron sequences in eukaryotes has indicated that a number of complementary base pairs flank the branch site A (Breathnach et al. 1978; Breathnach and Chambon 1981; Dodgson and Engel 1983; Keller and Noon 1984; Padgett et al. 1986). However, the paired nucleotide two positions upstream of the branch site A appears always to be a pyrimidine: U in ~75% of eukaryotic sequences studied thus far (always opposing an A in the consensus sequence of U2 snRNA) and C in the remaining 25% of the branch site sequences (paired with G) (Lim and Burge 2001; Kol et al. 2005; Gao et al. 2008; Corvelo et al. 2010). Thus, a R_{U2}-Y_{intron} complementary base pair (A7-U22 in the NMR structure and this study) is maintained at this position in all eukaryotes studied to date.

Based on this observation, we used NMR studies to measure the impact of altered minor groove constituents and geometry on the branch site A position. Our measurements indicated unambiguously that reversing the orientation of the A_{U2}-U_{intron} pair resulted in the branch site A (and also branch site 2ap in analogous experiments) adopting a stacked intrahelical position.

The suitability of 2ap as a structural probe of adenosine conformation has been demonstrated by a number of groups (Sowers et al. 1986; Fagan et al. 1996; Kulinski et al. 1996; Zagorowska and Adamiak 1996; Newby and Greenbaum

2002a). Since our NMR data indicated that 2ap did not perturb the structural features of the branch site in the duplexes examined, we exploited the fluorescent properties of 2ap as a probe of branch site conformation for a number of other duplex sequences. Our earlier data had shown that fluorescence of 2ap substituting for the branch site A in ψ BP_{2ap} was high, indicative of extrusion of the residue from the helix (Newby and Greenbaum 2002a). This finding was corroborated by the NMR results reported here.

Based upon similar geometry in the minor groove, we would expect that a G_{U2}-C_{intron} base pair, which occurs in a minority of eukaryotic sequences, would support similar interactions as seen with the A_{U2}-U_{intron} base pair. As predicted, replacement of the A-U base pair with a G-C base pair displayed high 2ap fluorescence, consistent with extrahelical position of the branch site A. Interestingly, fluorescence of 2ap in a duplex in which a G-C pair replaced A-U was even greater than for the A-U pair in that position, consistent with even greater exposure to solution. This effect could be the result of greater thermal stability of a G-C pair than for an A-U pair in this position as a result of enhanced hydrogen bonding and stacking interactions (Freier et al. 1981), as well as of slight alterations in helical stacking parameters, such as helical twist and differences in base-base orientation between A-U and G-C base pairs, which may affect the exposure of the fluorophore and, therefore, the intensity of the fluorescent signal.

In contrast, reversal of the positions of the R_{U2}-Y_{intron} pair in the *S. cerevisiae* sequences (with or without the ψ) resulted in low 2ap fluorescence, implying that a greater proportion of the ensemble reverted to an intrahelical position of the branch point 2ap in the m ψ BP_{2ap} and muBP_{2ap} duplexes, as was shown for m ψ BP and m ψ BP_{2ap} by the NMR studies. Similarly, substitution of R_{U2}•R_{intron} (G•A) or Y_{U2}•Y_{intron} (U•C) pairs for the native R_{U2}-Y_{intron} pair also resulted in relatively low 2ap fluorescence, indicative of a predominantly stacked conformation for the branch site A. Thus, minor groove geometry, and not only presence of a purine in the U2 snRNA strand, appears to be important in stabilization of the extrahelical branch site residue.

We also investigated the impact of base pair identity on the intra- or extrahelical position of the 5' neighboring A for several of the duplexes. Crystallographic studies of an RNA duplex representing the human U2 snRNA-intron pairing in the absence of the conserved ψ modification indicated that the branch site adenosine was stacked into the helix but that its 5' neighbor, also an adenosine, was extruded from the helix and was involved in interhelical stacking (Berglund et al. 2001) or, in some cases, surrounded by solvent (Lin and Kielkopf 2008). In some species, although not in yeast (the sequence examined in the study of Berglund et al. [2001]), the neighboring residue can act as the nucleophile in the first step of splicing. For this reason, we also measured fluorescence of helices in which 2ap was substituted for the 5' neighboring adenosine (2ap23, in these

studies). In full agreement with our NMR studies of the ψ -modified helix presented here, as well as our previous studies, our 2ap fluorescence data indicate that the adenosine residue adjacent to the branch site in these constructs is not exposed to solvent. Similar results in each of the mutant sequences implied that, regardless of the intra- or extrahelical conformation of the branch site adenosine, its 5' neighbor appeared to adopt an intrahelical conformation in solution.

Taken together, our data indicate that, even if hydrogen bond formation is not obligatory, the specific geometry associated with the presence of a R_{U2}-Y_{intron} pair in the conserved position, along with the ψ modification in its phylogenetically conserved location on the U2 snRNA strand across from the branch site, is necessary for the extrahelical position of the branch site residue. Interestingly, in addition to a general conservation of the R_{U2}-Y_{intron} pair interacting with the branch site A, the adjacent pair (three positions 5' of the branch site) is also very predominantly a R_{U2}-Y_{intron} pair (Lim and Burge 2001). Details of stacking interactions and/or the specific placement of water molecules noted by Lin and Kielkopf (2008) associated with this sequence may contribute to the favorability of the branch site motif.

Mutations associated with components of the branch site motif result in decreased splicing efficiency. Mutations of the branch site adenine suppress splicing, with an A→C mutation causing the least deleterious effect (Query et al. 1996). It is possible that substitution of C may be tolerated (Hornig et al. 1986) because it has an amino group on the Watson-Crick face of the base and may, therefore, be able to form similar hydrogen bonding or other interactions as those formed by the analogous face of A, although with slightly different geometry.

Wide conservation of the A_{U2}-U_{intron} base orientation suggests that the orientation is critical to the branch site motif formation and function. Splicing assays performed in yeast to effect a reversal of the A_{U2}-U_{intron} pair to U_{U2}-A_{intron} (m ψ BP duplex) show <10% splicing efficiency compared to the wild type (McPheeters and Abelson 1992), a finding that we propose is at least partly the result of the intrahelical position of the branch site A in the presence of the reversed U_{U2}-A_{intron} base pair. In contrast, however, inhibition of splicing as a result of mutation in the three intron nucleotides 5' of the branch site A in a SV40/human system were rescued by compensatory mutations in U2 snRNA that maintained complementary pairing, without an apparent effect of orientation of the R_{intron}-Y_{U2} base pair (Wu and Manley 1989), which may reflect subtle mechanistic differences between the yeast and mammalian spliceosomes. We emphasize, however, that only limited correlations can be made when extrapolating conclusions from a cellular to an in vitro system due to the complexity and dynamic nature of the spliceosomal assembly.

A number of factors may play a role in recognition of the branch site and activity. Among them, electrostatic features of the ψ -modified branch site duplex (Xu et al. 2005) may

impact on recognition and function of the branch site. Smith et al. (2009) have recently shown that the branching activity in yeast spliceosomes is also affected by the distance of the branch site A from the U2-U6 helix Ia, suggesting that long range interactions with remote sections of the U2-U6 snRNA complex may play a role in recognition and/or positioning of the branch site adenosine. Specific protein interactions within the branch site region are essential for recruitment of the U2 snRNP and splicing activity (Berglund et al. 1997; Liu et al. 2001). Perturbation of binding between U2 snRNP protein SF3b 155 and sequences flanking the pre-mRNA branch site by a small molecule inhibitor, spliceostatin A, disrupts alternative splicing patterns, perhaps by promoting nonproductive RNA pairing, thus underscoring the role of branch site duplex formation in assembly of the U2 snRNP-branch site complex (Corrionero et al. 2011) that may explain its anti-cancer activity (Kaida et al. 2007; Kotake et al. 2007). Thus, an intricate balance involving multiple recognition motifs and interactions, including conserved base modifications and local RNA interactions as well as protein and long-range RNA interactions, appears to facilitate splicing activity.

MATERIALS AND METHODS

Design and synthesis of RNA helices

A series of RNA duplexes was designed to test the role of the R_{U2} - Y_{intron} base pair and the phylogenetically conserved ψ residue ($\psi 35$ in the *S. cerevisiae* U2 snRNA sequence) on the conformation of the branch site adenosine or its 5' neighbor in solution. RNA oligomers were designed to represent fragments of the native U2 snRNA and intron sequences from *S. cerevisiae* that pair to form the branch site helix; mutant sequences were designed with specific mutations at positions involved in formation of the base triple. 2ap-substituted sequences were designed to test the importance of the hydrogen bond donor in position 6 of the adenine ring on stabilizing the extrahelical branch site A conformation. U2 snRNA strands and intron strands comprised 9 and 10 nt, respectively, including 7 bp from the native duplex plus two additional Watson-Crick pairs added to increase thermal stability. It was shown previously that addition of these base pairs did not alter duplex conformation (Newby and Greenbaum 2001). Duplexes were formed by pairing equimolar amounts of U2 snRNA and intron strands.

We also performed fluorescence studies on duplexes in which the branch site adenosine (A24 in these studies) was replaced with 2ap in the intron and also substituted the adenosine 5' to the branch site A (A23) with 2ap to test the effect of mutations on the conformation of A23. A complementary duplex of 10 bp (U added to the U2 snRNA strand opposing the branch site A) was used as a control, as was a single-stranded intron sequence. All RNA oligomers were purchased from Dharmacon and were deprotected according to company protocols.

NMR spectroscopy

Samples of the ψ BP and 2ap-substituted ψ BP_{2ap}, as well as of ψ -modified mutated duplex (m ψ BP) and the 2ap-substituted

version m ψ BP_{2ap}, were purchased from Dharmacon, lyophilized after deprotection, and resuspended in 50 mM NaCl pH 6.8–7.2 and 0.1 mM EDTA, in 90% H₂O/10% ²H₂O (D₂O). For observation of nonexchangeable protons, samples were lyophilized three times in 99.96% D₂O and resuspended in 99.96% D₂O, 0.01 mg/mL DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid), the latter used as an internal referencing standard (Cambridge Isotope Laboratories, Inc.). NMR data were collected in micro-volume NMR tubes (Shigemi, Inc.).

NMR data were acquired on a 600 MHz Bruker Avance spectrometer (Dept. of Chemistry and Biochemistry, Hunter College of CUNY), equipped with the three-channel TXI-cryoprobe. Quadrature detection was achieved by using the States-TPPI method (Marion et al. 1989). Bruker Topspin 2.1 software was used to process two-dimensional experiments. SPARKY was used to visualize spectra and for chemical shift assignments. Spectra were apodized using sine squared function and zero-filled in both the direct and indirect dimensions.

Chemical shift assignments of exchangeable and nonexchangeable protons of the ψ BP, ψ BP_{2ap}, m ψ BP, and m ψ BP_{2ap} duplexes were made from NOESY spectra acquired at mixing times of 50–950 msec. For nonexchangeable protons, phase-sensitive NOESY spectra using a long and weak presaturation pulse for water suppression (NOESYphpr) were acquired at 17°C and 20°C and compared with those of ψ BP (Newby and Greenbaum 2001). C2'-endo (or other non-C3'-endo) sugar conformations were identified from the appearance of medium-to-strong H1'-H2' cross-peaks in TOCSY spectra collected at 70-msec mixing time. A one-dimensional proton-decoupled ³¹P spectrum of m ψ BP, also at 20°C, was collected in a Varian Inova 500 MHz spectrometer equipped with an indirect detection 5-mm H₂CX probe and referenced to 85% phosphoric acid.

Fluorescence studies

Wild-type and variant sequences of U2 snRNA and intron strands were combined in an aqueous buffer of 10 mM NaP_i, pH 6.4, 0.1 mM EDTA, and 1 M NaCl. The concentration of each strand was ~4.5 μ M, with a slight (<5%) excess of the non-2ap strand to minimize presence of free 10_{2ap} strand in solution. Melting transitions were measured for duplexes containing 2ap and A to confirm that the substitution did not perturb duplex stability. Samples (250 μ L) were suspended in 2 mm $\times$ 10 mm quartz fluorescence cells (Starna Cells, Inc.). Fluorescence excitation and emission profiles were scanned on a Cary Eclipse fluorescence spectrometer over a range of 200–850 nm. Emission scans were obtained at least five times for each sample using the observed maximum excitation wavelength 308 nm; maximal fluorescence emission was at ~370 nm. Experiments were repeated on three to eight samples of each duplex. All fluorescence data were processed in Microsoft Excel (Microsoft, Inc.) and Sigma Plot 8.0 for Windows (Microsoft, Inc.).

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Impact of base pair identity 5' to the spliceosomal branch site adenosine on branch site conformation

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