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Identification and characterization of a short 2′–3′ bond-forming ribozyme

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ABSTRACT

A large number of natural and artificial ribozymes have been isolated since the demonstration of the catalytic potential of RNA, with the majority of these catalyzing phosphate hydrolysis or transesterification reactions. Here, we describe and characterize an extremely short ribozyme that catalyzes the positionally specific transesterification that produces a 2′–3′ phosphodiester bond between itself and a branch substrate provided in *trans*, cleaving itself internally in the process. Although this ribozyme was originally derived from constructs based on snRNAs, its minimal catalytic motif contains essentially no snRNA sequence and the reaction it catalyzes is not directly related to either step of pre-mRNA splicing. Our data have implications for the intrinsic reactivity of the large amount of RNA sequence space known to be transcribed in nature and for the validity and utility of the use of protein-free systems to study pre-mRNA splicing.

Keywords: RNA catalysis; splicing; snRNAs

INTRODUCTION

The question of whether the active site of the spliceosome is composed partly or entirely of RNA, like that of the ribosome (Ban et al. 2000), remains unresolved (Collins and Guthrie 2000). Prompted by the demonstrated ribozyme activity of U6 snRNA-derived sequences (Tuschl et al. 1998, 2001) and the observation that RNAs with sequences based on those of human snRNAs and splicing substrates can form covalent bonds in vitro (Valadkhan and Manley 2001, 2003; Valadkhan et al. 2007), we sought to investigate whether *Saccharomyces cerevisiae* snRNAs would show such reactivity. *S. cerevisiae* lacks canonical SR proteins, which stimulate splicing in metazoan systems at least in part via the stabilization of RNA–RNA interactions (Shen and Green 2006, 2007), and its spliceosome therefore relies more heavily on the intrinsic stability of these interactions than do those of higher eukaryotes (Burge et al. 1999). We, therefore, reasoned that yeast snRNAs and splicing substrates may represent the optimal basis for a protein-free system to study pre-mRNA splicing.

One common issue with RNA-based systems, however, is the intrinsically reactive nature of RNA: reactions such as self-cleavage (Husken et al. 1996) and uncatalyzed RNA ligation (as discussed by Bartel and Szostak 1993) are commonplace and may have simple or even no structural requirements. Here, we describe the positionally specific and relatively efficient formation of a 2′–3′ bond between nucleotides in linker regions in our original snRNA- and branch-site-based constructs. The formation of this non-physiological bond does not require snRNA sequence beyond that required for substrate binding and can be observed using two short, synthetic RNA oligonucleotides containing a minimal hairpin motif. These results reiterate the reactivity of RNA toward phosphate transfer reactions and suggest that a large amount of potentially reactive RNA sequence space is likely to be explored in cells. The manner in which this ribozyme was identified also highlights the need for more stringent characterization and validation of RNA-only reactions currently interpreted as indicative of ribozyme catalysis by the spliceosome, which we discuss in an accompanying perspective (Smith and Konarska 2009).

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RESULTS AND DISCUSSION

An RNA based on fused *S. cerevisiae* U2 and U6 snRNA sequences slowly forms a covalent bond with a branch site oligonucleotide in vitro

In order to investigate the possibility that *S. cerevisiae* snRNAs may be able to catalyze reactions similar to those

observed with human snRNAs (Valadkhan and Manley 2001, 2003; Valadkhan 2007), we generated a construct comprising the 5′-terminal 46 nucleotides (nt) of U2 snRNA fused to the 3′ portion of U6 snRNA beginning at position G39, hereafter referred to as U26. The two snRNAs were joined via a loop of four uridine residues, and two extra G–C base pairs were added around this loop to encourage intramolecular folding (Fig. 1A). This construct was mixed at ~1 μM with a labeled oligonucleotide corresponding to the canonical *S. cerevisiae* UACUAAC branch site sequence with additional nucleotides upstream to stabilize binding to U2 snRNA, and an unlabeled 5′-splice-site (5′-SS) oligonucleotide with similarly hyper-stabilized binding to U6 snRNA in the register required for the first step of splicing (Fig. 1A; Kandels-Lewis and Seraphin 1993; Kim and Abelson 1996; Lesser and Guthrie 1993). The RNA mixture was heated to 95°C then slowly cooled to room temperature in 50 mM Tris-HCl (pH 7.2) containing 40 mM $MgCl_2$ and 200 mM KCl, left to react at room temperature, ethanol precipitated, and analyzed in a denaturing 20% polyacrylamide gel. A time course showed the slow generation of a specific low-mobility band over the course of many hours (Fig. 1B); the formation of this band was unaffected by the location of the label (5′ phosphate or 3′ pCp) on the BS RNA and by the presence or absence of the 5′-SS RNA (Fig. 1C), indicating that it contained the entire BS and no 5′-SS sequence. The slow mobility of the product suggested that it likely contained some or all of U26. We therefore carried out RNase H degradation on the purified product using oligonucleotides directed against either end of U26 and observed an increase in mobility with an oligo against the 5′ but not the 3′ end (Fig. 1D). The product therefore retains the 5′ end of U26 but has lost the 3′ end. Taken together, these observations are suggestive of a reaction between an internal nucleophile in BS and an internal phosphodiester bond in U26.

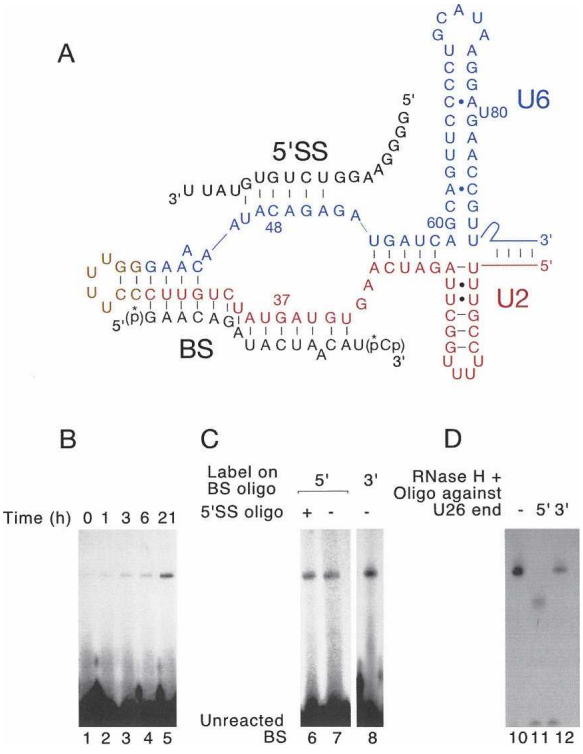

FIGURE 1. Slow formation of a covalently linked product containing the 5′ end of an snRNA-based construct and both ends of a branch-site-based oligonucleotide. (*A*) Schematic of constructs based on *S. cerevisiae* snRNAs and splicing substrates: U6 snRNA sequence is shown in blue, U2 snRNA sequence in red, and splicing substrates in black. Potential base pairs, even when mutually exclusive, are indicated. (*B*) The substrates depicted in *A* form a slowly migrating product over the course of several hours. (*C*) Product formation does not depend on the presence of the 5′ SS oligonucleotide, and the product contains both ends of the BS oligonucleotide. (*D*) RNase H digestion of purified product with oligonucleotides complementary to the 5′ or 3′ ends of the U26 construct indicates that the product contains the 5′ but not the 3′ end of U26.

Conserved U6 snRNA sequences are not required for reactivity toward BS

The single sharp band observed following reaction of BS and U26 suggested a positionally specific reaction. In addition, the observed internal cleavage of U26 is suggestive of a transesterification; we, therefore, wished to investigate the dependence of our observed reactivity on snRNA sequences known to be important for pre-mRNA splicing. We generated constructs in which either or both of the ACAGAGA and AGC motifs in U6, which are universally conserved and essential for viability (Burge et al. 1999), were mutated to runs of adenosine residues (Fig. 2A). The reactivity of these constructs was indistinguishable from that of U26 containing wild-type ACAGAGA and AGC (Fig. 2B). In addition, we made a series of deletion constructs in which U26 was truncated from its 5′ (Fig. 2C, Δ1–3) or 3′ ends (Fig. 2C, Δ4–6) and expressed from a cloning vector (pDrive, Qiagen). U26 RNAs truncated at their 5′ ends showed strong reactivity until most of the BS binding site had been removed (Fig. 2D, lanes 2–4), with further truncation presumably precluding binding of the BS oligonucleotide. 3′ end truncations/substitutions removed U6 sequence upstream of a constant vector sequence preceding a T7 terminator. Truncations beginning at and downstream of ACAGAGA retained reactivity (Fig. 2D, cf. lanes 1 and 5,6), and a construct containing only 2 nt of U6 sequence still produced a trace amount of product (Fig. 2D, lane 7). The mobilities of products derived from truncated U26 molecules are consistent with retention of only the 5′ end of the molecule; this rules out a 2′–5′ linkage, and, consistent with this, the mobility of the product was not affected by treatment with recombinant debranching enzyme (data not shown). (We note that DBR1 displays a degree of sequence specificity [Nam et al. 1994] and that the failure of an RNA molecule to

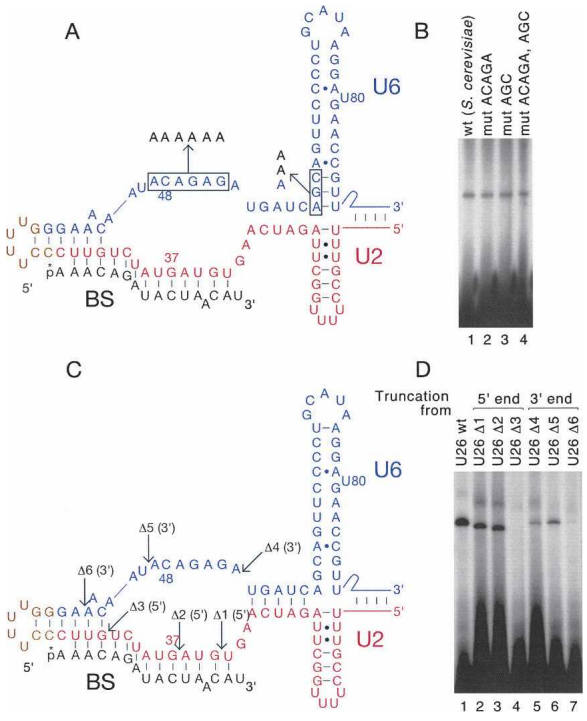

FIGURE 2. Virtually no snRNA sequence is required for U26 reactivity. (*A*) Schematic indicating mutations in the ACAGAGA and AGC motifs of the U6 portion of the U26 construct: These motifs are universally conserved and essential for viability. The BS oligonucleotide used in this figure contains a 5′-terminal adenosine and was used due to its increased reactivity relative to that with a 5′-terminal guanosine used in Figure 1. The effect on reactivity of the identity of the 5′-terminal base is addressed in Figure 3B. (*B*) Mutation of either or both of the ACAGAGA and AGC motifs to runs of A's has no effect on the formation of the U26–BS product. (*C*) Schematic indicating truncations made from the 5′ end (1–3) or 3′ end (4–6) of the U26 construct. These truncations were made in the context of constant surrounding vector (pDrive, Qiagen) sequence. (*D*) Some truncated U26 constructs retain reactivity toward BS. Deletion construct 6 shows little reactivity and construct 3 none, the latter likely due to an inability to bind the BS oligonucleotide. The observed variation in product size is consistent with retention of only the 5′ end of U26 in the product.

act as a substrate for this enzyme, therefore, does not constitute proof that no 2′–5′ bond is present). The reactivity of our truncated constructs suggests (conservatively) that the scissile bond in U26 lies between positions G37 of U2 and C48 of U6. Subsequent experiments were therefore performed with a synthetic RNA oligonucleotide corresponding to the region of U26 from U2 position G37 to U6 position C48 (Fig. 3A).

The product comprises the entire BS RNA joined via its 5′-terminal nucleotide to a 5′ fragment of U26 via a 2′–3′ phosphodiester bond

Rough mapping by RNase digestion using internally labeled BS oligonucleotides suggested that the reactive group in BS was close to the 5′ end of the molecule (data not shown). 5′-end-labeled BS with a uridine as its 5′-terminal nucleoside was reacted with the minimal U26 oligonucletide depicted in Figure 3A, and this product was gel purified. Purified BS–U2 product was digested to completion with P1 nuclease, which cuts 5′ of each nucleotide to yield pN, or RNase T2, which cuts 3′ of each nucleotide to yield Np, and pNp in the case of a phosphorylated 5′-terminal nucleotide; digestion products were separated by two-dimensional thin layer chromatography as previously described (Padgett et al. 1984). The labeled phosphate from unreacted BS purified from the same gel as the product comigrated as expected with pU and pUp markers after P1 and T2 digestions, respectively (Fig. 3C, i,iii). P1 and T2 digestions of the BS–U2 product, however, produced species that did not comigrate with any of our mononucleotide markers, but which migrated similarly to one another (Fig. 3C, ii,iv). Neither P1 nor T2 can cleave at a branch nucleotide (Padgett et al. 1984), so the failure of these nucleases to produce labeled pU and pUp, respectively, from BS–U2 is indicative of branching from the 5′-terminal nucleotide of BS. Branching from further downstream nucleotides would not result in aberrant labeled P1/T2 digestion products. While this initial TLC analysis did not clarify the nature of the covalent bond between BS and U26, we reasoned that the most parsimonious chemistry was a 2′–3′ phosphodiester bond between the 2′ hydroxyl of the 5′-terminal uridine of BS and a phosphate group from U26. Such 2′–3′ branched molecules would be expected to produce similar products on digestion with P1 nuclease/RNase T2 (these products are shown schematically in Fig. 3C, ii,iv). Because the P1 and T2 digestion products of such a BS–U26 product are expected to differ only in the location of a single phosphate group (Fig. 3C), they should carry an identical net charge of −6. Further TLC analysis of the P1 and T2 digestion products using DEAE plates indicated that both did carry a −6 charge (Fig. 3C, v—the slight mobility difference is due to the 5′ or 3′ location of the unlabeled terminal phosphate); only a branched RNA molecule could carry more than four charges after P1 or T2 digestion, and only a 2′–3′ branch would generate P1 and T2 digestion products with the same charge. The bond between BS and U26 can therefore be assigned as a 2′–3′ phosphodiester. We obtained analogous results for all mapping experiments using full-length U26 and a BS oligonucleotide with a 5′-terminal G, indicating the generality of this product (data not shown).

In addition to our TLC analysis, further data support the assignment of this reaction as a 2′–3′ transesterification. The reaction proceeds regardless of the 5′ end phosphorylation state of the BS oligonucleotide (Fig. 1C), and a 5′-terminal phosphate on BS remains sensitive to phosphatase treatment in the product (data not shown); this rules out an involvement for the 5′ phosphate itself, meaning that the nucleophile must be a group either on the 5′-terminal

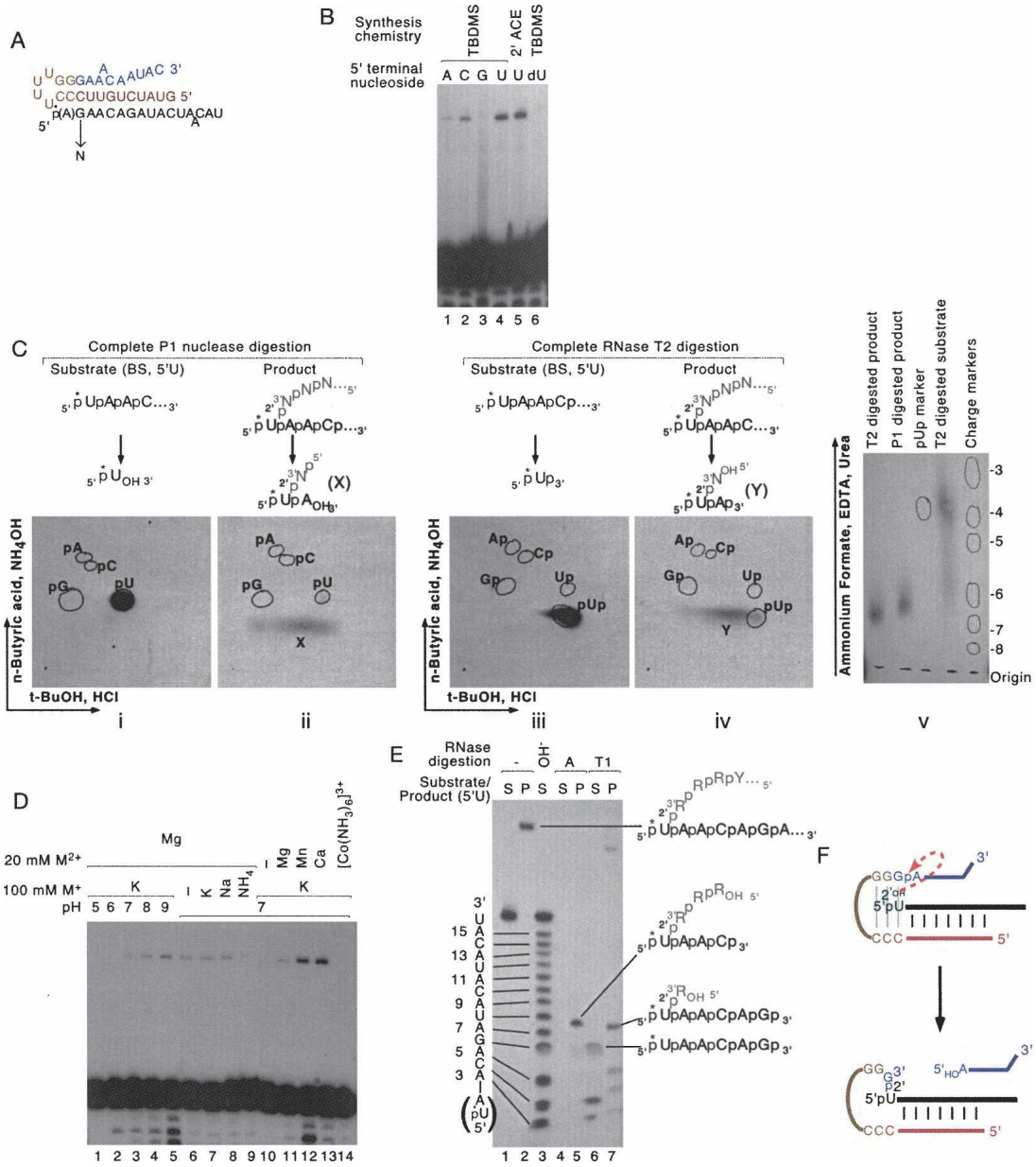

FIGURE 3. Characterization of BS–U26 reactivity and mapping of the reactive sites. (*A*) Schematic indicating the sequences of constructs used in this figure. (*B*) The 2′ hydroxyl of the 5′-terminal nucleotide of BS is strictly required for reactivity toward U26. BS oligonucleotides with diverse 5′ ends can react; reaction efficiency varies with the identity of this nucleotide, but not in a manner consistent with a functional group from the base acting as a nucleophile. Reactivity is not due to a synthesis artifact, as oligonucleotides made via different synthesis routes (TBDMS and 2′ ACE) react with identical efficiency. (*C*) TLC characterization of the product: (*i–iv*) 2D TLC analysis of P1 nuclease/RNase T2 digestions of unreacted BS substrate (*i,iii*) or reaction product (*ii,iv*) with a labeled 5′ terminal U. The migration of unlabeled markers is indicated, and expected labeled digestion products are shown. BS-derived sequence is shown in black and unmapped U26 sequence in gray. (*v*) DEAE TLC analysis of P1 nuclease/RNase T2 digestions of the 5′U reaction products to assay net charge; the positions of an unlabeled pUp marker and markers generated by partial alkaline hydrolysis of unreacted substrate are indicated. (*D*) pH and metal ion dependence of the reaction between [5′U] BS and the minimal U26 oligonucleotide. (*E*) RNase digestion of [5′U] BS (S) and its product with U26 (P) identifies 5′YRRRN3′ as the 3′-most nucleotides of U26 appended to BS; the sequence of BS and the sizes of hydrolysis fragments are indicated *beside* the ladder in lane *3*. BS sequence is shown in black and U26 sequence in gray. (*F*) Schematic of the reaction with sites of reactivity and polarity of phosphodiester linkages detailed.

base or its associated ribose/phosphate. Changing the 5′-terminal base of BS has a profound effect on reactivity (Fig. 3B, lanes 1–4), with the 5′U being the most reactive and the 5′G the least. However, the relative efficiencies of reaction that we observe are inconsistent with attack by a functional group on the base, and we believe them to be related to a basic structural requirement for this reaction. Reactivity due to a contaminant or incompletely deprotected group

arising from chemical oligonucleotide synthesis was ruled out by the identical reactivity of oligonucleotides produced via TBDMS and 2′ACE synthesis (Fig. 3B, lanes 4,5). The 2′ hydroxyl of the 5′-terminal nucleotide therefore remains as the only possible nucleophile for this reaction. Indeed, substitution of the normally reactive 5′ uridine with deoxyuridine abolished reactivity (Fig. 3B, lanes 4,6).

Transesterification reactions are strongly dependent on metal ions, requiring divalent cations for nucleophile activation and leaving group stabilization (Steitz and Steitz 1993). Consistent with the BS–U26 reaction being a transesterification, no product is observed in the absence of divalent metal ions, but magnesium, manganese, or calcium can all support reactivity (Fig. 3D, lanes 10–13). Two metal ion catalysis requires inner shell coordination of reactive groups by the divalent metal ion; such coordination is not possible with cobalt hexamine—a structural mimic of a fully hydrated Mg^{2+}—and the absence of reactivity in the presence of cobalt hexamine (Fig. 3D, lane 14), therefore, further supports the assignment of this reaction as a transesterification. Finally, the increase in efficiency observed with increasing pH (Fig. 3D, lanes 1–5) is suggestive of the expected requirement for nucleophile deprotonation.

Mapping the scissile phosphate within U26

Together, our data demonstrate the formation of a 2′–3′ phosphodiester bond between BS and U26. Such a bond is currently unprecedented in any physiological system, but has previously been observed in ribozyme catalysis (Tuschl et al. 1998). Incompatible strand polarities precluded mapping the site of reactivity in U26 by primer extension, and the U26 concentrations required to generate detectable levels of product rendered specific labeling experiments impractical. Indirect identification of the scissile bond was, however, possible via RNase digestion of products containing end-labeled BS.

In order to allow mapping of the scissile bond within U26, purified product resulting from reaction between a 5′ uridine end-labeled BS and the minimal U26 oligonucleotide was digested with RNases A or T1 (Fig. 3E). Typically RNase T1 cuts 3′ of guanosines, but under our conditions of high enzyme concentration it cuts 3′ of all purines with only a slight preference for guanosine. T1 digestion of the product indicated that that U26 was joined to BS via a purine, as each band resulting from digestion of the product showed a decrease in mobility corresponding to 1 nt relative to the analogous band resulting from the digestion of unreacted substrate (Fig. 3E, lanes 6,7—digestion products are shown schematically beside the gel). Digestion of the product with RNase A, which cuts 3′ of pyrimidines, generated a species that migrated as a 7-mer (Fig. 3E, lane 5). Branching from the terminal U of BS would inhibit RNase A cleavage at this position, so the first cut in BS must be made 3′ of C4; therefore the 7-mer digestion product indicates that the last four most 3′ nucleotides of the retained U26 fragment must be (with the RNase A cleavage site indicated by an asterisk) 5′Y*RRR3′–2′BS (shown schematically in Fig. 3E). The minimal U26 oligonucleotide contains only one YRRRN sequence—the UGGGA running from the terminal U of the U_4 loop to U6 A40—indicating that the scissile phosphate is that between the third G and the A in this motif. We therefore conclude that the U26-derived strand of the product comprises the entire U2 portion and the CCCUUUUGGG stem–loop (reactants/products are shown schematically in Fig. 3F). The released 3′ end of U26 is expected to migrate among degradation products and can therefore not be followed.

Despite the simple ionic requirements of this reaction (Fig. 3D, lanes 6–14) and the ability of BS oligonucleotides with diverse 5′ ends to react (Fig. 3B), at least some degree of higher-order structure appears to be important. Mutations in the hairpin region of the U26 oligonucleotide, including compensatory changes as well as those that disrupt the helix, strongly inhibit or abolish reactivity toward all tested BS substrates (data not shown), suggesting that the juxtaposition of nucleophile and electrophile requires structure beyond simple base-pairing. The sensitivity of the U26 hairpin to mutation precludes detailed analysis of such structural requirements. However, given that the 5′-terminal nucleotide (G) of BS is capable of pairing to the 3′-terminal nucleotide (C) of U2 sequence in U26 (Fig. 1A) and that the reactivity of BS oligonucleotides correlates inversely with their ability to form a base pair at this position (Fig. 3B, lanes 1–4), we hypothesize that nucleophilic attack occurs when the 5′ end of BS is single stranded.

Implications for the potential reactivity of cellular RNAs

The ease with which ribozymes, particularly those that catalyze RNA hydrolysis or transesterification reactions, can be generated by SELEX (Szostak et al. 2001) suggests that a large proportion of RNA sequence space is at least minimally catalytically active. Our data, which show the relatively efficient and positionally specific catalysis of a transesterification reaction, apparently by a small and simple structural motif, support this idea.

The use of tiling arrays and deep sequencing has recently indicated that a greater proportion of many organisms' genomes is transcribed than was previously thought (Graveley 2008); given the diversity of cellular RNA sequence that such widespread expression would generate, high intracellular RNA concentrations, and the potential for ribozyme catalysis by extremely simple structures, such intrinsic RNA catalysis may occur in vivo. Cells have likely evolved mechanisms to prevent and/or mitigate the effects of inappropriate RNA reactivity in vivo. These mechanisms

may include evolutionary selection against catalytically active sequences or structures, the rapid turnover of RNA such that sufficient time is not normally allowed for catalysis, and the coating of RNA molecules with proteins such as hnRNPs. It is, however, also possible that such ribozyme-generated RNAs may play physiological roles. The analysis of such potential products, as well as of enzymes involved in their further processing or degradation, may be complicated in eukaryotes by the background of lariats arising due to pre-mRNA splicing, as these will comprise the majority of nonlinear RNAs. In addition, ribozyme-derived products may contain bonds (such as 2′–3′ phosphodiesters) that do not lend themselves to conventionally used mapping techniques: For example, primer extension analysis of the 2′-linked strand of a 2′–3′ branched RNA is precluded by incompatible strand polarities.

Implications for the utility of protein-free systems as tools to study pre-mRNA splicing

The reactivity of protein-free RNA molecules based on snRNAs and splicing substrates has, over the past decade, been presented as evidence of ribozyme catalysis by the spliceosome (Tuschl et al. 1998, 2001; Valadkhan and Manley 2001, 2003; Valadkhan et al. 2007). Our data highlight the feasibility of direct and unambiguous characterization of the products of such in vitro reactions: In addition, they have caused us to reflect on the requirement for stringent validation of such systems, if such validation is indeed possible. We discuss these issues fully in an accompanying perspective (Smith and Konarska 2009).

MATERIALS AND METHODS

U26 constructs were generated by PCR or by the ligation of overlapping oligonucleotides with single A overhangs into the Qiagen pDrive PCR cloning vector, transcribed using T7 RNA polymerase, and gel purified before use. DNA oligonucleotides were purchased from Operon Biotechnologies and RNA from Oligos Etc. (TBDMS chemistry) or Dharmacon (2′ACE chemistry).

In general, reactions were assembled in 5–10 μL volumes, heated to 95°C, left to cool slowly to room temperature, and incubated for 12–24 h before ethanol precipitation and separation in 20% polyacrylamide/8 M urea gels. Products were excised from these gels and eluted at 4°C from crushed gel slices in 0.3 M NaOAc (pH 5.5), with tris-saturated phenol, then ethanol precipitated prior to analysis. RNase digestion and 2D TLC were performed as previously described (Padgett et al. 1984), except that the solvent used in the first TLC dimension contained *n*-butyric acid rather than isobutyric acid. The solvent for DEAE TLC was 0.2 M ammonium formate, 1 mM EDTA, 9 M urea.

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REFERENCES

Ban, N., Nissen, P., Hansen, J., Moore, P.B., and Steitz, T.A. 2000. The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. *Science* **289:** 905–920.

Bartel, D.P. and Szostak, J.W. 1993. Isolation of new ribozymes from a large pool of random sequences [see comment]. *Science* **261:** 1411–1418.

Burge, C.B., Tuschl, T.H., and Sharp, P.A. 1999. Splicing of precursors to mRNAs by the spliceosomes. In *The RNA world*, 2nd ed. (eds. R.F. Gesteland et al.), pp. 525–560. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Collins, C.A. and Guthrie, C. 2000. The question remains: Is the spliceosome a ribozyme? *Nat. Struct. Biol.* **7:** 850–854.

Graveley, B.R. 2008. Molecular biology: Power sequencing. *Nature* **453:** 1197–1198.

Husken, D., Goodall, G., Blommers, M.J., Jahnke, W., Hall, J., Haner, R., and Moser, H.E. 1996. Creating RNA bulges: Cleavage of RNA in RNA/DNA duplexes by metal ion catalysis. *Biochemistry* **35:** 16591–16600.

Kandels-Lewis, S. and Seraphin, B. 1993. Involvement of U6 snRNA in 5′ splice site selection. *Science* **262:** 2035–2039.

Kim, C.H. and Abelson, J. 1996. Site-specific crosslinks of yeast U6 snRNA to the pre-mRNA near the 5′ splice site. *RNA* **2:** 995–1010.

Lesser, C.F. and Guthrie, C. 1993. Mutations in U6 snRNA that alter splice site specificity: Implications for the active site. *Science* **262:** 1982–1988.

Nam, K., Hudson, R.H., Chapman, K.B., Ganeshan, K., Damha, M.J., and Boeke, J.D. 1994. Yeast lariat debranching enzyme. Substrate and sequence specificity. *J. Biol. Chem.* **269:** 20613–20621.

Padgett, R.A., Konarska, M.M., Grabowski, P.J., Hardy, S.F., and Sharp, P.A. 1984. Lariat RNA's as intermediates and products in the splicing of messenger RNA precursors. *Science* **225:** 898–903.

Shen, H. and Green, M.R. 2006. RS domains contact splicing signals and promote splicing by a common mechanism in yeast through humans. *Genes & Dev.* **20:** 1755–1765.

Shen, H. and Green, M.R. 2007. RS domain-splicing signal interactions in splicing of U12-type and U2-type introns. *Nat. Struct. Mol. Biol.* **14:** 597–603.

Smith, D.J. and Konarska, M.M. 2009. A critical assessment of the utility of protein-free splicing systems. *RNA* **15:** (this issue). doi: 10.1261/rna.1322709.

Steitz, T.A. and Steitz, J.A. 1993. A general two-metal-ion mechanism for catalytic RNA. *Proc. Natl. Acad. Sci.* **90:** 6498–6502.

Szostak, J.W., Bartel, D.P., and Luisi, P.L. 2001. Synthesizing life. *Nature* **409:** 387–390.

Tuschl, T., Sharp, P.A., and Bartel, D.P. 1998. Selection in vitro of novel ribozymes from a partially randomized U2 and U6 snRNA library. *EMBO J.* **17:** 2637–2650.

Tuschl, T., Sharp, P.A., and Bartel, D.P. 2001. A ribozyme selected from variants of U6 snRNA promotes 2′,5′-branch formation. *RNA* **7:** 29–43.

Valadkhan, S. 2007. The spliceosome: Caught in a web of shifting interactions. *Curr. Opin. Struct. Biol.* **17:** 310–315.

Valadkhan, S. and Manley, J.L. 2001. Splicing-related catalysis by protein-free snRNAs. *Nature* **413:** 701–707.

Valadkhan, S. and Manley, J.L. 2003. Characterization of the catalytic activity of U2 and U6 snRNAs. *RNA* **9:** 892–904.

Valadkhan, S., Mohammadi, A., Wachtel, C., and Manley, J.L. 2007. Protein-free spliceosomal snRNAs catalyze a reaction that resembles the first step of splicing. *RNA* **13:** 2300–2311.